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ORIGINAL RESEARCH

A critical update on endothelial nitric oxide synthase gene variations in women with idiopathic recurrent spontaneous abortion: genetic association study, systematic review and meta-analyses

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ABSTRACT: A number of case–control studies investigated the association between idiopathic recurrent spontaneous abortion (IRSA) and variations in the gene encoding endothelial nitric oxide synthase (*NOS3*), but yielded contradictory results. Our aim was to test the association of the *NOS3* variable number of tandem repeats (VNTR) in intron 4 and +894 G/T single-nucleotide polymorphism (SNP) with IRSA in Slovenian women (148 IRSA and 149 control women), conduct a systematic review of literature on the association between *NOS3* gene variations and IRSA, and perform meta-analyses of studies that met the inclusion criteria, defined by virtue of the European Society for Human Reproduction and Embryology evidence-based guidelines for recurrent spontaneous abortion. Genotyping was performed using PCR and restriction fragment length polymorphism methods. The systematic review of literature (English language) was conducted using PubMed and Scopus databases, to 1 November 2014. We determined no association of IRSA with the VNTR in intron 4 and +894 G/T SNP in Slovenian women. Furthermore, 16 case–control studies were identified on the association between 15 *NOS3* gene variations and IRSA. However, significant inconsistencies exist in the selection criteria of patients and controls between studies. The meta-analysis of VNTR in intron 4 was performed on five studies (894 patients, 944 controls), whereas the meta-analysis of +894 G/T SNP included six studies (1111 patients, 1121 controls). The association with IRSA was significant for the +894 G/T SNP under the dominant genetic model (GT+TT versus GG) based on fixed (odds ratio (OR) = 1.54, 95% confidence interval (CI) = 1.28–1.86, $P = <0.01$) and random effects models (OR = 1.54, 95% CI = 1.03–2.31, $P = 0.03$). In conclusion, the GT and TT genotypes of the +894 G/T SNP in women might contribute to a predisposition to IRSA. Additional genetic association and functional studies in different populations with larger numbers of participants and a uniformly defined IRSA are needed to clarify the contribution of *NOS3* +894 G/T gene variation to IRSA.

Key words: endothelial nitric oxide synthase / evidence-based medicine / genetic polymorphism / miscarriage

Introduction

Recurrent spontaneous abortion (RSA) is a reproductive disorder that has been variously defined in the scientific literature. However, according to the European Society for Human Reproduction and Embryology (ESHRE) evidence-based guidelines for the clinical evaluation and medical treatment of RSA, the condition is defined as three or more consecutive spontaneous abortions (SAs) before the 20th week of gestation (the upper limit of gestational week for SA might vary according to state laws) (Jauniaux *et al.*, 2006). These guidelines are based on data of RCTs and meta-analyses, and recommend the basic investigations of a couple with RSA to include personal, obstetric and family history, exposure to toxins, full blood count, antiphospholipid antibodies, parental karyotype and pelvic ultrasound and/or hysterosalpingogram. The etiology of RSA is unknown in ~50% of couples and is referred to as idiopathic RSA (IRSA) (Porter and Scott, 2005; Jauniaux *et al.*, 2006). Genetic predisposition was proposed as a contributing factor and a large number of gene variations were tested in women with IRSA

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(Rull *et al.*, 2012). Considering that almost 90% of IRSA occurs before the 12th week of gestation (Porter and Scott, 2005), the frequently studied candidate genes are those involved in angiogenesis and vascular function.

Fetoplacental blood vessels lack autonomic innervation; therefore the regulation of vascular functions at the fetomaternal interface is mediated by endothelial cell-derived vasoactive mediators (Toda *et al.*, 2013). Among these is the endothelial nitric oxide synthase (eNOS), one of the three isoforms of NOS, which are involved in the production of nitric oxide (NO). The eNOS is synthesized in endothelial cells and other cell types, including those of the endometrium and placenta. In the endometrium, its expression is cyclic with highest levels during the window of implantation and continues during pregnancy, suggesting a role in endometrial perfusion, receptivity, implantation and placental function (Sladek *et al.*, 1997; Khorram *et al.*, 1999; Najafi *et al.*, 2012). Physiological functions of eNOS-derived NO are vasodilation and activation of endothelial progenitor cells, as well as the inhibition of platelet aggregation, inflammation and vascular smooth muscle proliferation (Förstermann and Sessa, 2012).

Alterations in eNOS and NO activity were detected in plasma, platelets and endometrium of women with IRSA compared with control women (Raffaelli *et al.*, 2010; Najafi *et al.*, 2012; Banerjee *et al.*, 2013; Makino *et al.*, 2004). Considering the altered levels of eNOS and NO in IRSA women, as well as the importance of eNOS and NO in angiogenesis, control of vascular tone and anti-thrombotic actions in endometrium during (pre)decidualisation and in placenta during early pregnancy, variation in the gene encoding for eNOS (*NOS3*) has been proposed as a potential factor of predisposition for IRSA. Association of IRSA was tested in several studies, which yielded contradictory results. Furthermore, two meta-analyses assessed whether variable number of tandem repeats (VNTR) in intron 4 and +894 G/T single nucleotide polymorphism (SNP; rs1799983) contribute to IRSA (Su *et al.*, 2011; Cao *et al.*, 2014).

The present study had three aims. The first was to test the association of *NOS3* VNTR in intron 4 and +894 G/T SNP with IRSA in Slovenian women in a case–control study. The second aim was to conduct a systematic review of literature on the association between *NOS3* gene variations and IRSA. Finally, we aimed to perform meta-analyses only of those studies that met the strict inclusion criteria that we defined by virtue of ESHRE evidence-based guidelines for the evaluation of IRSA patients (Jauniaux *et al.*, 2006). Therefore, in the work presented herein, we integrate the results of our own and previously published studies, but also point out differences between publications on IRSA regarding the selection criteria of patients and controls.

Methods

Case–control study

Subjects

A case–control study was performed to examine the association of *NOS3* VNTR in intron 4 and +894 G/T SNP with IRSA in Slovenian women. All women were recruited through the Institute of Medical Genetics, Department of Obstetrics and Gynaecology, UMC Ljubljana, Slovenia. Written informed consent for participation in this study was obtained from all participants. The study was approved by Slovenian and Croatian National Ethics' Committees. The IRSA group consisted of 148 women with a history of three or more consecutive SAs of unknown etiology before 22nd week of gestation (91.9% in the first 12 weeks and 8.1% between Weeks 13 and 22), with a mean age of 33 years (range 23–46). Exclusion criteria were: endocrine or metabolic disorders (including diabetes mellitus), antiphospholipid syndrome (APS), autoimmune disease or other systemic diseases, previous venous or arterial thrombosis or uterine anatomical abnormalities detected by ultrasonography and/or hysteroscopy. All couples had normal karyotypes. A total of 97 (65.5%) women had no live births (primary IRSA), whereas 51 (34.5%) women had at least one live born child (secondary IRSA). Three SAs were present in 136 (91.9%) women and 4–10 SAs in 12 (8.1%) women. The control group consisted of 149 unrelated, healthy women with at least two live births, and no history of SA or any other pregnancy complication.

