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Original Article

Reproductive genetics

A genetic variant in the 3′-UTR of *PIWIL4* confers risk for extreme phenotypes of male infertility by altering miR-215 and miR-136 binding affinity

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ABSTRACT

STUDY QUESTION: What is the functional impact of the rs508485 genetic polymorphism, located in the 3′-untranslated region (UTR) region of the *PIWIL4* gene, on non-obstructive azoospermia (NOA)?

SUMMARY ANSWER: The rs508485 genetic variant contributes to the pathogenesis of extreme patterns of NOA by modulating *PIWIL4* expression through microRNA (miRNA) interactions.

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WHAT IS KNOWN ALREADY: Male infertility represents a significant global health challenge with profound societal and economic consequences. One of the most severe forms of male infertility is NOA, which is characterized by severe spermatogenic failure (SPGF) of idiopathic origin in most cases. Cumulating knowledge increasingly suggests that this idiopathic form of NOA may represent a multifactorial condition involving complex interactions between genetic and environmental factors. The PIWI protein subfamily, particularly PIWIL4, plays a pivotal role in spermatogenesis by processing PIWI-interacting RNAs, which silence retrotransposons to protect genomic integrity. Genetic variations in this gene have been found to be associated with susceptibility to NOA.

STUDY DESIGN, SIZE, DURATION: A case–control study was conducted in a European cohort including 1516 infertile men with SPGF and 2451 fertile controls. Logistic regression and functional assays were employed to investigate the functional role of the rs508485 polymorphism in *PIWIL4*.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants were genotyped for the rs508485 polymorphism. Associations between the polymorphism and NOA phenotypes, including Sertoli cell-only (SCO) syndrome and testicular sperm extraction (TESE) outcomes, were assessed. *In silico* tools predicted miRNA binding effects, which were subsequently validated using luciferase reporter assays.

MAIN RESULTS AND THE ROLE OF CHANCE: The T allele of rs508485 was significantly associated with the SCO phenotype ($P = 2.69E-03$, OR = 1.34) and unfavourable TESE outcomes ($P = 1.09E-03$, OR = 1.54). *In silico* analyses predicted that the rs508485 variant might alter binding sites in the 3′-UTR region of PIWIL4 for different miRNAs, such as hsa-miR-215-3p and hsa-miR-136-3p. Functional validation using luciferase assays confirmed that these miRNAs differentially bind to the T and C alleles of this polymorphism, influencing *PIWIL4* regulation.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The study is limited to a single genetic polymorphism and functional assays were performed *in vitro*. Additional studies are required to validate these findings across diverse populations and explore additional genetic interactions.

WIDER IMPLICATIONS OF THE FINDINGS: These findings highlight the critical role of miRNA regulation in extreme forms of male infertility by influencing the expression of essential spermatogenesis genes, such as *PIWIL4*. Our study sheds light on the genetic mechanisms underlying spermatogenesis and suggests potential therapeutic targets for NOA.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Spanish Ministry of Economy and Competitiveness through the Spanish National Plan for Scientific and Technical Research and Innovation (grant: PID2020-120157RB-I00 funded by MICIU/AEI/10.13039/501100011033; and grant: PID2023-152215OB-I00 funded by MICIU/AEI/10.13039/501100011033 and ERDF, EU). M. C.-M is beneficiary of a Juan de la Cierva 2022 postdoctoral fellowship (reference number JDC2022-048561-I) funded by MCIN/AEI/10.13039/501100011033 and European Union NextGenerationEU/PRTR. S.L. received support from "Generalitat de Catalunya" (grant 2021SGR052). S.L. is sponsored by the "Researchers Consolidation Program" from the SNS-Dpt. Salut Generalitat de Catalunya (Exp. CES09/020). S.G.-M. was funded by grant ref. FPU23/02674. S.S. was supported by funds from the Portuguese Foundation for Science and Technology (FCT; 10.54499/DL57/2016/CP1363/CT0019). The authors declare no competing interests.

Keywords: male infertility / NOA / SCO / TESE / *PIWIL4* / piRNA / miRNA / SNP

Introduction

Infertility is currently recognized as a significant global public health concern by the World Health Organization (WHO). This condition affects ∼10–15% of couples worldwide, with male factor infertility accounting for around half of these cases (Massart *et al.*, 2012; Tournaye *et al.*, 2017; World Health Organization, 2017). In this regard, non-obstructive azoospermia (NOA), defined as the total absence of sperm in the ejaculate due to a severe spermatogenic failure (SPGF), represents the most extreme and one of the most common causes of male infertility, affecting 10% of infertile men and 1% of the male population (Cocuzza *et al.*, 2013; Tournaye *et al.*, 2017). While several Mendelian causes have been identified for NOA, the aetiology of this condition remains unknown for around 75% of affected men. Increasing evidence clearly suggests that this idiopathic form of NOA represents a complex trait influenced by interactions between genetic and environmental factors (Cervan-Martin *et al.*, 2020, 2022, 2024).

P-element-induced wimpy testis (PIWI) proteins, part of the Argonaut protein subfamily, include PIWIL1, PIWIL2, PIWIL3, and PIWIL4 in humans. These proteins are predominantly expressed in germline cells and play a critical role in silencing retrotransposons through binding to PIWI-interacting RNAs (piRNAs), a class of small non-coding RNAs (Aravin *et al.*, 2007a; Ozata *et al.*, 2019). This silencing is achieved via mechanisms such as DNA methylation and histone modification, both of which are essential for spermatogenesis (Hartig *et al.*, 2007; Klattenhoff and Theurkauf, 2008; Juliano *et al.*, 2011). Although piRNA expression has been detected in both testis and ovaries, only male knock-out mice for orthologous *PIWI* genes develop infertility, likely due to upregulation of transposable elements in the germline (Aravin *et al.*, 2007b; He *et al.*, 2009). Specifically, several studies have revealed that *PIWIL4* deficiency in male mice leads to meiotic arrest (MA) during spermatogenesis, resulting in male sterility (Deng and Lin, 2002; Kuramochi-Miyagawa *et al.*, 2004; Carmell *et al.*, 2007). Regarding common genetic variations in the *PIWIL4* gene, several studies have reported associations of different single-nucleotide polymorphisms (SNPs), including rs508485, with a higher risk of male infertility due to SPGF in populations of different ancestries (Gu *et al.*, 2010; Munoz *et al.*, 2014; Kamaliyan *et al.*, 2017, 2018). However, the functional implications of the two allelic variants of rs508485 in the pathophysiology of male infertility remain unclear. There is ongoing debate regarding its association with specific patterns of SPGF, such as NOA, severe oligozoospermia (SO, fewer than 5 million spermatozoa per millilitre of ejaculate), or even a subgroup of NOA patients with a histological phenotype of MA (Gu *et al.*, 2010; Munoz *et al.*, 2014; Kamaliyan *et al.*, 2017, 2018). Although some authors have hypothesized that the rs508485 variant affects mRNA stability or alters the binding affinity of regulatory microRNAs (miRNAs), the precise molecular mechanism in which this SNP exerts its influence remains ambiguous (Kamaliyan *et al.*, 2018).

