ASSOCIATION OF *PRMT6, PEX10* AND *SOX5* GENETIC VARIANTS WITH IDIOPATHIC MALE INFERTILITY: EVIDENCE FROM NORTH MACEDONIAN POPULATION AND AN UPDATED META-ANALYSIS

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PRMT6, *PEX10* and *SOX5* genetic variants were identified as male infertility-associated loci in a genome-wide association study and further validated in various populations. Still, the results of previous case-control studies varied, which could be due to differences in participants' ethnic backgrounds. The main purpose of the present study was to evaluate the supposed association of these variants with idiopathic male infertility in

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North Macedonian population. Furthermore, we aimed to conduct the systematic quantitative data synthesis which includes the results of previous studies on the same issue in other European and non-European populations. A total of 137 men from North Macedonia diagnosed with idiopathic infertility and 130 age-matched fertile controls were included in the present case-control study. PCR-RFLP method was used for genotyping. Meta-analysis was performed by OpenMeta-analyst statistical software. Variants rs10842262 in *SOX5*, rs2477686 in *PEX10* and rs12097821 in *PRMT6* showed the lack of statistically significant differences in genotype distributions between men diagnosed with idiopathic infertility and the control group. Still, rs10842262 allele G frequency was significantly increased in men with poor sperm concentration ($P= 0.024$, OR = 2.10, 95%CI 1.08–4.06). Meta-analysis further showed the association of rs10842262 and rs12097821 with the risk of idiopathic male infertility. Our results obtained in North Macedonian population supported the previous reports on the involvement of rs10842262 in the genetic basis of male infertility. The meta-analysis confirmed the association of rs10842262 and rs12097821 with male infertility occurrence. Still, additional studies are needed to support the present findings.

Keywords: male infertility, rs10842262, rs12097821, rs2477686, meta-analysis

INTRODUCTION

According to recent statistics on reproductive issues, the male factor was identified as a cause or a contributory factor in about 50% of cases of infertility (AGARWAL *et al*., 2021). Therefore, the etiology of male infertility has become one of the major research fields in reproductive medicine, considering the implications of findings related to causes of male reproductive dysfunction for potential therapeutic approaches. Still, to a large percent, the main factors contributing to male infertility remain unknown, defining these cases as idiopathic (KOTHANDARAMAN *et al*., 2016; AGARWAL *et al*., 2021). Since genetic factors were related to the impairment in testicular function, various studies focused on the genetic basis of male infertility. Beside rare chromosomal abnormalities and structural variations recognized as causative factors, various genetic loci with low to moderate penetrability were identified as risk variants contributing to the susceptibility to male reproductive disorders (MYAMOTO *et al*., 2015; XAVIER *et al*., 2021).

The genome-wide approach resulted in the identification of risk variants for non-obstructive azoospermia (NOA) within genomic loci 1p13.3, 1p36.32, and 12p12.1 (HU *et al*., 2011). This initial genome-wide association study (GWAS) conducted in Chinese population demonstrated the statistically significant risk of NOA associated with genetic variant rs12097821 located upstream of Protein Arginine Methyltransferase 6 gene (*PRMT6*). Yet, the effect size of this genetic variant was relatively modest. Similar results were found for rs2477686 located near Peroxisomal Biogenesis Factor 10 gene (*PEX10*), as well as for rs10842262, an intronic variant of SRY-related HMG-box gene 5 (*SOX5*) (HU *et al*., 2011). All of the mentioned genes located within the risk-associated regions were experimentally implicated in testicular development and spermatogenesis, suggesting the potential functional mechanisms of action of NOA-risk variants through effects on the regulation of expression and/or function of near-by genes (CHEN *et al*.,

2010; LUO *et al*., 2015; DAIGLE *et al*., 2015; ZHANG *et al*., 2017; CHEN *et al*., 2021; ZHENG *et al*., 2021).

After the initial GWAS, several studies aimed to validate the results on the effects of rs12097821, rs2477686 and rs10842262 on NOA susceptibility, as well as to test the association of these genetic variants with other types of idiopathic male infertility (SATO *et al*., 2013; ZOU *et al*., 2014; TU *et al*., 2015; LIU *et al*., 2017; GU *et al*., 2019; VUČIĆ *et al*., 2020; CERVÁN-MARTÍN *et al*., 2021). However, the results of these replicative studies are conflicting, suggesting the potential influence of confounding factors, such as ethnicity. Also, some of the differences could reflect the differences in the effects related to type of infertility, defined according to the results of semen analysis. To date, only one study conducted in Serbia analyzed the effect of rs12097821, rs2477686 and rs10842262 on idiopathic male infertility in Slavic populations (VUČIĆ *et al*., 2020). In the present case-control study, we intended to validate the effects of these genetic variants in an independent cohort of infertile men from North Macedonia, presuming the similarities in the genetic background. Furthermore, in order to elucidate the potential contribution of rs12097821, rs2477686 and rs1084226 to the occurrence of male infertility, as well as to resolve the effects of ethnic background and/or specific abnormality of semen parameters, we conducted the updated meta-analysis.

MATERIALS AND METHODS

Association study

The study group comprised 267 participants from North Macedonian population who provided peripheral blood samples, as well as the written informed consent for their inclusion in the study. The case group included 137 men diagnosed with idiopathic infertility in 2018 at Acibadem Sistina Hospital, Skopje, North Macedonia. Control group consisted of age-matched healthy volunteers who fathered at least one child. Exclusion criteria for the case group were diagnosis of orchitis, obstruction of vas deferens, chromosomal abnormalities, as well as microdeletions of the AZF region within Y chromosome. Semen analysis in infertile men was conducted according to WHO guidelines (WORLD HEALTH ORGANIZATION, 2021) and patients were stratified into subgroups based on the abnormalities in semen concentration, sperm motility or morphology. Patients' basic data on age, serum testosterone levels and semen parameters are given in Table 1.

