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NOS3 gene variants and male infertility: Association of 4a/4b with oligoasthenozoospermia

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Summary

Results of recent studies confirmed that oxidative stress negatively affects sperm motility and causes sperm DNA damage. Produced by nitric oxide synthase 3 (NOS3), nitric oxide is considered to be one of the important mediators of oxidative stress in testis tissue. The aim of this study was to assess the possible association of three genetic variants (rs2070744, rs1799983 and intron variant 4a/4b) in *NOS3* gene and infertility occurrence in two groups of infertile men (idiopathic azoospermia and oligoasthenozoospermia) and fertile controls. Genotypes for the single-nucleotide genetic variants rs1799983 and rs2070744 were determined by PCR-RFLP, while genotyping of intron 4 variant 4a/4b was performed by gel electrophoresis of PCR products. Statistical analysis was performed by SNPStats software. No significant association between the three genetic variants of the *NOS3* gene and infertility risk was determined comparing allele and genotype frequencies among group of patients diagnosed with azoospermia and the control group. Nevertheless, there was a significant positive association between 4a/4b and infertility in the group of males diagnosed with oligoasthenozoospermia, under overdominant genetic model. Our findings suggest that tandem repeat variant within intron 4 of the *NOS3* gene is associated with an increased risk of infertility in men diagnosed with idiopathic oligoasthenozoospermia.

KEYWORDS
4a/4b, Male infertility, NOS3 gene, rs1799983, rs2070744

1 | INTRODUCTION

Multiple studies have provided experimental results suggesting the involvement of oxidative stress (OS) in dysregulation of spermatogenesis. Based on these findings, OS was hypothesised to be one of the potential causes of idiopathic infertility (Bansal & Bilaspuri, 2010; Gharagozloo & Aitken, 2011; Saleh & Agarwal, 2002; Tremellen, 2008). Being a major component of OS in testis tissue, nitric oxide (NO) was recognised as one of important mediators involved in regulation of reproductive function (Rosselli, Keller, & Dubey, 1998). In physiologically small concentrations, NO acts as an antioxidant with protective role in spermatogenesis, while the elevated concentrations of this molecule exploit negative effects on motility and morphology of spermatozoa (Huang, Jones, & Khorram, 2006; Jurado & Rosselli, 2007). Furthermore, higher levels of NO cause sperm DNA damage, apoptosis and interference in sperm–oocyte fusion (Gunes, Al-Sadaan, & Agarwal, 2015; Saleh et al., 2003; Wu, Huang, Tsai, Lui, & Liu, 2004).

The main source of NO in testicular tissue and spermatozoa is the endothelial isoform of NOS synthase (NOS3), which is encoded by *NOS3* gene (Moncada & Higgs, 1993). Therefore, genetic variants located within this gene potentially affecting its function represent suitable candidates for association studies on male infertility. To date, single-nucleotide genetic variants (SNVs) in coding (RefSeqGene on chromosome 7; GenBank accession number NG_011992.1; NCBI SNP cluster ID rs1799983; SNP change G894T) and noncoding regions of *NOS3* (RefSeqGene on chromosome 7; GenBank accession

number NG_011992.1; NCBI SNP cluster ID rs2070744; SNP change T-786C), as well as the intron 4 variable number tandem repeat polymorphism (4a/4b) (RefSeqGene on chromosome 7; GenBank accession number NG_011992.1; NCBI SNP cluster ID rs61722009; tandem repeat (AGGGGTGAGGAAGTCTAGACCTGCTGC)), have been evaluated for association with male infertility in multiple populations (Bianco et al., 2013; Buldreghini et al., 2010; Safarinejad, Shafiei, & Safarinejad, 2010; Song et al., 2015; Ying, Pu, Liu, & A, 2013; Yu et al., 2014; Yun, Park, Song, & Lee, 2008). Results obtained in these studies were found to be discordant, implying the necessity for case–control studies on the same subject in additional European and non-European populations.

