OXFORD

human reproduction

Human Reproduction, 2024, **00(0)**, 1–11
https://doi.org/10.1093/humrep/deae007
Original Article

Reproductive genetics

Changes in environmental exposures over decades may influence the genetic architecture of severe spermatogenic failure

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ABSTRACT

STUDY QUESTION: Do the genetic determinants of idiopathic severe spermatogenic failure (SPGF) differ between generations?

SUMMARY ANSWER: Our data support that the genetic component of idiopathic SPGF is impacted by dynamic changes in environmental exposures over decades.

WHAT IS KNOWN ALREADY: The idiopathic form of SPGF has a multifactorial etiology wherein an interaction between genetic, epigenetic, and environmental factors leads to the disease onset and progression. At the genetic level, genome-wide association studies (GWASs) allow the analysis of millions of genetic variants across the genome in a hypothesis-free manner, as a valuable tool for identifying susceptibility risk loci. However, little is known about the specific role of non-genetic factors and their influence on the genetic determinants in this type of conditions.

STUDY DESIGN, SIZE, DURATION: Case-control genetic association analyses were performed including a total of 912 SPGF cases and 1360 unaffected controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All participants had European ancestry (Iberian and German). SPGF cases were diagnosed during the last decade either with idiopathic non-obstructive azoospermia (n = 547) or with idiopathic non-obstructive oligozoospermia (n = 365). Case-control genetic association analyses were performed by logistic regression models considering the generation as a covariate and by *in silico* functional characterization of the susceptibility genomic regions.

MAIN RESULTS AND THE ROLE OF CHANCE: This analysis revealed 13 novel genetic association signals with SPGF, with eight of them being independent. The observed associations were mostly explained by the interaction between each lead variant and the age-group. Additionally, we established links between these loci and diverse non-genetic factors, such as toxic or dietary habits, respiratory disorders, and autoimmune diseases, which might potentially influence the genetic architecture of idiopathic SPGF.

LARGE SCALE DATA: GWAS data are available from the authors upon reasonable request.

LIMITATIONS, REASONS FOR CAUTION: Additional independent studies involving large cohorts in ethnically diverse populations are warranted to confirm our findings.

WIDER IMPLICATIONS OF THE FINDINGS: Overall, this study proposes an innovative strategy to achieve a more precise understanding of conditions such as SPGF by considering the interactions between a variable exposome through different generations and genetic predisposition to complex diseases.

Received: October 31, 2023. **Revised:** January 8, 2024. **Editorial decision:** January 16, 2024.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the "Plan Andaluz de Investigación, Desarrollo e Innovación (PAIDI 2020)" (ref. PY20_00212, P20_00583), the Spanish Ministry of Economy and Competitiveness through the Spanish National Plan for Scientific and Technical Research and Innovation (ref. PID2020-120157RB-I00 funded by MCIN/ AEI/10.13039/501100011033), and the 'Proyectos I+D+i del Programa Operativo FEDER 2020' (ref. B-CTS-584-UGR20). ToxOmics-Centre for Toxicogenomics and Human Health, Genetics, Oncology and Human Toxicology, is also partially supported by the Portuguese Foundation for Science and Technology (Projects: UIDB/00009/2020; UIDP/00009/2020). The authors declare no competing interests.

TRIAL REGISTRATION NUMBER: N/A.

Keywords: male infertility / exposome / GWAS / spermatogenic failure / complex trait / genetics

Introduction

Human complex diseases have a multifactorial etiology wherein a combination of diverse risk factors, including genetic, epigenetic, and environmental factors, drives their onset and prognosis. Consequently, the observed clinical phenotypes are a result of the relatively minor or moderate individual contributions from multiple genetic loci, mediated through intricate gene–gene and gene–environment interactions that exert significant overall effects (Civelek and Lusis, 2014).

At the genetic level, single-nucleotide polymorphisms (SNPs) play a pivotal role in the susceptibility to complex diseases, and the genome-wide association study (GWAS) approach is a highly valuable strategy for analyzing their effects. GWASs enable the simultaneous examination of millions of variants across the genome in a hypothesis-free manner, which has allowed an unprecedented advance in biomedical discoveries over the last decade (Loos, 2020). However, the pathogenic mechanisms underlying the reported GWAS associations are yet to be fully understood in most cases due to the inherent limitations of this methodology. First, the majority of GWAS datasets still lack the necessary statistical power to identify the whole genetic heritability of the studied traits. Secondly, complex traits often exhibit a wide range of clinically diverse phenotypes, and traditional case-control GWASs may not adequately capture the multifaceted etiology of such heterogeneity. Thus, it may be worthwhile to reassess the study design of GWASs and consider more comprehensive case-case comparisons (Ishigaki, 2022). Additionally, there is a diversity of potential statistical models that could explain the missing heritability of complex traits using available GWAS data, through the examination of the interaction between genetic variants and cultural and environmental factors (Cloninger *et al.*, 1979; Arnedo *et al.*, 2015; Selzam *et al.*, 2017; Feldman and Ramachandran, 2018).

