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ARTICLE

Insertion/deletion polymorphism in intron 16 of ACE gene in idiopathic recurrent spontaneous abortion: case-control study, systematic review and meta-analysis

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Abstract The insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin I-converting enzyme gene (*ACE*) has been extensively studied as a predisposing factor for idiopathic recurrent spontaneous abortion (IRSA). A case-control study including 149 women with ≥3 spontaneous abortions and 149 controls was performed to test the association of *ACE* I/D polymorphism with IRSA. A systematic review was conducted of previous case-control studies, with strict selection criteria for meta-analyses. We also aimed to evaluate the potential differences in summary estimates between studies defining IRSA as ≥2 and ≥3 spontaneous abortions. Genotyping was performed by PCR, and systematic review conducted using PubMed and Scopus. There was no association of the polymorphism with IRSA in Slovenian women. Sixteen case-control studies, showing substantial differences regarding IRSA definition and selection criteria for women were identified. Meta-analysis was performed and included four studies defining IRSA as ≥2 spontaneous abortions and the current study, which defined IRSA as ≥3 spontaneous abortions. Based on random effects model, meta-analysis conducted on 1192 patients and 736 controls showed no association with IRSA under dominant$_{(DD+IDvsII)}$ and recessive$_{(DDvsID+II)}$ genetic models. Well-designed studies are needed to evaluate the role of *ACE* I/D polymorphism in IRSA defined as ≥3 spontaneous abortions.

KEYWORDS: angiotensin I-converting enzyme, evidence-based medicine, genetic variability, insertion/deletion polymorphism, pregnancy, recurrent spontaneous abortion

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Introduction

The renin-angiotensin system (RAS) is an important regulator of human pregnancy, during which the maternal and fetal circulating RAS interact with various tissue RAS, including those of the ovaries and fetoplacental unit (Irani and Xia, 2011; Lumbers and Pringle, 2014). At the systemic level, the RAS is involved in the regulation of blood pressure and volume, haemostasis and homeostasis of water and electrolytes, whereas at the fetoplacental unit it influences implantation, placentation, angiogenesis and uteroplacental blood flow (Irani and Xia, 2011; Lumbers and Pringle, 2014; Nielsen et al., 2000). Therefore, abnormalities in the RAS have been suggested as a potential contributing mechanism to different pregnancy complications, including idiopathic recurrent spontaneous abortion (IRSA).

According to the evidence-based guidelines for the investigation and treatment of RSA, the condition is characterized by three or more (≥3) consecutive spontaneous abortions (Jauniaux et al., 2006), although many scientific groups include couples with two or more (≥2) spontaneous abortions in their research. The aetiology of RSA cannot be determined in approximately 60% of cases. Considering that the expression of genes encoding RAS components, including renin, angiotensinogen (AGT), angiotensin I-converting enzyme (ACE), AGT II type 1 and 2 receptors (AGT1R, AGT2R), is in part influenced by gene variations (Jeunemaitre, 2008), a large number of studies investigated their association with IRSA. The insertion/deletion (I/D) of a 287 bp sequence in intron 16 of the *ACE* gene is the most commonly tested variation; however, there is no conclusive evidence on its role in the pathogenesis of IRSA.

Angiotensin I-converting enzyme is a zinc metallopeptidase that converts the inactive angiotensin I to active angiotensin II (ANG II), and in addition cleaves bradykinin and several other peptides (Masuyer et al., 2014). Although the *ACE* I/D polymorphism in intron 16 is associated with alterations in circulating and tissue concentrations of ACE, evidence suggests that it is not a functional polymorphism, but is linked with other, yet unknown, intragenic functional variations (Sayed-Tabatabaei et al., 2006). Nevertheless, the DD genotype is accompanied by increased serum ACE and plasminogen activator inhibitor-1 (PAI-1) concentrations compared with the II genotype (Rigat et al., 1990; Tiret et al., 1992).

According to the first genetic-association study, the assumption for a possible association between the *ACE* I/D polymorphism in intron 16 and IRSA was based on the well-known roles of ANG II in the control of vascular tone and fibrinolysis, which, if disturbed, might lead to abnormal development of placental vasculature and disturbances of haemostasis (Fatini et al., 2000). Afterwards, a series of studies in different populations continued the search for a potential association, but results did not confirm one. A qualitative and quantitative synthesis of results was performed in three studies; however, we observed that the process of study selection was not performed in accordance with the proposed eligibility criteria (Su et al., 2013; Wang et al., 2013; Yang et al., 2012). Therefore, we performed a case-control study to test the association of *ACE* I/D polymorphism in intron 16 with IRSA in Slovenian women and conducted a thorough systematic review of previous case-control studies. Finally, based on precisely chosen inclusion criteria for meta-analyses, we aimed to evaluate whether there is a difference in summary estimates between studies in which IRSA is defined as ≥2 and ≥3 spontaneous abortions.