DNA extraction and molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes by standard procedures using commercially available kit (Qiagen_FlexiGene kit; QIAGEN GmbH, Hilden Germany) as described by the manufacturer. Genotyping of VNTR in intron 4 was performed by allele-specific oligonucleotide PCR (ASO-PCR) using the following primers: 5′- AGGCCCTATGGTA GTGCCTTT-3′ and 5′- TCTCTTAGTGCTGTGGTCAC-3′. The wild-type allele (B) generated a 420 base pair band, whereas the mutant allele (A) generated a 393 base pair band. Genotyping of +894 G/T SNP was performed using PCR and restriction fragment length polymorphism (RFLP) methods. The following primers were used for PCR amplification: 5′- CATGAGGC TCAGCCCCAGAAC-3′ and 5′- AGTCAATCCCTTTGGTGCTCAC-3′, and yielded a 206 base pair long PCR product. PCR products were digested with the restriction enzyme MboI (New England Biolabs, Ipswich, MA, USA) at 37°C for 15–20 min. In the presence of the variant allele (T), the product was cleaved into two fragments of 119 and 87 base pairs. All PCRs were carried out in thermal cyclers (Mastercycler personal, Eppendorf, Hamburg, Germany and 2720 Thermal Cycler, Applied Biosystems, Carlsbad, CA, USA). The PCR and RFLP products were visualized under ultraviolet light after electrophoresis on 1 and 3% agarose gels, respectively, stained with GelRed™ (Olerup SSP®, Saltsjöbaden, Sweden).

Systematic review and meta-analyses

Search strategy

The systematic review of literature was conducted using PubMed and Scopus electronic databases, which were searched for publications on the association between *NOS3* gene variations and IRSA up to 1 November 2014. Considering that the exclusion of non-English language studies changes the effect estimates less than 5% (Jüni *et al.*, 2002), we applied a language restriction and only reports in the English language were taken into consideration. The following key words were used: *recurrent miscarriage*, *recurrent pregnancy loss*, *recurrent spontaneous abortion* or *habitual abortion* in combination with *endothelial nitric oxide synthase*, *eNOS* or *NOS3*. In addition, we searched references of retrieved articles. The search for publications was performed independently by two authors (N.P., S.O.) and all retrieved articles were compared to avoid duplication. Any disagreements were discussed and resolved with consensus.

Study selection

All of the case–control studies in the English language analyzing the association between *NOS3* gene variations and IRSA were included in the systematic review. However, meta-analyses were performed only for those gene variations that were tested in at least five studies that met the following strict inclusion criteria: (i) case–control study in which genotyping was performed in women with IRSA and control women; (ii) RSA defined as three or more SAs; (iii) diagnosis of IRSA based on ESHRE evidence-based guidelines (exclusion of APS in women, karyotyping of both partners for exclusion

of chromosome abnormalities, ultrasonography and/or hysteroscopy for exclusion of uterine anatomical abnormalities) (Jauniaux *et al.*, 2006); (iv) control group defined as women with at least one live birth and no pregnancy complications (no SA mandatory); (v) genotyping of *NOS3* gene variations performed by ASO-PCR, PCR-RFLP or sequencing; (vi) genotype frequencies reported; (vii) no deviation of genotype frequencies from Hardy–Weinberg equilibrium (HWE) in the control group.

Data extraction

For each study included in the systematic review, and afterwards in meta-analyses, the following data were extracted: authors, year of publication, ethnicity and number of patients and controls, IRSA definition, diagnostic evaluations performed in IRSA women, inclusion and/or exclusion criteria for controls, *NOS3* gene variations analyzed, methods used for genotyping, genotypes and allele frequencies. Additionally, we calculated HWE for genotype frequencies of VNTR in intron 4 and +894 G/T SNP in patient and control groups for each study.

Statistical analysis

Genetic association study

All statistical analyses were carried out using Statistica for Windows, version 10 (StatSoft, Inc., Tulsa, OK, USA) and MedCalc for Windows, version 13.3.3 (MedCalc Software, Mariakerke, Belgium), with the exception of statistical power, which was calculated using DSS Researcher's Toolkit (www.dssresearch.com/toolkit/spcalc/power_p2.asp), as well as deviations from HWE, which were tested using the Simple Hardy–Weinberg Calculator – Court Lab (Washington State University College of Veterinary Medicine, Pullman, WA, USA). Differences in genotype and allele frequencies between patients and controls were tested using Pearson's chi square (X^2) test. The associations of the two variations with the risk of IRSA were estimated by odds ratios (OR) and their 95% confidence intervals (CI). *P*-values <0.05 were considered statistically significant.

Meta-analyses

Meta-analyses were performed using Comprehensive Meta-Analysis, version 2.2.064 (Biostat, Inc., Englewood, NJ, USA). For each study, individual and summary ORs and associated 95% CI were calculated under dominant and recessive models, using fixed and random effects model. Tests for heterogeneity were performed for each meta-analysis using Cochran's Q test. Sensitivity analysis was performed by removing one study at a time to evaluate the relative influence of each study on the pooled estimate. Publication bias was tested using the funnel plot and Egger's regression test. A *P*-value <0.05 was considered significant for all analyses. All *P*-values were two-tailed.

Results

Genetic association study

The power of the present study was 80% to detect a 2-fold increase in VNTR A allele frequency and 90% to detect a 1.5-fold increase in +894 T allele. The distributions of VNTR in intron 4 and +894 G/T SNP genotypes in IRSA and control women were in HWE (discussed below). Genotypes and alleles frequencies of VNTR in intron 4 and +894 G/T SNP in IRSA and control women are presented in Table I, whereas the association with IRSA under different genetic models is shown in Table II. There were no statistically significant differences in the distribution of genotypes and alleles frequencies between IRSA and control women or association with IRSA under any genetic model. Additionally, genotypes and alleles frequencies were similar between women with primary and secondary IRSA, and we found no association of either variation with primary and secondary IRSA under any genetic model (data not shown).

Table I Genotype and allele frequencies of the *NOS3* VNTR in intron 4 and +894 G/T SNP in women with IRSA and control women.