Based on the considerations exposed above, we conducted the present study to investigate the possible association between the *PIWIL4*-rs508485 SNP and NOA in a well-powered and characterized cohort of European descent. We aimed to elucidate the functional implications of this variant in the pathogenesis of this form of male infertility.

Materials and methods

Study population

Two independent case–control populations of European descent were analysed in this study, comprising a total of 1516 infertile men due to SPGF and 2451 unaffected controls. The discovery cohort included Iberian and German individuals (1274 cases and 1951 controls) and the replication cohort was composed only of Iberian men (242 cases and 500 controls).

All participants provided informed written consent and DNA samples were irreversibly anonymized prior to enrolment. The study procedures adhered to the principles outlined in the Declaration of Helsinki and received approval from the institutional Ethics Committee 'CEIM/CEI Provincial de Granada' (Andalusia, Spain), ensuring full compliance with government policies and ethical standards.

The discovery cohort data utilized in this study were derived from a previously published genome-wide association study (GWAS), where detailed clinical and demographic information was provided (Cervan-Martin *et al.*, 2022). The same inclusion criteria were applied for the replication cohort. Briefly, this study included men diagnosed with SPGF of idiopathic origin based on WHO guidelines (World Health Organization, 2021). Diagnosis was established through comprehensive clinical assessment, including physical examination, endocrine profiling, and histological evaluation when available, to confirm the total absence of sperm in the ejaculate. To ensure a well-defined NOA cohort, known causes of infertility were systematically excluded, including anatomical disorders, testicular neoplasms, obstructive cases, congenital bilateral absence of vas deferens, as well as genetic abnormalities such as karyotype anomalies and Y chromosome microdeletions, both of which were determined through standard genetic screening. Around half of the NOA cohort underwent a testicular sperm extraction (TESE) procedure from a testis biopsy, allowing classification into three different histological subgroups: hypospermatogenesis (HS, production of an extremely low number of sperm cells), MA (incomplete differentiation of the germline), and Sertoli cell-only (SCO) (total absence of germ cells in the seminiferous tubules). Moreover, NOA patients were also divided into two additional subgroups based on the TESE outcome (i.e. successful and unsuccessful TESE). The control group consisted of fertile men, matched with cases based on geographical origin and average age, who had fathered one or more healthy children naturally. Supplementary Table S1 presents comprehensive details regarding the study cohort.

Genotyping, quality controls, and imputation

DNA samples from the replication cohort were extracted from peripheral blood mononuclear cells and genotyped using the Infinium Global Screening Array-24 v3.0 (GSA) on an iScan System (Illumina, Inc). This procedure followed the same protocol outlined for the samples from the discovery cohort, as detailed in our previous study (Cervan-Martin *et al.*, 2022).

The SNP rs508485, selected from the discovery cohort using GWAS data (Cervan-Martin *et al.*, 2022), was genotyped in the replication cohort using the TaqMan™ allelic discrimination technology on a 7900HT Fast Real-Time PCR System with a predesigned TaqMan probe (reference assay ID: C__1365626_10). The SDS 2.3 software was used for allelic discrimination (Thermo Fisher Scientific™).

Statistical analysis

Logistic regression models were used to compare all case groups (SPGF, NOA, MA, HS, SCO, and TESEneg) against unaffected controls, incorporating covariates such as principal components and country of origin and assuming additive, dominant and recessive effects. Additionally, a combined analysis of both cohorts was performed via inverse variance-weighted meta-analysis under a fixed-effects model. The possible heterogeneity of the estimated odds ratios (OR) between studies was evaluated using I^2 and Cochran's Q tests. The *P*-value for statistical significance was set at $P = 0.05$.

Functional relevance of the rs508485 polymorphism

To assess the functional impact of *PIWIL4*-rs508485 on the spermatogenic process, functional annotations of the human genome overlapping the SNP under study (rs508485) and all its proxies (polymorphisms within the same linkage disequilibrium (LD) block, $R^2 \geq 0.8$) were retrieved from public databases. Initially, we identified proxies for the associated rs508485 variant in the European population using LDlink (Machiela and Chanock, 2015). To enhance our understanding of the regulatory potential of the selected variants, we extracted detailed functional annotation data from multiple publicly available resources including HaploReg v.4.2. (Ward and Kellis, 2016), RegulomeDB (Dong and Boyle, 2019), Open Target Genetics Portal (Ghoussaini *et al.*, 2021), GTEx (v8) (GTEx Consortium, 2020), and SNPNexus (Oscanoa *et al.*, 2020).

Prediction of miRNA target sequences

A comprehensive bioinformatic analysis was performed to predict the target sequences of miRNAs that were altered by the rs508485 risk allele located in the 3′-untranslated region (UTR) region of *PIWIL4*. For this purpose, the following bioinformatics tools were utilized: TargetScan (McGeary *et al.*, 2019), PolymiRTS (Bhattacharya *et al.*, 2014), miRBase (Kozomara *et al.*, 2019), and miRmap (Vejnar and Zdobnov, 2012), which assess the binding potential based on sequence complementarity and seed region match. Only candidate target sites identified by at least two prediction algorithms were selected for further analysis. Additionally, RNAhybrid (Kruger and Rehmsmeier, 2006) was employed to determine the minimum free energy (MFE) required for the hybridization of miRNAs to their predicted target sites (lower MFE values indicate enhanced stability of formed structures).