Materials and methods

Case-control study

Subjects

In order to test the genetic association between *ACE* I/D polymorphism in intron 16 and IRSA, a case-control study in Slovenian women was conducted. One hundred and forty-nine women with a history of ≥3 consecutive spontaneous abortions of unknown aetiology before the 22nd week of gestation, with the same partner, and 149 control women were included in the study. Exclusion criteria for IRSA women were: endocrine disorders, antiphospholipid syndrome (APS), autoimmune or systemic diseases, venous or arterial thrombosis, uterine anatomical abnormalities detected by ultrasonography and/or hysteroscopy, as well as chromosome abnormalities in either partner. A total of 98 (65.8%) women had no live births (primary IRSA) and 51 (34.2%) had at least one live born child (secondary IRSA). Couples with IRSA were described in more detail elsewhere (Pereza et al., 2012). The control group consisted of unrelated, healthy women with at least two live births, and no history of spontaneous abortion or any other pregnancy complication. All women were recruited through the Clinical Institute of Medical Genetics (UMC Ljubljana, Slovenia) and gave written informed consent for participation in the study. The study was approved by Slovenian (152/07/09; 2 September 2009) and Croatian National Ethics' Committees (641–01/07–01/19; 28 February 2007).

DNA extraction and molecular analysis

Genomic DNA was extracted from peripheral blood leucocytes using a commercially available kit (Qiagen_FlexiGene kit; QIAGEN GmbH, Hilden Germany). Genotyping was performed by allele-specific PCR as described previously (Rigat et al., 1992). All PCR were carried out in thermal cyclers (Mastercycler personal, Eppendorf, Hamburg, Germany and 2720 Thermal Cycler, Applied Biosystems, Carlsbad, CA, USA). The PCR products were visualized under ultraviolet light after electrophoresis on 2% agarose gels stained with GelRed™ (Olerup SSP®, Saltsjöbaden, Sweden).

Systematic review and meta-analysis

Search strategy

The systematic review was carried out using PubMed and Scopus electronic databases, which were searched for publications in the English language on the association between *ACE* I/D polymorphism in intron 16 and IRSA up to 1 January 2015. The following keywords were used: "recurrent miscarriage", "recurrent pregnancy loss", "recurrent spontaneous abortion" + "angiotensin converting enzyme", "ACE" + "polymorphism", "mutation". In addition, we searched references of retrieved articles. The literature search was performed by two authors independently and retrieved pub-

lications were compared to avoid duplication. Any disagreements were discussed and resolved with consensus.

Study selection

The systematic review included all of the retrieved case-control studies analysing the association of *ACE* I/D polymorphism in intron 16 with IRSA, and written in the English language. The criteria for inclusion in meta-analysis were: (i) case-control study in which genotyping was performed in IRSA women and control women; (ii) RSA defined as ≥2 or ≥3 spontaneous abortions; (iii) exclusion of known causes of IRSA according to the evidence-based guidelines for the investigation and medical treatment of RSA provided by the European Society of Human Reproduction and Embryology (ESHRE; 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 spontaneous abortions; (v) genotyping performed by PCR or sequencing; (vi) genotype frequencies reported; and (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 the following data were extracted: authors, year of publication, population and number of patients and controls, RSA definition, exclusion criteria for IRSA women, inclusion criteria for control women, methods used for genotyping, genotype and allele frequencies. HWE for genotype frequencies in patient and control groups for each study were also calculated.

Statistical analysis

Case-control study

Statistical analyses were carried out using Statistica for Windows, version 10 (StatSoft, Inc., Tulsa, OK, USA) and MedCalc for Windows, version 14.12.0 (MedCalc Software, Mariakerke, Belgium). In addition, statistical power was calculated using DSS Researcher's Toolkit (https://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx), and deviations from HWE were tested using the Simple Hardy-Weinberg Calculator–Court Lab (Washington State University College of Veterinary Medicine, Pullman, WA, USA). Pearson's chi-squared (χ^2) test was used to test for differences in genotype and allele frequencies between study groups. Association of genotypes and alleles with IRSA was estimated by odds ratios (OR) and 95% confidence intervals (CI) under dominant, recessive and co-dominant genetic models. *P*-values <0.05 were considered statistically significant.

Meta-analysis

Meta-analysis was performed using Comprehensive Meta-Analysis, version 2.2.064 (Biostat, Inc., Englewood, NJ, USA). Individual and summary OR and associated 95% CI were calculated under dominant and recessive genetic models using random effects model. Cochran's Q test was used to assess heterogeneity, whereas funnel plot and Egger's regression test were used to evaluate publication bias. Sensitivity analysis was performed by removing one study at a time to evaluate the relative influence of each study on the pooled estimate. *P*-values <0.05 were considered statistically significant.

Results

Case-control study

The power of the present study was 100% to detect a twofold increase in *ACE* I allele frequency. No statistically significant differences were found in the distribution of genotype and allele frequencies between IRSA and control women or between women with primary and secondary IRSA (**Table 1**). Genotype frequencies were in HWE in all groups. Nor did the study determine statistically significant associations of *ACE* I/D polymorphism in intron 16 with IRSA under any genetic model (**Table 2**).

Systematic review

The PRISMA flow diagram including the details for the study searching is shown in **Figure 1**. Sixteen case-control studies were identified on the association between *ACE* I/D polymorphism in intron 16 and IRSA (**Table 3**). All articles were written in the English language. Molecular analysis was appropriate in all studies. Twelve studies defined IRSA as ≥2 spontaneous abortions (Aarabi et al., 2011; Buchholz et al., 2003; Bukreeva et al., 2009; Corbo et al., 2011; Dossenbach-Glaninger et al., 2008; Goodman et al., 2009; Kim et al., 2014; Ozdemir et al., 2012; Poursadegh Zonouzi et al.,

Table 1 Genotype and allele frequencies of *ACE* I/D polymorphism in intron 16 in IRSA women, control women and women with primary and secondary IRSA.