Therefore, the aim of this study was to assess the possible association between genetic variants rs2070744, rs1799983 and 4a/4b of *NOS3* gene and male infertility in Serbian population.

2 | MATERIALS AND METHODS

2.1 | Subjects

A total of 131 (ranging from 23 to 49 years of age) infertile men including 84 patients diagnosed with idiopathic azoospermia and 47 patients with oligoasthenozoospermia were recruited as a case group. All patients in the case group passed a standard clinical examination procedure which included: physical examination, ultrasound examination, semen analysis, serum hormone analysis, cytogenetic and genetic tests in order to exclude existence of chromosome aberrations and Y chromosome microdeletions. Infertile men diagnosed with the diseases that are known to affect spermatogenesis or with the obstruction of the genitourinary system were excluded from this study. Patients with chromosomal abnormalities and microdeletions of AZF regions on Y chromosome were excluded as well. Ascertainment of infertility was performed by semen analysis according to WHO guidelines (Cooper et al., 2010). Patient's age, serum hormone levels and semen parameters were showed in Table 1. Control group involved 131 fertile men (ranging from 26 to 52 years of age) who have fathered at least one child which was confirmed by paternity testing. After explanation of the study objectives, all participants gave a written informed consent to participate in the study. Experiments were conducted in accordance with the Helsinki Declaration of 1975. This study was approved by the Ethics Committee of Clinical Centre Serbia, Belgrade, Serbia (Number 68/5).

2.2 | Genotype determination

Genomic DNA was extracted from buccal swab samples using a commercial kit for DNA isolation (*Qiagen*, Hilden, Germany). DNA fragments that include genetic variants rs1799983 and rs2070744 were amplified using primer sets previously published by Colombo et al. (2002) and Nakayama et al. (1999) respectively (Colombo et al., 2002; Nakayama et al., 1999). The primers used for amplification of DNA fragments that include intron 4 VNTR were 5′-CTA TGG TAG TGC CTT GGC TGG AGG-3′(sense) and 5′-ACC GCC CAG GGA AGC TCC GCT-3′(antisense), designed by Primer3 software (Untergasser et al., 2012).

Genotyping of genetic variants rs1799983 and rs2070744 was performed by PCR-RFLP analysis. PCR products were digested overnight using restriction enzymes, and digestion products were analysed by electrophoresis on 3% agarose gel; MboI was used for rs1799983 and MspI for rs2070744. The T allele of rs1799983 produced two fragments (87 and 119 bp) in response to MboI restriction enzyme digestion, while allele G of the same variant corresponded to uncut PCR product. The C allele of rs2070744 produced three fragments (35, 46 and 156 bp) in response to MspI enzyme, while allele T of the same variant produced two fragments (81 and 156 bp).

Genotyping of genetic variant 4a/4b was performed by electrophoresis of PCR products on 3% agarose gel. The 169- and 196-bp amplified products of PCR corresponded to alleles 4a and 4b respectively.

TABLE 1 Clinical characteristics of infertile men with Oligoasthenozoospermia and Azoospermia

Clinical characteristics (mean)	Oligoasthenozoospermia (*n* = 47)	Azoospermia (*n* = 84)
Age		
Years	40.5	37.3
Serum hormones		
FSH(mIU/ml)	13.78	17.36
LH(mIU/ml)	5.62	7.53
PRL(pmol/L)	341.24	247.24
T(nmol/L)	9	8.05
Inhibin B(pg/ml)	83.9	78.86
Sperm parameters		
Volume (ml)	3.56	2.94
Total sperm number ($\times 10^6$)	6.45	/
Sperm density($\times 10^6$/ml)	2.3	/
Motility (%)	11	/

FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; T, testosterone.

2.3 | Sample size calculations

A sample power calculation was conducted for 4a/4b VNTR intron variant, using the Quanto software, version 1.2.4 (Gauderman & Morrison, 2006). We tested a gene only hypothesis using unmatched case–control design. Minor allele frequency for 4a/4b VNTR was 0.17; sample size (47 cases and 131 controls); disease prevalence 0.1; and odds ratio 2.42. Assuming these parameters our study had 0.71 to 0.85 power to detect association according to dominant or log-additive model of inheritance respectively.