In this sense, it is important to emphasize the crucial impact of the environmental triggers on the emergence of complex diseases. An increasing number of studies have established a correlation between numerous environmental risk factors or exposures and the development of this type of condition (Toskala and Kennedy, 2015; Bellou *et al.*, 2016; Badimon *et al.*, 2019). Thus, in 2005, the concept of 'exposome' emerged to define all environmental exposures experienced by an individual from conception to demise (Wild, 2005). The study of the exposome is indispensable to comprehensively capture the diversity and range of exposures to synthetic chemicals, dietary constituents, psychosocial elements, and physical factors, alongside their corresponding biological responses (Vermeulen *et al.*, 2020). In addition, given the increasing recognition of the dominant role of non-genetic factors in numerous diseases, it is essential to comprehend the exposome on a scale comparable to the current understanding of the human genome. This understanding would enable us to gain insight into gene–environment interactions in conditions in which the etiology is far from being completely understood (Vermeulen *et al.*, 2020).

Numerous longitudinal studies have explored the exposome changes in recent years. In the last decade, relevant factors for health, such as a traditional diet, have been swapped for new habits. Young people appear to be particularly affected by these changes (Kearney, 2010; Fang *et al.*, 2023). For instance, the Spanish population seems to have adopted a less diverse diet, showing a trend toward an increasingly 'Westernised' diet from the traditional Mediterranean diet (Partearroyo *et al.*, 2019). Another critical factor that has changed significantly in recent times is air pollution levels. Overall, both air pollution levels and the associated morbidity and mortality have considerably decreased in Europe over the last three decades. However, due to the substantial growth of the European population, air pollution remains a significant public health concern today (Maynard, 2015; Juginovic *et al.*, 2021).

Nowadays, the link between the exposome and male infertility is a topic of interest in the field of human reproduction, mostly due to the suspicion that environmental factors may be responsible for the alarming reduction in semen quality and other indicators of male reproductive health (Skakkebaek *et al.*, 2016; Levine *et al.*, 2023). Additionally, the decrease in sperm count is linked to a reduction in testosterone levels and an increase in occurrences of testicular cancer and anomalies in male genitalia, particularly in developed societies (Skakkebaek *et al.*, 2016). Although male infertility appears to be influenced by four main causes (i.e. biological/physiological/genetic factors, behavioral/lifestyle risks, environmental factors, and sociodemographic risks), substantial evidence on the specific causes within each category is still lacking, creating gaps for future research and experiments (Okonofua *et al.*, 2022). Two of the most extreme phenotypes of male infertility are severe non-obstructive oligozoospermia (NOSO, very low concentration of spermatozoa in semen) and non-obstructive azoospermia (NOA, complete lack of sperm in the ejaculate due to non-obstructive causes), which are characterized by severe spermatogenic failure (SPGF) caused, in some patients, by Mendelian genetic alterations (such as point mutations in genes crucial to male gametogenesis, Y-chromosome microdeletions, and karyotype abnormalities) (Gunes and Esteves, 2021). However, the etiology remains unknown in most SPGF cases and there is increasing evidence suggesting that an important proportion of idiopathic male infertility represents a complex trait, in which common genetic variation in the human genome may contribute to genetic susceptibility in combination with environmental factors (Cervan-Martin *et al.*, 2020).

In a previous study, we performed the first GWAS of SPGF in a large case-control cohort of European origin, which yielded highly promising findings that support the concept of idiopathic SPGF as a complex trait (Cervan-Martin *et al.*, 2022a). Using the data generated in such study, we decided to further investigate the possible impact of the exposome in the predisposition to this condition through its interaction with the genetic variation in the human genome. Our hypothesis was that SPGF could be influenced by environmental exposures that may change over

decades, thus modifying the genetic architecture of the multifactorial form of SPGF across generations.

Materials and methods

Study population

The study cohort comprised all individuals of our previous SPGF GWAS with documented date of birth (i.e. 912 SPGF cases and 1360 unaffected men from the Iberian Peninsula and Germany) (Cervan-Martin *et al.*, 2022a). All cases were diagnosed either with idiopathic NOA, if a complete absence of spermatozoa was observed in the ejaculate (n = 547), or with idiopathic NOSO, if they exhibited <5 million spermatozoa per milliliter of semen (n = 365), as described elsewhere (Cervan-Martin *et al.*, 2022b). Before enrolling in the study, all participants provided informed written consent, and their DNA samples were irreversibly anonymized. The research procedures adhered to the principles outlined in the Declaration of Helsinki and were approved by the Ethics Committee 'CEIM/CEI Provincial de Granada' (Andalusia, Spain) during the session held on 26 January 2021 (approval number: 1/21). Additionally, each participating center obtained ethical approval and complied with the regulations set by their respective local regulatory authorities.

All the available information about the main clinical features of our study cohort is shown in Supplementary Table S1.

Study design and statistical analysis

In the present study, and following the STREGA reporting guidelines (Little *et al.*, 2009), we reanalyzed the data of our SPGF GWAS (Cervan-Martin *et al.*, 2022a) using age as a proxy for exposure to different environmental factors over time. We stratified the study cohort into two age groups: Group 1 included a population of men over 40 years old in 2023, i.e. those born before 1983 (including 452 cases and 936 controls), and Group 2 comprised a population of men under 40 years old (including 460 cases and 424 controls), i.e. those born after 1983. This division based on age groups relies on the premise that populations of similar age will somehow have a similar exposome that might differ between different age groups, allowing us to evaluate our working hypothesis under the study design illustrated in Fig. 1.