	Genotype frequencies/n *(%)*					*Allele frequencies*/n *(%)*		
	DD	*ID*	*II*	χ^2; P	*HWE*/χ^2; P	*D*	*I*	χ^2; P
IRSA	43 (28.9)	75 (50.3)	31 (20.8)	2.72; 0.257	0.03;0.871	161 (54.0)	137 (46.0)	0.68; 0.409
Control	55 (36.9)	62 (41.6)	32 (21.5)		3.24;0.072	172 (57.7)	126 (42.3)	
Primary IRSA	31 (31.6)	48 (49.0)	19 (19.4)	1.14; 0.564	0.00;0.957	110 (56.1)	86 (43.9)	0.78; 0.377
Secondary IRSA	12 (23.5)	27 (52.9)	12 (23.5)		0.18;0.674	51 (50.0)	51 (50.0)	

HWE, Hardy-Weinberg equilibrium; IRSA, idiopathic recurrent spontaneous abortion; χ^2, chi-squared test.

Table 2 Association of *ACE* I/D polymorphism in intron 16 with IRSA under different genetic models.

Genetic model		W_{IRSA} *versus* W_C		$W_{Primary\ IRSA}$ *versus* $W_{Secondary\ IRSA}$	
		OR (95% CI)	P	*OR (95% CI)*	P
Dominant:	DD + ID vs. II	1.04 (0.60–1.81)	0.887	1.28 (0.56–2.90)	0.555
Recessive:	DD vs. ID + II	0.69 (0.43–1.13)	0.140	1.50 (0.69–3.26)	0.302
Co-dominant:	DD vs. II	0.81 (0.43–1.52)	0.508	1.63 (0.61–4.36)	0.329
	DD vs. ID	0.65 (0.38–1.09)	0.101	1.45 (0.64–3.29)	0.370
	II vs. ID	0.80 (0.44–1.45)	0.466	0.89 (0.37–2.11)	0.792
Alleles:	D vs. I	0.86 (0.62–1.19)	0.364	1.28 (0.79–2.07)	0.315

CI, confidence interval; IRSA, idiopathic recurrent spontaneous abortion; OR, odds ratio; W_{IRSA}, IRSA women; W_C, control women; $W_{Primary/Secondary\ IRSA}$, women with primary or secondary IRSA.

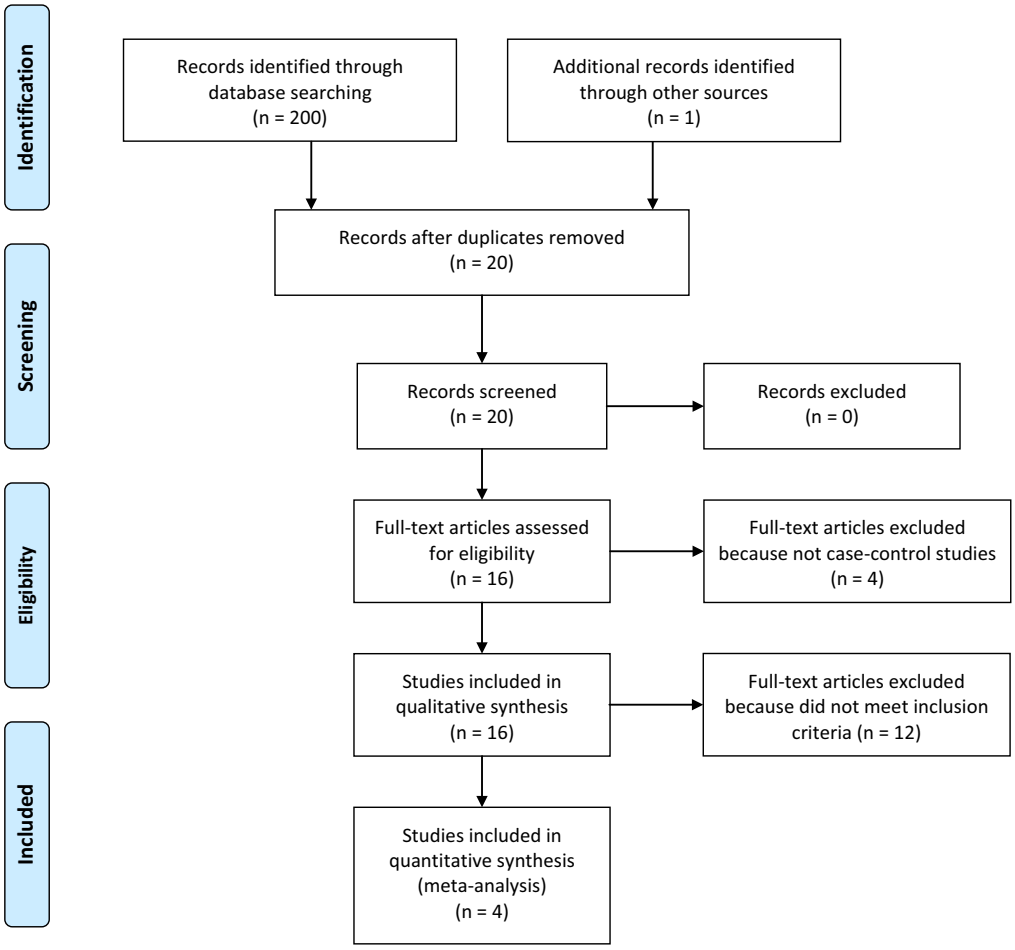

Figure 1 PRISMA flow diagram showing the process of literature searching and selection criteria for inclusion in the systematic review and meta-analysis.

