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ACE gene and male infertility: a South Slavic case-control study and multi-omics data integration

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ABSTRACT

Components of the renin-angiotensin system (RAS) are expressed in both female and male reproductive tracts, with angiotensin I converting enzyme (ACE) being an important component for male reproductive function, as shown in animal models. The most studied *ACE* polymorphism is the Alu insertion-deletion (I/D), which has been proposed to have a negative effect on male fertility. Given the conflicting evidence in the literature, we conducted a multicentric case-control study to investigate the association between the *ACE* Alu I/D polymorphism and impaired spermatogenesis. Using PCR amplification and agarose electrophoresis, we genotyped the *ACE* gene Alu I/D polymorphism in 745 South Slavic men. The study group consisted of 457 patients with impaired spermatogenesis, 239 with non-obstructive azoospermia (NOA) and 218 with oligoasthenoteratozoospermia (OAT) and a control group of 288 fertile men. No association was found between the Alu I/D polymorphism and these semen phenotypes, suggesting that it is not associated with NOA or severe OAT in this cohort. To provide a broader regulatory context, we also developed an integrative atlas of *ACE* regulatory elements by in silico multi-omics analysis using genomics databases and bioinformatics tools. Data integration revealed various regulatory mechanisms at multiple omics levels, including genomics, epigenomics, miRNAomics, transcriptomics, proteomics and epiproteomics. These include genomic variants with predicted deleterious effects, a CpG island, microRNAs (miRNAs) and post-translational modifications (PTMs). In addition, protein interaction analysis revealed that ACE is indirectly linked to several proteins previously associated with male infertility and is also targeted by miRNA previously associated with oligozoospermia. This comprehensive, multi-faceted approach, combining genetic association analysis with bioinformatics, provides insights into *ACE* regulation in its broader molecular context. These results emphasize the importance of further integrative multi-omics and systems biology research to better understand the role of ACE in male reproductive function.

List of abbreviations: ACE: Angiotensin I converting enzyme; ACE2: Angiotensin converting enzyme 2; AGT: Angiotensinogen; AGTR1: Angiotensin II receptor type 1; AGTR2: Angiotensin II receptor type 2; CI: confidence interval; D: deletion; HWE: Hardy-Weinberg Equilibrium; I: insertion; MAS1: MAS1 proto-oncogene, G protein-coupled receptor; miRNA: microRNA; MTI: miRNA-target interaction; OR: odds ratio; p: p-value; RAS: renin-angiotensin system; ROS: reactive oxygen species; SNP: single-nucleotide polymorphism; X^2: Chi-square test; CADD: Combined

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Annotation Dependent Depletion; SIFT: Sorting Intolerant From Tolerant; PolyPhen: Polymorphism Phenotyping; REVEL: The Rare Exome Variant Ensemble Learner; MetaLR: Meta logistic regression

Introduction

Male infertility affects approximately 2.5–12% of the adult population (Agarwal, Mulgund et al. 2015). Its diagnosis is challenging due to the heterogeneous nature of the condition and the interplay of genetic and environmental factors (reviewed in Axelsson et al. 2010). Irregularities at various omics levels have been observed in infertile men (Podgrajsek et al. 2024). Nevertheless, only a limited number of genetic factors, such as chromosomal abnormalities and Y chromosome microdeletions, are routinely tested in infertile men (reviewed in Krausz and Riera-Escamilla 2018; Cioppi et al. 2021). Common genetic alterations may contribute to infertility to a lesser extent, but due to their much higher frequency in the population, their contribution cannot be completely excluded (Aston et al. 2010).

The presence of components of the renin-angiotensin system (RAS) in the male reproductive tract suggests a possible link between reproductive functions and RAS (reviewed in Vinson et al. 1997; Leung and Sernia 2003; Gianzo and Subirán 2020). The RAS includes components such as AGT, ACE, ANG II and AGTR1, which have been associated with various fertility-related processes including sperm motility, oocyte fertilization and early embryonic development (Gianzo and Subirán 2020).

Angiotensin I converting enzyme (ACE) is a key enzyme of the RAS system. It exists in two isoforms: the larger somatic isoform (sACE), which is expressed in various tissues and the shorter testicular isoform (tACE), also known as the germinal isoform (gACE), which is expressed exclusively in the adult testis and is encoded by exons 13–26 (reviewed by Khurana and Goswami 2022). Animal studies investigating the role of *ACE* in male infertility have highlighted the critical function of *tACE*. Mice deficient in both isoforms exhibited sperm transport and fertilization defects, while mice lacking only the somatic isoform (*sACE*) remained fertile, confirming the essential role of *tACE* and not *sACE* in normal male reproduction (Hagaman et al. 1998).

Studies suggested that *ACE* polymorphisms might contribute to male infertility, although the results have been inconsistent. A Chinese association study examining five *ACE* gene single nucleotide polymorphisms (SNPs) (C10514T, T10527C, A10578G, C23152A and G23202A) in Singaporean Chinese infertile men found no significant association with male infertility (Liao and Roy 2002). In contrast, Li et al. (2014) demonstrated that the rs4316 polymorphism in *ACE* is associated with the absence of germinal *ACE* in sperm and poor fertilization outcomes in IVF. In addition to SNPs, another frequently investigated variant of *ACE* is the Alu insertion/deletion (I/D) polymorphism. Alu elements are short, repetitive sequences dispersed throughout the human genome and this polymorphism involves the insertion or deletion of a 287-base pair (bp) Alu sequence in intron 16 of the *ACE* gene (reviewed in Baudin 2002). It was reported that men with the D/D and I/D genotypes exhibited abnormal semen parameters, thereby strengthening the association between the I/D polymorphism in *ACE* and male infertility (Zalata et al. 2012). However, Pehlivan et al. (2008) observed no significant differences in the genotype or allele frequencies between infertile men and controls.