Cell cultures

HEK-293T cells (obtained from ATCC® and provided by Dr José Luis Garcia-Perez, GENYO, Spain) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Gibco), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen). Cell cultures were maintained in humidified incubators at 37°C with 5% CO_2. Mycoplasma contamination was ruled out monthly using a PCR-based assay (Minerva).

Plasmid constructs

In order to enhance the possible effect of the *PIWIL4*-rs508485 risk variant, five copies of the 3′-UTR region of *PIWIL4* were cloned into the psiCHECK2 vector. Specifically, 56 bp segments of this region were selected to minimize secondary structures, based on predictions generated by the RNAfold tool (Lorenz *et al.*, 2011). These segments, containing either the T or C allele of the rs508485 polymorphism, along with a mutated miRNA binding sequence as a negative control are described in Supplementary Table S2. All constructs were synthesized in pUC57 (GenScript) and cloned into psiCHECK2 using XhoI and NotI restriction enzyme sites (New England Biolabs). Correct insertions were confirmed by restriction digestion and Sanger sequencing.

miRNAs mimics

miRIDIAN miRNA mimics for hsa-miR-215-3p, hsa-miR-136-3p, and a scrambled control (Supplementary Table S3) were purchased from Dharmacon. Mimics were resuspended in 1× siRNA Buffer (Fisher Scientific) to a working concentration of 20 μM and stored at −80°C until use.

Transfection and luciferase reporter assays

HEK-293T cells were seeded (1×10^5 per well in 24-well plates) 24 h prior to transfection. Cells at 80–90% confluence were transfected with 10 ng of each psiCHECK2 plasmid and varying concentrations (10, 25, and 50 nM) of selected miRNA mimics using DharmaFECT reagents (Dharmacon).

Dual luciferase assays were conducted to confirm specific miRNA binding using the Dual Luciferase Reporter Assay System (Promega) and GloMax® 96 Microplate Luminometer (Promega), following manufacturer's instructions. Three experiments per condition with two measurements per sample were performed. Luciferase activity was measured as relative luminescence units (renilla/firefly luciferase) and normalized to scrambled controls. Statistical significance was determined using a two-tailed Student's *t*-test.

Results

Testing for association of rs508485 with male infertility due to SPGF

We initially compared the allele frequencies of the rs508485 SNP between the overall SPGF group (encompassing both NOA and SO patients) and the control group to assess its role in SPGF susceptibility. No statistically significant differences were found across genetic models (Table 1, Supplementary Table S4). Subsequently, and taking advantage of the detailed patient phenotyping, we compared the NOA group as well as the different NOA subgroups, defined by specific histological patterns (i.e. HS, MA, and SCO) and unsuccessful TESE outcome (TESEneg), against the control group. Notably, *PIWIL4*-rs508485 was associated with both TESEneg and SCO, showing significant *P*-values for the recessive and additive models, respectively, in the combined European discovery cohort of Iberians ($P_{TESEneg,\ rec}$=3.01E-02, OR $(CI)_{TESEneg,\ rec}$=1.54 (1.04–2.28); $P_{SCO,\ add}$=4.10E-02, OR $(CI)_{SCO,\ add}$=1.35 (1.01–1.81)) and Germans ($P_{TESEneg,\ rec}$=1.03E-02, OR $(CI)_{TESEneg,\ rec}$=1.70 (1.13–2.54); $P_{SCO,\ add}$=3.82E-02, OR $(CI)_{SCO,\ add}$=1.36 (1.02–1.81)) (Table 1, Supplementary Table S4).

To validate these findings, we conducted an additional case–control analysis in an independent Iberian cohort. While this replication analysis did not yield significant *P*-values due to smaller subgroup sizes, effect sizes similar to the discovery cohort were observed for the SCO versus control and TESEneg versus control comparisons (OR = 1.25 and OR = 1.17, respectively) (Table 1, Supplementary Table S4). Interestingly, the meta-analysis combining both cohorts substantially increased the statistical significance observed in the discovery cohort for SCO (P_{META}=2.69E-03, OR_{META}=1.34, CI_{META}=1.11–1.63) and TESEneg (P_{META}=1.09E-03, OR_{META}=1.54, CI_{META}=1.19–2.00) conditions, with no significant heterogeneity observed between studies (Table 1, Supplementary Table S4).

In silico characterization of the rs508485 polymorphism and its proxies

With the aim of investigating the biological impact of the rs508485 SNP in SCO development and TESE outcome, we analysed the functional evidence and predicted effects for the risk variant and its proxies (R^2>0.8) in the European population of the 1KGPh3 project (1000 Genomes Project Consortium *et al.*, 2015).

Four proxy variants were identified according to LDlink (Machiela and Chanock, 2015) (Supplementary Fig. S1). Notably, all variants influenced the *PIWIL4* gene, being rs508485 located in the 3′-UTR region, both the rs621425 and rs621922 polymorphisms in the 3′ downstream region, and the rs504010 and rs1491388272 variants in intronic positions of the gene (Supplementary Table S5). Among these, rs504010 and rs508485 exhibited the strongest evidence of pathogenicity based on CADD, RegulomeDB, and FunSeq2 scoring (Supplementary Table S5). In addition, all variants overlapped with epigenetic marks such as H3K36me3 and H3K27me3, while rs621425 also overlapped with a CTCF-binding site (Supplementary Table S5), as indicated by ChIP-seq data from ENCODE (Davis *et al.*, 2018). Further analyses predicted disruptions in transcription factor binding for the SNPs rs508485, rs504010, and rs621922, with affected transcription factors related to male infertility, spermatogenesis or having a predominant expression in the testis, including MEIS1 or GATA, among others (Supplementary Table S5). According to the transcriptome data of the GTEx project (GTEx Consortium, 2020), the proxies rs508485, rs621425, rs504010, and rs621922 were annotated as expression and splicing quantitative trait loci, thereby influencing the expression and splicing, respectively, of nearby genes in the testis (Supplementary Table S5, Supplementary Fig. S2). Furthermore, the Reactome pathway analysis linked rs508485, rs504010, and rs1491388272 to pathways such as 'gene silencing by RNA', 'piRNA biogenesis', and 'gene expression (transcription)' (Supplementary Table S5).