NOS3	IRSA women *N* (%)	Control women *N* (%)	X^2	*P*-value
VNTR in intron 4				
Genotype				
BB	118 (79.7)	121 (81.2)	0.20	0.906
AB	26 (17.6)	25 (16.8)		
AA	4 (2.7)	3 (2.0)		
Allele				
B	262 (88.5)	267 (89.6)	0.18	0.672
A	34 (11.5)	31 (10.4)		
+894 G/T SNP				
Genotype				
GG	74 (50.0)	65 (43.6)	1.62	0.444
GT	54 (36.5)	65 (43.6)		
TT	20 (13.5)	19 (12.8)		
Allele				
G	202 (68.2)	195 (65.4)	0.53	0.467
T	94 (31.8)	103 (34.6)		

Table II Association of *NOS3* VNTR in intron 4 and +894 G/T SNP with IRSA under different genetic models.

NOS3 genetic model		IRSA versus control women	
		OR (95% CI)	*P*
VNTR in intron 4			
Dominant:	AA + AB versus BB	1.10 (0.62–1.95)	0.748
Recessive:	AA versus AB + BB	1.35 (0.30–6.15)	0.696
Co-dominant:	AA versus BB	1.37 (0.30–6.24)	0.686
	AA versus AB	1.28 (0.26–6.31)	0.760
	BB versus AB	0.94 (0.51–1.72)	0.835
Alleles:	A versus B	1.12 (0.67–1.87)	0.672
+894 G/T			
Dominant:	TT + GT versus GG	0.77 (0.49–1.22)	0.271
Recessive:	TT versus GT + GG	1.07 (0.54–2.10)	0.846
Co-dominant:	TT versus GG	0.92 (0.45–1.88)	0.829
	TT versus GT	1.27 (0.61–2.61)	0.522
	GG versus GT	1.37 (0.84–2.24)	0.209
Alleles:	T versus G	0.88 (0.63–1.24)	0.467

CI, confidence interval; OR, odds ratio.

Systematic review

The flow diagram showing the details for the study searching is shown in Fig. 1. We identified 16 case–control studies on the association between *NOS3* gene variations and IRSA, which we meticulously evaluated according to several criteria (Table III). Two additional studies were in Chinese and were excluded from the review (Fan *et al.*, 2007; Gao *et al.*, 2009). Thirteen studies analyzed the VNTR in intron 4 (Table IV), 11 studies analyzed the +894 G/T SNP (Table V), 7 studies analyzed the -786 T/C SNP, 2 studies analyzed rs1007311, and 11 variations were each analyzed once in 3 studies. Molecular analysis was appropriate in all studies.

In the next step we divided the 16 studies into two groups based on the definition of IRSA: the first one comprised studies defining IRSA as two or more SAs (Table IIIA), and the second as at least three SAs. The latter was further divided into two subgroups according to mandatory diagnostic evaluations performed in IRSA women and definition of the control group: one that contained studies with certain limitations and the other one that contained studies in which proper diagnostic evaluations were performed and were included in meta-analyses (Table IIIB and C).

Idiopathic RSA was defined as two or more SAs in three studies (Table IIIA). The mandatory diagnostic evaluations in IRSA women were appropriately performed, but the selection criteria for the control group in the study by Makino *et al.* (2004) are arguable because according to their data 65.8% of women were never pregnant.

In the remaining 13 studies and the present study, IRSA was defined as three or more SAs. Seven studies had limitations (Table IIIB), whereas the other six had appropriate diagnostic evaluations performed in IRSA women, as well as adequately defined control groups (Table IIIC). Regarding the seven studies with limitations, three of them had no patient criteria inclusion and exclusion described (Al Sallout and Sharif, 2010; Shin *et al.*, 2010; El-Gharably and Sharif, 2013), while in two other studies APS was not excluded in IRSA women (Karvela *et al.*, 2008; Zammiti *et al.*, 2008). In the study by Oztürk *et al.* (2011) the number of SA as a criteria for IRSA definition was not described in the section Subjects, although IRSA was defined as three or more SAs in the Introduction. Furthermore, the control group inclusion and exclusion criteria were not appropriately defined in three studies (Zammiti *et al.*, 2008; Oztürk *et al.*, 2011; Abulata *et al.*, 2014). In addition, in the

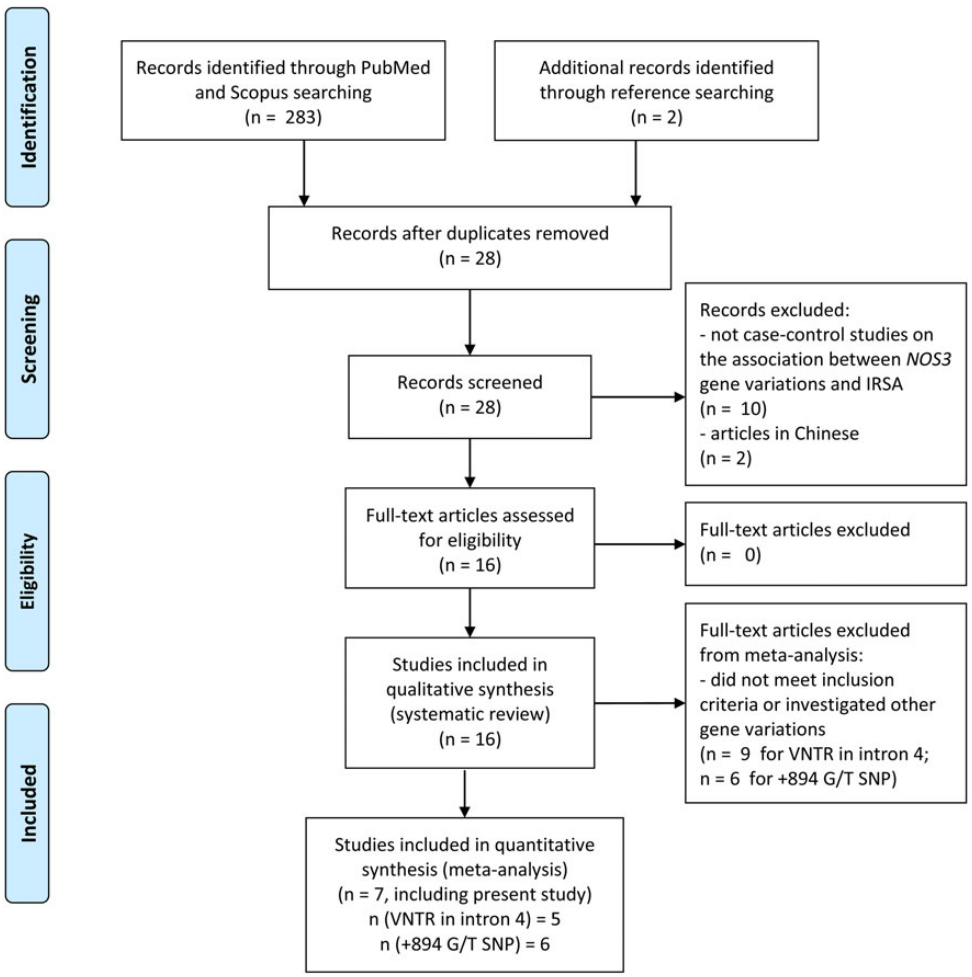

Figure 1 PRISMA flow diagram detailing the inclusion and exclusion criteria, and the number of studies included and excluded at each step of the literature search.