The statistical analyses were performed using R packages. The effects of the different covariates on the case-control status were tested using generalized linear models (GLMs) as implemented in the glm() function included in the stats package (version 3.6.2). The SNP covariate was defined by allele dosage, age-group was established as a categorical covariate, and different covariates were included in the models described in Table 1. Briefly, Model 1 included the SNP, geographical origin (Spain or Germany), and the first 10 genetic principal components as covariates, assuming an additive effect model for genetic risk. Model 2 included all the covariates in Model 1 plus the age-group as a new covariate. Model 3 included the all covariates in Model 2, but we additionally incorporated an interaction term between the age-group and the SNP. The significance of the effects for the SNP and age-group covariates were subsequently calculated in each model. Finally, the contribution of the interaction term to increase the goodness of fit of Model 3 was tested using an ANOVA comparison and establishing Model 2 as the null model. Significant effects for the covariates in the GLMs were defined by a nominal *P*-value <0.05, and a *P*-value <0.0001 was established as a significance threshold for the goodness of fit ANOVA test based on the Bonferroni correction method for the number of independent tests.

SNP to gene prioritization of associated loci

We assessed the functional implications of the selected top signals by using various bioinformatics tools and exploring publicly available annotation data of the human genome.

Initially, we analyzed whether the associated variants located in the same chromosome were proxies for each other using LDmatrix (i.e. their alleles showed values of D' = 1 and $r^2 > 0.8$) (Supplementary Fig. S1) (Machiela and Chanock, 2015). For those variants that were proxies, we evaluated their potential to have a regulatory function according to the forge scores. Within the same haplotypic block, the variant with the highest forge score was selected for downstream functional analysis.

Then, we employed BRAVO (https://bravo.sph.umich.edu/freeze5/hg38/), GTEx (v8) (GTEx Consortium, 2020), and Open Target Genetics Portal (Ghoussaini *et al.*, 2021) to extract detailed information regarding the associated variants. Subsequently, using the Open Target Genetics Portal (Ghoussaini *et al.*, 2021), we identified the genes functionally implicated by these variants. To further identify functional evidence suggesting a significant role of these genes in fertility, we consulted different databases. We used the GeneCards platform (Stelzer *et al.*, 2016) to explore the function of each gene and to determine whether they had been previously implicated in biological processes relevant to male fertility, such as blood–testicular barrier maintenance or spermatogenesis, among others. Furthermore, we leveraged the data available in the Human Protein Atlas (Uhlen *et al.*, 2015; Karlsson *et al.*, 2021) to analyze whether these genes are expressed in the testicle and to determine, based on single-cell data available on this platform, the specific testicular cell types where they were most highly expressed.

Moreover, we conducted an exhaustive bibliographic search to review existing literature and to identify any evidence suggesting that these genes might indeed play a role in male infertility or were relevant to testicular function. We applied various search terms, including the names of the candidate genes, as well as terms like 'male infertility' or 'testis', to broaden our investigation.

Finally, we conducted a prioritization of genes based on suggestive evidence of playing a role in male reproductive function (either by exploring the data from GeneCards (Stelzer *et al.*, 2016) or by performing bibliographic searches), and on their reported expression in the testis according to the Human Protein Atlas (Uhlen *et al.*, 2015; Karlsson *et al.*, 2021). The selected genes were included in an extensive search in the GWAS Catalog (Sollis *et al.*, 2023) in an attempt to find any possible link between such genes and potential risk factors for male infertility due to idiopathic SPGF. Traits were prioritized based on their frequency of occurrence, the number of genes associated with them, and the number of independent studies in which the gene-trait relationship was reported.

Results

Testing for association with idiopathic spermatogenic failure

Thirteen associations were identified by applying a stepwise selection of SNPs, as follows: (i) variants showing a significant nominal association with SPGF, this is, a SNP *P*-value <0.05 in Model 1 (fulfilled by 347 017 variants); (ii) variants showing also a significant nominal effect of the interaction between the SNP and the age-group covariate on SPGF susceptibility, i.e. *P*-value <0.05 for the interaction term in Model 3 (fulfilled by 18 317 of the previously selected SNPs); (iii) variants also showing a study-wide