2.4 | Statistical analysis

Genotype and allele frequencies of all three genetic variants were calculated, and deviation from the Hardy–Weinberg equilibrium was assessed using exact test within SNPStats software package. A statistical test based on logistic regression within SNPStats program was used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs). Association between alleles and genotypes of the three genetic variants and idiopathic infertility in males was tested using five different genetic models: codominant, dominant, recessive, overdominant and log-additive. When testing the codominant genetic model, heterozygous genotypes, as well as homozygotes for minor allele, were compared to referent genotype, which is homozygote for major allele. In dominant model testing, combined heterozygous and minor allele homozygous genotypes were compared to referent genotype. Similarly, in recessive model tests, minor allele homozygotes were compared to the combined heterozygotes and major allele homozygotes. When testing the association under overdominant model, heterozygotes were compared to the combined homozygotes. The log-additive model testing assumed analysing the additive effect of minor allele of logarithmic scale. Akaike information criterion (AIC) was used to determine the best-fitting genetic model for association analysis (Bozdogan, 1987; Solé, Guinó, Valls, Iniesta, & Moreno, 2006).

3 | RESULTS

Genotyping of rs2070744, rs1799983 and 4a/4b was successful in 99.6% (262/263) of subjects. Distributions of genotypes in the control group and among cases were in accordance with those expected under the Hardy–Weinberg equilibrium (Tables 2 and 3). When rs2070744, rs1799983 and 4a/4b genotype distributions among infertile cases and controls were compared, no evidence of association was obtained for any genetic model tested (Table 3).

In the group of infertile men diagnosed with azoospermia, none of the alleles or genotypes of the three genetic variants that we analysed was found to be associated with male infertility in any of the tested genetic models. Nevertheless, statistical trend for association between alleles and genotypes of rs2070744 and infertility was observed for two genetic models tested (p = .094, for log-additive model, OR = 0.69, 95% CI = (0.45–1.07); p = .094, for dominant model (TC+CC versus TT genotype), OR = 0.61, 95% CI = (0.35–1.09)) (Table 4). In the group of infertile men diagnosed with oligoasthenozoospermia, heterozygous genotype of intron 4 VNTR (a/b) compared to combined homozygotes (a/a+b/b), significantly increased the risk for infertility occurrence, according to overdominant genetic model (p = .038). The OR obtained in this comparison was estimated to 2.42 with 95% CI being 1.00-5.88. Furthermore, for genetic variant rs2070744 statistical trend of association with infertility occurrence under dominant genetic model (TC+CC versus TT genotype) was obtained (p = .084, OR = 1.99, 95% CI = (.88-4.50)) (Table 5).

4 | DISCUSSION

Prevalence of infertility, defined as the inability of a couple to achieve pregnancy after one year of unprotected sexual intercourse, ranges from 5% to 15% worldwide. Male infertility as the contributing factor is present in approximately 40% of infertile couples (Boivin, Bunting, Collins, & Nygren, 2007). Over the years, numerous studies revealed that despite complex nature of the disease, infertility has substantial molecular genetic basis with high prevalence of chromosome aberrations and single gene mutations (Ferlin et al., 2007). Despite great advances in clinical diagnostics and predictive genetic testing, causative factor of infertility in a large number of patients remains unidentified, classifying them as men with idiopathic infertility (Quaas & Dokras, 2008). Results of numerous studies showed that these men could have normal results of routine semen analysis but significantly higher seminal reactive oxygen species (ROS) levels and lower antioxidant potential than fertile controls (Agarwal, Makker, & Sharma, 2008).