Table III Characteristics of studies on the association between *NOS3* gene variations and IRSA.

Authors	Ethnicity of women	IRSA women							Control women			*NOS3* gene variation tested		
		N	IRSA definition	Primary or secondary IRSA	Ultrasonography/ hysteroscopy	Karyotyping of both partners	APS	Other exclusion criteria	*N*	Live births	No pregnancy complications[a]	VNTR in intron 4	+894 G/T SNP	Other
(A)			Two or more:											
Buchholz *et al.* (2004)	German	179	Consecutive SA before 25th week of gestation	ND	+	+	+	• abnormal thyroid function • polycystic ovary syndrome • deficiencies of antithrombin, protein C and S	126	1	+	+		
Makino *et al.* (2004)	Japanese	85	SA before 10th week of gestation	ND	+	+	+	• abnormal immunologic tests for natural killer cell activity • abnormal thyroid function • diabetes mellitus • hyperprolactinemia • infections	76	26 with live births 50 without live births	+	+		
Dutra *et al.* (2014)	Brazilians	145	Consecutive SA before 24th week of gestation with the same partner	Primary	+	+	+	• abnormal hormonal status	135	2	+		+	-786 T/C SNP
(B)			Three or more:											
Al Sallout and Sharif (2010)	Palestinians	99	Consecutive SA before 25th week of gestation	ND		ND[b]		• infections	99	1	+	+		
Karvela *et al.* (2008)	Greeks	126	SA before 20th week of gestation with the same partner	ND	+	+	ND	• ND	130	2	+	+	+	

Zammiti *et al.* (2008)	Tunisians	350	Consecutive SA before 30th week of gestation	ND	+	+	ND	• infections • diabetes mellitus • abnormal thyroid function • hyperprolactinemia • erythroblastosis fetalis-Rh disease • immune thrombocytopenic purpura • foeto-maternal alloimmune thrombocytopenia	200	ND[c]	+	+	+	-786 T/C SNP
Shin *et al.* (2010)	Koreans	340	Consecutive SA	ND		ND[b]		• ND	115	1	+	+	+	-786 T/C SNP
Oztürk *et al.* (2011)	Turkish	60[d]	ND[e]	ND	+	+	+	• abnormal thyroid function • diabetes mellitus • inflammatory diseases	60[f]	ND[g]	+	+	+	
El-Gharably and Sharif (2013)	Palestinians	45	SA before 20th week of gestation	ND		ND[b]		• ND	45	2	+	+	+	-786 T/C SNP
Abulata *et al.* (2014)	Egyptians	50	SA before 20th week of gestation with the same partner	ND	+	+	+	• infections • abnormal hormonal status	50	ND	+	+		-786 T/C SNP
(C)			Three or more:											
Tempfer *et al.* (2001)	Austrians	105	Consecutive SA before 20th week of gestation with the same partner	ND[h]	+	+	+	• infections • abnormal hormonal status	91	2	+	+		
Hefler *et al.* (2002)		130							67				+	
Suryanarayana *et al.* (2006)	South Indians	145	First trimester SA	Primary	+	+	+	• infections	99	1	+	+	+	rs1007311
Parveen *et al.* (2011)	North Indians	200	SA	Primary	+	+	+	• abnormal hormonal status • prothrombotic risk factors (activated protein-C resistance, factor V Leiden and prothrombin mutations) • luteal-phase insufficiency • diabetes mellitus • abnormal thyroid function • infections	300	2	+	+	+	rs1007311 rs79468462 rs9282804 rs617606555

Continued

Table III *Continued*

Authors	Ethnicity of women	IRSA women							Control women			*NOS3* gene variation tested		
		N	IRSA definition	Primary or secondary IRSA	Ultrasonography/ hysteroscopy	Karyotyping of both partners	APS	Other exclusion criteria	*N*	Live births	No pregnancy complications[a]	VNTR in intron 4	+894 G/T SNP	Other
Almawi *et al.* (2013)	Bahrain Arabs	296	SA during the first trimester of gestation with the same partner	Primary: 217 (73.3%) secondary: 79 (26.7%)	+	+[i]	+[j]	• Rh blood group incompatibility • systemic autoimmune disease • diabetes mellitus • abnormal thyroid function • infections • abnormal liver function	305	2	+	+	+	-786 T/C SNP rs3918188 rs743507
Luo *et al.* (2013)	Chinese	340[k]	Consecutive SA	ND	+	+	+	• endocrine disorder • autoimmune disorder • infections	115[l]	1	+		+	-786 T/C SNP rs11771443 rs1541861 rs3918188 rs2853792 rs2853796 rs7830
Present study	Slovenians	148	Consecutive SA before 22nd week of gestation with the same partner	Primary: 97 (65.5%) secondary: 51 (34.5%)	+	+	+	• abnormal hormonal status • infections	149	2	+	+	+	

(A) Studies defining IRSA as two or more SA; (B) studies defining IRSA as three or more SA, but with certain limitations; (C) studies defining IRSA as three or more SA, included in meta-analysis.
APS, antiphospholipid syndrome; ND, not described; SA, spontaneous abortion.
[a]Including SA.
[b]Inclusion and exclusion criteria not described.
[c]Only the mean number of children is mentioned in the paper.
[d]Genotype data for VNTR in intron 4 available for 54, not 60 patients as indicated in the paper.
[e]The number of SAs is not defined in the section Subjects, although IRSA is defined as three or more SAs in the Introduction.
[f]Genotype data for VNTR in intron 4 available for 70, not 60 controls as indicated in the paper.
[g]Authors indicate that the control group consisted of multipara women, but did not specify the number of live births.
[h]Authors define primary IRSA as no history of a pregnancy carried beyond 20 weeks of gestation and secondary IRSA as a history of at least one pregnancy carried beyond 20 weeks of gestation.
[i]Performed in 38.9% of the initial number of patients.
[j]Performed in 67.6% of the initial number of patients.
[k]Genotype data for +894 G/T SNP available for 192, not 340 patients as indicated in the paper.
[l]Genotype data available for 201, not 115 controls as indicated in the paper.

Table IV Genotype frequencies obtained in studies on the association between *NOS3* VNTR in intron 4 and IRSA.