Given the conflicting evidence regarding the role of the *ACE* Alu I/D polymorphism in male infertility, the aim of our study was to investigate its potential association with impaired male fertility through a multicentric case-control study involving South Slavic infertile men and proven fertile controls. To obtain a comprehensive overview of *ACE* regulation, we developed an integrative atlas incorporating data from various omics layers to further explore their potential relevance for future research.

Results

In this study, we investigated the association of the *ACE* Alu I/D polymorphism with impaired spermatogenesis and explored the regulation of *ACE* across multiple omics levels. The case-control analysis revealed no significant differences in *ACE* Alu I/D polymorphism between cases and controls. However,

the results of the integrative multi-omics atlas of *ACE* regulatory elements suggest that *ACE* can be regulated across various omics layers, highlighting its possible involvement in broader molecular mechanisms.

Case-control study

The analysis revealed no statistically significant deviation from Hardy-Weinberg equilibrium in the genotype distribution of the control group ($p = 0.5387$). Furthermore, no significant differences in allele or genotype frequencies were found between patients with non-obstructive azoospermia (NOA) and controls, patients with oligoasthenoteratozoospermia (OAT) and controls, or between all patients with impaired spermatogenesis and controls. The genotype and allele frequencies of the *ACE* Alu I/D polymorphism in cases and controls are shown in Table 1 and Figure 1. Furthermore, no significant differences were observed in the allelic, dominant and recessive models (Table 2). We intended to perform subgroup analyses by population to assess possible ethnic differences. However, an additional analysis was conducted only for the Slovenian cohort, as the sample size for participants from other countries was too small to allow reliable comparisons between ethnic groups. Similarly, no significant differences in genotype or allele frequencies were found in any of the tested genetic models.

Integrative multi-omics atlas of *ACE* regulatory elements

An overview of the results of the integrative atlas of *ACE* regulatory elements is presented in Figure 2A. Data were extracted from 14 genomics databases and bioinformatics tools. We compiled relevant molecular data related to *ACE* across multiple omics levels, including genomics (DNA level), epigenomics, miRNAomics, transcriptomics (RNA level), proteomics and epiproteomics (post-translational modifications; PTMs).

While our primary genetic association analysis focused on the *ACE* Alu I/D polymorphism in intron 16, we extended the bioinformatics part of this study by extracting clinically significant and potentially pathogenic variants using six established prediction tools available through the Ensembl browser: SIFT, PolyPhen, CADD, REVEL, MetaLR and Mutation Assessor (Figure 2B). The analysis identified seven missense variants, six of which were classified as deleterious by all six tools. One of these variants (rs2029861365) was also annotated as a splice region variant. According to the Ensembl database, the clinical significance of these variants is currently unknown. However, the *ACE* gene also contains variants that are classified as pathogenic, including rs983649759 (inframe deletion and insertion), rs367797185 (stop gain) and rs1219522144 (frameshift) as well as a likely pathogenic missense variant, rs148491967. These results suggest that other *ACE* variants may have functional relevance and should be prioritized in future case-control studies or functional assays to elucidate their potential role in male infertility.

Table 1. Frequency of *ACE* Alu I/D genotypes and alleles in male infertility patients and controls.

		Patients N (%)		Controls N (%)
	All	AZO	OAT	
	$N = 457$	$N = 239$	$N = 218$	$N = 288$
I/D genotype frequency				
DD	154 (33.7 %)	80 (33.5 %)	74 (34 %)	83 (28.8 %)
ID	208 (45.5 %)	104 (43.5 %)	104 (47.7 %)	148 (51.4 %)
II	95 (20.8 %)	55 (23 %)	40 (18.3 %)	57 (19.8)
X^2p	2.684	3.246	1.5232	
	$p = 0.2614$	$p = 0.1974$	$p = 0.4669$	
HWE in controls				$p = 0.5387$
Allele frequency				
D	516 (56.46 %)	264 (55.23 %)	252 (57.8 %)	314 (54.51 %)
I	398 (43.54 %)	214 (44.77 %)	184 (42.2 %)	262 (45.49 %)
X^2p	0.5396	0.0541	1.086	
	$p = 0.4626$	$p = 0.8161$	$p = 0.2974$	

Abbreviations: HWE: Hardy-Weinberg Equilibrium; X^2: Chi-square test. $p < 0.05$.

