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Whole-exome sequencing and *Drosophila* modelling reveal mutated genes and pathways contributing to human ovarian failure

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Abstract

Background Ovarian failure (OF) is a multifactorial, complex disease presented by up to 1% of women under 40 years of age. Despite 90% of patients being diagnosed with idiopathic OF, the underlying molecular mechanisms remain unknown, making it difficult to personalize treatments for these patients in the clinical setting. Studying the presence and/or accumulation of SNVs at the gene/pathway levels will help describe novel genes and characterize disrupted biological pathways linked with ovarian failure.

Methods Ad-hoc case-control SNV screening conducted from 2020 to 2023 of 150 VCF files WES data included Spanish IVF patients with (***n*** = 118) and without (***n*** = 32) OF (< 40 years of age; mean BMI 22.78) along with GnomAD (***n*** = 38,947) and IGSR (***n*** = 1,271; 258 European female VCF) data for pseudo-control female populations. SNVs were prioritized according to their predicted deleteriousness, frequency in genomic databases, and proportional differences across populations. A burden test was performed to reveal genes with a higher presence of SNVs in the OF cohort in comparison to control and pseudo-control groups. Systematic in-silico analyses were performed to assess the potential disruptions caused by the mutated genes in relevant biological pathways. Finally, genes with orthologues in ***Drosophila*** melanogaster were considered to experimentally validate the potential impediments to ovarian function and reproductive potential.

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Results Eighteen genes had a higher presence of SNVs in the OF population (FDR < 0.05). ***AK2***, ***CDC27***, ***CFTR***, ***CTBP2***, ***KMT2C***, and ***MTCH2*** were associated with OF for the first time and their silenced/knockout forms reduced fertility in ***Drosophila***. We also predicted the disruption of 29 sub-pathways across four signalling pathways (FDR < 0.05). These sub-pathways included the metaphase to anaphase transition during oocyte meiosis, inflammatory processes related to necroptosis, DNA repair mismatch systems and the MAPK signalling cascade.

Conclusions This study sheds light on the underlying molecular mechanisms of OF, providing novel associations for six genes and OF-related infertility, setting a foundation for further biomarker development, and improving precision medicine in infertility.

Keywords Ovarian failure, Primary ovarian insufficiency, Ovarian function, Whole-exome sequencing, Single nucleotide variant, Variant prioritization, Functional analysis, Infertility, Human assisted reproduction, ***Drosophila melanogaster***

Background

Ovarian failure (OF) is a complex and multifactorial disorder usually characterized by an accelerated depletion of the follicular reserve before the age of natural menopause in healthy women [1]. OF affects 1% of women under 40 years of age and 0.1% of women under 30 years of age. While 90% of screened OF cases are diagnosed as idiopathic [2, 3], experts predict that 10–25% of cases are caused by genetic factors, such as the presence of deleterious mutations or variants that reduce functions of key genes [4, 5]. Indeed, genetic effects are estimated to account for at least 50% of the inter-individual variability in menopausal age and potentially influence the intergenerational persistence of OF within families [6]. However, the pathogenesis of OF remains poorly understood and there are still many healthcare and research gaps to be addressed [7]. Particularly, the late detection of OF at a stage of irreversible infertility limits women's fertility options, reinforcing the necessity for continued research and development of potential biomarkers for early diagnosis in the scope of human assisted reproduction [2].

Multiple studies have focused on the heritable factors of OF by analyzing the relationships between members of the same family [7–9]. Nevertheless, advances in next-generation sequencing technologies have radically transformed human genomics by enabling a comprehensive assessment of individual genetic variation and its association with OF, while making use of larger populations [10, 11]. These advances have also prompted the use of variant-association approaches to identify genes with a significant presence of single nucleotide variants (SNVs), which are suspected to be responsible for manifesting a certain phenotype absent in a population of healthy individuals [12, 13]. These methods aggregate the genomic information between genes into a genetic score that can be compared to reveal statistical differences between populations [14, 15]. However, variants impact complex diseases beyond the genetic level, due to groups of genes and proteins sharing functions or participating in the same biological process referred to as pathways, comprised of sub-pathways [16, 17]. As genes and proteins underlying complex phenotypes are postulated to physically interact with one another and alter signalling pathways and protein-to-protein interactions, functional approaches based on damage propagation algorithms have been developed to predict systemic disruptions due to the presence of SNVs across genes of the pathway [18–20].

Various animal models, including mice and *Drosophila melanogaster*, have been employed to study and validate the association of potential genes with a role in OF onset or progression [21]. Specifically, genes involved with DNA repair responses, the effect of the NOBOX gene, cohesins for chromosome segregation, and the members of the MCM family for the regulation of the oocyte meiosis were investigated [22–25]. In this regard, *Drosophila* is an excellent biomedical model for evaluating how certain genes may impact fertility. For instance, genes with a direct impact on the quantity of offspring produced as well as the ovarian and egg chamber morphology have recently been identified and extrapolated to human fertility [21, 26].

This study aimed to characterize genes associated with human OF and unravel disrupted biological pathways, innovatively integrating the gene- and pathway-level burden from SNVs presence. The populations used consisted of Spanish patients with or without OF along with pseudo-control populations from public genomic variation resources. We hypothesized that idiopathic OF could be a condition resulting from the presence of moderate mutations, as the effect of highly detrimental variants would result in a more pronounced and identifiable phenotype like in patients with syndromic OF. Thus, we did not restrict the study to very rare variants, and we considered the presence of multiple variants across genes through burden testing. Prioritized genes were validated in *Drosophila* mutant strains to determine the effects on reproductive potential (i.e., ovarian morphology, oocyte maturation and number of offspring) and infer their role in human female reproduction.

Methods

Ethics approval and consent to participate

The study was approved by the Institutional Review Board of the Instituto Valenciano de Infertilidad and Hospital Universitario y Politecnico La Fe (HUP La Fe) in Valencia, Spain (1709-PAM-090-PR). Written informed consent was obtained from all participants. The whole-exome sequences of the OF and control samples were uploaded to PRJNA1010150.