Authors	VNTR in intron 4 genotype frequency/*N* (%)									
	IRSA women					Control women				
	BB	AB	AA	Total	HWE	BB	AB	AA	Total	HWE
(A)										
Buchholz *et al.* (2004)	123 (68.7)	52 (29.1)	4 (2.2)	179	0.582	82 (65.1)	39 (30.9)	5 (4.0)	126	0.893
Makino *et al.* (2004)	70 (82.4)	15 (17.6)	0 (0)	85	0.372	62 (81.6)	14 (18.4)	0 (0)	76	0.376
(B)										
Al Sallout and Sharif (2010)	65 (65.0)	30 (30.0)	4 (4.0)	99	0.819	67 (67.7)	32 (32.3)	0 (0)	99	0.055
Karvela *et al.* (2008)	95 (75.4)	30 (23.8)	1 (0.8)	126	0.407	95 (73.1)	31 (23.8)	4 (3.1)	130	0.460
Zammiti *et al.* (2008)	231 (66.0)	97 (27.7)	22 (6.3)	350	0.009	146 (73.0)	46 (23.0)	8 (4.0)	200	0.084
Shin *et al.* (2010)	275 (80.9)	63 (18.5)	2 (0.6)	340	0.427	90 (78.2)	24 (20.9)	1 (0.9)	115	0.662
Oztürk *et al.* (2011)	0 (0)	16 (29.6)	38 (70.4)	54	0.201	6 (8.6)	10 (14.3)	54 (77.1)	70	<0.001
El-Gharably and Sharif (2013)	43 (95.6)	2 (4.4)	0 (0)	45	0.879	45 (100)	0 (0)	0 (0)	45	0.881
Abulata *et al.* (2014)	22 (44.0)	19 (38.0)	9 (18.0)	50	0.191	40 (80.0)	10 (20.0)	0 (0)	50	0.432
(C)										
Tempfer *et al.* (2001)	65 (61.9)	39 (37.1)	1 (0.9)	105	0.190	69 (75.8)	22 (24.2)	0 (0)	91	0.190
Suryanarayana *et al.* (2006)	101 (69.7)	43 (29.6)	1 (0.7)	145	0.114	71 (71.7)	28 (28.3)	0 (0)	99	0.101
Parveen *et al.* (2011)	133 (66.5)	50 (25.0)	17 (8.5)	200	<0.001	214 (71.3)	74 (24.7)	12 (4.0)	300	0.091
Almawi *et al.* (2013)	177 (59.8)	114 (38.5)	5 (1.7)	296	0.005	176 (57.7)	109 (35.7)	20 (6.6)	305	0.576
Present study	118 (79.7)	26 (17.6)	4 (2.7)	148	0.098	121 (81.2)	25 (16.8)	3 (2.0)	149	0.223

(A) Studies defining IRSA as two or more SA; (B) studies defining IRSA as three or more SA, but with certain limitations; (C) studies defining IRSA as three or more SA, included in meta-analysis.
HWE, Hardy–Weinberg equilibrium.

study by Oztürk *et al.* (2011), genotype frequencies significantly deviate from HWE in the control group, and are opposite to all other studies, indicating a possible genotyping error (Table IV). Finally, differences in the number of patients and controls reported in text and tables were detected in two studies (Table III; Oztürk *et al.*, 2011; Luo *et al.*, 2013).

Meta-analysis of VNTR in intron 4

Five studies, including the present study, met the criteria for inclusion in the meta-analysis of VNTR in intron 4 (Table IIIC and Fig. 1) (Tempfer *et al.*, 2001; Suryanarayana *et al.*, 2006; Parveen *et al.*, 2011; Almawi *et al.*, 2013). These five studies included 894 patients and 944 controls. The association between VNTR in intron 4 and IRSA was not statistically significant under the dominant (AA + AB versus BB) nor recessive genetic model (AA versus AB + BB), based on both fixed and random effects models (Fig. 2). There was no evidence of among-study heterogeneity (Cochran's Q test: $X^2 = 4.73$; df = 4; $P = 0.316$; Higgins statistics: $I^2 = 15.47\%$) or publication bias ($t = 1.51$; df = 3.00; $P = 0.227$) under the dominant genetic model. However, the among-study heterogeneity was statistically significant under the recessive genetic model (Cochran's Q test: $X^2 = 12.58$; df = 4; $P = 0.013$; Higgins statistics: $I^2 = 68.2\%$), whereas no publication bias was detected ($t = 0.08$; df = 3.00; $P = 0.939$).

Meta-analysis of +894 G/T SNP

Six studies, including the present study, met the criteria for inclusion in the meta-analysis of +894 G/T SNP, with a total of 1111 patients and 1121 controls (Table IIIC and Fig. 1) (Hefler *et al.*, 2002; Suryanarayana *et al.*, 2006; Parveen *et al.*, 2011; Almawi *et al.*, 2013; Luo *et al.*, 2013). The association between +894 G/T SNP and IRSA was statistically significant under the dominant genetic model (GT + TT versus GG) based on both fixed and random effects models (Fig. 3). The among-study heterogeneity was statistically significant (Cochran's Q test: $X^2 = 21.83$; df = 5; $P = 0.001$; Higgins statistics: $I^2 = 77.1\%$), while there was no evidence of publication bias ($t = <0.01$; df = 4.00; $P = 0.999$). On the contrary, there was no association between +894 G/T SNP and IRSA under the recessive genetic model (TT versus GT + GG) based on both fixed and random effects models (Fig. 3). We detected no among-study heterogeneity (Cochran's Q test: $X^2 = 8.9$; df = 5; $P = 0.111$; Higgins statistics: $I^2 = 44.1\%$) or publication bias ($t = 0.15$; df = 4.00; $P = 0.886$).

Discussion

In this study, we tested the association of *NOS3* VNTR in intron 4 and +894 G/T SNP with IRSA in Slovenian women, conducted a systematic review of literature on the role of *NOS3* gene variations in IRSA, and performed meta-analyses of VNTR in intron 4 and +894 G/T SNP. The meta-analyses demonstrated that the VNTR in intron 4 in women does not contribute to IRSA, whereas the variant (T) allele of the +894 G/T SNP is associated with IRSA when considered under the dominant genetic model.

Table V Genotype frequencies obtained in studies on the association between *NOS3* +894 G/T SNP and IRSA.