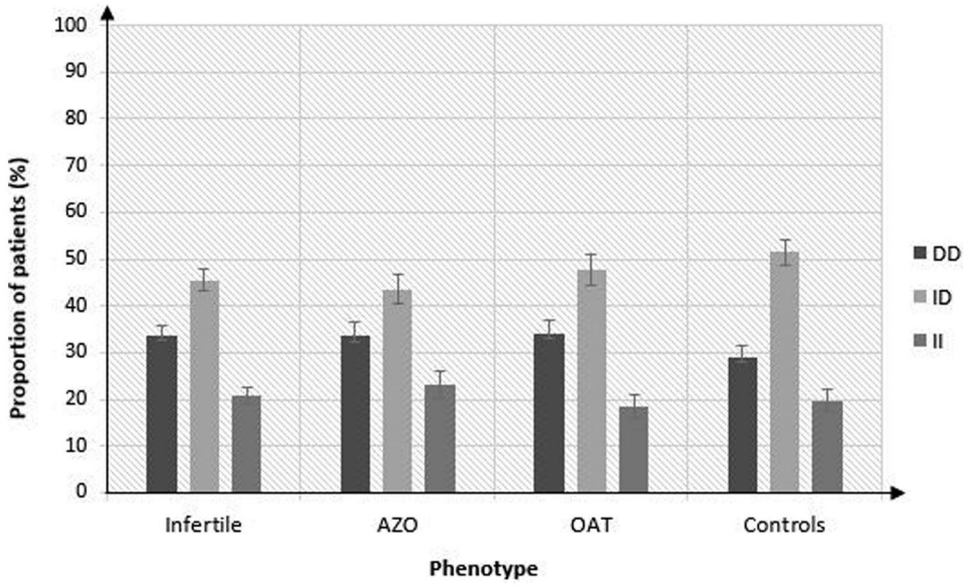


Figure 1. Distribution of *ACE* Alu I/D polymorphism genotypes in infertile men and controls.
Bar chart show the proportions of DD, ID and II genotypes of the *ACE* gene in four groups: combined infertile men (Infertile), men with non-obstructive azoospermia (AZO), men with severe oligoasthenoteratozoospermia (OAT) and fertile controls (Controls). Genotype frequencies are presented as percentages.
Legend: DD**:** deletion/deletion, ID**:** insertion/deletion, II: insertion/insertion

Table 2. Results of association analysis of *ACE* I/D polymorphism with male infertility under different genetic models.

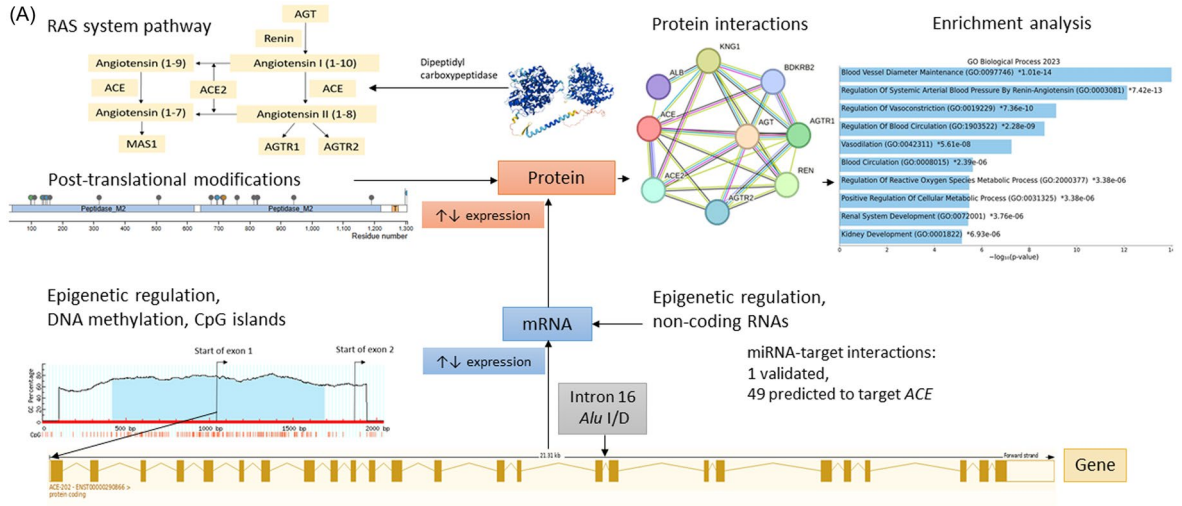
Genotype or allele	DD vs. ID + II Recessive model	DD+ID vs. II Dominant model	Allele D vs. allele I Allelic model
Infertile men			
OR (95% CI)	1.2553 (0.9112–1.7294)	0.9403 (0.6511 to 1.3579)	1.0818 (0.8771–1.3342)
p-Value	$p = 0.1642$	$p = 0.7425$	$p = 0.4626$
AZO			
OR (95% CI)	1.2427 (0.8580–1.7999)	0.8255 (0.5435 to 1.2539)	1.0293 (0.8067– 1.3134)
p-Value	$p = 0.2502$	$p = 0.3686$	$p = 0.8161$
OAT			
OR (95% CI)	1.2692 (0.8689–1.8541)	1.0981 (0.7008 to 1.7205)	1.1428 (0.8891–1.4689)
p-Value	$p = 0.2175$	$p = 0.6831$	$p = 0.2975$

Abbreviations: CI: confidence interval, OR: odds ratio, $p < 0.05$.

We investigated the epigenetic regulation of *ACE*, focusing on DNA methylation patterns and the identification of miRNAs potentially targeting the gene. Our analysis revealed a 1,266 bp CpG island extending from the upstream region of *ACE* to intron 1 with a CG content of over 80%. It was also experimentally confirmed that the miRNA hsa-miR-335-5p targets *ACE*. In addition, 49 miRNAs were computationally predicted to target *ACE*, including five with a target score of 90 or above in the miRDB database: hsa-miR-4492, hsa-miR-4498, hsa-miR-5001-5p, hsa-miR-762 and hsa-miR-4789-3p.

In addition, we investigated the epiproteomic level and retrieved 22 PTMs of ACE from the PhosphositePlus database, including N-glycosylation (at residues N111, N146, N160, N318, N509, N677, N695, N714, N760, N942, N1191), acetylation (K100, K136), phosphorylation (Y140, Y151, T697, T716, S824), ubiquitylation (Q99, K718) and methylation (R814, R826). N-glycosylation was the most common PTM. A higher density of PTMs was observed around amino acid positions 100 and 700 (Figure 2A).

Two isoforms of *ACE* have previously been reported: the testicular and the somatic isoform (Lattion et al. 1989). Currently, 21 human and four mouse *ACE* transcripts are annotated in the Ensembl database (Supplementary Figure S1). As reported in the literature, ACE is differentially expressed at both the transcript and protein levels (Rockett et al. 2004; Pencheva et al. 2021). Using AlphaFold to predict the 3D structure of ACE, we found that the protein consists of two distinct domains.