Study design and participants

Ad-hoc case-control SNV screening of whole-exome sequencing (WES) case-control study was conducted between 2020 and 2023 at IVI Foundation. The study included 118 women diagnosed with OF (without strictly differentiating clinical subtypes like primary ovarian insufficiency and diminished ovarian reserve) and 32 healthy women as controls. Participant recruitment and diagnostic criteria, DNA extraction, and WES protocols were described in our previous study [11]. Briefly, women presenting with amenorrhea for over six months, serum anti-Müllerian hormone (AMH) values < 0.3 ng/mL, follicle stimulating hormone (FSH) values > 20 IU, and an antral follicle count (AFC) < 5 via transvaginal ultrasound were diagnosed as having OF, in accordance with the European Society of Human Reproduction and Embryology (ESHRE) criteria. Alternatively, controls had serum AMH values > 1.5 ng/mL, FSH < 10 IU, and an AFC > 10. All patients were < 40 years old during recruitment. The study design is summarized in Fig. 1.

Ovarian failure-related variant prioritization

Genomic datasets of both the case and control populations were uploaded to OpenCGA, a state-of-the-art platform for genomic analysis part of OpenCB [27]. Datasets were annotated with (i) population frequency data from GnomAD (n = 38,947 female samples) and The International Genome Sample Resource (IGSR) databases (n = 1,271 female samples) [28, 29], both accessed in December 2021; and (ii) deleteriousness scores from functional predictors REVEL and CADD [30, 31]. REVEL is a machine learning ensemble specifically developed to predict the deleteriousness of missense variants, considering conservation of amino-acid or nucleotides and biochemical properties of the amino-acid modifications, that is more sensible for loss of function variants [31, 32]. CADD was developed as a machine learning framework that considers evolutionary constraint, epigenetic measurements, functional predictions, surrounding sequence context and gene model annotations. Several criteria were applied to ensure we kept high-quality and biologically relevant SNVs. Briefly, variant calling parameters (genotype quality > 90, allele depth > 20, from GATK software), and type of mutation (missense, nonsense, and splice variants) were used to filter the SNVs. Next, genes associated to OF were highlighted based on the SNVs presence in the population, using the following filters: (i) SNV deleteriousness score prediction [CADD > 20 for non-missense, both CADD > 20 and REVEL > 0.75 for missense; [33]], and [2], following the example set by variant prioritization studies [21], population genomic parameters (allele frequency, minor allele frequency, etc.) for all female samples from the IGSR and GnomAD were retrieved from OpenCGA, and used during variant prioritization to retain:

- variants absent in these databases;
- uncommon-rare SNVs (minor allele frequency [MAF] < 0.5 in both GnomAD and IGSR), which were significant and in a higher proportion in our OF group (false discovery rate [FDR] < 0.05), after performing a fisher test.

Identification of genes that frequently carry the prioritized variants

Prioritized variants were considered for a gene-based burden analysis using the Rvtest tool [34]. Genes with a significantly higher presence of SNVs in the OF group (n = 118) compared to the healthy controls included in this study (n = 32) and the pseudo-control IGSR-European population (n = 258 European female samples; a total n = 290 female controls) were prioritized for subsequent analyses. Variants were annotated with the database from the MANE project, which consists of selecting one transcript at each protein-coding locus across the genome that is representative of biology at that locus [35]. FDR-adjusted p-values were obtained for each gene and compared between populations.

Prediction of SNV impact in signalling pathways and the interactome

The GenePy scorer was used to calculate gene scores according to the MAF in GnomAD and predicted deleteriousness for filtered SNVs (using CADD for non-missense; REVEL for missense) [36]. To identify compromised pathways and sub-pathways in the OF population, we calculated the genomic impact to signalling pathways extracted from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (n = 121, accessed August 2021; Supplementary Table S1) [37]. In parallel, Hornet software [38] was used to calculate the impact to protein-to-protein interactions of the human interactome [39] The technical aspects of both functional analyses are presented in the Supplementary Methods.