Nitric oxide, produced by NOS3 enzyme in Sertoli and Leydig cells, is one of the mediators of OS in testicular tissue. Several studies have investigated the effect of high seminal NO levels on sperm motility by comparing semen parameters between group of men with asthenozoospermia and normozoospermic controls (Balercia et al., 2004; Buldreghini et al., 2010; Song et al., 2015). It has been demonstrated

TABLE 2 p-values obtained for Hardy–Weinberg equilibrium in a group of cases and among controls using exact test

	Cases		Controls
Genetic variant	Azoospermia (n = 84)	Oligoasthenozoospermia (n = 47)	Fertile males (n = 131)
rs1799983	0.17	0.29	0.32
rs2070744	0.33	0.34	0.83
4a/4b	0.74	0.099	0.76

TABLE 3 Association of three genetic variants within *NOS3* gene and male infertility

			Cases	Controls	Logistic regression		
Genetic variant	**Genetic model**	**Genotype**	***n* = 131**	***n* = 131**	**OR (CI 95%)**	***p*-value**	**AIC**
rs1799983							
	Codominant	GG	57 (43.5%)	66 (50.4%)	1.00	.54	368
		GT	60 (45.8%)	53 (40.5%)	0.76 (0.46-1.27)		
		TT	14 (10.7%)	12 (9.2%)	0.74 (0.32-1.73)		
	Dominant	GG	57 (43.5%)	66 (50.4%)	1.00	.26	366
		GT+TT	74 (56.5%)	65 (49.6%)	0.76 (0.47-1.23)		
	Recessive	GG+GT	117 (89.3%)	119 (90.8%)	1.00	.68	367
		TT	14 (10.7%)	12 (9.2%)	0.84 (0.37-1.90)		
	Overdominant	GG+TT	71 (54.2%)	78 (59.5%)	1.00	.38	366.4
		GT	60 (45.8%)	53 (40.5%)	0.80 (0.49-1.31)		
	Log-additive	–	–	–	0.82 (0.57-1.19)	.3	366.1
rs2070744							
	Codominant	TT	52 (39.7%)	57 (43.5%)	1.00	.56	368
		TC	63 (48.1%)	63 (48.1%)	0.91 (0.55-1.52)		
		CC	16 (12.2%)	11 (8.4%)	0.63 (0.27-1.47)		
	Dominant	TT	52 (39.7%)	57 (43.5%)	1.00	.53	366.8
		TC+CC	79 (60.3%)	74 (56.5%)	0.85 (0.52-1.40)		
	Recessive	TT+TC	115 (87.8%)	120 (91.6%)	1.00	.31	366.2
		CC	16 (12.2%)	11 (8.4%)	0.66 (0.29-1.48)		
	Overdominant	TT+CC	68 (51.9%)	68 (51.9%)	1.00	1	367.2
		TC	63 (48.1%)	63 (48.1%)	1.00 (0.62-1.62)		
	Log-additive	–	–	–	0.83 (0.57-1.21)	.34	366.3
4a/4b							
	Codominant	bb	92 (70.2%)	89 (67.9%)	1.00	.46	367.6
		ab	33 (25.2%)	39 (29.8%)	1.22 (0.71-2.11)		
		aa	6 (4.6%)	3 (2.3%)	0.52 (0.13-2.13)		
	Dominant	bb	92 (70.2%)	89 (67.9%)	1.00	.69	367
		ab+aa	39 (29.8%)	0.85 (0.48-1.51)	1.11 (0.66-1.88)		
	Recessive	bb+ab	125 (95.4%)	128 (97.7%)	1.00	.3	366.2
		aa	6 (4.6%)	3 (2.3%)	0.49 (0.12-2.00)		
	Overdominant	bb+aa	98 (74.8%)	92 (70.2%)	1.00	.41	366.5
		ab	33 (25.2%)	39 (29.8%)	1.26 (0.73-2.17)		
	Log-additive	–	–	–	1.00 (0.64-1.56)	1	367.2

OR, odds ratio; CI, confidence interval; AIC, Akaike information criteria.