Finally, using the Open Targets Genetics Portal (Ghoussaini *et al.*, 2021), we analysed which traits were associated with the rs508485 variant in the UK Biobank, FinnGen, and/or GWAS Catalog summary statistics repositories. Remarkably, the most associated trait with this polymorphism was the 'bioavailable testosterone levels', with a *P*-value of 1.40E-06 (Ruth *et al.*, 2020) (Supplementary Fig. S3).

miRNA targets affected by the rs508485 polymorphism

Given the location of the rs508485 variant within the 3′-UTR region of *PIWIL4*, we initially used TargetScan, miRmap, and PolymiRTS to identify miRNAs with target sites that include this variant or are proximal to it. The identified miRNAs were hsa-miR-215-3p, hsa-miR-6773-3p, hsa-miR-4522-3p, hsa-miR-3664-5p, and hsa-miR-136-3p (Fig. 1). These computational tools assign a 'score' metric, that reflect the biological significance of each miRNA. Among these, the miRNAs hsa-miR-136-3p and hsa-miR-6773-3p exhibited the highest scores, indicative of their potential functional relevance. Subsequently, miRmap and RNAhybrid tools were employed to investigate the binding stability between the 3′-UTR sequence of the *PIWIL4* gene for each miRNA previously identified. All miRNAs exhibited increased stability in binding to the C allele of rs508485 compared to the T allele, except for hsa-miR-136-3p, which exhibited binding only in the presence of the T allele (Fig. 1). Furthermore, the difference in MFE values between the C and T alleles (ΔMFE) was computed. Remarkably low ΔMFE values were observed for all miRNAs, except for hsa-miR-136-3p, wherein, as previously mentioned, no binding occurred in the presence of the C allele, leading to *de novo* target formation in the presence of the T allele (Fig. 1).

Among all the miRNAs examined through the *in silico* analyses, rs508485 is located outside the recognition seed region in two of them (hsa-miR-3664-5p and hsa-miR4522-3p), thereby diminishing their relevance for this study. In the case of hsa-miR-6773-3p, while rs508485 localizes within the seed region, this miRNA lacks

Table 1. Associations of rs508485 genetic variant with severe SPGF subtypes when comparing with the control group under the considered models in the Iberian and German discovery cohorts and/or in the meta-analysis by the inverse variance method with the Iberian replication cohort.

					Iberian (discovery)		German (discovery)		Iberian (replication)		Meta-analysis			
SNP ID	Position (GRCh38)	EA	Pheno	Model	P	OR [95% CI]	P	OR [95% CI]	P	OR [95% CI]	P	OR [95% CI]	Q	I^2
rs508485	11:94621313	T	TESEneg	Recessive	**3.01E−02**	1.54 [1.04–2.28]	**1.03E−02**	1.70 [1.13–2.54]	6.68E−01	1.17 [0.58–2.36]	**1.09E−03**	1.54 [1.19–2.00]	0.67	0.00
			SCO	Additive	**4.10E−02**	1.35 [1.01–1.81]	**3.82E−02**	1.36 [1.02–1.81]	4.36E−01	1.25 [0.72–2.17]	**2.69E−03**	1.34 [1.11–1.63]	0.96	0.00

The results of the three independent studies are also shown. Significant *P*-values are highlighted in bold.
CI, confidence interval; EA, effect allele; GRCh38, Genome Reference Consortium Human Build 38; HS, hypospermatogenesis; ID, identifier; MA, maturation arrest; NOA, non-obstructive azoospermia; OR, odds ratio; *P*, *P*-value; Pheno, phenotype; SCO, Sertoli cell-only; SPGF, spermatogenic failure; SNP, single-nucleotide polymorphism; TESEneg, negative outcome in testicular sperm extraction; sQTL, Splicing Quantitative Trait Locus.