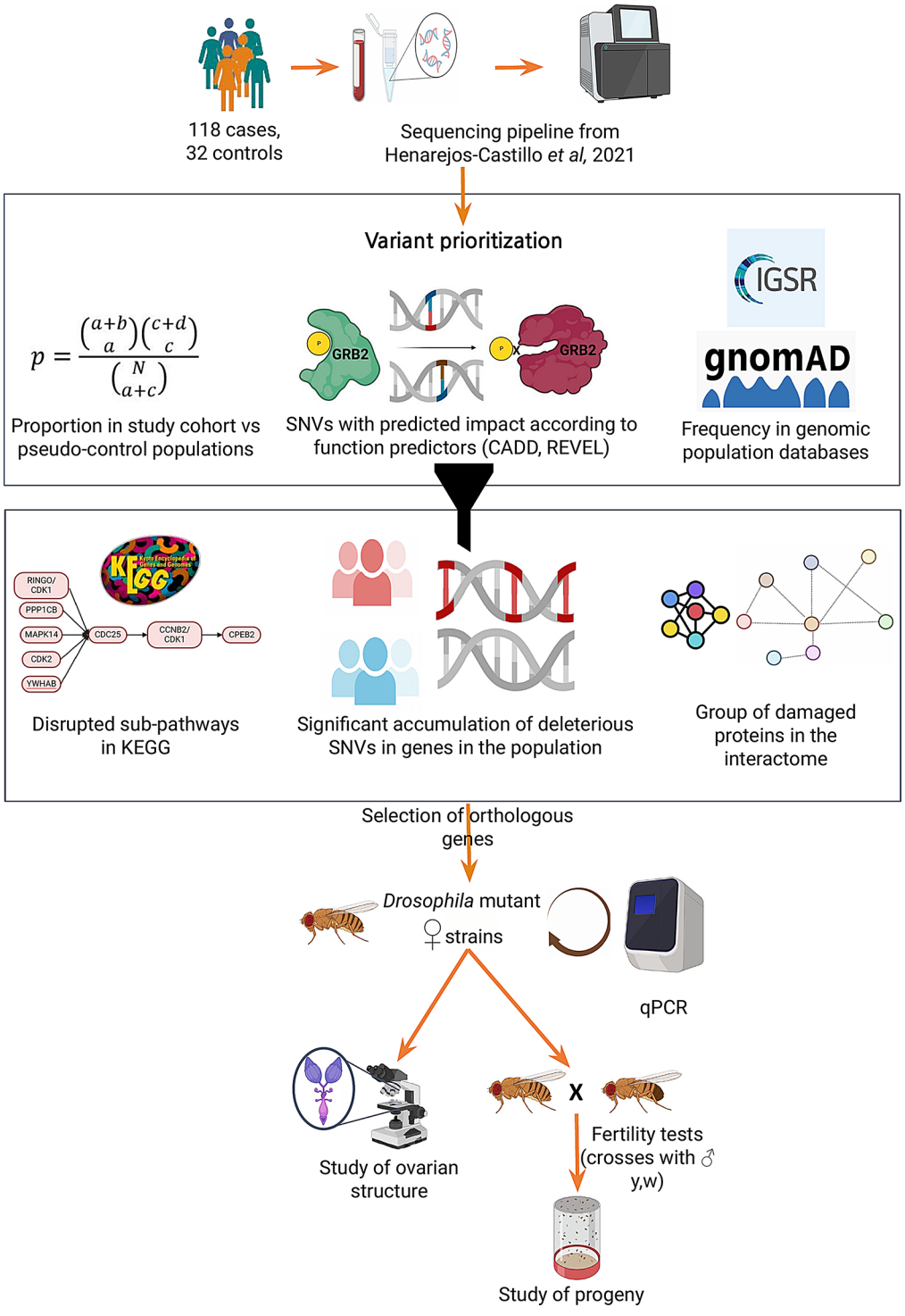


Fig. 1 Schematic representation of the bioinformatic and experimental pipeline. Whole-exome sequencing data from 118 women with ovarian failure (cases) and 32 healthy controls collected in a previous study (Henarejos-Castillo et al., 2021) was uploaded to the OpenCGA platform to prioritize single nucleotide variants (SNVs) according to their predictive deleteriousness (REVEL and CADD predictors), minor allele frequency in the general population (IGSR and GnomAD databases), and statistical differences assessed by Fisher tests between the cases and control populations (including pseudo-control populations using female samples from IGSR and GnomAD). Next, a burden test prioritized genes with a significant presence of SNVs in the OF population compared to controls. To validate the impact of compromised genes with deleterious SNVs on female fertility, *Drosophila* females with mutations in orthologues of the prioritized genes were generated via RNAi-mediated silencing and gene-knockout and compared with control females (*y*, *w*). Silencing and knockout efficiency was evaluated by RT-qPCR, and the flies reproductive potential was assessed by histological analysis of ovarian morphology, proportion of mature oocytes and number of offspring produced

In vivo validation of fertility impairments using *Drosophila melanogaster*

Genes with a higher frequency of SNVs in the OF population, known functions reported in predicted disrupted pathways [extracted from Genecards [40]] and orthologues in *Drosophila melanogaster* (source: FlyBase database, accessed June 2022) were considered for in vivo validation. *Drosophila* stocks used in this study were obtained from the Bloomington *Drosophila* Stock Center (USA) and are listed in Supplementary Table S2. Additional details regarding the *Drosophila* model, like culture, diet, and mating, are presented in the Supplementary Methods. The *nanos*-GAL4 and *C135*-GAL4 strains were employed to express dsRNA for RNAi-mediated silencing of prioritized genes in germ cells or follicle cells, respectively [21]. When available, knockout mutant stocks for prioritized genes were used instead. Primers are listed in Supplementary Table S3. The efficiency of gene silencing was evaluated with qRT-PCR, comparing expression of the prioritized genes in the mutant strains to that in wild-type controls (*y*, *w*). To assess the implications of the prioritized mutated genes on ovarian function, ovaries from mutant females were harvested and analysed by fluorescence microscopy to evaluate ovarian and egg chamber morphology, and proportion of mature oocytes. Finally, mutant females were mated to control males, and the offspring were quantified to assess their reproductive potential.

Statistical analyses

Briefly, a Fisher test was employed in the variant prioritization process to determine significant SNVs according to their proportion in the OF population compared to IGSR and GnomAD. Burden test was performed with CMC method (multivariate test which leverages variants in genes to associate to case-control status) [41] using Rvtests tool to identify genes with a higher presence of SNVs across the OF population. The variant profile per gene present in the cohort of cases ($n=118$) from the study was compared to the profile presented by the study controls ($n=32$) in addition to the pseudo-control European population ($n=268$) from the IGSR (total control population $n=290$). P-values were obtained for each gene and contrast between populations, and FDR was subsequently applied. For KEGG pathway analysis, the Wilcoxon test was implemented to identify sub-pathways that exhibited a higher degree of SNV impact. Functional enrichments were performed using the overrepresentation analysis. For the *Drosophila* analysis, significant differences between means were assessed using a Student t-test when two experimental groups were compared or Analysis of Variance (ANOVA) followed by a Tukey's post-hoc analysis when more than two groups were compared. In all cases, the differences were considered statistically significant when the p-value <0.05 in a single hypothesis contrast or FDR <0.05 in the case of multiple testing. Data are expressed as means ± standard deviation.