Authors	*NOS3* +894 G/T SNP genotype frequency/*N* (%)									
	IRSA women					Control women				
	GG	GT	TT	Total	HWE	GG	GT	TT	Total	HWE
(A)										
Dutra *et al.* (2014)	84 (58.0)	53 (36.5)	8 (5.5)	145	0.924	69 (51.1)	57 (42.2)	9 (6.7)	135	0.397
(B)										
Karvela *et al.* (2008)	53 (42.1)	57 (45.2)	16 (12.7)	126	0.912	62 (47.7)	58 (44.6)	10 (7.7)	130	0.478
Zammiti *et al.* (2008)	256 (73.1)	83 (23.7)	11 (3.2)	350	0.190	157 (78.5)	39 (19.5)	4 (2.0)	200	0.398
Shin *et al.* (2010)	266 (78.2)	60 (17.7)	14 (4.1)	340	<0.001	103 (89.6)	12 (10.4)	0 (0)	115	0.555
Oztürk *et al.* (2011)	42 (70.0)	18 (30.0)[a]		60	NA	28 (46.7)	32 (53.3)[a]		60	NA
El-Gharably and Sharif (2013)	26 (57.8)	19 (42.2)[a]		45	NA	22 (48.9)	23 (51.1)[a]		45	NA
(C)										
Hefler *et al.* (2002)	60 (46.2)	57 (43.8)	13 (10.0)	130	0.920	32 (47.8)	27 (40.3)	8 (11.9)	67	0.537
Suryanarayana *et al.* (2006)	91 (62.8)	47 (32.4)	7 (4.8)	145	0.770	69 (69.7)	27 (27.3)	3 (3.0)	99	0.856
Parveen *et al.* (2011)	155 (77.5)	42 (21.0)	3 (1.5)	200	0.936	278 (92.7)	22 (7.3)	0 (0)	300	0.510
Almawi *et al.* (2013)	154 (52.1)	109 (36.8)	33 (11.1)	296	0.046	194 (63.6)	95 (31.1)	16 (5.3)	305	0.334
Luo *et al.* (2013)	118 (61.5)	72 (37.5)	2 (1.0)	192	0.012	154 (76.6)	41 (20.4)	6 (3.0)	201	0.122
Present study	74 (50.0)	54 (36.5)	20 (13.5)	148	0.054	65 (43.6)	65 (43.6)	19 (12.8)	149	0.664

(A) Studies defining IRSA as two or more SA; (B) studies defining IRSA as three or more SA, but with certain limitations; (C) studies defining IRSA as three or more SA, included in meta-analysis.
NA, not applicable.
[a]Frequencies for GT and TT genotypes were not shown separately.

Characteristics of studies on the association of *NOS3* gene variations with IRSA

A total of 16 studies were published on the association between 15 *NOS3* gene variations and IRSA. Only a minority of studies comply with the evidence-based guidelines, whereas most show inconsistencies in design, primarily in patients and controls selection criteria. The first major disparity we observed concerns the definition of IRSA, especially regarding the minimum number of SAs needed for diagnosis. IRSA was defined as two or more SAs in three studies, although no available guideline recommends scientific research under this criterion (American College of Obstetricians and Gynecologists, 2002; Christiansen *et al.*, 2005; Laurino *et al.*, 2005; Porter and Scott, 2005; Jauniaux *et al.*, 2006; Practice Cmmittee of the American Society for Reproductive Medicine, 2012). Furthermore, all 16 studies differed considerably in reporting the order of SAs, upper limit of gestational age required for the diagnosis of SA, classification of couples into primary and secondary aborters, and the information on whether the SAs were with the same partner. The second major objection regards the abundance of unwarranted diagnostic evaluations performed in IRSA women regardless of clinical indications or evidence provided in the scientific literature, even when the basic clinical evaluation was not performed. In addition, three studies had no patient inclusion and exclusion criteria described (Al Sallout and Sharif, 2010; Shin *et al.*, 2010; El-Gharably and Sharif, 2013). Finally, the selection of women for the control group differed to a great extent between studies. Considering that no universal criteria for controls exist (Rull *et al.*, 2012), we set the minimum criteria as at least one live birth and no pregnancy complications, including SA.

The role of *NOS3* gene variations in IRSA

Among the 15 *NOS3* gene variations tested in IRSA women, the VNTR in intron 4, +894 G/T SNP and -786 T/C SNP were investigated in more than two studies. The remaining 11 variations were tested once and 1 was tested twice, precluding us from making conclusions on their association with IRSA. The -786 T/C SNP (rs2070744) was investigated in seven studies, but meta-analysis was not performed because only two studies met the inclusion criteria.

The VNTR in intron 4 consists of five (wild-type B allele) or four (mutant A allele) copies of a 27-bp repeat. The VNTR was shown to act as an enhancer/repressor regulating *NOS3* expression, and the 27-bp repeats are the source of a 27-nt small RNA, a short intronic repeat small RNA, which functions as a negative feedback regulator and maintains the stable expression of *NOS3* (Wang *et al.*, 2002; Zhang *et al.*, 2008). The mutant (A) allele produces lower levels of the 27-nt small RNA, leading to higher *NOS3* expression. However, the association with changes in plasma NO, nitrite and nitrate concentrations remains inconclusive (Tsukada *et al.*, 1998; Salimi *et al.*, 2008). Our genetic association study and meta-analysis indicate that the VNTR in intron 4 does not contribute to IRSA.

The +894 G/T SNP is located within the *NOS3* open reading frame at exon 7 (Wattanapitayakul *et al.*, 2001). This missense SNP corresponds to a substitution of glutamate to aspartate at amino acid position 298. Various reports associated this SNP with altered functional properties of eNOS and basal levels of NO (Wattanapitayakul *et al.*, 2001; Veldman *et al.*, 2002). It was also shown that the presence of the T allele does not affect *NOS3* steady state transcription in endothelial