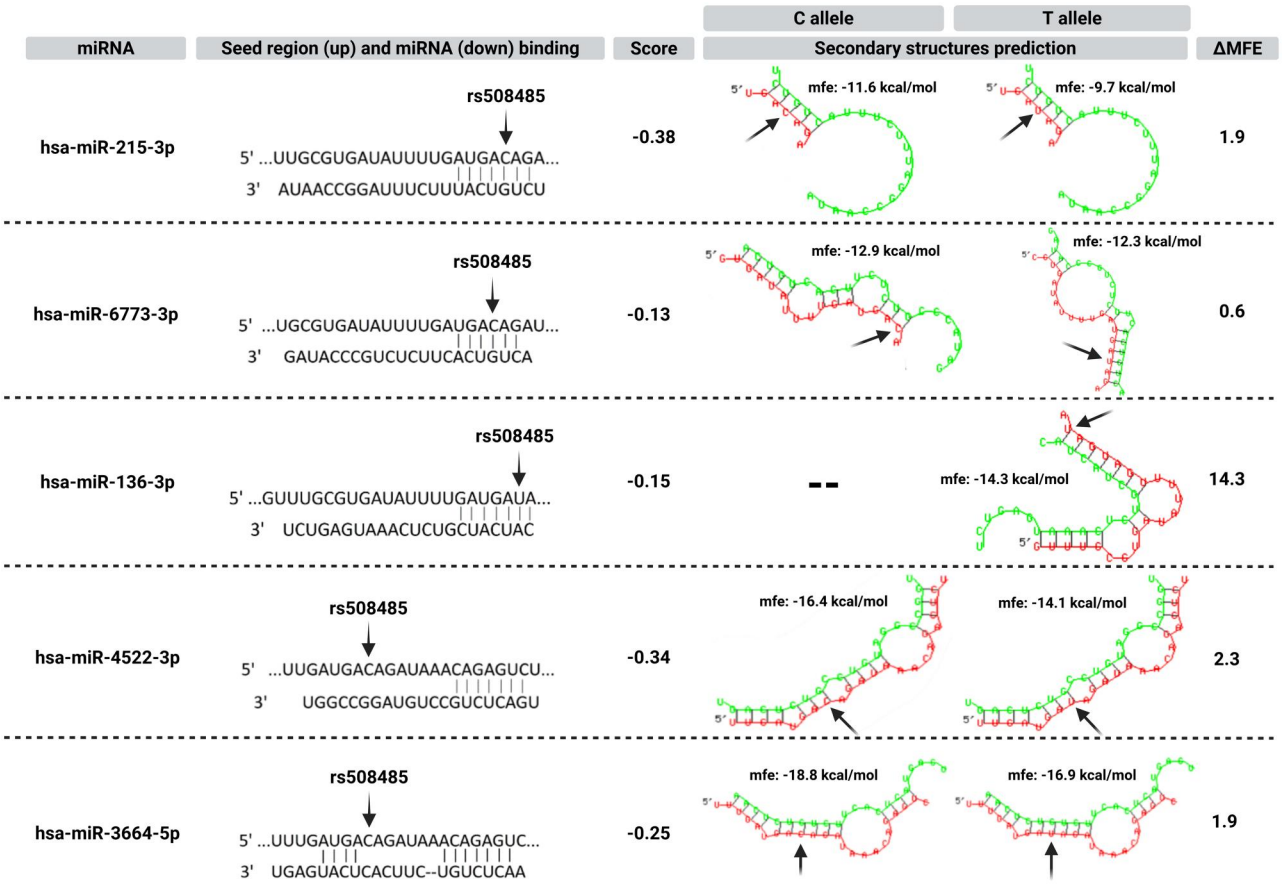

Figure 1. Effect of the rs508485 polymorphism on the binding affinity of various microRNAs (miRNAs) to the 3′-untranslated region of the *PIWIL4* gene. The target RNA and the different miRNAs sequences are shown, with binding base pairs in the seed region represented by vertical line. The polymorphism location within the target sequence is indicated by a black arrow. Predicted scores from TargetScan are displayed, where higher scores (less negative values) indicate stronger predicted repression. The figure also includes secondary structures, minimum free energy (MFE), and minimum free energy differences (ΔMFE) as calculated by the RNAhybrid tool. Lower MFE values indicate stronger miRNA binding affinity, while larger ΔMFE values reflect greater changes in miRNA binding affinity. Figure created in BioRender. Bossini-Castillo, L. (2025) https://BioRender.com/i18e878.

established functionality and it is not expressed in the testis. However, both hsa-miR-215-3p and hsa-miR-136-3p include the polymorphism within their seed regions. Furthermore, they are expressed in the testis (Ludwig *et al.*, 2016) and have been associated with the development of male infertility (Cheng *et al.*, 2017; Zhang *et al.*, 2021). Consequently, the functional assays were exclusively performed with hsa-miR-215-3p and hsa-miR-136-3p miRNAs, in conjunction with a scrambled miRNA as negative control; the sequences are shown in Supplementary Table S3.

Functional validation of the effect of the rs508485 polymorphism on miRNA binding affinity

To further investigate the binding interactions of both hsa-miR-215-3p and hsa-miR-136-3p miRNAs with the seed regions of the *PIWIL4* 3′-UTR according to presence of any of the two rs508485 alleles (T or C), we performed dual-luciferase reporter assays. Different miRNA concentrations (10, 25, and 50 nM) were tested to avoid non-specific effects resulting from cellular instability caused by excessively high miRNA concentrations.

The results showed that the hsa-miR-136-3p miRNA did not bind to the target sequence in the presence of the C allele, corroborating our previous *in silico* predictions (Fig. 2). Conversely, hsa-miR-215-3p exhibited a concentration-dependent decrease in luciferase activity, achieving ∼60% specific inhibition at 50 nM compared to the scrambled miRNA control (Fig. 2). For the *PIWIL4*-3′-UTR region containing the T allele of the rs508485 polymorphism, has-miR-136-3p miRNA achieved ∼75% specific inhibition at 50 nM, whereas hsa-miR-215-3p showed no significant impact on luciferase activity (Fig. 2). As anticipated, no variation in luciferase activity was observed for either miRNA when the mutated *PIWIL4* 3′-UTR sequence was used, due to the absence of binding sites resulting from the loss of the seed regions (Fig. 2).

Discussion

Understanding the genetic basis of SPGF is crucial for enhancing clinical management and genetic counselling for infertile patients (Sharma *et al.*, 2021). SPGF often results from a complex interplay of genetic, epigenetic, and environmental factors (Cervan-Martin *et al.*, 2020). While protein-coding genes were traditionally implicated in spermatogenesis, recent studies emphasize the importance of non-coding RNAs, particularly piRNAs and their associated PIWI proteins, in regulating male germ line development (Gou *et al.*, 2017; Mann *et al.*, 2023). Mutations and polymorphisms in both coding and non-coding genes can significantly impact spermatogenesis, highlighting the need for comprehensive genetic analyses to identify biomarkers for this multifaceted condition.

In the present study, we analysed the role of the rs508485 polymorphism, located in the 3′-UTR region of the *PIWIL4* gene, in the predisposition to SPGF. This SNP has been previously