Table 1. Clinical characteristics of infertile study participants. Results are presented as mean±SD

Clinical characteristic	NOA	Other types of infertility
N	12	125
Age (years)	36.0±5.8	39.9±8.7
Testosterone (ng/ml)	5.70±3.08	4.9±1.23
Sperm parameters		
Ejaculate volume (ml)	3.96±2.18	3.00±1.56
Total sperm number (10^6)	-	219.4±167.7
Sperm density (10^6/ml)	-	74.7±52.8
Progressive motility (%)	-	33.1±13.9
Rapid progressive motility (%)	-	17.5±8.4
Average progression grade (0-4)	-	2.58±0.78
Vitality (%)	-	47.4±17.7

The research was conducted with the approval of Ethics Committee of this medical institution (08-5110/01, May 2nd 2018, Acibadem Sistina Hospital, Skopje, North Macedonia) and in accordance with the Helsinki declaration of 1975.

Commercial DNA extraction kit (Blood DNA Isolation Mini Kit, Norgen Biotek Corp., Canada) was used for the extraction of genomic DNA from peripheral blood samples following the manufacturer's instructions. Genotyping was performed by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method, as previously described in VUČIĆ *et al*., (2020). Namely, regions surrounding rs12097821, rs2477686, and rs10842262 were amplified using the same primers set as in previous study (VUČIĆ *et al*., 2020), in reaction mixtures of 15 μL which included forward and reverse primer (0.2 μM each, Thermo Fisher Scientific, Waltham, MA, USA), 200 μM of each deoxyribonucleoside triphosphate (Fermentas, Hanover, MD, USA), 1.5 μL of 10× PCR buffer A (containing 15 mM $MgCl_2$, Kapa Biosystems, Woburn, MA, USA), 0.04 U/μL of Taq DNA polymerase (Kapa Biosystems), nuclease-free water (Serva, Westbury, NY, USA) and 10-20 ng of genomic DNA. The following conditions were used for PCR amplification: initial denaturation at 97ºC for 3 min; 35 cycles consisting of denaturation at 95ºC for 1 min, primer annealing at 60ºC for 1 min and extension at 72ºC for 1 min; final extension at 72ºC for 10 min. Amplified fragments were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide (EtBr) staining. 10 μL of PCR products were digested overnight with 1 U of adequate restriction enzyme (VUČIĆ *et al*., 2020) in a 15 μL reaction mixture at optimal temperature recommended by the manufacturer (Fermentas, Hanover, MD, USA). Digested products were separated by electrophoresis on 3% agarose gel stained with EtBr. For rs12097821, the expected lengths of fragments resulting from restriction digest with AluI were 100 bp, 78 bp and 39 bp for TT genotype, 178 bp, 100 bp, 78 bp and 39 bp for TG genotype and 178 bp and 39 bp for GG genotype. The expected lengths of fragments resulting from restriction digest of amplified region surrounding rs2477686 with FspBI were 173 bp and 73 bp for CC genotype, 246 bp, 173 bp and 73 bp for CG genotype and 246 bp for GG genotype. For rs10842262, restriction digest with FspBI resulted in 138 bp and 87 bp fragments for GG genotype, 225 bp, 138 bp and 87 bp fragments for GC genotype and 225 bp long fragment for CC genotype.

Deviations of genotype distributions from Hardy-Weinberg equilibrium (HWE) were assessed using the exact test implemented in SNPStats software (Institut Català d'Oncologia, Barcelona, Spain) (SOLÉ *et al*., 2006). The same software was used to test the associations of rs12097821, rs2477686, and rs10842262 with idiopathic infertility by applying logistic regression method with the adjustment for age differences. Odds ratios (ORs) with corresponding confidence intervals (CIs) were used as effect size measures. All the tests were two-sided and *P* values <0.05 were considered to be statistically significant. Five different genetic models were tested (log-additive, codominant, dominant, recessive and overdominant) and the best-fitting models were determined by using Akaike information criterion (AIC).

Meta-analysis

Potentially relevant articles for the meta-analysis were identified by searching the literature database PubMed using the search strategy based on the combination of keywords: gene/protein name or polymorphism identifier ("PRMT6" or "PEX10" or "SOX5" or

"rs12097821" or "rs2477686" or "rs10842262"); term "polymorphism" or related terms ("variant", "allele", "genotype"); term "infertility" or "sterility" or related terms referring to different types of male infertility ("azoospermia", "oligospermia", "oligozoospermia", "asthenospermia", "asthenozoospermia", "teratospermia", "teratozoospermia", "oligoasthenozoospermia", "asthenoteratozoospermia", "oligoteratozoospermia"). During the database search, language restriction was not applied. Reference lists of the retrieved original articles and previous reviews were thoroughly examined in order not to miss additional potentially relevant studies. The search was limited to publication date before 2022 (database inception to December 31st 2021).

The eligibility of the retrieved studies was assessed based on the predetermined criteria: a) study analysed the association of rs12097821, rs2477686 or rs10842262 with idiopathic male infertility; b) case-control study design; c) sufficient data provided on allele/genotype counts for the calculations of effect sizes; d) detailed information provided about the study design, recruitment of participants, selection criteria for controls, diagnostic procedure, ethnicity of participants, genotyping and statistical methodology, as well as other relevant methodological data. Among the predetermined exclusion criteria were the retraction of articles, poor quality of study design and major errors in presented results. The results of studies with several datasets were segregated into multiple entries for the quantitative data synthesis. The data considered relevant for the extraction from eligible studies included: first author's name with the publication year, country and the ethnicity of participants, recruitment method, number of participants, diagnostic procedure and genotyping methods, allele/genotype counts/frequencies.