(B)

Variant ID	Chr: bp	Alleles	Evidence	Clin. Sig.	Conseq. Type	AA	AA co-ord	SIFT	Poly-Phen	CADD	REVEL	MetaLR	Mutation Assessor	Transcript
rs1266264733	17:63481112	G/C	gnom AD	-	missense variant	W/S	290	0	1	33	0.722	0.591	0.967	ENST00000290866.10
rs1599141410	17:63482599	G/A		-	missense variant	E/K	418	0.01	0.994	32	0.683	0.598	0.941	ENST00000290866.10
rs2147534927	17:63483160	T/C	-	-	missense variant	W/R	492	0	1	31	0.816	0.523	0.963	ENST00000290866.10
rs2029861365	17:63483850	T/C		-	missense variant splice region variant	Y/H	530	0	0.995	33	0.772	0.593	0.921	ENST00000290866.10
rs748643856	17:63483958	G/A	Exac gnom AD	-	missense variant	G/R	566	0	1	33	0.706	0.529	0.967	ENST00000290866.10
rs1243492273	17:63484347	G/C/T	gnom AD	?	missense variant	G/V	576	0	0.999	35	0.903	0.826	0.967	ENST00000290866.10
rs1157631466	17:63490976	G/C	gnom AD	?	missense variant	W/C	888	0	0.984	33	0.699	0.59	0.941	ENST00000290866.10

Figure 2. Integrative atlas of *ACE* gene function, regulation and variability.
(A) Functional overview of the *ACE* gene, including its role in the (RAS) pathway, protein structure, post-translational modifications, protein-protein interactions, gene ontology enrichment analysis and epigenetic regulation via CpG islands and non-coding RNAs (miRNA interactions). The location of the ACE Alu I/D polymorphism in intron 16 is shown.
(B) Table of potentially deleterious variants identified in the *ACE* gene based on six prediction tools (SIFT, PolyPhen, CADD, REVEL, MetaLR and Mutation Assessor). Variants include missense and splice region variants. Red values indicate a high predicted deleterious or damaging effect according to each tool's scale (e.g. SIFT = 0, PolyPhen = 1, CADD ≥ 30). Orange values in the Mutation Assessor column represent a medium functional impact.
Legend: RAS: renin-angiotensin system. ACE: angiotensin I converting enzyme; ACE2: angiotensin converting enzyme 2; AGT: angiotensinogen; AGTR1: angiotensin II receptor type 1; AGTR2: angiotensin II receptor type 2; MAS1: MAS1 proto-oncogene, G protein-coupled receptor.

Further analysis using the STRING tool revealed that ACE interacts with eight proteins, most of which are primarily involved in the RAS signaling pathway. Although ALB, KNG1 and BDKRB2 are not directly part of the RAS pathway, they are closely related to the maintenance of blood vessels and pressure. This is confirmed by the enrichment analysis, which shows the primary association of ACE and its interacting proteins with biological processes related to the regulation of blood vessel diameter and the maintenance of blood pressure (Figure 2A). Extending the STRING analysis to the first neighbors in the protein interaction network led to the identification of 12 additional proteins (KLK4, MMEL1, NR3C2, MAS1L, CYP11B2, MME, ATP6AP2, PRCP, APLN, EDN1, F2 and BDKRB1) (Figure 3).

Discussion

In this case-control study, we investigated the potential role of the *ACE* Alu I/D polymorphism in impaired spermatogenesis. Comparison of genotype frequencies between 457 men with impaired spermatogenesis (239 with NOA and 218 with severe OAT) and 288 fertile controls revealed no statistically significant differences in allele or genotype frequencies or in any of the tested genetic models (allelic, dominant, or recessive). These results suggest that the *ACE* Alu I/D polymorphism

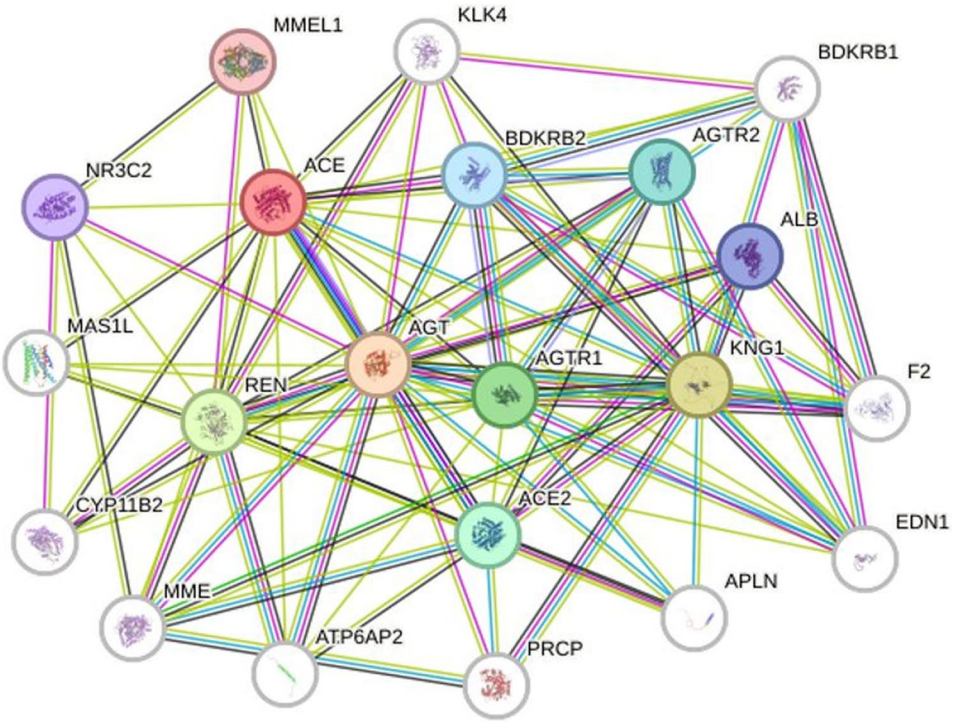


Figure 3. Protein-protein interaction network of ACE visualized using the STRING tool. The network shows eight direct interacting proteins (ALB, KNG1, BDKRB2, AGT, AGTR1, AGTR2, ACE2 and REN) and 12 first-neighbor proteins **(**KLK4, MMEL1, NR3C2, MAS1L, CYP11B2, MME, ATP6AP2, PRCP, APLN, EDN1, F2 and BDKRB1).

is not associated with the risk of severe forms of impaired spermatogenesis, including NOA and severe OAT, in this cohort.