2013; Vettriselvi et al., 2008; Yenicesu et al., 2010; Zhang et al., 2011), and the remaining four defined it as ≥3 spontaneous abortions (Al Sallout and Sharif, 2010; Bagheri et al., 2010; Choi et al., 2011; Fatini et al., 2000).

In the group of studies defining IRSA as ≥2 spontaneous abortions, inclusion criteria for control groups and IRSA women according to ESHRE criteria (Jauniaux et al., 2006) were appropriately defined in six studies (Buchholz et al., 2003; Kim et al., 2014; Ozdemir et al., 2012; Poursadegh Zonouzi et al., 2013; Vettriselvi et al., 2008; Yenicesu et al., 2010); however, genotype frequencies significantly deviate from HWE in the study by Vettriselvi et al. (2008), and it was not possible to calculate genotype and allele frequencies from the manner in which they were presented in the original paper by Yenicesu et al. (2010). The other six studies either had no patient selection criteria described (Bukreeva et al., 2009; Corbo et al.,

Table 3 Characteristics of IRSA and control women included in case-control studies on the association between *ACE* I/D polymorphism in intron 16 and IRSA.

Authors	*Population*	*Number of patients and controls*	*RSA definition*	*Exclusion criteria in IRSA women*				*Inclusion criteria for control women*	
				APS	*CA*	*UA*	*Other*	*SA*	*Number of children*
Buchholz *et al.*, 2003	German	P: 184 W C: 127 W	≥2 consecutive SA before 25 WOG	+	+	+	endocrine disorders, protein C, S and antithrombin III deficiencies	0	≥1
Dossenbach-Glaninger *et al.*, 2008	Austrian	P: 49 W C: 48 W	2 consecutive or 3–6 non-consecutive SA between 8 and 12 WOG	ND	+/−[a]	+	endocrine and autoimmune disorders, infections, inflammatory pelvic disease, arterial hypertension, pregnancy complications or assisted reproduction, liver abnormalities	0	≥1
Vettriselvi *et al.*, 2008	Indian	P: 104 W C: 120 W	≥2 consecutive SA before 20 WOG, with the same partner[b]	+	+	+	endocrine and autoimmune disorders, infections, pregnancy complications	0	≥2
Bukreeva *et al.*, 2009	German	P: 177 W C1: 527 W C2: 553 W	≥2 SA in first trimester	ND	ND	ND		C1: VTE, UPD, ATD C2: without SA, VTE, UPD, ATD	
Goodman *et al.*, 2009	American	P: 120 W C: 84 W	≥2 consecutive SA	+	+	+	autoimmune disorders	≤1	≥2
Yenicesu *et al.*, 2010	Turkish	P: 272 W, 152 M C: 56 Co	≥2 consecutive SA between 5 and 12 WOG, with the same partner[b]	+	+	+	endocrine and autoimmune disorders, urogenital infections	0	≥2
Aarabi *et al.*, 2011	Iranian	P: 63 W C: 114 W	≥2 consecutive SA before 25 WOG	ND	ND	+	endocrine and autoimmune disorders, urogenital infections, inflammatory pelvic disease	0	ND
Corbo *et al.*, 2011	Italian	P: 18 W[c] C: 47 W	≥2 SA	ND	ND	ND		ND	ND
Zhang *et al.*, 2011	Chinese	P: 127 W C:132 W	≥2 consecutive SA in early pregnancy[b]	+	+	+	endocrine and autoimmune disorders, infections, hereditary thrombophilia	ND	ND[d]
Ozdemir *et al.*, 2012	Turkish	P: 543 W, 327 M C: 106 Co	≥2 consecutive SA between 5 and 12 WOG, with the same partner[b]	+	+	+	endocrine and autoimmune disorders, urogenital infections	0	≥1
Poursadegh Zonouzi *et al.*, 2013	Iranian	P: 89 W C: 50 W	≥2 consecutive SA	+	+	+	endocrine disorders, urogenital infections	0	≥2
Kim *et al.*, 2014.	Korean	P: 227 W C: 304 W	≥2 consecutive SA before 20 WOG	+	+	+	endocrine and autoimmune disorders	0	≥2
Fatini *et al.*, 2000	Italian	P: 59 W C: 70 W	≥3 SA between 7 and 12 WOG	+	-	-	endocrine disorders, arterial or venous thromboembolism, pregnancy complications, hypertension	ND	ND
Al Sallout and Sharif, 2010	Palestinian	P: 100 W C: 100 W	≥3 consecutive SA before 25 WOG	+	ND	ND	infections	0	≥1
Bagheri *et al.*, 2010	Iranian	P: 50 W C: 63 W	≥3 SA before 20 WOG, with the same partner	+	+	+	endocrine and immune disorders, infections, protein C, S and antithrombin III deficiencies	ND	≥2
Choi *et al.*, 2011	South Korean	P: 251 W C: 126 W	≥3 consecutive SA before 20 WOG[e]	ND	+	+	endocrine and autoimmune disorders, infections, arterial or venous thromboembolism, family history of IRSA	0	≥1
Present study	Slovenian	P: 149 W C: 149 W	≥3 SA before 22 WOG, with the same partner	+	+	+	endocrine and autoimmune disorders, arterial or venous thrombosis	0	≥2

APS, antiphospholipid syndrome; ATD, atherothrombotic disease; C, controls; CA, chromosome abnormalities; Co, couples; IRSA, idiopathic recurrent spontaneous abortion; M, men; ND, not described; P, patients; SA, spontaneous abortion; UA, uterine anomalies; UPD, uteroplacental dysfunction; VTE, venous thromboembolism; W, women; WOG, weeks of gestation.