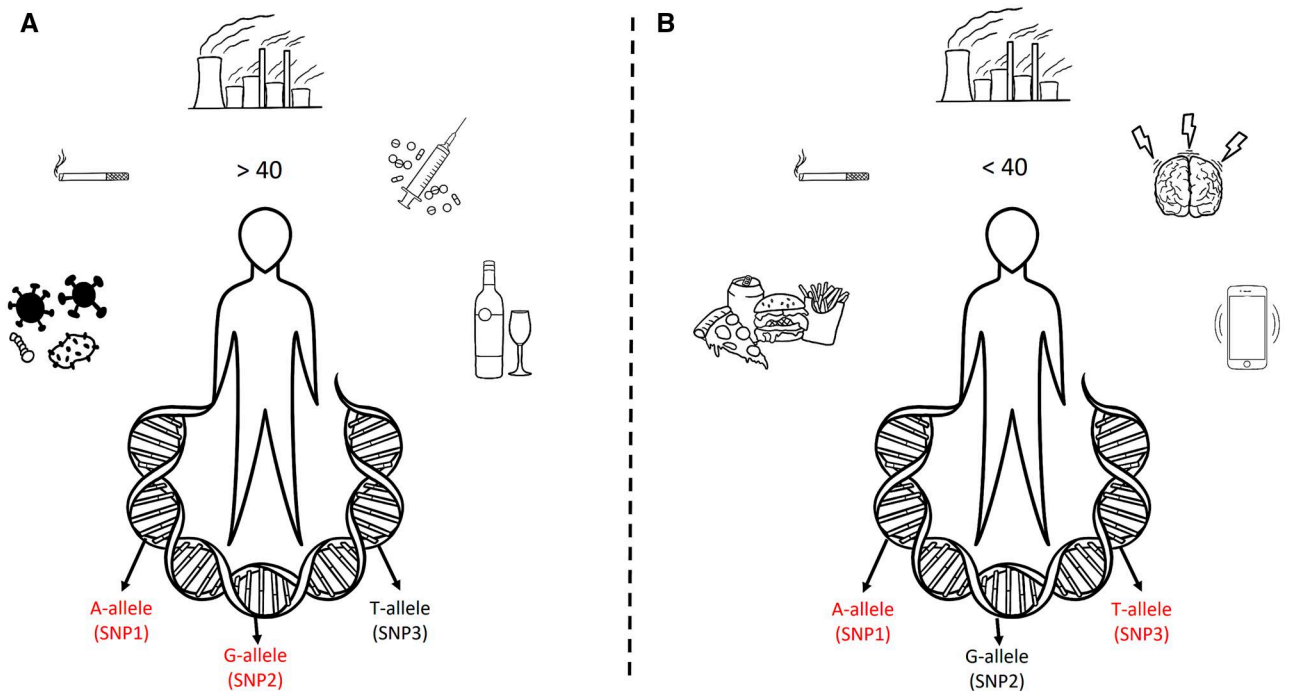

Figure 1. Schematic representation of the possible influence of environmental factors on the genetic architecture of complex diseases. (A) Exposure to environmental factors that men born during the 70s and the early 80s have faced during their lifetime and specific associated variants for their generation. (B) Exposure to environmental factors that men born during the late 80s and the 90s may have faced during their lifetime and specific associated variants for their generation. Risk alleles in each age-group are highlighted in red. SNP, single-nucleotide polymorphism.

Table 1. Statistical models applied in this study.

Model	Formula
1	$Y\ (\text{status}) \sim \beta_1{*}\text{SNP} + \beta_2{*}\text{CountryOfOrigin} + \beta_{(3\ \text{to}\ 13)}{*}\text{PC}(1\ \text{to}\ 10)$
2	$Y\ (\text{status}) \sim \beta_1{*}\text{SNP} + \beta_2{*}\text{CountryOfOrigin} + \beta_{(3\ \text{to}\ 13)}{*}\text{PC}(1\ \text{to}\ 10) + \beta_{14}{*}\text{AgeGroup}$
3	$Y\ (\text{status}) \sim \beta_1{*}\text{SNP} + \beta_2{*}\text{CountryOfOrigin} + \beta_{(3\ \text{to}\ 13)}{*}\text{PC}(1\ \text{to}\ 10) + \beta_{14}{*}\text{AgeGroup} + \beta_{15}{*}\text{SNP}{*}\text{AgeGroup}$
ANOVA model comparison	Model 3 vs Model 2 (null) = P-value <0.0001

PC, principal component; SNP, single-nucleotide polymorphism.

significant contribution of the interaction between the SNP and the age-group covariate to better explain the variance between SPGF cases and controls. In this last selection step, we used an ANOVA test to compare the goodness of fit of the model considering the interaction between SNP and age-group (Model 3) versus the model lacking this interaction term (Model 2, which was established as the null model), and we found a significant improvement for 13 SNPs (*P*-value <0.0001) (Table 2, Supplementary Table S2).

The interaction between the SNP and the age-group seemed to play a crucial role in explaining the 13 observed associations, as the interaction term showed highly significant *P*-values for all of them (Table 2). However, the minor allele effects for five of them were related to an increased SPGF susceptibility in the youngest age-group ($\beta_{SNP*age} > 0$), while the remaining eight SNPs showed an increased SPGF susceptibility in the oldest age-group ($\beta_{SNP*age} < 0$) (Table 2).

SNP to gene prioritization of associated loci

Of the 13 selected variants, nine were intronic, three were intergenic, and one was a non-coding transcript exon variant. The 13 variants corresponded to eight independent signals (Supplementary Fig. S1). Hence, for each signal, we identified the haplotype region including the lead variant and its proxies ($r^2 > 0.8$). Then, we defined the most probable affected genes as implemented in the 'SNP to gene' pipeline in the Open Targets Genetic portal (in which the associated haplotype region is referred to using the lead SNP ID), as shown below.

Regarding eQTL effects, rs115408081 was reported to influence *LINGO1* gene expression in the testicular tissue of the GTEx population (GTEx Consortium, 2020). Additionally, we observed that rs75938373 and rs61997636 were both associated with sex hormone-binding globulin levels adjusted for body mass index according to Open Target Genetics (Ghoussaini *et al.*, 2021). Furthermore, rs61997636 also showed an association with arm fat mass, while rs74514513 exhibited associations with traits such as calcium levels, HDL cholesterol levels, apolipoprotein A1 levels, and creatinine levels. The rs144324356 variant was also associated with strong meridian, weak meridian, mean platelet volume, white blood cell count, and mean platelet (thrombocyte) volume. We did not find any significant associations with traits for the remaining variants.