Heterogeneity tests and quantitative data synthesis were performed using statistical software OpenMeta-analyst (The Center for Evidence-based Medicine, Brown University, Providence, RI, USA) and MetaGenyo (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research, (GENYO), Granada, Spain) (WALLACE *et al*., 2021; MARTORELL-MARUGAN *et al*., 2017). Effect sizes were presented as ORs and their 95% CIs. Cochran's Q test and the inconsistency index (I^2) were applied for between-study heterogeneity assessment (*P* value <0.1 or $I^2 \geq 50\%$ corresponded to significant heterogeneity). Random-effects statistical model was used for pooling results by the method of DERSIMONIAN and LAIRD (1986) when heterogeneity was statistically significant. Otherwise, Mantel-Haenszel method of weighting was used for meta-analysis under the fixed-effect model (MANTEL and HAENSZEL, 1959). Quantitative data synthesis was performed for several genetic models (allelic, dominant, recessive and overdominant). Ethnicity of participants and the results of semen analysis were used as criteria for classification of studies in subgroup meta-analyses. When the number of entries to the pooled analysis was more than 5, the presence of potential publication bias was estimated by the visual inspection of funnel plots and by performing the Egger's test.

RESULTS AND DISCUSSION

Results - association study

Genotyping of the analyzed genetic variants rs10842262, rs2477686 and rs12097821 was successful for all the participants involved in the study. Genotype frequencies in the group of

137 infertile men, as well as in 130 fertile controls, are summarized in Table 2. The acquired genotype distributions in fertile control subjects were not shown to significantly deviate from HWE (P= 0.38, P=0.076 and P=0.69 for rs10842262, rs2477686 and rs12097821, respectively).

Table 2a. Association of genetic variants rs10842262, rs2477686 and rs12097821 with male infertility

SNP	Genetic model	No of controls (%)	No of cases (%)	cases *vs* controls		
				OR (95% CI) [a]	*P* value [a]	AIC
rs10842262						
	Codominant					
	CC	36 (27.7)	28 (20.4)	1.00		
	CG	70 (53.9)	84 (61.3)	1.54 (0.86-2.77)	0.35	373.8
	GG	24 (18.5)	25 (18.2)	1.34 (0.63-2.83)		
	Dominant					
	CC	36 (27.7)	28 (20.4)	1.00	0.16	372
	CG+GG	94 (72.3)	109 (79.6)	1.49 (0.85-2.62)		
	Recessive					
	CC+CG	106 (81.5)	112 (81.8)	1.00	0.96	374
	GG	24 (18.5)	25 (18.2)	0.99 (0.53-1.83)		
	Overdominant					
	CC+GG	60 (46.1)	53 (38.7)	1.00	0.22	372.4
	CG	70 (53.9)	84 (61.3)	1.36 (0.83-2.21)		
	Log-additive					
	-	-		1.18 (0.82-1.72)	0.37	373.2
rs2477686						
	Codominant					
	GG	45 (34.6)	56 (40.9)	1.00		
	GC	54 (41.5)	48 (35)	0.71 (0.41-1.24)	0.49	374.5
	CC	31 (23.9)	33 (24.1)	0.86 (0.46-1.60)		
	Dominant					
	GG	45 (34.6)	56 (40.9)	1.00	0.29	372.8
	GC+CC	85 (65.4)	81 (59.1)	0.77 (0.47-1.26)		
	Recessive					
	GG+GC	99 (76.2)	104 (75.9)	1.00	0.96	374
	CC	31 (23.9)	33 (24.1)	1.01 (0.58-1.78)		
	Overdominant					
	GG+CC	76 (58.5)	89 (65)	1.00	0.27	372.8
	GC	54 (41.5)	48 (35)	0.76 (0.46-1.25)		
	Log-additive					
	-	-	-	0.90 (0.66-1.23)	0.53	373.6

Table 2b. Association of genetic variants rs10842262, rs2477686 and rs12097821 with male infertility

SNP	Genetic model	No of controls (%)	No of cases (%)	cases *vs* controls		
				OR (95% CI) [a]	*P* value [a]	AIC
rs12097821						
	Codominant					
	GG	100 (76.9)	105 (76.6)	1.00		
	GT	29 (22.3)	30 (21.9)	0.99 (0.55-1.76)	0.86	375.7
	TT	1 (0.8)	2 (1.5)	1.90 (0.17-21.34)		
	Dominant					
	GG	100 (76.9)	105 (76.6)	1.00	0.96	374
	GT+TT	30 (23.1)	32 (23.4)	1.02 (0.58-1.79)		
	Recessive					
	GG+GT	129 (99.2)	135 (98.5)	1.00	0.59	373.7
	TT	1 (0.8)	2 (1.5)	1.91 (0.17-21.33)		
	Overdominant					
	GG+TT	101 (77.7)	107 (78.1)	1.00	0.94	374
	GT	29 (22.3)	30 (21.9)	0.98 (0.55-1.74)		
	Log-additive					
	-	-	-	1.05 (0.62-1.78)	0.86	373.9

[a] adjusted for age

Abbreviations: OR- odds ratio; CI- confidence interval; AIC- Akaike information criteria.