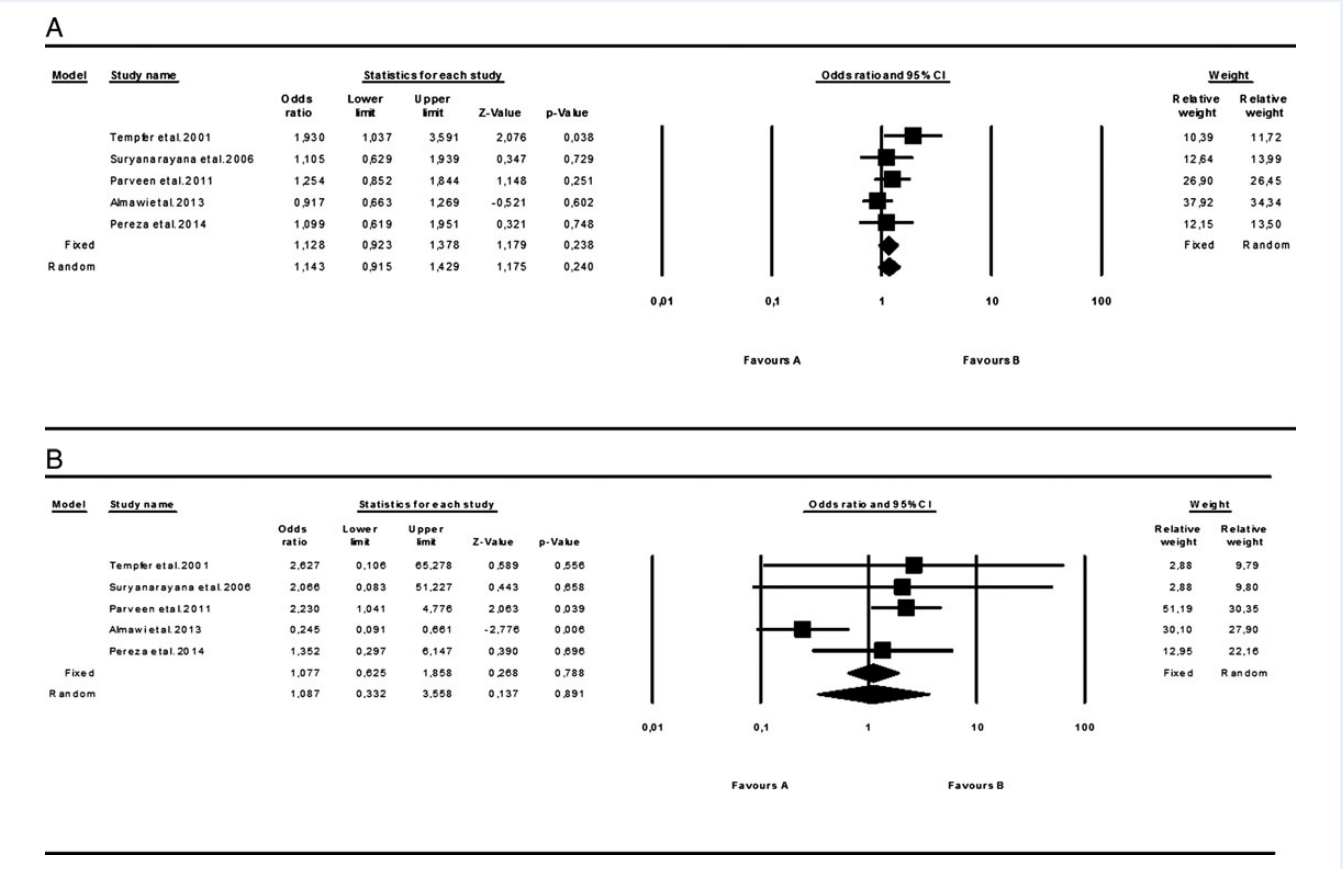

Figure 2 Association of VNTR in intron 4 and IRSA under dominant (**A**) and recessive (**B**) genetic models. Results of individual and summary OR estimates with 95% CIs of each study are shown. Horizontal lines represent 95% CIs and vertical lines the value of the summary OR.

cells, but rather affects the function of eNOS under shear stress (Joshi *et al.*, 2007). The T allele leads to decreased eNOS activity and alterations in localization at the endothelial caveolae, reducing the response to shear stress, impairing enzyme regulation and NO bioavailability (Joshi *et al.*, 2007). In addition, the T allele generates protein products with differing susceptibility to cleavage (Tesauro *et al.*, 2000). In the present study, we found no association between the SNP and IRSA in Slovenian women. However, the results of meta-analysis show that women homozygous and heterozygous for the variant (T) allele have a 1.5-fold increased risk of IRSA compared with women homozygous for the G allele. The discrepancy between the results of our case–control study and meta-analysis regarding the +894 G/T SNP might be the consequence of population differences and/or the number of participants included in case–control studies. Significant associations between this SNP and IRSA were determined in North Indian, Bahrain Arab and Chinese women, whereas the association was not confirmed in European Caucasians (Slovenians and Austrians) and South Indians. In addition, the three studies with positive associations had the largest number of participants.

The pathophysiologic significance of VNTR in intron 4 and +894 G/T SNP in IRSA has not been well investigated. Only two studies evaluated the impact of these variations on plasma NO levels in IRSA women and found no association (Oztürk *et al.*, 2011; El-Gharably and Sharif, 2013). However, both studies had major limitations (Table III). In the study by Oztürk *et al.* (2011) the control group was not well defined, and genotype frequencies significantly deviated from HWE and were opposite to all other studies, indicating a possible genotyping error, whereas the study by El-Gharably and Sharif (2013) had no patient inclusion and exclusion criteria described. Therefore, future studies should focus on the evaluation of the functional significance of +894 G/T SNP in IRSA women, regarding alterations in eNOS mRNA and protein levels and activity, as well as NO, nitrites and nitrate concentrations in plasma and endometrium.

Comparison to previous meta-analyses

In all three meta-analysis, the association with IRSA was significant only for the +894 G/T SNP under the dominant genetic model. However, the summary OR was higher in our meta-analysis ($OR_{random} = 1.54$, 95% CI = 1.03–2.31, $P = 0.033$) than in the paper by Su *et al.* (2011). Comparison with the results presented by Cao *et al.* (2014) is discussed below. Regardless of similar results, previous meta-analyses have several shortcomings regarding their design.

First, in both of the previous meta-analyses the inclusion and exclusion criteria for study selection were not based on any guideline for the evaluation of IRSA and the condition was defined as two or more SAs. In the meta-analyses by Su *et al.* (2011) the criteria for proven causes of RSA were not chosen appropriately and the process of study selection was