Subsequently, using the Variant-to-Gene (V2G) tool of Open Target Genetics (Ghoussaini *et al.*, 2021), we identified 62 candidate genes potentially involved in SPGF development (Fig. 2 and Supplementary Table S3). According to GeneCards (Stelzer *et al.*, 2016), 21 of them play a role in biological processes relevant to male fertility, such as blood–testicular barrier maintenance or

Table 2. Genetic variants associated with spermatogenic failure (SPGF) in this study.

			Model 1			Model 2						Model 3									
			SNP			SNP			Age-group			SNP			Age-group			SNP*Age-group			Model 3 vs Model 2
SNP coordinates (GRCh38)	SNP ID	MA	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value[1]
chr2:60077559	rs72818509	T	7.60E−03	0.45	0.17	8.52E−03	0.44	0.17	1.10E−04	0.42	0.11	6.71E−01	−0.09	0.22	5.24E−03	0.31	0.11	2.19E−05	1.91	0.45	2.59E−06
chr8:130011544	rs144324356	G	3.59E−02	−0.20	0.09	3.26E−02	−0.20	0.09	9.07E−05	0.42	0.11	2.03E−01	0.16	0.12	1.13E−07	0.63	0.12	2.37E−05	−0.80	0.19	2.18E−05
chr10:54467592	rs57701320	G	3.84E−02	−0.30	0.14	3.72E−02	−0.30	0.14	9.60E−05	0.42	0.11	5.73E−05	−0.89	0.22	6.94E−03	0.30	0.11	5.20E−05	1.27	0.31	2.68E−05
chr10:54470332	rs79733930	T	4.82E−02	−0.28	0.14	4.68E−02	−0.28	0.14	9.63E−05	0.42	0.11	9.47E−05	−0.85	0.22	6.41E−03	0.30	0.11	7.81E−05	1.23	0.31	4.30E−05
chr10:54477211	rs77137582	T	2.69E−02	−0.32	0.14	2.65E−02	−0.32	0.14	9.75E−05	0.42	0.11	6.23E−05	−0.89	0.22	5.58E−03	0.31	0.11	1.04E−04	1.22	0.31	5.87E−05
chr11:83072772	rs3763812	T	8.34E−03	0.30	0.11	8.09E−03	0.30	0.11	9.63E−05	0.42	0.11	5.41E−06	0.66	0.15	7.30E−07	0.57	0.11	8.61E−05	−0.91	0.23	8.31E−05
chr13:98781888	rs75938373	A	2.51E−02	0.36	0.16	2.36E−02	0.36	0.16	9.36E−05	0.42	0.11	3.45E−01	−0.21	0.22	7.07E−03	0.30	0.11	3.56E−05	1.56	0.38	1.12E−05
chr14:104147826	rs61997636	A	7.73E−03	−0.22	0.08	8.51E−03	−0.22	0.08	1.09E−04	0.42	0.11	5.73E−01	0.06	0.11	1.79E−07	0.64	0.12	1.00E−04	−0.67	0.17	9.41E−05
chr15:77833084	rs111364930	G	2.65E−02	0.36	0.16	2.51E−02	0.37	0.16	9.42E−05	0.42	0.11	1.65E−05	0.96	0.22	1.89E−06	0.53	0.11	9.63E−05	−1.28	0.33	9.18E−05
chr15:77834415	rs115408081	A	1.03E−02	0.39	0.15	1.01E−02	0.39	0.15	9.71E−05	0.42	0.11	3.68E−06	0.93	0.20	1.22E−06	0.55	0.11	5.77E−05	−1.21	0.30	5.66E−05
chr17:39420859	rs142908940	A	2.31E−02	−0.50	0.22	2.03E−02	−0.51	0.22	8.72E−05	0.42	0.11	4.12E−01	0.22	0.27	5.31E−06	0.50	0.11	1.76E−04	−1.76	0.47	9.43E−05
chr17:39477522	rs144031067	A	2.31E−02	−0.50	0.22	2.03E−02	−0.51	0.22	8.72E−05	0.42	0.11	4.12E−01	0.22	0.27	5.31E−06	0.50	0.11	1.76E−04	−1.76	0.47	9.43E−05
chr17:39502932	rs74514513	C	2.31E−02	−0.50	0.22	2.03E−02	−0.51	0.22	8.72E−05	0.42	0.11	4.12E−01	0.22	0.27	5.31E−06	0.50	0.11	1.76E−04	−1.76	0.47	9.43E−05

P-values, beta for the minor allele, and standard errors (SE) are shown for the different variables analyzed under the three considered models, i.e. single-nucleotide polymorphism (SNP), age-group, and SNP-age-group interaction (SNP*Age-group).

[1] The *P*-value for the improvement in the goodness of fit of Model 3 over Model 2 through an ANOVA comparison is also indicated. GRCh38, Genome Reference Consortium Human Build 38; MA, minor allele; MAF, minor allele frequency; ID, identifier.