The results of association tests, presented in Table 2, suggested the lack of statistically significant differences in genotype distributions between men diagnosed with idiopathic infertility and the control group. For any genetic model tested, *P* values failed to reach statistical significance. Nevertheless, when infertile subjects were stratified according to the abnormalities in semen parameters, the frequency of rs10842262 allele G was significantly increased in the group of men with poor sperm concentration, compared to controls ($P_{log-additive} = 0.024$, OR = 2.10, 95%CI 1.08–4.06) (Table 2). Also, rs10842262 GG genotype frequency in subjects with oligospermia was found to be about twice as high compared to the one determined for controls. According to AIC score, the best-fitting model of association was log-additive. It is also the only genetic model for which the statistical significance was determined, since for codominant, dominant and recessive models only the statistical trend towards significance ($0.05 \leq P < 0.1$) was reached ($P_{codom}=0.079$, $P_{rec}=0.062$, $P_{dom}=0.077$) (Table 3). In contrast to these results, when the same comparison was conducted for rs2477686 and rs12097821, the results were statistically insignificant (results not shown).

When genotype distributions of rs10842262, rs2477686 and rs12097821 were compared between men with abnormal sperm motility and fertile controls, no statistically significant

differences were found. Similarly, the *P* values obtained in the comparisons of rs10842262, rs2477686 and rs12097821 genotype frequencies between patients with abnormal sperm morphology and the control group did not reach statistical significance (results not shown). Since there were just 12 patients diagnosed with azoospermia in our study group, we considered that it was not statistically justified to compare genotype frequencies between such a small group of infertile men and the control group.

Table 3. Comparison of rs10842262 genotype distribution between subjects with low sperm concentration and fertile controls

SNP	Genetic model	No of controls (%)	No of cases (%)	cases *vs* controls		
				OR (95% CI) [a]	*P* value [a]	AIC
rs10842262						
	Codominant					
	CC	36 (27.7)	3 (12)	1.00		
	CG	70 (53.9)	13 (52)	2.23 (0.60-8.33)	0.079[b]	137.9
	GG	24 (18.5)	9 (36)	**4.50 (1.10-18.34)***		
	Dominant					
	CC	36 (27.7)	3 (12)	1.00	0.077[b]	137.8
	CG+GG	94 (72.3)	22 (88)	2.81 (0.79-9.96)		
	Recessive					
	CC+CG	106 (81.5)	16 (64)	1.00	0.062[b]	137.5
	GG	24 (18.5)	9 (36)	2.48 (0.98-6.29)		
	Overdominant					
	CC+GG	60 (46.1)	12 (48)	1.00	0.87	140.9
	CG	70 (53.9)	13 (52)	0.93 (0.39-2.19)		
	Log-additive					
	-	-	-	**2.10 (1.08-4.06)**	**0.024**	135.9

[a] adjusted for age

[b] statistical trend of significance

* statistically significant results are shown in bold

Abbreviations: OR- odds ratio; CI- confidence interval; AIC- Akaike information criteria.

Results - meta-analysis

After conducting the initial database searching, removal of duplicate records, as well as the eligibility assessment, 8 studies were identified as relevant for the intended meta-analysis of

the association of rs10842262, rs2477686 and rs12097821 with idiopathic male infertility. Among these records was a study by HU *et al*. (2011), which we excluded from the further analysis in order not to introduce bias, since it was the initial GWAS that identified rs10842262, rs2477686 and rs12097821 as putative variants associated with male infertility. Three of the retrieved studies included more than one group of infertile subjects, based on which multiple entries for meta-analysis were generated (GU *et al*., 2019; VUČIĆ *et al*., 2020; CERVÁN-MARTÍN *et al*., 2021). Also, two entries for data synthesis were made based on the results of a study that included two stages of the analysis (SATO *et al*., 2013). In total, 12 entries were found for the meta-analysis of the effect of rs2477686 and rs12097821, while 11 entries were included in the data synthesis related to rs10842262.

The results of the association between rs10842262 and idiopathic male infertility are presented in Figure 1. The results obtained through data-synthesis suggest that the increased risk of infertility is associated with the minor allele G of this genetic variant. The statistical significance was found for both allelic and recessive genetic models ($P<0.001$ and $P=0.002$ for allelic and recessive models, respectively). Still, the effect size of G allele according to allelic model was relatively modest, since the value of OR was 1.120 (95% CI 1.049-1.196). In the data synthesis that assumed recessive genetic model, the acquired OR was 1.203 (95% CI 1.068-1.356). After stratification according to ethnicity of participants, rs10842262 remained significantly associated with male infertility in the group of Asian ancestry, assuming allelic and recessive genetic models ($P_{allelic}<0.001$, $OR_{allelic}=1.206$, 95%CI 1.111-1.310; $P_{rec}<0.001$, $OR_{rec}=1.344$, 95%CI 1.130-1.599) (results not shown). Also, in the group of men with Asian origin, statistical significance was found for association of this genetic variant with male infertility occurrence under dominant genetic model ($P<0.001$, OR=1.244, 95%CI 1.113-1.391). Furthermore, statistically significant association under allelic and recessive genetic models was determined for the subgroup of studies that included men with non-obstructive azoospermia (NOA) ($P_{allelic}<0.001$, $OR_{allelic}=1.155$, 95%CI 1.070-1.246; $P_{rec}<0.001$, $OR_{rec}=1.283$, 95%CI 1.113-1.478) (results not shown).

For the other tested genetic variant, rs2477686, the results of the data synthesis remained statistically insignificant for all the genetic models tested (Figure 2). Although the stratification of entries was conducted according to the ethnicity of study participants and the results of spermogram, the subgroup meta-analyses did not show statistical significance (data not shown).