Several previous studies have attempted to evaluate the possible involvement of the *ACE Alu* I/D polymorphism in male infertility. One of these studies found a significant difference between infertile men and controls (Zalata et al. 2012), while in three studies no such association was observed (Kucera et al. 2001; Liao and Roy 2002; Pehlivan et al. 2008) (Table 3).

In addition to the *ACE* Alu I/D polymorphism, the data suggest that other *ACE* polymorphisms may not be associated with male infertility, as reported by Wang et al. (2019), who found no association between any of the four *ACE* SNPs and NOA (Wang et al. 2019). Together with the present study, the existing literature suggests that the *ACE* Alu I/D polymorphism does not play a significant role in the risk of male infertility. Although meta-analyses are a powerful tool for assessing the statistical significance of multiple studies (reviewed in Haidich 2010), their application to male infertility is more complex due to the variability of inclusion criteria for different phenotypes of male infertility. These include azoospermia, oligozoospermia, asthenozoospermia, teratozoospermia and combined forms such as oligoasthenozoospermia (Liao and Roy 2002; Kucera et al. 2001; Pehlivan et al. 2008; Zalata et al. 2012), which may lead to inappropriate comparisons.

The discrepancies between the studies could be explained by differences in sample size, ethnic background, infertility phenotypes and investigated polymorphisms. Although our results do not suggest a significant association between the Alu I/D polymorphism in *ACE* and male infertility in this cohort, they should be interpreted with caution. Notably, mice lacking both the somatic and testicular forms of *Ace* have reduced fertilization rates despite normal semen parameters (Hagaman et al. 1998), suggesting a possible role for *ACE* beyond spermatogenesis. It is also important to note that NOA and severe OAT represent semen phenotypes rather than definitive clinical diagnoses of infertility. Given the known overlap in semen quality between fertile and infertile men, our conclusions are specific to these two phenotypic categories and should not be generalized to all forms of male infertility. Further studies focusing on specific subgroups, such as men with normal semen profiles but failed fertilization, are needed to clarify this association.

Table 3. Overview of studies investigating the association between *ACE* polymorphisms and male infertility.

Study	Number of cases	Number of fertile men	Infertility phenotype	Origin	Investigated SNP	Outcome
Kucera et al. (2001)	46	88	AZO, severe OA, moderate OA	Czech	I/D	No statistically significant difference in allele D frequency between infertile and fertile men
Liao and Roy (2002)	90	84	AZO, A, T, O, AT, OA, OT, OAT	Singapore Chinese	C10514T[a], T10527C, A10578G, C23152A, G23202A	No statistically significant differences between infertile and fertile men
Pehlivan et al. (2008)	102	30	AZO, O	Turkey	I/D	No statistically significant difference between infertile and fertile men
Zalata et al. (2012)	303	102	A, AT, OAT	Egypt	I/D	D allele associated with impaired semen parameters, including reduced sperm count and motility.
Li et al. (2014)	90	50	Total fertilization failure and lower fertilization rates	China	rs4316	TT genotype: increased risk of fertilization failure
Wang et al. (2019)	121	256	NOA	Chinese Han	rs4316, rs4331, rs4343, rs4362	No significant association with NOA

Abbreviations: A: asthenozoospermia; AT: asthenoteratozoospermia; AZO: azoospermia; NOA: non-obstructive azoospermia; O: oligozoospermia; OA: oligoasthenozoospermia; OAT: oligoasthenoteratozoospermia; OT: oligoteratozoospermia; SNP: single nucleotide polymorphism; T: teratozoospermia.

[a]Allele C in absolute linkage disequilibrium with the *Alu* deletion, while T allele with the *Alu* insertion.

Additionally, gene-environment interactions may vary in different populations, affecting the reproducibility of findings. Our results contribute to the growing body of evidence suggesting that the association between *ACE* polymorphisms and male infertility is complex and possibly population-specific. These differences emphasize the need for larger, ethnically diverse cohorts, standardized phenotyping and meta-analyses to clarify inconsistent findings and determine the role of *ACE* in male fertility.

Since the outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), its cellular receptor, ACE2, has attracted considerable attention (reviewed in Younis et al. 2020). *ACE2* and its homologue *ACE* are both highly expressed in the testes, suggesting that dysregulation of either enzyme may contribute to impaired fertility. *ACE2* expression has been reported to be decreased in men with NOA (Liu et al. 2020). The presence of ACE2 in the testes could potentially facilitate viral invasion, which may contribute to male infertility, particularly through testicular inflammation (reviewed in Olaniyan et al. 2020). While some studies have reported associations between *ACE* polymorphisms and male infertility, no polymorphisms in *ACE2* have yet been associated with male infertility, indicating a gap in understanding the genetic contribution of the ACE2 pathway to male reproductive function.

Despite the lack of association between the *ACE* Alu I/D polymorphism and impaired spermatogenesis, the gene should not be disregarded as a candidate in the context of male infertility or other disorders of male reproductive function. Our integrative atlas of *ACE* regulatory elements suggests that *ACE* is subject to complex regulation across multiple omics levels. Several other potentially pathogenic variants are predicted to be deleterious by different computational tools. The results indicate that further studies on other *ACE* variants are needed.