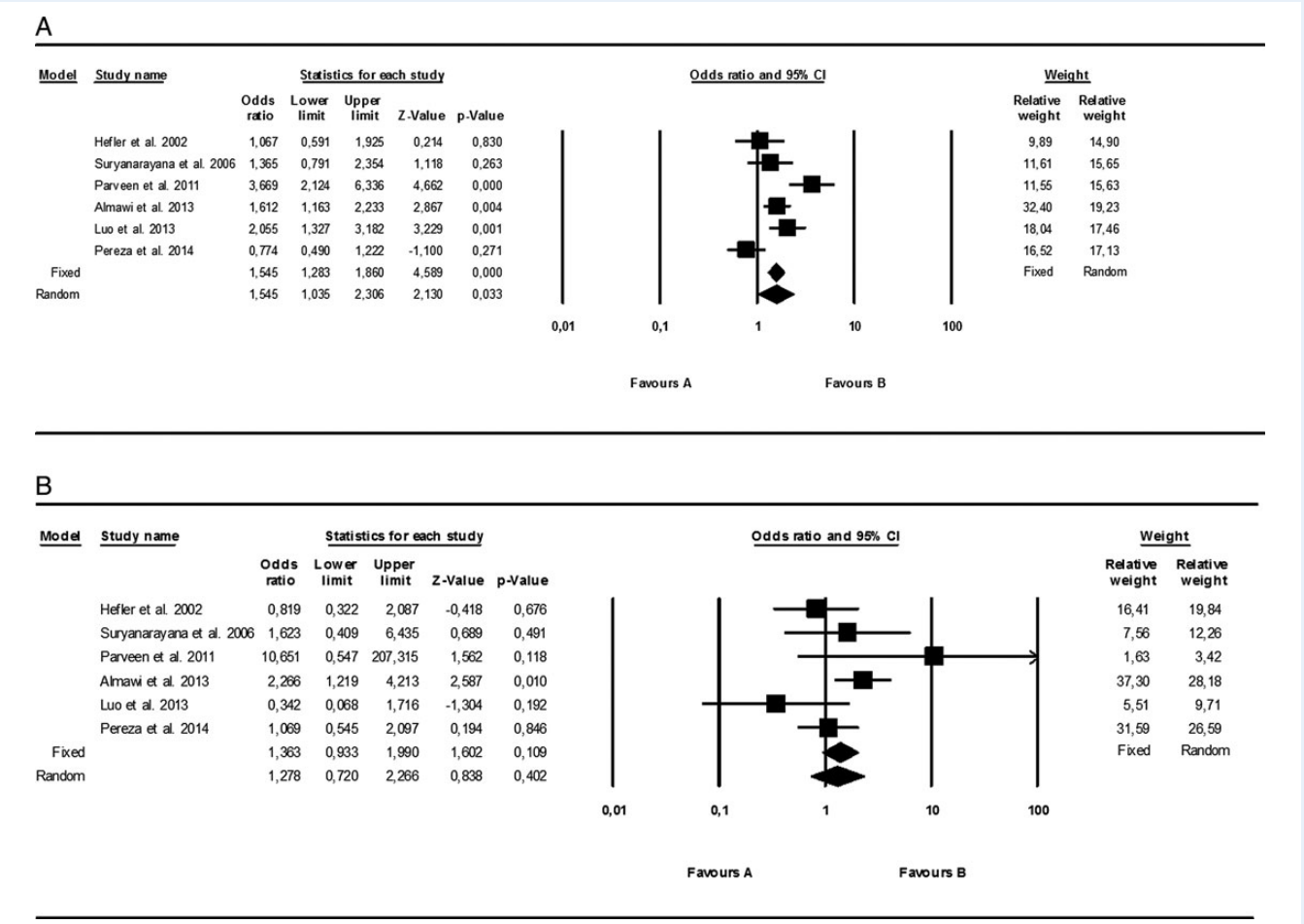

Figure 3 Association of +894 G/T single nucleotide polymorphism and IRSA under dominant (**A**) and recessive (**B**) genetic models. Results of individual and summary ORs estimates with 95% CIs of each study are shown. Horizontal lines represent 95% CIs and vertical lines the value of the summary OR.

not conducted in accordance with those criteria. The inclusion criteria for IRSA patients in the meta-analysis by Cao *et al.* (2014) are not detailed. In both papers, meta-analyses were performed for all case–control studies that were published up to a selected date.

Second, the quality of included studies was assessed using three (Cao *et al.*, 2014) or five (Su *et al.*, 2011) criteria, additionally graded as adequate, inadequate, not described or not stated. However, we noticed a contradiction in grading between criteria. For example, both papers state that the studies by Al Sallout and Sharif (2010) and Shin *et al.* (2010) had adequately described patients, but that patient selection criteria were not described in either study.

Third, the calculation of HWE is one of the major quality control checks in meta-analyses of genetic association studies. A deviation in the control group may bias the estimates of effects and the study should be excluded (Munafò and Flint, 2004). Unlike previous meta-analyses, we calculated HWE for all studies and excluded one study (Oztürk *et al.*, 2011) from further analysis.

Fourth, the reliability of the meta-analysis performed by Cao *et al.* (2014) is arguable for several other reasons. The total number of subjects included in meta-analyses presented in tables, results and abstract are different, and data for the study by Gao *et al.* (2009) differed from the original paper. The study by Oztürk *et al.* (2011) was not included in the meta-analysis of the +894 G/T SNP. Furthermore, the flowchart is unelaborated, not all studies included in meta-analyses were cited, and the case–control study by Gao *et al.* (2009) is cited as the source for the selection criteria.

Finally, we re-calculated the summary ORs for both gene variations in both meta-analyses. While we obtained identical results to those of Su *et al.* (2011), our observations differed from those of Cao *et al.* (2014). These discrepancies could be due to differences in statistical programs; however, this is unlikely given that Comprehensive Meta-Analysis and MIX software, used by Cao *et al.* (2014), provide identical numerical results (Bax *et al.*, 2007).

Limitations and strengths of the present study

One of the limitations of this meta-analysis is the among-study heterogeneity that was detected for the +894 G/T SNP under the dominant genetic model. Although it was not possible to recognize the source of heterogeneity considering the strict inclusion criteria, it may be the consequence of deviations from HWE in the group of patients in the studies by Almawi *et al.* (2013) and Luo *et al.* (2013), or population differences.

Additionally, given the small number of studies included in this meta-analysis, subgroup analysis by ethnicity was not performed.

Nevertheless, this study has several strengths. We tested the association of VNTR in intron 4 and +894 G/T SNP with IRSA in Slovenian women, thereby broadening the knowledge on the role of *NOS3* gene variations in an additional population. Also, the search for studies on the association between *NOS3* gene variations and IRSA was comprehensive and systematic. Attempts were made to avoid limitations in the original papers by applying strict criteria for study selection, which were based on ESHRE evidence-based guidelines. In this way, we increased the homogeneity of the study population, minimizing the possibility of selection bias.

Conclusions

The evidence presented in this paper indicates that the *NOS3* VNTR in intron 4 is not associated with IRSA, whereas the GT and TT genotypes of the +894 G/T SNP might be factors of predisposition to IRSA. Similar to the increasing number of critical reviews on IRSA, we detected significant differences in the inclusion and exclusion criteria for patients and controls between studies (Rull *et al.*, 2012; van den Boogard *et al.*, 2013; Christiansen, 2014). Additional genetic association studies in different populations, larger number of participants and a uniformly defined IRSA are needed to clarify the contribution of the *NOS3* +894 G/T SNP to IRSA. Future studies should also focus on the functional significance of +894 G/T SNP in IRSA.

Authors' roles

N.P.: design of the study, coordination, genotyping, systematic review, statistical analysis, manuscript writing (draft and revision); B.P.: sample collection, patient's clinical evaluation, critical discussion, revision; M.V.: sample collection and patient's clinical evaluation; M.K.: critical discussion, revision; S.O.: coordination, design of the study, systematic review, revision.

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Conflict of interest

None declared.

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