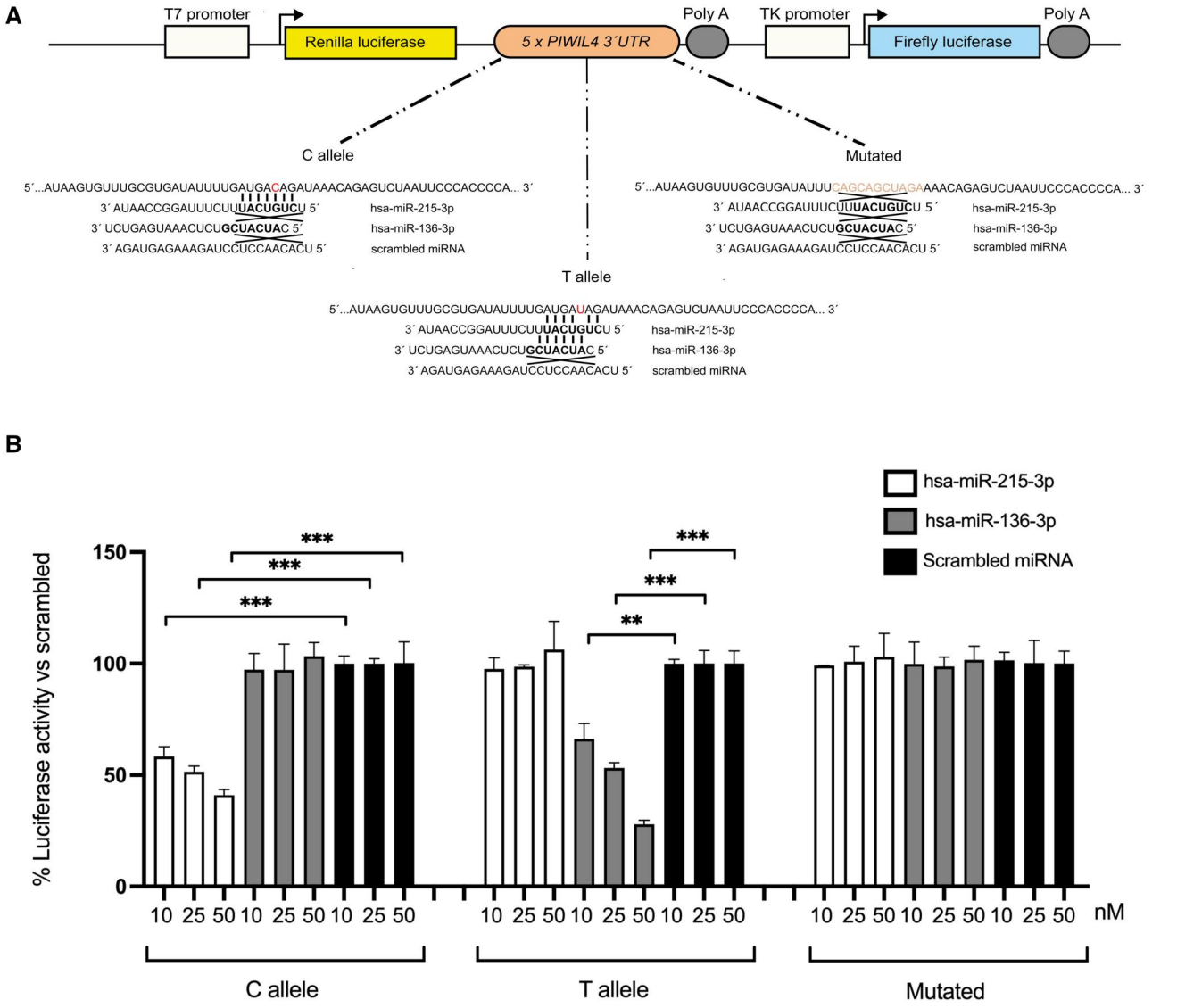

Figure 2. Influence of the rs508485 polymorphism on the binding affinity of hsa-miR-215-3p and hsa-miR-136-3p. (**A**) Schematic representation of psiCHECK-2 luciferase reporter constructs containing the 3′-untranslated regions (UTRs) of *PIWIL4*. The corresponding alleles are highlighted in red, microRNA (miRNA) seed sequences are in bold, and the mutated sequence is in brown. (**B**) Relative luciferase activity from dual-luciferase reporter assays. The percentage of luciferase activity normalized by the scrambled miRNA values versus the concentrations of hsa-miR-215-3p, hsa-miR-136-3p, and scrambled miRNAs (10, 25, and 50 nM) are represented for the presence of the T or C allele of rs508485 and a mutated sequence of *PIWIL4*-3′-UTR region. *5E−02 ≥ *P* > 5E−03; **5E−03 ≥ *P* > 5E−04; ***5E−04 ≥ *P*.

associated with certain patterns of male infertility (Gu *et al.*, 2010; Munoz *et al.*, 2014; Kamaliyan *et al.*, 2017, 2018). Our findings indicated that the T allele of rs508485 is a risk factor for SCO, the most severe form of male infertility, and may also predict unsuccessful TESE outcomes within a European genetic context. While the sample size of our discovery cohort provided sufficient statistical power to detect signals even in sub-phenotype groups of patients, the analysis performed in the replication cohort failed to replicate the observed associations due to a considerable reduction in the statistical power, particularly for the SPGF subgroups. Despite this, the allele effects remained consistent between the studied cohorts, with the T allele increasing the risk for SCO and TESE failure, suggesting an important role of the rs508485 genetic variant in the predisposition to the most severe forms of NOA. Indeed, the results of the meta-analysis showed an improvement in the statistical significance of the observed signals in both independent cohorts.

The results also shed light on the potential impact of the rs508485 genetic variant in SPGF development. The *in silico* analyses underscored its functional relevance, as indicated by its increased scores from multiple impact prediction algorithms and its correlation with active chromatin epigenetic marks. Additionally, the proximity of rs508485 to transcription factor binding sites, such as those for GATA (known to regulate Sertoli cell function and to maintain the spermatogonial stem cell niche; Kyronlahti *et al.*, 2011; Chen *et al.*, 2015) and MEIS1 (which is crucial for Sertoli cell-mediated regulation of male fertility; Sarkar *et al.*, 2021), further highlights its significance in spermatogenesis. Interestingly, rs508485 has been also reported to be associated with bioavailable testosterone levels (Ruth *et al.*, 2020). This steroid hormone, which is essential for testis development and normal spermatogenesis (Makela *et al.*, 2019), plays a critical role in male fertility and reproduction. Indeed, infertile patients with SPGF often exhibit reduced testosterone levels (Lardone *et al.*, 2018), and testosterone treatment is reported to induce elevated

piRNA expression in rats, suggesting that this hormone regulates testicular function through piRNA modulation (Kang *et al.*, 2014). All of this functional evidence reinforces the association between rs508485 and specific patterns of male infertility observed in our study cohort.