[a]Available for 50% of women.

[b]Primary IRSA.

[c]Authors indicate that all participants were enrolled without selection criteria.

[d]Multipara women were included, but the minimum number of children is not indicated.

[e]225 women with primary and 26 women with secondary IRSA.

2011), excluded the known causes of IRSA partially (Aarabi et al., 2011; Dossenbach-Glaninger et al., 2008) or did not define the inclusion criteria for the control group properly (Goodman et al., 2009; Zhang et al., 2011). In the study by Aarabi et al. (2011), genotype data were available for 94, not 114 control women as indicated in the paper. In three out of twelve studies, a statistically significantly higher frequency of the D allele was found in IRSA women compared with controls (**Table 4**) ($P = 0.006$ in Corbo et al., 2011; $P < 0.038$ in Zhang et al., 2011; $P < 0.001$ in Ozdemir et al., 2012).

In the second group of studies, in which IRSA was defined as ≥3 spontaneous abortions, none of the four studies selected both the IRSA and the control group appropriately: the mandatory diagnostic evaluations in IRSA women were partially performed in three studies (Al Sallout and Sharif, 2010; Choi et al., 2011; Fatini et al., 2000), and the inclusion criteria for the control group were unsuitable in the studies by Bagheri et al. (2010) and Fatini et al. (2000). The only reliable genotype data for women with ≥3 spontaneous abortions is mentioned in the study by Kim et al. (2014), who defined IRSA as ≥2 spontaneous abortions but presented the results for women with ≥3 spontaneous abortions separately. Statistically significantly higher frequency of the D allele was determined in IRSA women compared with controls in two studies ($P < 0.001$ in Zhang et al., 2011; $P = 0.019$ in Fatini et al., 2000), whereas the I allele was more frequent in IRSA women in one study ($P = 0.025$ in Choi et al., 2011) (**Table 4**).

Meta-analysis

The inclusion criteria for meta-analysis were met in four studies in which IRSA was defined as ≥2 spontaneous abortions (Buchholz et al., 2003; Kim et al., 2014; Ozdemir et al., 2012; Poursadegh Zonouzi et al., 2013). Considering that women with ≥3 spontaneous abortions also satisfy the definition of ≥2 spontaneous abortions, our study was included in this meta-analysis, which was then performed on a total of five studies. Quantitative synthesis was performed just for this group of studies because only two studies in which IRSA was defined as ≥3 spontaneous abortions fulfilled the aforementioned criteria (present study; Kim et al., 2014).

The four studies in which IRSA was defined as ≥2 spontaneous abortions and the present study in which IRSA was defined as ≥ 3 spontaneous abortions included 1192 patients and 736 controls (**Tables 3 and 4**). Based on random effects model, the association of *ACE* I/D polymorphism in intron 16 with IRSA was not statistically significant under the dominant (DD + ID versus II) or recessive genetic models (DD versus ID + II) (**Figures 2 and 3**). High among-study heterogeneity was observed under both dominant (Cochran's Q test: $\chi^2 = 21.79$; df = 4; $P = 0.000$; Higgins statistics: $I^2 = 81.65\%$) and recessive genetic models (Cochran's Q test: $\chi^2 = 19.12$; df = 4; $P = 0.001$; Higgins statistics: $I^2 = 79.08\%$). No publication bias was detected in either genetic model (dominant: $t = 0.40$; df = 3; $P = 0.718$; recessive: $t = 0.66$; df = 3; $P = 0.555$).

Discussion

In the present study an extensive examination of the role of *ACE* I/D polymorphism in intron 16 in IRSA women was performed through a case-control study, systematic review and meta-analysis.

The results of the case-control study suggest that *ACE* I/D polymorphism in intron 16 is not associated with IRSA in Slovenian women. Although it was not possible to include this study in a meta-analysis of studies defining IRSA as ≥3 spontaneous abortions, the results are comparable with those obtained in the only study in which women with ≥3 spontaneous abortions and control women were selected appropriately (Kim et al., 2014). Although the distribution of genotype frequencies between these studies is slightly different, this could probably be attributed to population differences.

The second aim of this study was to perform a search of the available scientific literature on the association of *ACE* I/D polymorphism in intron 16 with IRSA. We recently pointed to significant inconsistencies between studies regarding the selection criteria for patients and controls, as well as the fact that only a minority of studies comply with the available guidelines for IRSA provided by different professional societies (Pereza et al., 2015). Unfortunately, the results of such inadequately designed studies, including meta-analyses, may be misleading, creating unfounded assumptions on associations with IRSA. The present systematic review, in which 16 case-control studies were identified that investigated the *ACE* I/D polymorphism in intron 16 in IRSA women, confirms our previous conclusions on discrepancies between studies. The most notable differences observed concern IRSA definition, designation and exclusion of known causes of IRSA, and selection criteria for control women. We would like to emphasize that the studies in which the criteria for the selection of IRSA patients were not described (Bukreeva et al., 2009; Corbo et al., 2011), or known causes of IRSA were only partially eliminated (Aarabi et al., 2011; Al Sallout and Sharif, 2010; Choi et al., 2011; Dossenbach-Glaninger et al., 2008) were included in previously conducted meta-analyses (Su et al., 2013; Wang et al., 2013; Yang et al., 2012). In addition, the current systematic review shows that studies in which RSA is defined as ≥2 spontaneous abortions constitute the majority of case-control studies on the association between *ACE* I/D polymorphism in intron 16 and IRSA (12/16 or 75%). Although the reasons for choosing the less strict definition of RSA are beyond the scope of this article, we recommend that research groups that define IRSA as ≥2 spontaneous abortions present results for women/couples with ≥3 spontaneous abortions separately.