The presence of a CpG island extending from the upstream region of the gene into intron 1 suggests possible epigenetic regulation, consistent with previous reports on the influence of DNA methylation on *ACE* expression (Zill et al. 2012). This potential epigenetic regulation is also supported by miRNA-target interaction (MTI) analysis, which identified one experimentally validated and 49 predicted interactions with *ACE*. According to miRTarBase (Cui et al. 2025), hsa-miR-335-5p has been experimentally validated to target *ACE*. In addition, this miRNA was reported to be differentially expressed in men with oligozoospermia and associated with sperm concentration (Salas-Huetos et al. 2015). The decreased expression of hsa-miR-335-5p observed in the oligozoospermia group suggests that impaired regulation may be associated with reduced sperm count, potentially contributing to male infertility.

At the transcriptional level, Rockett et al. (2004) reported differential expression of *Ace* in the testes of infertile compared to fertile mice. At the protein level, Pencheva et al. (2021) reported

reduced expression of tACE in the neck and midpiece of ejaculated and capacitated sperm from infertile men compared to fertile controls. While early studies focused primarily on the testicular and somatic isoforms of *ACE*, the Ensembl database now lists 21 transcripts in human and four in mouse, reflecting a greater degree of alternative splicing and isoform diversity that may contribute to tissue-specific regulation and function. These results suggest that alterations in *ACE* regulation at both the transcript and protein levels may be associated with pathologies in both species. Furthermore, PTMs from the PhosphoSitePlus database, a resource for experimentally validated PTM data (Hornbeck et al. 2012), suggest that ACE undergoes multiple modifications, including N-glycosylation, acetylation, phosphorylation, ubiquitylation and methylation, highlighting the complexity of its post-translational regulation. Based on these findings, it would be valuable to investigate whether SNPs at or near these sites could lead to the gain or loss of specific PTMs.

According to the STRING database, ACE interacts with eight proteins mainly involved in blood vessel maintenance and the regulation of blood pressure. Components of the RAS are involved in sperm physiology and have been reported to play a role in sperm motility, capacitation, acrosome reaction and fertilization (reviewed by Gianzo and Subirán 2020). Although the enrichment analysis did not reveal any direct involvement in male infertility (Figure 2A), these processes may indirectly influence reproductive function. For example, vasodilation or vasoconstriction can regulate testicular blood flow, potentially affecting spermatogenesis. In addition, reactive oxygen species (ROS) are associated with impaired male fertility due to their role in lipid peroxidation in sperm membranes and DNA fragmentation (reviewed in Tremellen 2008; Hussain et al. 2023). Oxidative stress has a negative effect on sperm motility, fertilization and embryo development and may contribute to infertility and an increased risk of miscarriage (reviewed in Tremellen 2008).

Extending the STRING analysis to the first neighbors in the protein-protein interaction (PPI) network provided a broader view of potential functional associations and led to the identification of twelve additional proteins, eight of which were previously associated with male infertility. Variants in *KLK4* were found in infertile men, suggesting a possible association with fertility disorders (Precone et al. 2020). Another relevant gene, *MMEL1*, was found to be differentially methylated in asthenozoospermic men compared to fertile controls (Qin et al. 2019). According to the Mouse Genome Informatics (MGI) database (Baldarelli et al. 2024), homozygous *MMEL1*-deficient mice are infertile and exhibit impaired fertilization. The role of *MAS1L* is still unclear. One study identified this locus as a candidate for male fertility traits and found an association between rs724078 and semen parameters (Kosova et al. 2012), while another study failed to replicate this association in a Japanese population (Sato et al. 2015). *MME*, a membrane metallo-endopeptidase, has been reported to be overexpressed in infertile men (Agarwal, Ayaz et al. 2015). It has been hypothesized that *ATP6AP2* may influence sperm fertility potential (Gianzo et al. 2021). This result is confirmed by data from the MGI database (Baldarelli et al. 2024), which report that *Atp6ap2*-deficient mice are infertile. In addition, a negative correlation was observed between *PRCP* and sperm motility (Becker et al. 2023), while *APLN* is involved in inflammatory pathological conditions and has been reported to be increased in infertile men, especially those with varicocele or genital tract infections (Moretti et al. 2023). *EDN1* has been reported to be elevated in infertile men with varicose veins compared to controls (Gyftopoulos et al. 2011).

Although the Alu I/D polymorphism in *ACE* was not associated with NOA or severe OAT in the present study and most previous association studies also reported no association with male infertility (Kucera et al. 2001; Liao and Roy 2002; Pehlivan et al. 2008), a broader network analysis incorporating data from first-neighbor protein interactions and MTIs revealed connections between network proteins, miRNAs and male infertility. These extended interactions may reveal additional regulatory links between ACE and biological pathways relevant to vascular function and possibly to male reproductive health.

We acknowledge that some data in our bioinformatics study are indirect and should be interpreted with caution. Protein-protein interaction data, such as those from STRING, reflect predicted or known associations and do not imply causality. The observed associations may represent broader biological processes rather than a direct role in male reproduction and should be considered as a hypothesis, requiring further experimental validation. Based on our results, the integration of multi-omics data and the application of systems biology approaches could provide a more comprehensive understanding of the role of *ACE* in various human diseases.

Future research should investigate SNPs predicted to be deleterious, particularly SNPs located at PTM sites or those potentially influencing PPIs involving ACE. Investigation of SNPs located in regulatory regions that can create or disrupt miRNA binding sites could further elucidate the post-transcriptional regulation of *ACE*. Given the complexity of *ACE* regulation, studies on epigenetic mechanisms such as DNA methylation and histone modifications are also needed. The regulation of *ACE* by miRNAs and other non-coding RNAs (ncRNAs) also merits further investigation. Future studies should also characterize the expression patterns, regulatory mechanisms and functional significance of the newly annotated *ACE* isoforms, particularly in reproductive tissues, to better understand their potential contribution to male fertility. A multi-omics and systems biology approach will be essential for a comprehensive understanding of *ACE* function and its impact on human disease.