Table 1 Genes with a significant presence of SNVs in patients with ovarian failure

Gene symbol	Position in genome	Nº SNV	Adjusted *p*-value
CDC27	17:47117703–47,189,422	9	5.35E-55
ANKRD20A1	9:67832765–67,920,552	2	5.35E-55
IGSF3	1:116574399–116,667,755	1	3.64E-54
KMT5A	12:123384132–123,409,353	1	5.36E-53
FAM104B	X:55,143,102–55,161,310	2	1.08E-38
KMT2C	7:152134922–152,436,644	4	5.09E-33
MTCH2	11:47617315–47,642,607	5	1.48E-25
CTBP2	10:124984317–125,161,170	20	1.12E-24
AK2	1:33007940–33,080,996	2	1.54E-19
ANKRD36B	2:97492663–97,589,877	1	7.02E-17
WDR89	14:63597039–63,641,871	4	1.60E-11
ESRRA	11:64305497–64,316,743	2	2.43E-08
MST1	3:49683947–49,689,501	2	1.68E-07
ZNF717	3:75678660–75,785,583	9	3.59E-07
TEKT4	2:94871430–94,876,823	1	4.18E-03
CFTR	7:117287120–117,715,971	4	5.75E-03
HLA-DRB1	6:32578769–32,589,848	1	1.30E-02
MLH1	3:36993350–37,050,846	2	2.51E-02

Results

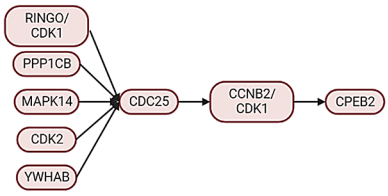
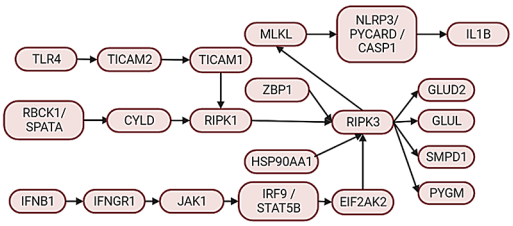
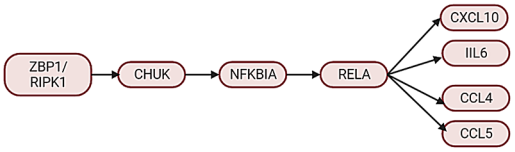
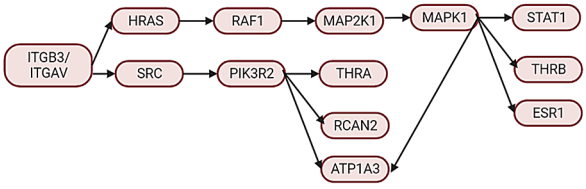
Disrupted genes and pathways in human OF

A total of 1,768 SNVs were prioritized for having potentially deleterious effects (Supplementary Figure S1). Eighteen genes were found to have a significant presence of deleterious SNVs in the OF group with respect to controls (FDR <0.05) (Table 1). The specific SNVs affecting each of these 18 genes were all found in heterozygous state and fell into the MANE selected transcript for each gene (Supplementary Table S4). The extent of the genomic impact in OF was reflected through four main pathways encompassing 29 KEGG sub-pathways (FDR <0.05). Specifically, the mutated genes were found to be related to oocyte meiosis ($n=5$ sub-pathways), necroptosis ($n=12$ sub-pathways), cytosolic DNA-sensing ($n=4$ sub-pathways), and thyroid hormone signalling ($n=8$ sub-pathways) (Table 2A). Similarly, the functional analysis through the human interactome unveiled a group of 376 affected proteins, from which 21 were significantly involved in DNA mismatch repair, Fanconi anemia and estrogen signalling pathways from KEGG (FDR <0.05; Table 2B).

Experimental validation using *Drosophila* orthologues

Next, we used a *Drosophila* model to validate whether disruption of prioritized genes due to the presence of SNVs affected fertility. After confirming the expression of orthologous genes in the *Drosophila* ovaries,

(A) Significantly dysregulated signalling pathways and sub-pathways in patients with ovarian failure (FDR-adjusted p-value < 0.05). The KEGG sub-pathways are represented as small biological networks.

KEGG Pathway ID	Pathway name	Sub-pathways	p-adjusted
hsa04114	Progesterone-modulated Oocyte Meiosis		0.037
hsa04217	Necroptosis		0.043
hsa04623	Cytosolic DNA-sensing pathway		0.026
hsa04919	Thyroid hormone signaling		0.0245

(B) Enriched KEGG pathways by genes affected by the presence of SNVs in the human interactome.

Mismatch repair	*MSH6*
	MLH1
	MSH3
	EXO1
Fanconi anemia pathway	*FANCA*
	FANCD2
	MLH1
	FANCI
	BRCA1
	BRCA2
	BRIP1
Estrogen signaling pathway	*KRT25*
	KRT28
	KRT24
	KRT14
	KRT26
	KRT18
	KRT32
	KRT38
	KRT36
	TFF1

Table 2 Disrupted molecular pathways and sub-pathways in patients with ovarian failure

including those not previously related to OF, we selected six candidate genes: *ak2*, *cdc27*, *cftr*, *ctbp*, *kmt2c*, and *mtch* (Supplementary Table S5). All patients presented at least seven variants across these genes with over half the cohort of cases carrying 10 or more variants, though *AK2*, *MTCH2* and *CFTR* variants were rarer (found in 4, 3, and 8 cases of 118, respectively) in counterpart to *CTBP2*, *KMT2C* and *CDC27* (81, 101 and 104 cases of 118, respectively). For *ak2*, *ctbp*, *kmt2c*, and *mtch*, mutant flies were generated with the *GAL4/*UAS system using UAS-RNAi constructs and *nanos*-GAL4 or *c135*-GAL4 drivers to silence gene expression in germ cells or follicle