Considering that rs508485 is located within the 3′-UTR region of *PIWIL4*, we hypothesized that this SNP affects the post-transcriptional regulation of *PIWIL4* by modifying miRNA binding affinity or accessibility. Dysregulation of gene expression by miRNAs, either through gene silencing by degrading mRNA or by disrupting their translation through base sequence complementarity with the target mRNA, has been linked to different diseases including neurodegenerative disorders, cancer, and cardiovascular conditions (Vishnoi and Rani, 2017). In the context of male infertility, miRNAs have been reported to play a critical role in the spermatogenic process (Procopio *et al.*, 2017), and abnormal expression levels of miRNAs in semen samples have been associated with a reduced sperm count, low motility, and morphological abnormalities (Barbu *et al.*, 2021). Our *in silico* analysis suggested that rs508485 influences miRNA binding affinity for *PIWIL4* transcripts. Specifically, hsa-miR-215-3p miRNAs form a more stable secondary structure when binding to the 3′-UTR region of *PIWIL4* in the presence of the C allele, whereas the T allele introduces a novel binding site for hsa-miR-136-3p. Dual-luciferase reporter assays confirmed these interactions, showing reduced luciferase activity associated with the C allele and hsa-miR-215-3p, and comparable reductions for the T allele and hsa-miR-136-3p.

Interestingly, hsa-miR-215-3p miRNA was found to be underexpressed in patients with SCO compared to fertile controls (Munoz *et al.*, 2015; Zhang *et al.*, 2021), and in individuals with Klinefelter syndrome (who are normally affected by NOA) (Sui *et al.*, 2012). This miRNA is important for regulating cell cycle progression and cellular differentiation (Bueno and Malumbres, 2011; Jones *et al.*, 2015; Vychytilova-Faltejskova and Slaby, 2019; Jia *et al.*, 2022), both of which are critical events in spermatogenesis. Notably, miR-215 is overexpressed in the testes during puberty and adulthood in boars and dears (Xu *et al.*, 2018), suggesting that it may be crucial for spermatogonial differentiation in post-pubertal spermatogenesis.

In contrast, overexpression of hsa-miR-136-3p has been observed in SCO patients compared to unaffected men (Cheng *et al.*, 2017). This miRNA is specifically expressed in spermatogonia (McIver *et al.*, 2012) and, consequently, in the prepubertal testes of mammals (Liu *et al.*, 2022). However, its precise role in male germ cell production remains unclear. It is important to note that *PIWIL4* is expressed from early stages of spermatogenesis (~14 weeks post-fertilization) until puberty (Guo *et al.*, 2021). This timing aligns with the expression patterns of hsa-miR-215-3p and hsa-miR-136-3p miRNAs, as previously described (McIver *et al.*, 2012; Xu *et al.*, 2018; Guo *et al.*, 2021; Liu *et al.*, 2022) (Fig. 3). Taking all of the above into account, we strongly believe that slight alterations in the binding affinity of these miRNAs, caused by the risk allele of rs508485 in *PIWIL4*, lead to dysregulation of pathways involving this gene at crucial stages of germ cell development and differentiation. In this sense, we hypothesize that, in normal spermatogenesis, involving the rs508485 C allele, hsa-miR-136-3p does not recognize *PIWIL4* transcripts. However, hsa-miR-215-3p may downregulate *PIWIL4* expression at the onset of puberty, thereby promoting spermatogonial differentiation and supporting the progression of spermatogenesis (Fig. 3). On the contrary, the T allele introduces a novel binding site for hsa-miR-136-3p, potentially downregulating *PIWIL4* expression early germ cell stages in infants. Simultaneously, the T allele reduces hsa-miR-215-3p binding, impairing its regulatory role in repressing *PIWIL4* expression during puberty and, consequently, hindering the progression of spermatogenesis. This disruption may lead to the activation of transposable elements in pro-spermatogonia, driven by piRNA pathway dysfunction (Aravin *et al.*, 2008; Kuramochi-Miyagawa *et al.*, 2008), ultimately resulting in germ cell depletion and SCO (Fig. 3). Remarkably, the T allele effects over *PIWIL4* expression seem to be aligned with the miRNAs