In addition to these directions, experimental validation of the bioinformatically predicted effects should also be carried out. While computational tools provide valuable predictions of pathogenicity and regulatory complexity, experimental approaches such as reporter gene assays, CRISPR/Cas9-mediated genome editing and *in vitro* characterization of *ACE* isoforms are essential for establishing the biological relevance of these results. Such functional assays will help to confirm causal relationships and increase the translational potential of this research.

While this study focused on genetic and bioinformatic analyses, future research should consider gene-environment interactions, as these may influence the effect of *ACE* variants on male fertility. Factors such as smoking and exposure to endocrine disruptors could interact with underlying genetic predispositions. The integration of genetic, environmental and lifestyle data will be necessary to understand the multifactorial nature of male infertility.

Limitations of the study

Some limitations of the study should also be acknowledged. One limitation is the focus on patients with a South Slavic ethnic background, which may limit the generalizability of the results to other populations. Genetic associations with polymorphisms may vary between populations due to differences in allele frequencies and gene-environment interactions. However, this focus was intentional, as South Slavic populations are underrepresented in genetic studies of male infertility.

Although it would be useful to examine possible differences between other ethnic groups, such analyses were not possible in our study due to the limited number of participants from other countries. The sample size of infertile men from Serbia, Montenegro, Croatia and Macedonia was too small for reliable country-specific analysis, especially when further subdivided by genotype. In addition, populations in this region are often ethnically mixed due to historical and demographic factors, making it difficult to draw clear genetic distinction between countries. A larger cohort would increase the statistical power of the study.

A limitation of our study is the clinical heterogeneity within the NOA and severe OAT groups. NOA is a broad phenotype encompassing various etiologies, including chromosomal abnormalities, Y chromosome microdeletions and endocrine dysfunction. Similarly, severe OAT can arise from various underlying defects affecting sperm concentration, motility, or morphology. As consistent clinical data were not available in all centres, we could not stratify these groups according to genetic or hormonal causes. This heterogeneity may have diluted possible associations between *ACE* variants and specific subtypes of male infertility and should be considered when interpreting our results.

Regarding the integrative atlas of *ACE* regulatory elements, it should be noted that the atlas is not yet complete and cell type-specific regulation may limit the generalizability of the results to different tissues and physiological contexts. Furthermore, as our study relied on bioinformatic predictions, it is important to acknowledge that these tools have limitations and experimental validation is required to confirm the functional significance of the identified elements.

Study highlights and implications

This study provides several important contributions to the understanding of *ACE* in the context of male infertility. First, we demonstrated that the *ACE* Alu I/D polymorphism is not significantly

associated with NOA and severe OAT in a South Slavic population. This finding adds to the evidence suggesting that this widely studied polymorphism may not be an important genetic risk factor for impaired spermatogenesis, at least in this population group.

Given the lack of genetic association and conflicting results in the literature, we expanded our analysis to examine the broader molecular and regulatory landscape of the *ACE* gene. By creating an integrative atlas of regulatory elements of the *ACE* gene, we identified a complex regulatory network across multiple omics levels, including genomics, epigenomics, miRNAomics, transcriptomics, proteomics and epiproteomics. This atlas revealed several possible mechanisms by which *ACE* could influence reproductive function beyond genetic variation.

Using six established pathogenicity prediction tools, we identified several *ACE* variants, including missense and splice region variants, predicted to be potentially deleterious. These variants represent promising targets for future functional validation studies. In addition, post-transcriptional regulation was highlighted as a key area of interest, involving both experimentally validated and predicted miRNA-target interactions with *ACE*. One of these miRNAs has previously been associated with male infertility, highlighting the potential regulatory role of miRNAs in sperm function and control of gene expression.

Post-translational regulation was also evident, with multiple modification sites identified on ACE, including glycosylation, phosphorylation, ubiquitylation and methylation. These results support the hypothesis that SNPs or structural variants affecting these modification sites may alter the function of the ACE protein, potentially impacting male fertility.

Furthermore, our PPI analysis revealed that several first-neighbor interacting proteins of ACE have already been associated with male infertility phenotypes in human or mouse models. This observation suggests that ACE may play an indirect role in the regulation of fertility through its interactions within broader protein networks.

Finally, the newly annotated *ACE* transcripts in human and mouse reflect a high degree of isoform diversity. This transcript variability could contribute to tissue-specific gene regulation and function, particularly in the reproductive system.

Taken together, these results highlight the importance of examining *ACE* within a broader molecular and regulatory context. Our multi-omics and systems biology approach offers a valuable framework for investigating candidate genes in male infertility and may also be adapted to other complex traits and diseases. Future studies should include experimental validation of *ACE* gene variants and their regulatory mechanisms.

Conclusion

In conclusion, our findings suggest that the *ACE* Alu I/D polymorphism is not a risk factor for the development of NOA or severe OAT in the South Slavic population. However, our in silico multi-omics analysis of the *ACE* gene suggests that further studies using different omics approaches are needed to clarify the role of *ACE* in male infertility. Although no direct association with the polymorphism was observed, a miRNA and most first-neighbor proteins in the ACE interaction network have previously been linked to male infertility, suggesting a potential indirect role of ACE through post-transcriptional and protein interaction mechanisms. This broader systems-level approach may help to uncover mechanisms by which *ACE* or its molecular network contributes to male infertility and related diseases.