cells, respectively. For *cdc27* and *cftr*, knockout mutants caused by transposon insertions were available and thus used. All the strains used in the study presented a normal development, hence ensuring that alterations found in further experiments are not related to development defects. First, we confirmed that all RNAi and knockout strains showed a significant decrease in gene expression, except *nanos-GAL4/-UAS-kmt2c-RNAi* (Fig. 2A). Next, we aimed to evaluate if these prioritized genes could be relevant to female fertility. We found an evident significant decrease in offspring number of *ctbp*, *mtch*, *kmt2c* deficient and *cftr*-knockout female flies; however, this decrease, although significant, was observed to a lesser extent in *ak2* deficient and *cdc27* knockout flies (Fig. 2B). Subsequently, we investigated if the mutations in the candidate genes could lead to ovarian defects. To do this, we extracted ovaries of 3-day-old female flies of the different mutant strains. According to results obtained in the fertility test, we found that ovaries from *ctbp*, *mtch*, *kmt2c* deficient and *cftr* knockout females presented morphological distinctions and lacked mature oocytes compared to the ovaries of wild-type controls (Fig. 3A). No evident morphological alterations were observed in the ovaries from *ak2* deficient and *cdc27* knockout female flies that showed a milder decrease in fertility (Fig. 3A). To determine how *ak2* deficiency and *cdc27* knockout might be implicated in fertility impairments, we decided to evaluate the oocyte maturation rate in ovary preparations. The oocyte maturation rate was significantly reduced in *cdc27*-knockout flies, suggesting a potential involvement of *cdc27* in oocyte maturation. No differences were noted in *ak2* deficient flies, suggesting the reduced offspring number could not be attributed to ovarian defects or a reduced oocyte maturation rate, and other unidentified factors may be involved (Fig. 3B). Figure 4 presents a schematic summary of the six novel human OF-related biomarkers (*CDC27*, *CFTR*, *KMT2C*, *CTBP2*, *AK2*, *MTCH2*) along with the disrupted sub-pathways and biological processes determined through this work.

Discussion

Idiopathic OF is theorized to result, in part, from genetic factors; nonetheless, its pathogenesis remains largely unknown. Identifying the genes affected by OF-related deleterious mutations and elucidating the molecular pathways they dysregulate helps advance the

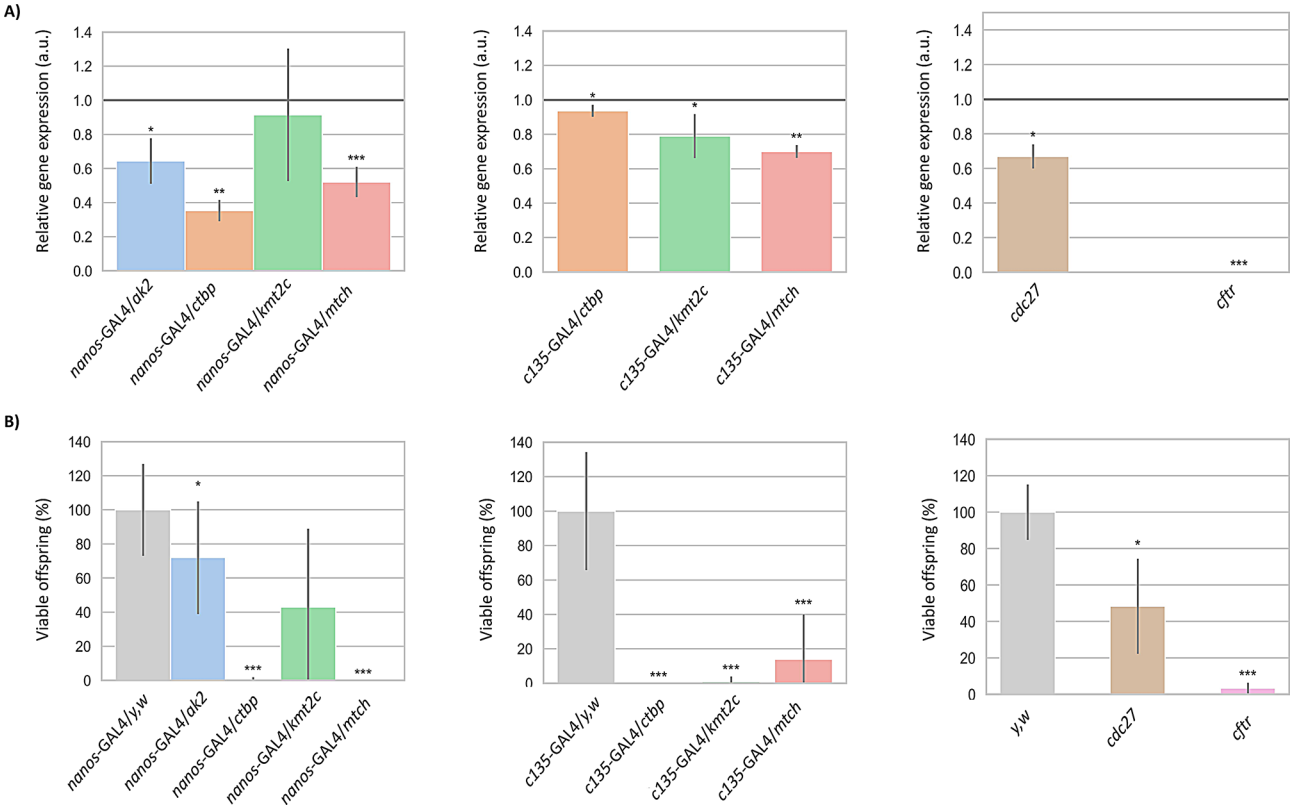


Fig. 2 Gene expression with predicted deleterious mutations in *Drosophila* ovaries and their effects on female fertility. (**A**) Relative mRNA expression of prioritized OF-related genes in 3-day-old female flies with the corresponding gene silencing or knockout. Gene expression levels were normalized to tubulin and are expressed as fold-changes in expression compared to controls (mean of four replicates ± standard deviation). (**B**) Quantification of the viable offspring produced by female flies of each genotype. Data was expressed as percentages over wild-type controls (mean of 23 replicates ± standard deviation). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