that excessive concentrations of NO in semen of asthenozoospermic patients have overall negative effect on spermatozoa kinetic characteristics and consequently, a negative role in the reduction of sperm motility (Balercia et al., 2004). In addition, genotype-dependent influence of genetic variants located in *NOS3* gene on gene expression, protein concentrations and enzyme activity in cultured human endothelial cells and placental tissue was confirmed, suggesting the functional significance of these genetic variants (Song et al., 2003; Wang et al., 2000). Also, Song et al. showed significant association between minor allele of the 4a/4b variant and asthenozoospermia. This result was supported by measuring NOS3 mRNA expression levels in semen samples, which was upregulated in patients in comparison with controls (Song et al., 2015). Another study showed that mRNA of NOS3 was more expressed in peripheral blood leucocytes of asthenozoospermic infertile men versus those of fertile normozoospermic men, and a positive correlation between the concentration of NO and the percentage of immotile spermatozoa. (Buldreghini et al., 2014).

Since association between *NOS3* gene variants and male infertility has been analysed in several case–control studies, we aimed to determine if there is a possible association of three genetic variants in *NOS3* gene with male infertility in Serbian population. To date, the effect of these three genetic variants on male infertility was assessed in seven

TABLE 4 Association of three genetic variants within *NOS3* gene and male infertility in the group of patients diagnosed with azoospermia

			Azoospermia	Controls	Logistic regression		
Genetic variant	**Genetic model**	**Genotype**	***n* = 84**	***n* = 131**	**OR (CI 95%)**	***p*-value**	**AIC**
rs1799983							
	Codominant	GG	35 (41.7%)	66 (50.4%)	1.00	.38	291.8
		GT	42 (50%)	53 (40.5%)	0.67 (0.38-1.19)		
		TT	7 (8.3%)	12 (9.2%)	0.91 (0.33-2.52)		
	Dominant	GG	35 (41.7%)	66 (50.4%)	1.00	.21	290.1
		GT+TT	49 (58.3%)	65 (49.6%)	0.70 (0.40-1.22)		
	Recessive	GG+GT	77 (91.7%)	119 (90.8%)	1.00	.83	291.7
		TT	7 (8.3%)	12 (9.2%)	1.11 (0.42-2.94)		
	Overdominant	GG+TT	42 (50%)	78 (59.5%)	1.00	.17	289.8
		GT	42 (50%)	53 (40.5%)	0.68 (0.39-1.18)		
	Log-additive	-	-	-	0.83 (0.54-1.26)	.38	290.9
rs2070744							
	Codominant	TT	27 (32.1%)	57 (43.5%)	1.00	.23	290.7
		TC	47 (56%)	63 (48.1%)	0.63 (0.35-1.15)		
		CC	10 (11.9%)	11 (8.4%)	0.52 (0.20-1.38)		
	Dominant	TT	27 (32.1%)	57 (43.5%)	1.00	.094[a]	288.9
		TC+CC	57 (67.9%)	74 (56.5%)	0.61 (0.35-1.09)		
	Recessive	TT+TC	74 (88.1%)	120 (91.6%)	1.00	.4	291
		CC	10 (11.9%)	11 (8.4%)	0.68 (0.27-1.68)		
	Overdominant	TT+CC	37 (44%)	68 (51.9%)	1.00	.26	290.4
		TC	47 (56%)	63 (48.1%)	0.73 (0.42-1.26)		
	Log-additive	–	–	–	0.69 (0.45-1.07)	.094[a]	288.9
4a/4b							
	Codominant	bb	54 (64.3%)	89 (67.9%)	1.00	.59	292.6
		ab	26 (30.9%)	39 (29.8%)	0.91 (0.50-1.66)		
		aa	4 (4.8%)	3 (2.3%)	0.46 (0.10-2.11)		
	Dominant	bb	54 (64.3%)	89 (67.9%)	1.00	.58	291.4
		ab+aa	30 (35.7%)	0.85 (0.48-1.51)	0.85 (0.48-1.51)		
	Recessive	bb+ab	80 (95.2%)	128 (97.7%)	1.00	.33	290.7
		aa	4 (4.8%)	3 (2.3%)	0.47 (0.10-2.15)		
	Overdominant	bb+aa	58 (69%)	92 (70.2%)	1.00	.85	291.7
		ab	26 (30.9%)	39 (29.8%)	0.95 (0.52-1.71)		
	Log-additive	–	–	–	0.82 (0.50-1.34)	.42	291.1