Material and methods

Patients

The study population consisted of 745 male partners of infertile couples recruited from genetic centers across five countries: Slovenia (Ljubljana), Croatia (Rijeka), Macedonia (Skopje), Montenegro (Podgorica) and Serbia (Belgrade). The genetic centers included the Clinical Institute of Genomic Medicine, University Medical Centre Ljubljana, Ljubljana, Slovenia; Center of Genomic Medicine and Immunology, Clinical Center of Montenegro, Podgorica, Montenegro; Institute of Human Genetics, Faculty of

Medicine, University of Belgrade, Belgrade, Serbia; Research Centre for Genetic Engineering and Biotechnology "Georgi D. Efremov" Macedonian Academy of Sciences and Arts, Skopje, Macedonia and the Department of Medical Biology and Genetics, Faculty of Medicine, University of Rijeka, Rijeka, Croatia.

Male individuals were included from September 2022 onward during their genetic evaluation. Samples originating abroad were transferred to Slovenia as part of collaborative research. Of the 745 participants, 457 had impaired spermatogenesis, including 239 with NOA and 218 with severe OAT. Of this group, 367 men were from Slovenia, 31 from Serbia, 29 from Montenegro, 17 from Croatia and 13 from Macedonia. The control group consisted of 288 fertile men from Slovenia, each with at least one offspring. The samples were obtained from the genetic biobank of the Clinical Institute of Genomic Medicine, University Medical Centre Ljubljana, Slovenia. Informed consent was obtained from all participants, who were all of South Slavic origin.

Participants were selected based on current guidelines from the European Association of Urology and American Urological Association for genetic testing of infertile men, which recommend evaluation in cases of azoospermia and severe oligozoospermia (sperm concentration <5 million/ml) (Brannigan et al. 2024; EAU Guidelines 2025). NOA was defined as the complete absence of spermatozoa in at least two semen analyses, without evidence of ductal obstruction. Severe OAT was defined based on WHO 2010 criteria, involving reduced sperm concentration (<5 million/ml), motility (<32% progressive) and abnormal morphology. However, reproductive hormone levels, testicular volume, karyotyping, Y chromosome microdeletion analysis and histological evaluation were not consistently available for all patients and were not used for subgroup stratification.

DNA isolation and molecular analysis

Genomic DNA was extracted from peripheral blood samples using the chemagic™ 360 instrument (PerkinElmer, Waltham, Massachusetts, USA). We analyzed the Alu I/D polymorphism of the *ACE* gene using PCR amplification and agarose electrophoresis, following previously established protocols (Rigat et al. 1992; Peterlin et al. 2000).

Statistical analysis

The association between the allelic and genotype frequencies of Alu I/D polymorphism and male fertility status was assessed using the Chi-square test (χ^2). The deviation of genotype distribution in the control from those predicted by Hardy-Weinberg equilibrium (HWE) was tested. For the different genetic models (allelic, dominant and recessive), the odds ratio (OR) and the respective 95% confidence intervals (CI) were calculated. Differences were considered significant at $p < 0.05$.

Integrative multi-omics atlas of *ACE* regulatory elements

To investigate the broader role of *ACE*, we incorporated data from various omics approaches to explore its potential involvement in male infertility. For retrieval of the *ACE* structure and exploring sequence variants, we used the Ensembl genome browser (https://www.ensembl.org/index.html), release 114 (Dyer et al. 2025). Six tools were used for retrieval variants with the highest predicted deleterious effect: SIFT, PolyPhen, CADD, REVEL, MetaLR and Mutation Assessor. MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Li and Dahiya 2002) was utilized to determine the presence of CpG islands in the *ACE* gene using the canonical transcript ENST00000290866.10. The parameters for the determination of CpG islands in *ACE* were: window > 200 bp, Observed/Expected CpG > 0.6, and GC% > 50%. MicriRNAs, experimentally validated to target *ACE* were extracted from miRTarBase, version 10.0 (https://mirtarbase.cuhk.edu.cn/) (Cui et al. 2025) and miRNAs predicted to target *ACE* were retrieved from the miRDB database (https://mirdb.org/mirdb/index.html) (Chen and Wang 2020). Protein post-translational modifications were retrieved from the PhosphoSitePlus Database V6.7.7 (https://www.phosphosite.org/homeAction) (Hornbeck et al. 2012). Protein interactions

involving *ACE* were analyzed using the STRING database, version 12.0 (https://string-db.org/) (Szklarczyk et al. 2023) using interactions with the highest confidence (0.9). Additionally, *ACE* and its interacting proteins with a confidence of 0.9 were examined for enriched biological processes using the Enrichr tool (https://maayanlab.cloud/Enrichr/) (Kuleshov et al. 2016). To explore its first interaction neighbors, the function "add more nodes to current network" in STRING was used, using a confidence threshold of 0.7. The AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/) (Varadi et al. 2022), accessed in February 2025, was used to predict the structure of ACE.

Ethical approval

The study was approved by the national medical ethics committee (ref. number: 0120-213/2022/6). Written informed consent was obtained from participants included in the study.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contributions

CRediT: **Tanja Kunej**: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing; **Rebeka Podgrajsek**: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing; **Helena Jaklic**: Methodology, Formal analysis, Writing – review & editing; **Alenka Hodzic**: Writing – review & editing; **Martin Stimpfel**: Writing – review & editing; **Olivera Miljanovic**: Resources, Writing – review & editing; **Momcilo Ristanovic**: Resources, Writing – review & editing; **Ivana Novakovic**: Resources, Writing – review & editing; **Dijana Plaseska-Karanfilska**: Resources, Writing – review & editing; **Predrag Noveski**: Resources, Writing – review & editing; **Sasa Ostojic**: Resources, Writing – review & editing, **Alena Buretic-Tomljanovic**: Resources, Writing – review & editing, **Antun Grskovic**: Resources, Writing – review & editing; **Borut Peterlin**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing.

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