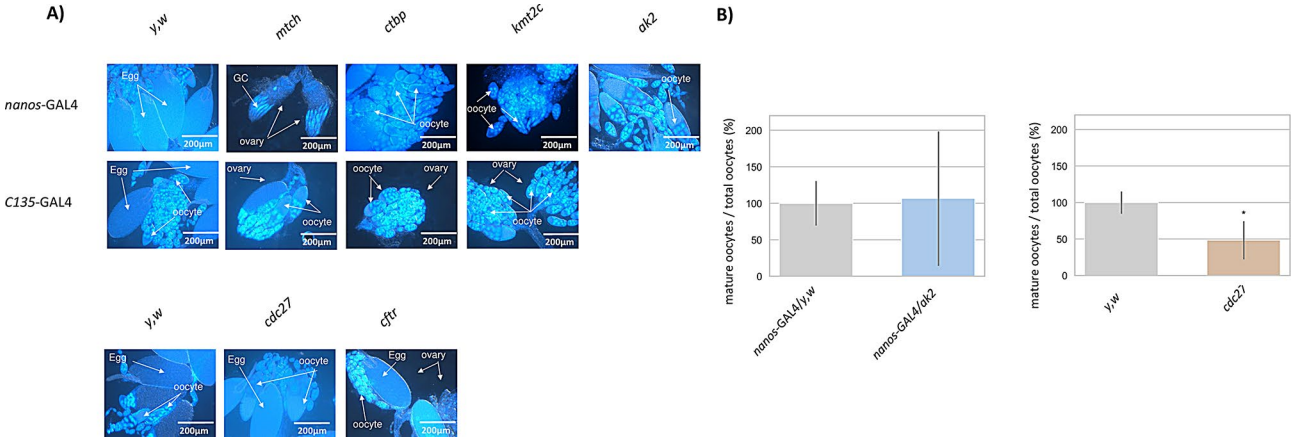


Fig. 3 *Drosophila* mutants model ovarian failure. (**A**) Representative images of DAPI-stained *Drosophila* ovaries show three to 4-day-old female flies with alterations in egg chamber morphology compared to control females (*y*, *w*). *Drosophila* lines are indicated on the left of the panel and the genes that were silenced by RNAi or that were knocked out by transposon insertions are indicated on the top. Fluorescent images were captured at 40x magnification. (**B**) Oocyte maturation rate in *ak2* deficient and *cdc27* mutant female ovaries. Data are expressed as percentages (%) over the respective controls (mean of minimum three replicates ± standard deviation). Arrows are included to show the structures in the pictures: eggs, oocytes, germ cells (GC), ovaries. *$p < 0.05$

development of predictive biomarkers for early diagnosis of the disease. This study integrated insights from comprehensive and state-of-the-art genomic variant prioritization methods to predict genes with a potential role in human OF. Eighteen genes were found to have a significantly higher SNV mutation rate in our OF cohort ($n=118$) in comparison to healthy controls ($n=32$) and the additional pseudo-control population from the IGSR-European population ($n=258$). In-parallel, functional analyses performed with KEGG and the human interactome further reinforced the strength of the SNV prioritization performed herein by highlighting the disruption of OF-related biological processes. Together with these results, we refine our selection to six novel OF biomarkers (*AK2*, *CDC27*, *CFTR*, *CTBP2*, *KMT2C*, and *MTCH2*) for in vivo validation using orthologous genes expressed in *Drosophila*. Our results showed that deleterious mutations in these six genes had detrimental effects on *Drosophila's* fertility. Combined with our in-silico prioritizations, these findings suggest, for the first time, a potential association between these genes and OF. However, further research is needed to confirm this association and elucidate the underlying pathomechanisms of the disease and impairment of ovarian function.

Among the candidate OF-related biomarkers, *CDC27* plays a role in cell cycle progression [40] and was found to compromise oocyte maturation in *Drosophila*. In humans, *CDC27* is expressed in the ovary [42]. The SNVs carried by this gene, which encodes a subunit of the anaphase-promoting complex (APC/C), could disrupt the metaphase to anaphase transition in developing human oocytes, and influence the degradation of the maturation-promoting-factor (MPF) complex, composed of CDK1 and Cyclin *B* subunits [43]. Under normal circumstances, a negative feedback loop initiated by *CDK1* activates the APC/C and degrades the MPF to allow the oocyte to enter meiosis II [44]. Analyzing the impact of the SNVs in the progesterone-modulated meiosis progression pathway, we found several genes enriched in processes related to the formation of the MPF complex (i.e., *CPEB2*, *MAP2K1*, *MAPK1/3*, *RINGO*, *CDK1*, *CDK2*, *MAPK12*, *PPP1CA*, *CDC25C*, *YWHAB*, and *CCNB2*) suggesting multiple disruptions in the metaphase to anaphase transition. A recent study associated *CPEB1*, which KEGG considers as an alternative to *CPEB2* in the meiosis progression pathway, to primary ovarian insufficiency [45]. As oocytes are arrested in prophase I until puberty, when surges of LH activate the MPF [46], genetic disruptions due to deleterious SNVs may lead to premature OF.

Variants in mitochondrial activity and respiratory chain genes induce apoptosis and have previously been linked to OF and Perrault Syndrome [24, 47]. However, no such associations have been reported for the mitochondrial genes *AK2* and *MTCH2*. In women, *AK2* RNA and protein expression is confirmed in the ovary [42] while the respective murine and bovine orthologues have been associated with oocyte developmental competence [48]. In our study, no flies were obtained when silencing *ak2* in follicles, cyst cell and eye discs, showing that *ak2* expression in these tissues is essential for female *Drosophila* reproduction. Alternatively, *Drosophila* lines with *ak2* silenced in germ cells did produce fewer offspring, but analysis of their ovaries did not reveal evident morphological alterations. Thus, further experiments will be required to determine how *ak2* silencing in germ cells reduces ovarian function in *Drosophila*, and its potential role in human ovaries. Meanwhile, *MTCH2* has been associated to pro-apoptotic events under normal