The meta-analysis of the association between rs12097821 and idiopathic male infertility yielded statistical significance for allelic, dominant and recessive genetic models (Figure 3) $P_{allelic}=0.006$, $P_{dom}=0.020$ and $P_{rec}=0.026$). Still, ORs obtained for allelic and dominant models suggested only slight effect of rs12097821 allele T ($OR_{allelic}=1.113$, 95%CI 1.032-1.201; $OR_{dom}=1.116$, 95%CI 1.018-1.223). On the other hand, when assuming recessive genetic model, the OR obtained through data synthesis was 1.261 (95%CI 1.029-1.545) (Figure 3), rising to 1.311 (95%CI 1.056-1.627) in Asians (data not shown).

The results of Egger's test and the visual inspection of funnel plots constructed for all the pooled analyses in the present study did not demonstrate the presence of publication bias (results not shown).
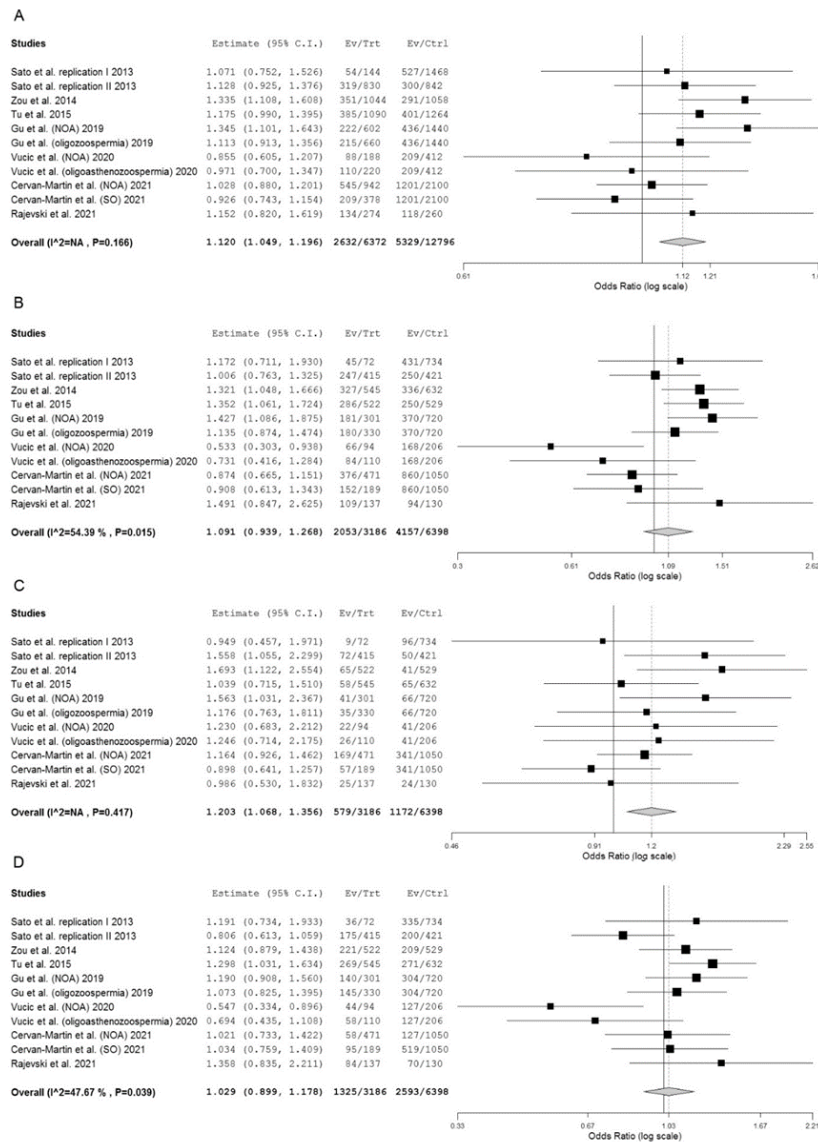
Figure 1. Meta-analysis of the association between rs10842262 and idiopathic male infertility. A) Allelic
model. B) Dominant model. C) Recessive model. D) Overdominant model. The results of each
study and the overall effect are presented as ORs with their corresponding 95% CIs in the forest
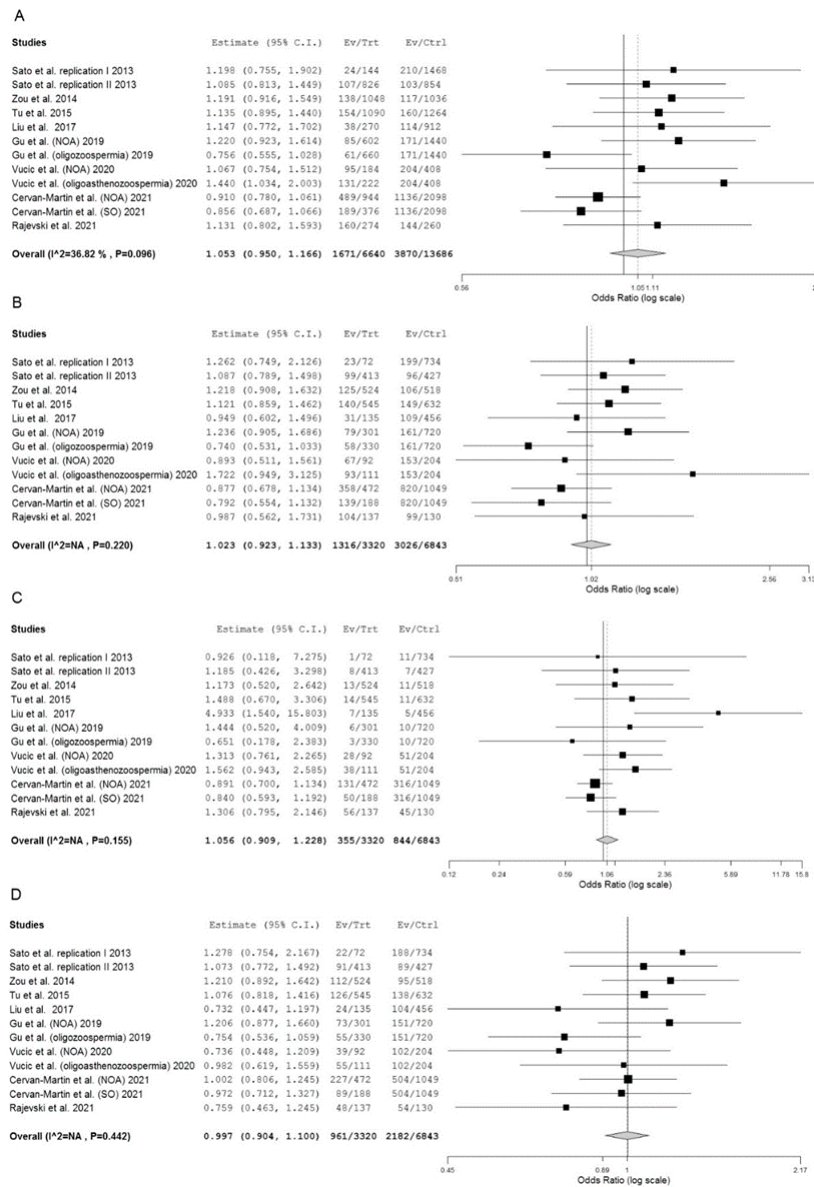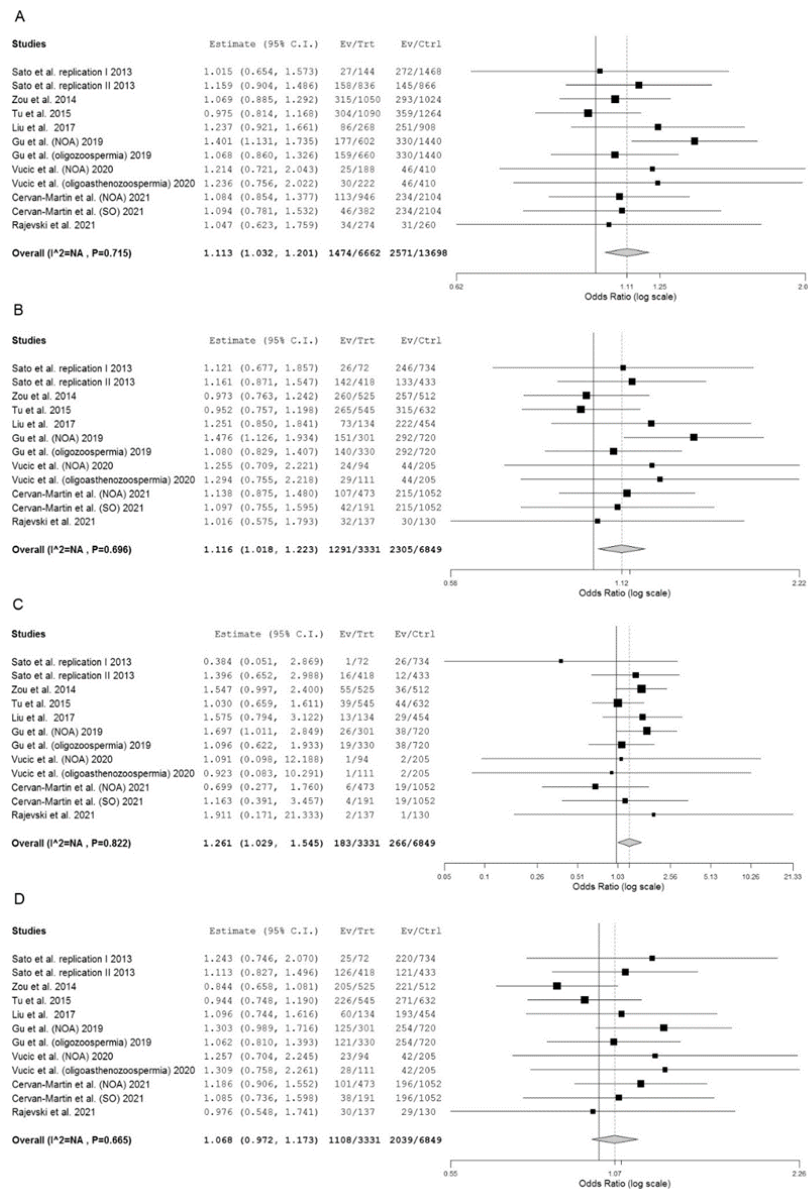plot. The presented *P* values are derived from heterogeneity tests