OR, odds ratio; CI, confidence interval; AIC, Akaike information criteria.
[a]Results showing statistical trend of association

case–control studies conducted in Brazilian, Italian, Korean, Chinese and Iranian population. Studies done in Chinese population included meta-analyses of all previously published researches (Bianco et al., 2013; Buldreghini et al., 2010; Safarinejad et al., 2010; Song et al., 2015; Ying et al., 2013; Yu et al., 2014; Yun et al., 2008). Taking into account potential interpopulational differences in genetic backgrounds, together with discordances in the results obtained from previous studies, we conducted an analysis of association between these genetic variants and male infertility in Serbian population. To our knowledge, this is the first study on this subject conducted in Serbia, as well as the second one that was performed in a population of European origin. Therefore, the main reason for conducting such a study was the necessity for additional case–control studies on the same subject in other European, as well as in non-European populations.

Two independent case–control studies in Han Chinese population analysed the association of rs2070744 with male infertility (Song et al., 2015; Ying et al., 2013). Ying et al. demonstrated strong association between minor allele C and TC genotype of rs2070744 and male

TABLE 5 Association of three genetic variants within *NOS3* gene and male infertility in the group of patients diagnosed with oligoasthenozoospermia

			Oligoasthenozoospermia	Controls	Logistic regression		
Genetic variant	**Genetic model**	**Genotype**	***n* = 47**	***n* = 131**	**OR (CI 95%)**	***p*-value**	**AIC**
rs1799983							
	Codominant	GG	22 (46.8%)	66 (50.4%)	1.00	.57	210.4
		GT	18 (38.3%)	53 (40.5%)	0.98 (0.48-2.02)		
		TT	7 (14.9%)	12 (9.2%)	0.57 (0.20-1.63)		
	Dominant	GG	22 (46.8%)	66 (50.4%)	1.00	.67	209.3
		GT+TT	25 (53.2%)	65 (49.6%)	0.87 (0.44-1.69)		
	Recessive	GG+GT	40 (85.1%)	119 (90.8%)	1.00	.29	208.4
		TT	7 (14.9%)	12 (9.2%)	0.58 (0.21-1.56)		
	Overdominant	GG+TT	29 (61.7%)	78 (59.5%)	1.00	0.8	209.4
		GT	18 (38.3%)	53 (40.5%)	1.09 (0.55-2.17)		
	Log-additive	–	–	–	0.82 (0.50-1.33)	.42	208.8
rs2070744							
	Codominant	TT	25 (53.2%)	57 (43.5%)	1.00	0.23	208.5
		TC	16 (34%)	63 (48.1%)	1.73 (0.84-3.56)		
		CC	6 (12.8%)	11 (8.4%)	0.80 (0.27-2.42)		
	Dominant	TT	25 (53.2%)	57 (43.5%)	1.00	.25	208.2
		TC+CC	22 (46.8%)	74 (56.5%)	1.48 (0.76-2.88)		
	Recessive	TT+TC	41 (87.2%)	120 (91.6%)	1.00	.39	208.8
		CC	6 (12.8%)	11 (8.4%)	0.63 (0.22-1.80)		
	Overdominant	TT+CC	31 (66%)	68 (51.9%)	1.00	.094[b]	206.7
		TC	16 (34%)	63 (48.1%)	1.80 (0.90-3.59)		
	Log-additive	–	–	–	1.14 (0.68-1.91)	.63	209.3
4a/4b							
	Codominant	bb	38 (80.8%)	89 (67.9%)	1.00	.1	207
		ab	7 (14.9%)	39 (29.8%)	2.38 (0.98-5.79)		
		aa	2 (4.3%)	3 (2.3%)	0.64 (0.10-3.99)		
	Dominant	bb	38 (80.8%)	89 (67.9%)	1.00	.084[b]	206.5
		ab+aa	9 (19.1%)	42 (32.1%)	1.99 (0.88-4.50)		
	Recessive	bb+ab	45 (95.7%)	128 (97.7%)	1.00	.5	209
		aa	2 (4.3%)	3 (2.3%)	0.53 (0.09-3.26)		
	Overdominant	bb+aa	40 (85.1%)	92 (70.2%)	1.00	.038[a]	205.2
		ab	7 (14.9%)	39 (29.8%)	2.42 (1.00-5.88)		
	Log-additive	–	–	–	1.55 (0.77-3.13)	.2	207.9