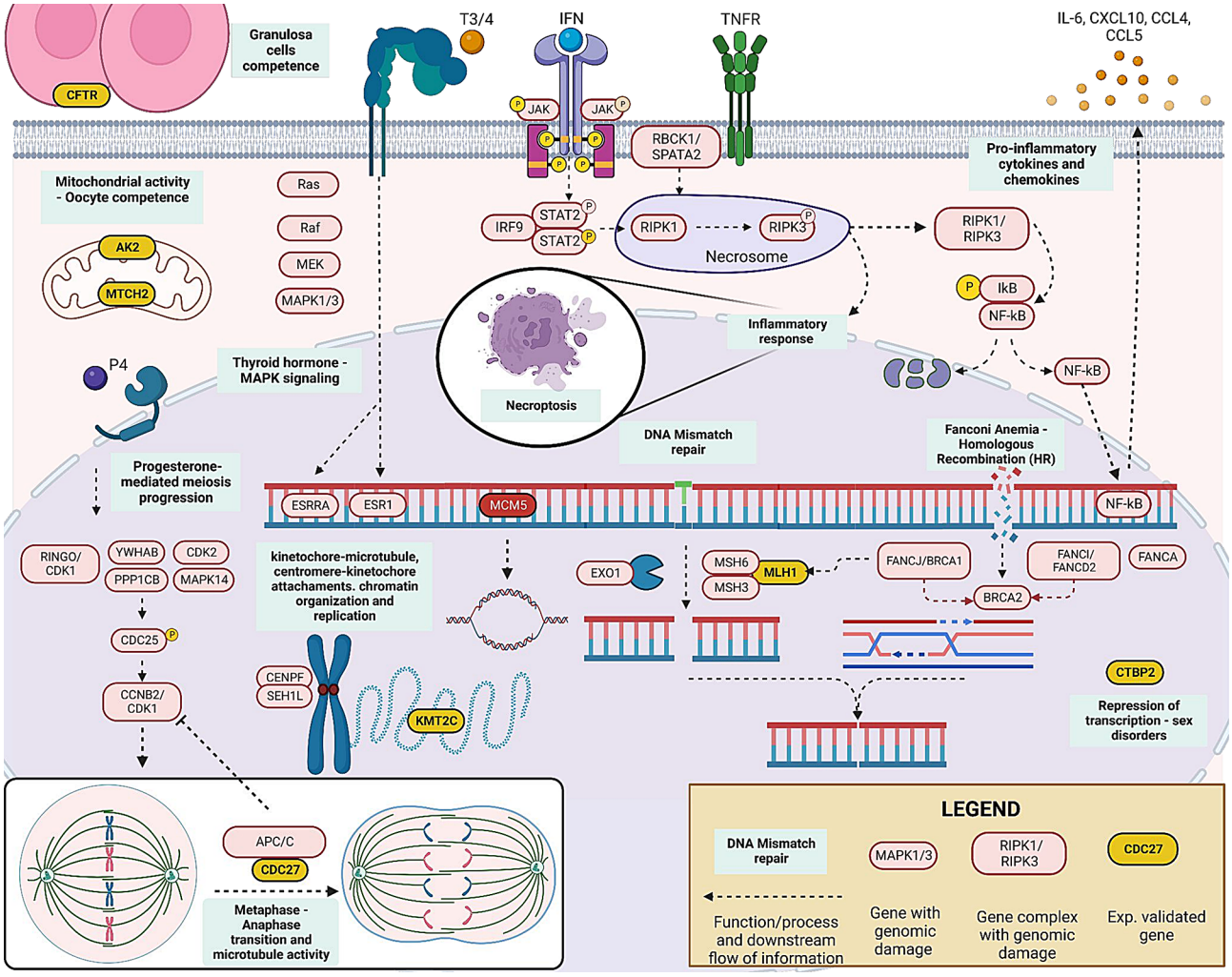


Fig. 4 The genomic landscape of human ovarian failure. Patients with ovarian failure presented single nucleotide variants (SNVs) in key genes (yellow) involved in DNA mismatch repair; homologous recombination; Fanconi anemia; the metaphase to anaphase transitions during meiosis; kinetochore-microtubule or centromere-kinetochore attachments and segregation during anaphase; chromatin organization; reduction of granulosa cell competency; perturbation of mitochondrial activities; pro-inflammatory responses; and necroptosis

conditions [40]. The observed decrease in *Drosophila* fertility suggests that these effects may potentially disrupt developing follicles. In addition, *MTCH2* was recently linked to ovarian cancer progression [49]. Additional studies are needed to investigate the possible involvement of these genes in the pathomechanisms of OF.

We also shown two genes with known nuclear functions, *KMT2C* and *CTBP2*, impaired *Drosophila* fertility. *KMT2C* reorganizes chromatin, acetylates histone H3K14 [50], and induced silencing of its orthologue hindered fly fertility. Notably, in humans, chromatin stability is necessary for correct follicular development [24, 51], thus mutations in *KMT2C* could lead to instabilities in follicle chromatin and affect follicle development. Furthermore, *KMT2C* expression has been confirmed in human ovary cell lines [52]. *CTBP2*, which regulates transcription, has been associated with sex-reversal disorders [40], it is expressed in human ovaries [42], and its silencing impaired *Drosophila* fertility, without provoking sex-reversal. Further studies are required to unveil how *CTBP2* can influence follicle development. Finally, *CFTR*, an ion transporter and associated to cystic fibrosis, has been linked to granulosa cell support [53], which is necessary for follicle and oocyte development. Additionally, *CFTR* expression is associated with proliferation of ovarian cancer [54] Its knockout in the fruit fly model, resulting in decreased fertility, suggests a crucial role of this gene that would be of special interest in OF biomarker research.