Figure 2. Heatmap of the functional relevance of the variants associated with severe spermatogenic failure in this study. Different colors are used to highlight specific features: blue for genes that have a potential role in male reproductive function according to the GeneCards database; a pink gradient to represent the relative bulk testis expression of the gene according to the Human Protein Atlas database (dark pink indicates higher expression); a green gradient to represent the cell types where the reported gene is expressed in the testis according to single-cell data available in the Human Protein Atlas database (the green gradient represents differences in the expression levels of each gene, with dark green indicating the highest expression level); yellow for genes with strong evidence of playing a role in male reproductive function according to the literature. GRCh38, Genome Reference Consortium Human Build 38; SNP, single-nucleotide polymorphism; ID, identifier.

spermatogenesis, among others (blue color in Fig. 2). Furthermore, we leveraged the data available in the Human Protein Atlas portal (Uhlen *et al.*, 2015; Karlsson *et al.*, 2021) finding that most of these genes were expressed in the testis. Specifically, 21 showed high gene expression, 20 showed medium gene expression, and 17 showed low gene expression (light to dark pink gradient in Fig. 2). Regarding their single-cell expression, we found that most of the candidate genes showed differential expression between the different germ cell types in which they are expressed, without observing an enrichment in any specific cell type regarding an increased or decreased expression of the entire set of candidate genes (light to dark green gradient in Fig. 2). Finally, by conducting an exhaustive bibliographic search, we found evidence suggesting that 27 of the 62 genes

were involved in male reproductive function (yellow in Fig. 2). Supplementary Table S3 contains all the detailed information used to generate Fig. 2.

Following the *in silico* functional analysis, our next goal was to prioritize genes with a higher probability of being involved in reproductive function based on the data presented in Fig. 2 and Supplementary Table S3, and following the criteria described in the Materials and methods section. This was a crucial step, as the main objective of our study was to evaluate the possible influence of environmental factors on the genetic architecture of SPGF. Thus, delving into the potential impact of genes affected by our associated variants on other traits could provide valuable insight into the etiology of idiopathic SPGF. This prioritization resulted in a total of 40 genes being included for the detailed search in the GWAS Catalog (Sollis *et al.*, 2023).

To analyze the data available in the GWAS Catalog (Sollis *et al.*, 2023), we downloaded the information gathered in this platform for the 40 selected genes. We then prioritized the top 10 traits that were most likely to have a potential correlation with SPGF development due to their association with several of the previously prioritized genes (Table 3). Interestingly, the top 10 traits could be classified into various categories, from toxic habits, such as smoking and alcohol consumption, to respiratory system-related terms like asthma, lung function, and coronavirus disease 2019 (COVID-19). Additionally, we observed that several loci had been previously associated with dietary habits, which were represented with terms such as cholesterol levels, body mass index, and type 2 diabetes. The list of putative exposures for SPGF also includes systemic lupus erythematosus, which is an autoimmune disease, and educational attainment. By examining the genes correlated with the predisposition to develop SPGF in our study, and observing their association with other traits based on the GWAS Catalog data (Sollis *et al.*, 2023), we established a potential connection between these top 10 traits and predisposition to develop SPGF.

Discussion

Our results suggest that changes in the exposome between generations could impact the genetic component of a complex form of male infertility due to SPGF. Indeed, we successfully identified eight independent genetic loci that are associated with SPGF susceptibility in age-specific study groups and established connections between such associations and different non-genetic factors.

Some of the SPGF variants with effect on the older age-group show a clear link to male infertility. For example, one of the related superpathways of *LINGO1* (whose expression is affected by rs115408081) is actin cytoskeleton regulation via Rho GTPases, which include important pathways for the maintenance of the blood–testis barrier. Other male reproductive function-related genes influenced by the SPGF associated variants identified here are *TDRD9* (affected by rs61997636), which is involved in spermatogenesis and has been implicated in spermatogenic impairment (Shoji *et al.*, 2009; Arafat *et al.*, 2017; Babakhanzadeh *et al.*, 2020; Kherraf *et al.*, 2022), and *ZPBP2* (affected by rs142908940), which is predicted to be involved in acrosome assembly and binding activity of sperm to the zona pellucida (Lin *et al.*, 2007; Ghanami Gashti *et al.*, 2021; Shen *et al.*, 2022).

Topics linked with lifestyle were also amongst the top ten related traits with the SPGF-associated variants, including cholesterol levels, type 2 diabetes, and body mass index (all three related with dietary habits), smoking and alcohol consumption (both related with bad lifestyle habits), and educational attainment. Interestingly, type 2 diabetes has been correlated with a higher risk of male infertility in numerous studies (Dai *et al.*, 2022; Diniz *et al.*, 2022; Leisegang, 2022; Kumar *et al.*, 2023). Likewise, alterations in cholesterol levels have also been previously associated with male infertility. Specifically, it has been reported that a correlation exists between oxidative stress caused by dyslipidemia and impaired sperm function (Saez and Drevet, 2019; Aitken *et al.*, 2022b). Both type 2 diabetes and cholesterol levels are strongly influenced by dietary habits (Schwingshackl *et al.*, 2017; Clifton, 2019) and several studies have reported a considerable change in dietary habits and lifestyle over the last few decades (Kearney, 2010; Partearroyo *et al.*, 2019; Fang *et al.*, 2023). Thus, the older age population is expected to have had different overall dietary habits and lifestyle during their early life compared to the younger age population, and we speculate that such disparities could reshape the genetic landscape of SPGF. Nevertheless, elevated body mass index, which have been associated with reproductive issues in both men and women (Talmor and Dunphy, 2015; Durairajanayagam, 2018), is associated in both the older and the younger age-group, suggesting that increased body mass index may be detrimental to fertility in different contexts independently from other exposome factors. Similarly, unhealthy lifestyle habits do not correlate with a specific age-group, having a negative impact on reproductive function independently of the generation. There is strong evidence that smoking or alcohol consumption have a damaging influence on male fertility (Wu *et al.*, 2016; Sansone *et al.*, 2018; Bisconti *et al.*, 2021; Finelli *et al.*, 2021; Basic *et al.*, 2023). Finally, although there is no clear relationship in the literature between educational attainment and a higher risk of reproductive disorders, it has been reported that socioeconomic status is related to a higher risk of developing mental and physical health problems (Kivimaki *et al.*, 2020).