OR, odds ratio; CI, confidence interval; AIC, Akaike information criteria.
[a]Statistically significant results.
[b]Results showing statistical trend of association.

infertility in men with azoospermia- and oligozoospermia, while Song et al. did not confirm this association analysing the group of men with asthenozoospermia, but the results of meta-analysis supported association of this genetic variant with male infertility in Asian and Caucasian populations. Furthermore, this association could not be confirmed in Brazilian population analysing men diagnosed with nonobstructive azoospermia and severe oligozoospermia (Bianco et al., 2013), nor in South Korean population (Yun et al., 2008). Safarinejad et al. (2010) revealed significant association between rs2070744 and male infertility in Iranian population, emphasising that the presence of CC genotype was significantly higher in the group of men diagnosed with azoospermia in comparison with the group with oligoasthenoteratozoospermia. In our study, we did not establish a statistically significant association between alleles and genotypes of rs2070744 and male infertility.

Findings of Buldreghini et al. (2010) suggested that the minor allele T of genetic variant rs1799983 may contribute to poor sperm motility of

patients with asthenozoospermia in Italian population. Also, in Iranian population minor allele T and TT genotype of rs1799983 were in significant correlation with male infertility (Safarinejad et al., 2010). Our results, as well as the results of studies conducted in Chinese, Brazilian and South Korean population, did not confirm this association.

The results of the present study suggested the association of heterozygous genotype of 4a/4b genetic variant with the increased risk of oligoasthenozoospermia in Serbian population. Recent studies of Ying et al. and Song et al. confirmed positive correlation between minor allele 4a and 4a/4b genotype of the 4a/4b genetic variant and male infertility in Han Chinese population, which is supported by the results of meta-analysis in both studies. Our results, suggesting the association of 4a/4b variant with oligoasthenozoospermia in a European population, are in accordance with the results previously obtained in Asian populations. Based on the analysis of sperm parameters depending on the genotypes of infertile subjects, Yun et al. indicated that sperm morphology is associated with this intron 4 VNTR. Furthermore, the study of Safarinejad et al. demonstrated negative correlations between semen parameters and allele 4a of the intron 4 VNTR. The results of a study conducted in Chinese population suggested that genetic variant 4a/4b negatively correlates with sperm motility and the number of spermatozoa in ejaculate (Song et al., 2015). These results further augment our findings and provide their potential biological explanation based on the impact on semen characteristics.

The main limitation of this study is its relatively small sample size. Nevertheless, our results provide additional evidence of the association between genetic variants in *NOS3* gene and male infertility, specifically oligoasthenozoospermia. Therefore, as in other studies on this issue, our findings may be helpful in identifying potential genetic markers of male infertility which could be used for constructing clinical algorithms. Furthermore, due to their potential functional significance these genetic markers could allow for better understanding of aetiology of oligoasthenozoospermia. Future case–control studies, as well as functional analyses, are needed to clarify the clinical significance of the *NOS3* genetic variants in male infertility.

5 | CONCLUSIONS

For the first time, we have demonstrated that genetic variant 4a/4b is associated with an increased risk of oligoasthenozoospermia in one European population, suggesting that intron 4 VNTR in the *NOS3* gene negatively affects sperm number and motility. Further functional and genetic association studies in other populations are required in order to validate our findings and ascertain the role of 4a/4b genetic variant in the aetiology of male infertility.

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CONFLICT OF INTEREST

The authors fully declare that there is no any financial or other potential conflict of interest.

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