Figure 2. Meta-analysis of the association between rs2477686 and idiopathic male infertility. A) Allelic model. B) Dominant model. C) Recessive model. D) Overdominant model. The results of each study and the overall effect are presented as ORs with their corresponding 95% CIs in the forest plot. The presented *P* values are derived from heterogeneity tests

Figure 3. Meta-analysis of the association between rs12097821 and idiopathic male infertility. A) Allelic
model. B) Dominant model. C) Recessive model. D) Overdominant model. The results of each
study and the overall effect are presented as ORs with their corresponding 95% CIs in the forest
plot. The presented *P* values are derived from heterogeneity tests

DISCUSSION

The results obtained in the initial GWAS on NOA implicated three autosomal polymorphisms, rs12097821, rs2477686 and rs10842262, in the genetic basis of male infertility (HU *et al*., 2011). Several validation studies were further conducted in Asian and Caucasian population, providing conflicting results on the effects of these variants (SATO *et al*., 2013; ZOU *et al*., 2014; TU *et al*., 2015; LIU *et al*., 2017; GU *et al*., 2019; VUČIĆ *et al*., 2020; CERVÁN-MARTÍN *et al*., 2021). Not only did these studies differ in terms of the ethnic origin of participants, significant variations are also found in the selection of case group(s), based on the exact diagnosis established according to the abnormalities in semen parameters related to sperm concentration, morphology and motility. Among these studies, only one analyzed the association of rs12097821, rs2477686 and rs10842262 with male infertility in a Slavic population (VUČIĆ *et al*., 2020). Therefore, our aim was to test the supposed genetic associations in another independent cohort of participants with Slavic genetic background, as well as to provide additional results in the Caucasian subgroup of studies on this issue. Furthermore, the pooled analysis was conducted in order to generate the pooled risk estimate, as well as to test the hypothesized influence of ethnicity and/or specific type of infertility.

Results of the present case-control study, regarding the effect of rs12097821 on the susceptibility to male infertility, significantly differ from the findings of the initial GWAS from China (HU *et al*., 2011). However, the lack of statistical significance obtained in the tests corresponding to this genetic variant in the current study matches the findings of all previous replicative studies, including our recent study in Serbian population (SATO *et al*., 2013; ZOU *et al*., 2014; TU *et al*., 2015; LIU *et al*., 2017; GU *et al*., 2019; VUČIĆ *et al*., 2020; CERVÁN-MARTÍN *et al*., 2021). In a study of GU *et al*. (2019), rs12097821 was not found to associate with male infertility in general, or with oligozoospermia risk, while the association with susceptibility to NOA remained significant even after applying Bonferroni correction. Therefore, among the reasons for the observed discordance in our results and the ones obtained by HU *et al*. (2011), could be the NOA-limited effect of rs12097821, which was not tested in the present study, due to a small percentage of infertile men diagnosed with NOA. Still, most of the previous studies in Asians tested the relation of rs12097821 with NOA, without considering the effects on other types of male infertility, and still obtained the results differing from those presented by HU *et al*. (2011). Taking into account the ethnic backgrounds, the results of the present study are in accordance with previous findings in Caucasians, which included results referring to different types of male infertility and also to multiple genetic models (VUČIĆ *et al*., 2020; CERVÁN-MARTÍN *et al*., 2021).

Similarly as for rs12097821, the present study demonstrated the lack of evidence for the association of rs2477686 with the susceptibility to idiopathic male infertility. The results remained insignificant even after the stratification of infertile men according to the type of infertility. Therefore, findings of the present case-control study which refer to rs2477686 are discordant with the results obtained in Serbian population for oligozoospemic men, suggesting the potential effect of the differences in genetic backgrounds, or the limitation of the present analysis to detect the effect of rs2477686 due to the relatively small number of infertile participants with low sperm count in semen analysis. The present results also differed from the findings of LIU *et al*. (2017), which showed the association of rs2477686 with

oligoasthenozoospermia, as well as with parameters of semen quality. Still, the number of patients diagnosed with oligoasthenozoospermia in their study was 22 (LIU *et al*., 2017). A considerably larger number of patients with the same diagnosis was included in the study of GU *et al*. (2019), which showed results matching to ours. The results on the effect of this genetic variant on NOA risk are also conflicting, since other studies in Asians (SATO *et al*., 2013; ZOU *et al*., 2014; TU *et al*., 2015; GU *et al*., 2019) failed to confirm the initial findings of HU *et al*. (2011). Our results could not be compared to the previously published, since we did not compare the distribution of rs2477686 genotypes between infertile men with NOA and fertile controls.

The only genetic variant displaying the statistically significant association with male infertility in the current case-control study is rs10842262. Even though we did not detect the association of this polymorphism with idiopathic male infertility in general, the increased risk of oligozoospermia associated with allele G. These results differed from our previous findings in Serbian population (VUČIĆ *et al*., 2020). Still, in the present study in North Macedonian men, genotype distributions were in accordance with HWE, while in VUČIĆ *et al*. (2020) statistically significant deviations were detected. The association of rs10842262 with oligozoospermia was also assessed in the Han Chinese (GU *et al*., 2019), but the results remained insignificant, suggesting the potential impact of ethnic differences. Another study involving Caucasian participants evaluated the effect of rs10842262 on spermatogenic disorders (CERVÁN-MARTÍN *et al*., 2021), showing the lack of evidence for the association with the evaluated phenotypes. Still, our present results could not be compared to theirs, since the study groups were not matching, due to the recruitment of men with a specific severe subtype of oligozoospermia by CERVÁN-MARTÍN *et al*. (2021).

As for the results of the present meta-analysis, minor allele T of rs12097821 was found to associate with the increased overall risk of male infertility. The obtained ORs for allelic, dominant and recessive genetic model are very similar to those acquired by VUČIĆ *et al*. (2020) and previously by TU *et al*. (2015). Still, TU *et al*. (2015) tested only allelic genetic model and included results of the initial GWAS, while in VUČIĆ *et al*. (2020), statistical significance was lost after the exclusion of the mentioned GWAS. These discordances were explained by the large overall effect of the GWAS, so we aimed to assess the association through meta-analysis with the exclusion of the results of HU *et al*. (2011), same as in VUČIĆ *et al*. (2020). Since we found similarities in effect sizes with the results from VUČIĆ *et al*. (2020), the differencies in statistical significance between the present meta-analysis and this previous one could be attributed to the increase in statistical power after the inclusion of additional studies. Therefore, the present results of data-synthesis suport the involvement of rs12097821 in the genetic basis of idiopathic male infertility.