On the other hand, it is noteworthy that some of the top 10 traits have a clear association with the immune system and inflammatory processes, such as asthma, systemic lupus erythematosus, lung function, or viral respiratory disorders. Cumulating knowledge clearly suggests that a subset of idiopathic SPGF cases may have an autoimmune origin (Hussein *et al.*, 2005; Spiess *et al.*, 2007; Hedger, 2011; Gong *et al.*, 2020). For example, different GWASs of SPGF in Europeans and Asians showed a significant association between specific SPGF patterns (such as NOA or Sertoli cell-only syndrome) and the human leukocyte antigen class II system (Hu *et al.*, 2011; Zhao *et al.*, 2012; Hu *et al.*, 2014; Cervan-Martin *et al.*, 2022a). Our data provide further evidence of the link between the immune system and idiopathic SPGF.

Respiratory problems have been shown to play a significant role in fertility. For instance, in the case of COVID-19, immediately after infection, semen quality appears to be suppressed through mechanisms that lower testosterone levels and disrupt all aspects of semen profile. These effects on semen quality seem to subside over time, thus affecting the population temporarily due to the infection and the inflammatory process, but independently of their age (Hajizadeh Maleki and Tartibian, 2021; Aitken, 2022a; Martinez *et al.*, 2023). However, due to the recent emergence of this disease, evaluating the impact of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposure on the genetic component of SPGF is not feasible yet. Future studies in the coming years are definitively warranted to evaluate this possible effect. Furthermore, air pollution, which clearly affects lung function (Kurt *et al.*, 2016; Bowatte *et al.*, 2017), has been

Table 3. Genome-wide association study (GWAS) Catalog annotation for the 40 prioritized genes through the single-nucleotide polymorphism (SNP) to gene analysis of this study.

Top 10 related traits	Genes associated with the top 10 traits (number of associations)	Study accession numbers of the gene-traits associations in GWAS Catalog
Smoking	*PCDH15 (12)*, *KLC1 (8)*, *KIF26A (6)*, *LINGO1 (5)*, *PPP1R1B (5)*, *BCL11A (5)*, *ZFYVE21 (4)*, *CDK12 (3)*, *STARD3 (2)*, *CYRIB (2)*, *FBXL20 (1)*, *PNMT (1)*, *PGAP3 (1)*	GCST007085, GCST007327, GCST007459, GCST007462, GCST007464, GCST007468, GCST007474, GCST009096, GCST90243968, GCST90243972, GCST90243980, GCST90243985, GCST90243986, GCST90243988
Asthma	*PGAP3 (24)*, *ZPBP2 (23)*, *IKZF3 (16)*, *MED1 (6)*, *FBXL20 (5)*, *GRB7 (3)*, *ERBB2 (3)*, *PPP1R1B (2)*, *STARD3 (2)*, *CDK12 (1)*, *PNMT (1)*, *TCAP (1)*	GCST002322, GCST002875, GCST007994, GCST007995, GCST008838, GCST008916, GCST009798, GCST009841, GCST009845, GCST009850, GCST011457, GCST011975, GCST90018575, GCST90095142, GCST90103427, GCST90244761
Alcohol consumption	*KIF26A (4)*, *CDK12 (4)*, *STARD3 (4)*, *FBXL20 (4)*, *PCDH15 (2)*, *HMG20A (2)*, *LINGO1 (2)*, *MED1 (1)*, *ASAP1 (1)*, *PEAK1 (1)*, *TCAP (1)*	GCST004956, GCST006633, GCST007461, GCST007472, GCST008757, GCST010544, GCST90032654, GCST90101760, GCST90132926, GCST90243984, GCST90243989
Body mass index	*LINGO1 (10)*, *KLC1 (9)*, *BCL11A (5)*, *TSPAN3 (4)*, *PCDH15 (3)*, *PEAK1 (2)*, *ZFYVE21 (1)*, *CYRIB (1)*, *HMG20A (2)*, *KIF26A (1)*	GCST002783, GCST004904, GCST005951, GCST006368, GCST007039, GCST008156, GCST008161, GCST009003, GCST009004, GCST009530, GCST009867, GCST009871, GCST012227, GCST90012108, GCST90012110, GCST90018947, GCST90020028, GCST90179150, GCST90255621, GCST90267268
Educational attainment	*BCL11A (18)*, *LINGO1 (14)*, *CYRIB (7)*, *KLC1 (6)*, *HMG20A (3)*, *PPP1R1B (3)*, *FBXL20 (2)*, *ASAP1 (1)*, *PCDH15 (1)*	GCST003676, GCST003677, GCST006442, GCST006571, GCST007037, GCST008595, GCST90011874, GCST90011875, GCST90027241, GCST90095190, GCST90100570, GCST90105038
COVID-19	*BCL11A (3)*, *IKZF3 (2)*, *ZPBP2 (1)*, *FBXO47 (1)*, *SLC15A1 (1)*	GCST90102529, GCST90102530, GCST90104034, GCST90104716, GCST90104723, GCST90255357, GCST90255367, GCST90255368, GCST90255369
Cholesterol levels	*STARD3 (34)*, *PNMT (2)*, *TCAP (2)*, *GRB7 (2)*, *MIEN1 (2)*	GCST000755, GCST002223, GCST004232, GCST006611, GCST007140, GCST007282, GCST008070, GCST008075, GCST008084, GCST008085, GCST008685, GCST009368, GCST010242, GCST90018956, GCST90018974, GCST90019510, GCST90092822, GCST90092824, GCST90092844, GCST90092845, GCST90092848, GCST90092892, GCST90092896, GCST90092897, GCST90093004, GCST90093009, GCST90239649, GCST90239651, GCST90239652, GCST90239655, GCST90239658, GCST90239673, GCST90244005
Lung function	*CDK12 (3)*, *PSMB3 (3)*, *FBXL20 (2)*, *MED1 (2)*, *TSPAN3 (2)*, *ASAP1 (1)*, *BCL11A (1)*	GCST004183, GCST006481, GCST006483, GCST007080, GCST007081, GCST007429, GCST007431, GCST90244094, GCST90267982
Systemic lupus erythematosus	*IKZF3 (4)*, *CDK12 (1)*, *ERBB2 (1)*, *HMG20A (1)*, *LINGO1 (1)*, *MED1 (1)*, *MIEN1 (1)*	GCST002069, GCST002463, GCST003155, GCST003156, GCST003622, GCST005752, GCST008729, GCST011096
Type 2 diabetes	*HMG20A (28)*, *LINGO1 (15)*, *CDK12 (3)*, *TSPAN3 (1)*	GCST001033, GCST001213, GCST001550, GCST002352, GCST004894, GCST005047, GCST009379, GCST010118, GCST010553, GCST010555, GCST010556, GCST010557, GCST90013693, GCST90018706, GCST90018926, GCST90132183, GCST90132184, GCST90132185, GCST90244709, GCST90250911