The third aim of this study was to perform the quantitative synthesis of results of studies with properly conducted diagnostic evaluations defined by ESHRE in IRSA women (couples) and a well-defined control group. We particularly wanted to evaluate whether there is a difference between summary estimates regarding the studies in which RSA is defined as ≥2 and ≥3 spontaneous abortions. Unfortunately, it was not possible to perform the comparison due to deficient selection criteria applied in original studies with the more rigorous definition. Consequently, a meta-analysis was performed that included four studies in which IRSA was defined as ≥2 spontaneous abortions and the current study, which defined IRSA as ≥3 spontaneous abortions, yielding negative results. When comparing this meta-analysis to those previously conducted, two major differences were observed (Su et al., 2013; Wang et al., 2013; Yang et al., 2012). The first difference is in the summary odds ratios. In contrast to our

Table 4 Genotype and allele frequencies obtained in case-control studies on the association between *ACE* I/D polymorphism in intron 16 and IRSA.

Authors	*Genotype frequencies [n (%)] II / ID / DD*	χ^2; P	*HWE; P-value*	*Allele frequencies [n (%)] I / D*	χ^2; P-value
Buchholz *et al.*, 2003	W_{IRSA}: 42 (22.8) / 83 (45.1) / 59 (32.1) W_C: 26 (20.5) / 71 (55.9) / 30 (23.6)	3.83; 0.147	0.222; 0.179	W_{IRSA}: 167 (45.4) / 201 (54.6) W_C: 123 (48.4) / 131 (51.6)	0.44; 0.505
Dossenbach-Glaninger *et al.*, 2008	W_{IRSA}[a]: 12 (24.5) / 20 (40.8) / 17 (34.7) W_C: 12 (25.0) / 29 (60.4) / 7 (14.6)	5.81; 0.055	0.220; 0.125	W_{IRSA}: 44 (44.9) / 54 (55.1) W_C: 53 (55.2) / 43 (44.8)	1.67; 0.196
Vettriselvi *et al.*, 2008	W_{IRSA}: 42 (40) / 39 (38) / 23 (22) W_C: 55 (46) / 38 (31) / 27 (23)	0.94; 0.630	0.022; < 0.001	W_{IRSA}: 123 (59.1) / 85 (40.9) W_C: 148 (61.7) / 92 (38.3)	0.20; 0.653
Bukreeva *et al.*, 2009	W_{IRSA}[b]: - W_{C1}: 136 (25.8) / 241 (45.7) / 150 (28.5) W_{C2}: 140 (25.3) / 254 (45.9) / 159 (28.8)	–	0.052; 0.059	W_{IRSA}[b]: – W_{C1}: 513 (48.7) / 541 (51.3) W_{C2}: 534 (48.3) / 572 (51.7)	–
Goodman *et al.*, 2009	W_{IRSA}: 31 (25.8) / 55 (45.8) / 34 (28.3) W_C: 22 (26.2) / 34 (40.5) / 28 (33.3)	0.73; 0.693	0.365; 0.088	W_{IRSA}: 117 (48.8) / 123 (51.2) W_C: 78 (46.4) / 90 (53.6)	0.13; 0.718
Yenicesu *et al.*, 2010[c]					
Aarabi *et al.*, 2011	W_{IRSA}[d]: 14 (22.2) / 30 (47.6) / 19 (30.2) W_C: 22 (23.4) / 47 (50.0) / 25 (26.6)	0.24; 0.888	0.741; 0.992	W_{IRSA}[d]: 58 (46.0) / 68 (54.0) W_C: 91 (48.4) / 97 (51.6)	0.09; 0.766
Corbo *et al.*, 2011	W_{IRSA}[e]: 0 (0) / 6 (33.0) / 12 (67.0) W_C[f]: 11 (23.0) / 20 (43.0) / 16 (34.0)	7.70; 0.021	0.396; 0.340	W_{IRSA}[e]: 6 (16.7) / 30 (83.3) W_C[f]: 42 (44.7) / 52 (55.3)	7.61; 0.006
Zhang *et al.*, 2011	W_{IRSA1}[e]: 49 (57.0) / 26 (30.2) / 11 (12.8) W_{IRSA2}[d]: 8 (19.5) / 23 (56.1) / 10 (24.4) W_C: 90 (68.2) / 34 (25.8) / 8 (6.0)	1[g]: 4.11; 0.128 2[h]: 31.92; < 0.001	0.021; 0.425; 0.064	W_{IRSA1}: 124 (72.1) /48 (27.9) W_{IRSA2}: 39 (47.6) / 43 (52.4) W_C: 214 (81.1) / 50 (18.9)	1[g]: 4.31; 0.038 2[h]: 34.04; < 0.001
Ozdemir *et al.*, 2012	W_{IRSA}: 71 (13.1) / 260 (47.8) / 212 (39.0) W_C: 33 (31.9) / 54 (50.0) / 19 (18.1)	29.33; < 0.001	0.531; 0.703	W_{IRSA}: 402 (37.0) / 684 (63.0) W_C: 120 (56.6) / 92 (43.4)	27.50; < 0.001
Poursadegh Zonouzi et al., 2013	W_{IRSA}: 23 (25.9) / 31 (34.8) / 35 (39.3) W_C: 7 (14.0) / 28 (56.0) / 15 (30.0)	6.23; 0.044	0.006; 0.291	W_{IRSA}: 77 (43.2) / 101 (56.8) W_C: 42 (42.0) / 58 (58.0)	0.01; 0.938
Kim *et al.*, 2014	W_{IRSA1}[e]: 83 (36.6) / 110 (48.5) / 34 (15.0) W_{IRSA2}[d]: 51 (35.7) / 71 (49.7) / 21 (14.7) W_C: 104 (34.2) / 148 (48.7) / 52 (17.1)	1[g]: 0.57; 0.752 2[h]: 0.43; 0.808	0.803; 0.643; 0.958	W_{IRSA1}[e]: 276 (60.8) / 178 (39.2) W_{IRSA2}[d]: 173 (60.5) / 113 (39.5) W_C: 356 (58.6) / 252 (41.4)	1[g]: 0.45; 0.501 2[h]: 0.23; 0.634
Fatini *et al.*, 2000	W_{IRSA}: 10 (16.9) / 21 (35.6) / 28 (47.5) W_C: 20 (28.6) / 30 (42.8) / 20 (28.6)	5.36; 0.069	0.098; 0.232	W_{IRSA}: 41 (35.0) / 77 (65.0) W_C: 70 (50.0) / 70 (50.0)	5.47; 0.019
Al Sallout and Sharif, 2010	W_{IRSA}: 9 (9.0) / 42 (42.0) / 49 (49.0) W_C: 12 (12.0) / 34 (34.0) / 54 (54.0)	1.51; 0.469	1.000; 0.081	W_{IRSA}: 60 (30.0) / 140 (70.0) W_C: 58 (29.0) / 142 (71.0)	0.01; 0.913
Bagheri *et al.*, 2010	W_{IRSA}: 7 (14.0) / 26 (52.0) / 17 (34.0) W_C: 12 (19.0) / 27 (42.9) / 24 (38.1)	1.05; 0.592	0.556; 0.380	W_{IRSA}: 40 (40.0) / 60 (60) W_C: 51 (40.5) / 75 (59.5)	0.00; 0.949
Choi *et al.*, 2011	W_{IRSA}: 77 (30.7) / 130 (51.8) / 44 (17.5) W_C: 35 (27.8) / 50 (39.7) / 41 (32.5)	11.20; 0.004	0.391; 0.022	W_{IRSA}: 284 (56.6) / 218 (43.4) W_C: 120 (47.6) / 132 (52.4)	5.05; 0.025