When it comes to rs2477686, the results of the present quantitative data synthesis are discordant with the previous findings of TU *et al*. (2015) and VUČIĆ *et al*. (2020). In contrast to previous results, we found no evidence to support the association of this genetic variant with the risk of male infertility occurance. Still, in TU *et al*. (2015), the results of data synthesis refer only to the association of rs2477686 with NOA, which could attribute the observed differences due to possible infertility type-specific effects, apart from the inclusion/exclusion of the results of initial GWAS. On the other hand, this previous meta-analysis included only participants of Asian descent, which raises the question about the potential influence of ethnicity on the results of data

synthesis. As for the differences between the present results and our previous findings published in VUČIĆ *et al*. (2020), the incorporation of the results of a large study in Caucasians could be among the reasons for losing statistical significance, together with the inclusion of additional results on the association of rs2477686 with oligospermia. Namely, in VUČIĆ *et al*. (2020), ethnicity-based subgroup meta-analysis showed differences in the effects of this genetic variant in Asians and Caucasians, as well as in NOA and oligospermia/oligoasthenozoospermia. Therefore, making conclusion about the involvement of rs2477686 in the genetic basis of male infertility requires additional studies and classifications of results according to ethnicity and the type of male idiopathic infertility.

The present meta-analysis replicated the results of previous data syntheses on the effects of rs10842262 on susceptibility to male infertility. As in VUČIĆ *et al*. (2020), statistical significance was reached for both allelic and recessive genetic models. Furthermore, for these genetic models, results remained statistically significant in Asians, as well is in NOA subgroup, which matched previous findings. Since the obtained results are also in accordance with earlier findings of TU *et al*. (2015), the robustnes of the data synthesis suggest that rs10842262 is the most promising candidate for potential biomarker of idiopathic male infertility among the tested genetic variants.

The main limitation of the present study is a relatively small number of participants. Also, the number of infertile men selected into subgroups according to the the abnormalities in semen parameters was small. For that reason, the results obtained in tests which involved these small subgroups of participants should be taken with caution. Still, both our findings from association study and from the data synthesis further support the involvement of rs10842262 in the molecular basis of idiopathic male infertility. The discordances between the results of the present study and the previous ones could be attributed to the differences in study designs, characteristics of participants, such as genetic origin and the specific diagnosis, as well as in study power to detect significant associations. In order to make the results of quantitative data synthesis more informative, further studies are needed with larger sample sizes, as well as with clinically well characterized participants bearing different types of semen abnormalities and with different ethnic backgrounds.

CONCLUSIONS

The present genetic association study showed the lack of statistically significant differences in rs10842262, rs2477686 and rs12097821 genotype distributions between men diagnosed with idiopathic infertility and the control group. Nevertheless, rs10842262 allele G frequency was significantly increased in men with poor sperm concentration and the meta-analysis further supported the involvement of rs10842262 in the genetic basis of male infertility. Even though the association of rs10842262 and rs12097821 with the risk of idiopathic male infertility was demonstrated through meta-analysis, rs10842262 was highlighted as the most promising candidate for potential male infertility biomarker among the tested variants. Still, additional studies are needed to support our findings, as well as to make clearer conclusion about the effects of tested variants while taking into account the ethnic backgrounds, specific characteristics of spermogram results and other potential confounders.

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ASOCIJACIJA VARIJANTI U GENIMA *PRMT6, PEX10* I *SOX5* SA IDIOPATSKIM MUŠKIM STERILITETOM: DOKAZI IZ POPULACIJE SEVERNE MAKEDONIJE I AŽURIRANA META-ANALIZA

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Izvod

Asocijacija varijanti u genima *PRMT6*, *PEX10* i *SOX5* sa muškim sterilitetom identifikovana je u studiji genetičke asocijacije na čitavom genomu i kasnije analizirana u studijama slučajeva i kontrola u različitim populacijama. Rezultati prethodnih studija su pokazali značajnu varijabilnost, što može biti posledica razlika u etničkom poreklu studijskih grupa. Osnovni cilj ovog istraživanja je analiza asocijacije navedenih genetičkih varijanti sa rizikom za pojavu idiopatskog muškog steriliteta u populaciji Severne Makedonije. Takođe, naš cilj je bio i sprovođenje sistematske kvantitativne sinteze podataka iz studija sa istom ili sličnom temom istraživanja sprovedenim u drugim evropskim i neevropskim populacijama. Ukupno 137 muškaraca sa idiopatskim sterilitetom iz Severne Makedonije i 130 fertilnih kontrola slične starosti uključeno je u studiju slučajeva i kontrola. Genotipizacaija je vršena PCR-RFLP metodom, dok je za meta-analizu korišćen statistički softver OpenMeta-analyst. Za varijante rs10842262 u *SOX5*, rs2477686 u *PEX10* i rs12097821 u *PRMT6* nije utvrđena statistički značajna razlika u distribucijama genotipova između grupe ispitanika sa idiopatskim sterilitetom i kontrolne grupe. Međutim, učestalost alela G varijante rs10842262 bila je značajno povećana kod muškaraca sa niskom koncentracijom spermatozoida ($P= 0.024$, OR = 2.10, 95%CI 1.08–4.06). Meta-analizom pokazana je asocijacija rs10842262, ali i rs12097821, sa rizikom za razvoj idiopatskog muškog steriliteta. Naši rezultati ustanovljeni u populaciji Severne Makedonije idu u prilog prethodnim navodima o učešću rs10842262 u genetičkoj osnovi muškog steriliteta. Ipak, dodatne studije su neophodne kako bi potvrdile značaj rezultata ovog istraživanja.