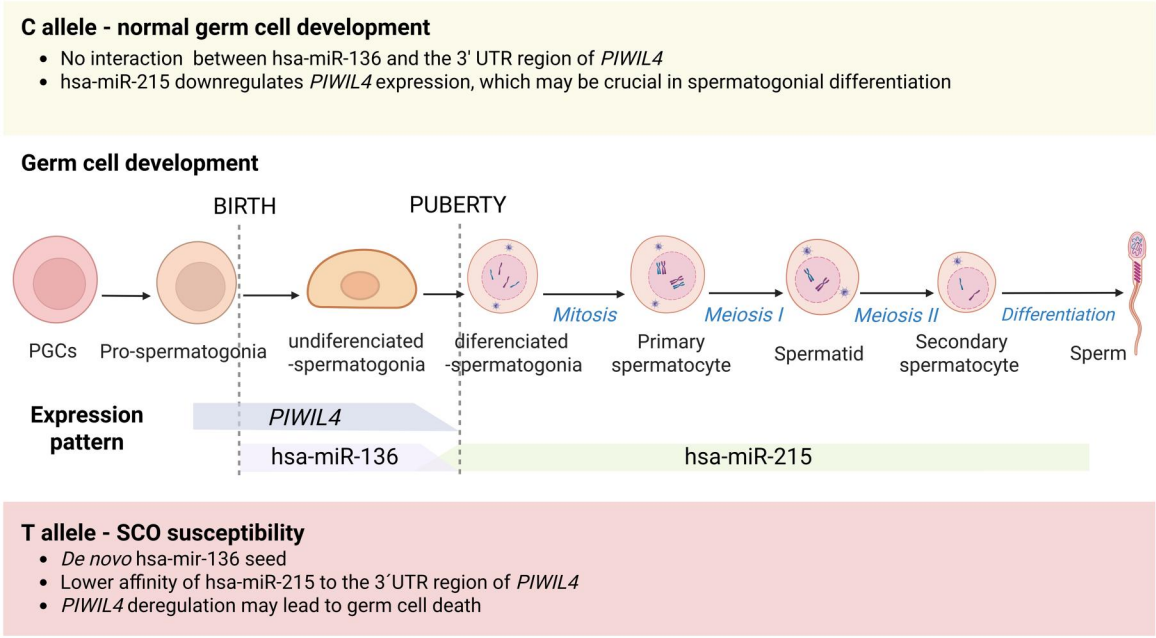

Figure 3. Schematic representation of the hypothetical role of rs508485 in human male germ cell development. Cell types of the male germ cell lineage and the expression pattern of *PIWIL4*, hsa-miR-215-3p, and hsa-miR-136-3p are shown. PGC, primordial germ cell; SCO, Sertoli cell-only. Figure created in BioRender. Bossini-Castillo, L. (2025) https://BioRender.com/rhoyx6n.

expression (up or down) effects in the SCO. This hypothesis is consistent with the association observed in our study cohort between this SNP and the SCO phenotype, and thus an unsuccessful TESE. Due to the complex aetiology of this disease, the rs508485 polymorphism cannot be considered a definitive biomarker for TESE success. However, its role in *PIWIL4* regulation via miRNA interactions highlights a broader genetic influence on spermatogenesis. From a clinical perspective, these findings may contribute to a more comprehensive patient assessment, where rs508485 genotyping could complement established clinical markers, improving the selection of NOA patients for sperm retrieval and enhancing pre-surgical counselling. In fact, disturbances in piRNAs expression profiles in testicular tissues of infertile men have been previously associated with TESE outcome (Cao *et al.*, 2018), and *MIWI2* knockout mice showed loss of germ cell lineages leading to SCO phenotype (Carmell *et al.*, 2007). Moreover, a recently published work also highlights that rare biallelic variants in protein-coding genes involved in piRNAs biogenesis led to defects in spermatogenesis and male infertility (Stallmeyer *et al.*, 2024). Thus, our study not only establishes rs508485 as a genetic susceptibility marker of SCO, but also identifies two miRNAs as potential novel regulators of *PIWIL4*, a critical player in retrotransposition control and spermatogenesis. Our findings align with previous reports suggesting an association between rs508485 and male infertility due to SPGF (Gu *et al.*, 2010; Munoz *et al.*, 2014; Kamaliyan *et al.*, 2017, 2018). However, our study provides additional evidence supporting a more specific role of this variant in SPGF, particularly in the SCO phenotype. While some studies have reported associations between rs508485 and SO (Gu *et al.*, 2010) or MA (Munoz *et al.*, 2014), our results did not support a broader implication of this SNP in other forms of SPGF. This divergence may be due to differences in cohort composition, genetic background, or phenotype characterization based on histopathological analysis across studies. Notably, our study includes the largest cohort of SPGF subtypes to date, which enhances the statistical power of our findings and reinforces their robustness compared to previous reports. Moreover, our functional assays strongly suggest that the impact of rs508485 is mediated through altered miRNA binding to *PIWIL4*, rather than affecting mRNA stability as previously hypothesized (Kamaliyan *et al.*, 2018). By providing functional validation of this mechanism and leveraging a more comprehensive cohort, our study clarifies the molecular role of this SNP and addresses the uncertainty in prior literature.

Nevertheless, this study has some limitations that should be considered when interpreting the results. First, approximately half of the NOA patients in our cohort lacked confirmatory histopathological diagnosis. In this regard, we would like to highlight that our key association was observed in the SCO subgroup, which represents the largest cohort of SCO patients evaluated so far. This strengthens the robustness of our findings despite the histopathological limitation. In any case, independent validation in well-characterized cohorts, including those from diverse ancestries, is warranted to confirm our findings. Additionally, while we provide functional evidence supporting the role of the rs508485 variant in *PIWIL4* regulation, our assays were conducted *in vitro* and focused on a single genetic polymorphism. Further studies using *in vivo* models and exploring additional genetic interactions are necessary to fully elucidate the molecular mechanisms underlying SPGF and to assess their potential implications for clinical practice.

In conclusion, our study provides valuable insights into the intricate molecular mechanisms underlying SCO, the most severe form of SPGF. We establish a link between the rs508485 polymorphism and both SCO and TESE outcome, highlighting the significant role of common genetic variations in the pathogenesis of complex conditions such as idiopathic male infertility. Specifically, by functional validation, we demonstrate that the rs508485 genetic variant may influence miRNA binding to piRNA pathway genes like *PIWIL4*, shedding light on its potential contribution to spermatogenesis defects. These findings advance our understanding of SCO aetiology and suggest promising avenues for developing targeted therapies. By exploring the interaction between genetic predisposition and disease manifestation, our study contributes to the progress of personalized treatment strategies in male infertility. However, further research involving populations of different ancestries, larger cohorts and comprehensive functional analyses, is essential to unravel the biological mechanisms involved in the development of extreme patterns of male infertility and improve patient care outcomes.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data generated in this study are either contained in the article file and its supplementary material or can be made available upon reasonable request to the corresponding author.

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Authors' roles

R.J.P.-M. and F.D.C. were involved in the conception, design, and supervision of the study. S.G.-M., M.C.-M., A.G.-J., A.I.R.-M., and L. B.-C. participated in the methodology. S.G.-M., M.C.-M., A.G.-J., and A.I.R.-M. performed the formal analysis. S.G.-M., M.C.-M., A. G.-J., A.I.R.-M., and M.B. were involved in the interpretation of the data. N.G., J.A.C., M.C.G., A.C., M.M., M.A.V., A.E., V.M., M.L.G.-P., N.G.-G., E.S., C.G., F.Q.-F., S.G., D.A., L.M.-G., Y.O.-G., I.P.-C., G.S. P., A.A., I.S.P., O.L.-R., L.B., S.S., J.G., A.M.L., and S.L. were responsible for study subjects and data recruitment. S.G.-M. and M.C.-M. were involved in the original draft preparation. All authors critically revised and approved the final manuscript.

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Conflict of interest

None declared.

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