HWE, Hardy-Weinberg equilibrium; χ^2, chi-square test; W_{IRSA}, IRSA women; W_C, control women.
[a]Data taken from Su et al. (2013).
[b]Genotype and allele frequencies were not shown separately for IRSA women.
[c]Numbers for genotype and allele frequencies were not shown.
[d]Women with ≥3 spontaneous abortions.
[e]Women with ≥2 spontaneous abortions.
[f]Women without spontaneous abortions.
[g]W_{IRSA1} versus W_c.
[h]W_{IRSA2} versus W_c.

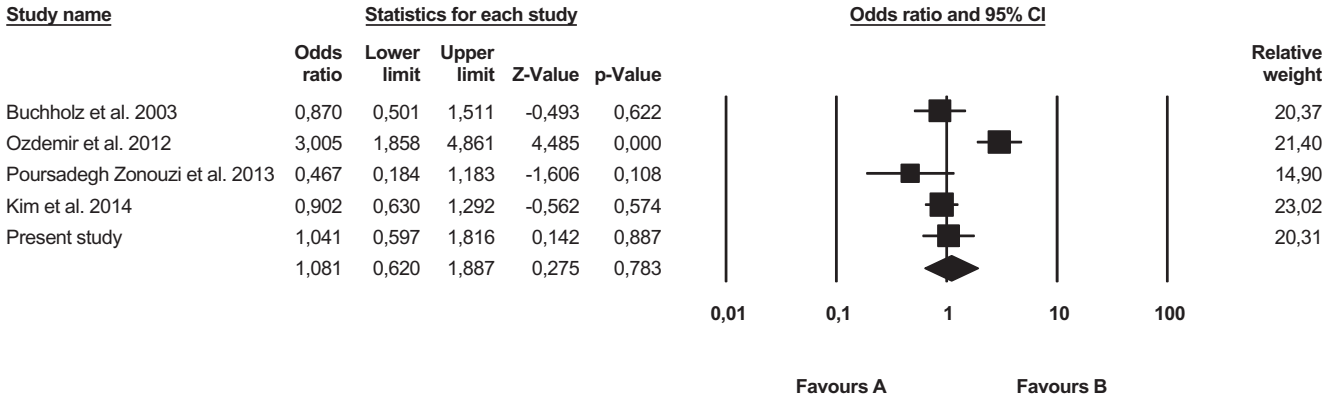

Figure 2 Forest plot for the meta-analysis of *ACE* I/D polymorphism in intron 16 under dominant genetic model (DD+ID versus II) using random effects model.