The top 10 associated traits are displayed, along with the corresponding genes and the number of associations (variant-gene) described for each gene (in parentheses). Additionally, the study accession numbers from the GWAS Catalog are provided for the different associations between genes and traits.

described to have a negative effect on seminal parameters (Carre *et al.*, 2017; Jurewicz *et al.*, 2018). In this sense, air pollution has also undergone substantial alterations in the last few decades (Maynard, 2015; Juginovic *et al.*, 2021). Interestingly, asthma, which is associated only with the older age-group, is directly correlated with air pollution (Guarnieri and Balmes, 2014; Pfeffer *et al.*, 2021). Hence, it is plausible that the observed association between this trait and the older age-group may be influenced by such changes in air pollution to which they have been exposed over the past decades. Additionally, asthma has also been linked to male infertility through various genes. Interestingly, *ZPBP2* (affected by rs142908940), is associated with both asthma and male infertility. Its encoded protein plays a crucial role in both the sperm–oocyte interaction during fertilization and asthma development. Specifically, in mouse models, *Zpbp2* mRNA has been shown to be expressed at low levels in immunological cell types pertinent to the pathogenesis of asthma, and exposure to dust mites leads has been reported to increase ZPBP2 levels in the airway epithelium (Miller *et al.*, 2018). In humans, numerous studies also points to *ZPBP2* as a key player in asthma development and immune response (Verlaan *et al.*, 2009; Akhabir and Sandford, 2011; Schmiedel *et al.*, 2016; Forsstrom *et al.*, 2023).

Systemic conditions related to immune imbalance, such as systemic lupus erythematosus (SLE), were also found amongst the associated traits. SLE seems to affect gonadal function by impairing spermatogenesis primarily due to antisperm antibodies and cyclophosphamide therapy (Tiseo *et al.*, 2016). This could potentially indicate the presence of shared molecular pathways between these two conditions. Therefore, although the idea of a comorbidity between male infertility and other autoimmune conditions has been previously proposed (Brubaker *et al.*, 2018), this is the first time that a link has been established between SLE and idiopathic SPGF.

Overall, our findings not only advance our understanding of idiopathic SPGF but also provide a new perspective for the study of complex traits. By using genetic association analyses and age as a proxy for the exposome, we were able to characterize generation-dependent differences in the genetic component of SPGF that might be influenced by changes in environmental exposures, and we achieved a more precise understanding of the physiopathology of this type of condition.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

Acknowledgements

We thank the National DNA Bank Carlos III (University of Salamanca, Spain) for supplying part of the control DNA samples from Spain and all the participants for their essential collaboration. The authors are also thankful for the support of the Unit of Excellence 'UNETE' from the University of Granada (reference UCE-PP2017-05).

Authors' roles

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

Funding

This work was supported by the 'Plan Andaluz de Investigación, Desarrollo e Innovación (PAIDI 2020)' (ref. PY20_00212, P20_00583), the Spanish Ministry of Economy and Competitiveness through the Spanish National Plan for Scientific and Technical Research and Innovation (ref. PID2020-120157RB-I00 funded by MCIN/ AEI/10.13039/501100011033), and the 'Proyectos I+D+i del Programa Operativo FEDER 2020' (ref. B-CTS-584-UGR20). ToxOmics-Centre for Toxicogenomics and Human Health, Genetics, Oncology and Human Toxicology, is also partially supported by the Portuguese Foundation for Science and Technology (Projects: UIDB/00009/2020; UIDP/00009/2020).

Conflict of interest

The authors declare no competing financial interests.

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