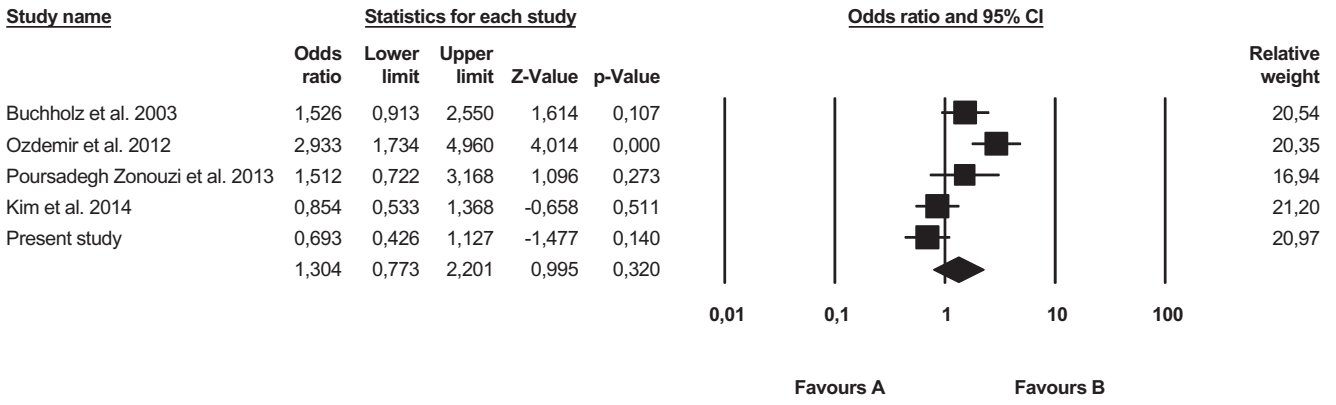

Figure 3 Forest plot for the meta-analysis of *ACE* I/D polymorphism in intron 16 under recessive genetic model (DD versus II+ID) using random effects model.

results, all three published meta-analyses detected a statistically significant association of *ACE* I/D polymorphism in intron 16 with IRSA defined as ≥2 spontaneous abortions, according to the dominant model (DD + ID versus II) using random effects model. However, the association was lost when women with ≥3 spontaneous abortions were analysed separately, which is contrary to expectations and difficult to explain because it would be logical for the association to grow stronger as the number of spontaneous abortions increases. The second difference concerns the inclusion criteria applied for the process of studies selection for meta-analysis. For instance, Wang et al. (2013) and Su et al. (2013) used the same criteria, in which protein C/S and antithrombin-III deficiencies are indicated as proven causes of IRSA, which has not been confirmed (Jauniaux et al., 2006). Also, in none of the three meta-analyses was the selection of studies performed in accordance with the proposed criteria. Only as an example, the protein C/S and antithrombin-III deficiencies were excluded in just two original case-control studies. Therefore, although meta-analyses are an important part of evidence-based medicine, the eligibility criteria should be thoroughly evaluated before accepting the conclusions. Although no association was found between *ACE* I/D polymorphism in intron 16 and women with ≥2 spontaneous abortions (including our study with the more rigorous definition of ≥3 spontaneous abortions), the result is based only on five studies, all of which were conducted in different populations. Thus, well-designed case-control studies are needed to evaluate the role of this variation in IRSA, especially IRSA defined as ≥3 spontaneous abortions.

The *ACE* I/D polymorphism in intron 16 has been investigated in a large number of studies as a factor of predisposition to various complex diseases, such as Alzheimer's disease, coronary heart disease and pre-eclampsia (Petrovic and Peterlin, 2004; Staines-Urias et al., 2012). The D allele and the DD genotype have emerged as potential risk factors for some of these diseases. However, as a consequence of contradictory results, strong evidence of such associations is still missing (Sayed-Tabatabaei et al., 2006). The

pathophysiologic consequences of this polymorphism in IRSA are almost completely unknown. The effect of different genotypes on plasma or local levels of ACE, other RAS components or peptides related to the activity of ACE or ANG II have not been sufficiently evaluated in IRSA women. Only one such study was conducted in which the association between *ACE* DD genotype and PAI-1 was tested, but the results were negative (Fatini et al., 2000). Accordingly, in addition to genetic-association studies, functional studies are needed to explain the role of *ACE* I/D polymorphism in intron 16 in IRSA on the systemic and local, uteroplacental levels. Until then we may only speculate about its pathophysiologic significance in IRSA, which could include alterations in ACE plasma or local levels, as well as affects on any functions that ACE and downstream peptides exert in normal pregnancy, such as regulation of trophoblast invasion, angiogenesis, fetoplacental circulation, haemostasis and synthesis of different enzymes.

The limitations of this meta-analysis are the small number of studies included in the quantitative synthesis, as well as the among-study heterogeneity that was detected under both dominant and recessive genetic models. For that reason, the meta-analyses was conducted using the random effects model. The heterogeneity may be the consequence of population differences, i.e. different distribution of genotype frequencies. On the other hand, we performed a case-control study with rigorous inclusion criteria for IRSA and control groups. The systematic review was comprehensive and selection criteria for inclusion in meta-analysis strict and based on ESHRE evidence-based guidelines for the diagnosis of IRSA, allowing studies with well-chosen participants to be selected.

In conclusion, we found no association of *ACE* I/D polymorphism in intron 16 with IRSA in Slovenian women. In the systematic review, 16 case-control studies were identified, showing notable differences regarding IRSA definition, as well as the selection criteria for IRSA and control women. Finally, the results of meta-analyses conducted on five well-designed studies did not provide evidence for an association between *ACE* I/D polymorphism in intron 16 and women with IRSA ≥2 spontaneous abortions.

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