Our in-silico analysis of biological processes affected by the presence of SNVs predicted the disruption of DNA repair and Fanconi anemia pathways, which have been previously associated with OF [25, 55–57]. For the genes that were part of our functional analysis (*MSH3*, *MSH6*,

BRCA1, *BRCA2*, *FANCI*, *FANCD2*, *EXO1*, *CENPF*, and *SEH1)*, a recent study associated *MSH6* with OF [58]. From what can we retrieve from our results, *FANCD2* and *FANCI* co-function in the Fanconi anemia core complex to repair DNA cross-links [59]. *BRCA2*, *BRCA1*, and *BRIP1* are key genes involved in homologous recombination and are primarily studied in the context of breast cancer [60]. Interestingly, *CENPF* and *SEH1* are involved in kinetochore-microtubule and centromere-kinetochore attachments during meiosis [40]. Proper attachments are essential for oocytes, which exhibit a higher frequency of spindle assembly errors [61]. Of note, *MLH1*, another significant gene included in the 18 genes with a higher presence of SNVs in the OF population, has been previously linked to OF and premature reproductive aging [62, 63]. As *MLH1* is involved in DNA repair damage, it predictably enriched the DNA repair and Fanconi anemia pathways in our analysis. Additionally, *MLH1* has already been proven to be vital for female fertility in *Drosophila* [64]. As this study was based on a non-targeted approach to discover new genes instead of further researching previously linked genes, the fact that a known gene was part of our results strengthens the overall findings of our study.

Further, we predicted that OF patients may have disruptions in sub-pathways related to cytosolic DNA-sensing and the necrosome. Interestingly, *RIPK1* and *RIPK2* participate in both these functions, regulating levels of tumor necrosis factor and interferon gamma, which are cytokines that mediate necroptosis in response to aberrant pro-inflammatory conditions [65, 66]. Previous studies examined the harmful effects of inflammation in OF, and how abnormal levels can be treated to avoid apoptosis and atresia of follicles [67, 68]. Of note, the inhibition of *TLR4*, encoding a transmembrane receptor that regulates necroptosis signalling pathways, was found to be associated with a better OF response [69]. These findings suggest that SNVs disrupting genes of the necroptosis and DNA-sensing pathways, involved in inflammatory processes, can potentially contribute to OF pathomechanisms. Finally, we predicted the involvement of sub-pathways within the thyroid hormone signalling pathway. Apart from thyroiditis being the most frequent autoimmune disorder associated with OF [70], we specifically highlight the *ITGA/B*, *RAS*, *RAF1*, *MEK*, and *ERK* genes that enriched this pathway, collectively forming the MAPK signalling sub-pathway, which plays a role in multiple signalling cascades. Indeed, the disruption of the RAS-MAPK cascade in granulosa cells leads to cell cycle arrest and hinders folliculogenesis [71].

Compared to a recent study with a big cohorts of cases (>1000) and empowered for association analyses without the need to use animal modelling [45], our study validated the ovarian expression of candidate genes *CDC27*, *AK2*, *MTCH2*, *KMT2C*, *CTBP2*, and *CFTR* genes, and the effects of their silencing or knockout on *Drosophila* female reproduction, associating these genes to OF for the first time. Although a limitation of our study is that we did not perform biochemical analyses to confirm the effects of the missense variants, our stringent in-silico prioritization approach enabled us to identify potentially impactful variants. In addition, all SNVs in candidate genes were in a heterozygous state. While we did not determine whether these mutations resulted in loss-of-function or gain-of-function effects, a limitation of the study, the incorporation of REVEL scores in our in-silico prioritization pipeline slightly favours the identification of loss-of-function mutations. This observation, combined with our initial hypothesis focusing on moderate rather than severe genomic effects, suggests that these mutations likely create a haploinsufficiency state for these genes, where reduced gene expression or activity levels may contribute to the pathogenesis of OF. In the case of *AK2*, *CTBP2*, *MTCH2*, and *KMT2C*, RNAi silencing lines with *Drosophila* modelling were employed, which would better reflect the heterozygous state of prioritized genes [72]. Regarding, *CDC27* and *CFTR*, however, the use of knockout lines might limit the extrapolation of our results to patients. Although *Drosophila* has been reported as an excellent model to study women's fertility and ovary function [64, 73, 74], we are aware that further experiments will be required in mice or human cells models aimed to fully extrapolate these results to women. In addition, our functional analysis highlighted the impact caused by SNV across the necroptosis and DNA-sensing pathways, specifically with inflammatory sub-pathways, potentially contributing to OF. Additionally, this functional analysis provided further in-silico validation to the SNV prioritization performed by also highlighting OF-related pathways, such as DNA-impact repair and oocyte meiosis progression. Notably, certain genes within these sub-pathways were not previously linked to OF, presenting novel routes for future investigations. Particularly, the SNVs carried by *CDC27* were predicted to disrupt MPF signalling pathways in human oocyte meiosis, suggesting a potential link between MPF dysregulations and OF. As our methodology focused on population genomics, the cumulative effects of these SNVs remain to be elucidated at the individual level. Nevertheless, taking all our in-silico and in vivo results together, it becomes apparent that the deleterious SNVs carried by key genes disrupt biological functions critical for fertility. These functions include the metaphase to anaphase transition in oocytes, chromosome attachments, chromatin organization, DNA mismatch repair systems, granulosa cell competency, mitochondrial activity, MAPK signalling cascades, and pro-inflammatory responses associated with necroptosis and cytosolic DNA-sensing. A genomic landscape of

SNVs affecting these functions can favour development or aggravation of OF.

Conclusion

In this study, we combined state-of-the-art genomic platforms, cutting-edge algorithms, revealing six novel genes with a significantly higher presence of deleterious SNV in heterozygosis in patients with OF. Additionally, we have shown that mutations in these genes *in Drosophila* lead to impaired fertility and affects natural ovarian morphology development. In humans, these six genes are expressed in the ovaries and are potentially involved in key oocyte developmental functions (i.e., metaphase to anaphase transition with *CDC27* and mitochondrial activities with *MTCH2*). We linked the functional impact of SNVs in patients with OF to important reproductive processes (namely the metaphase-anaphase transition in oocyte meiosis, inflammatory process related to necroptosis and cytosolic DNA-sensing, and the MAPK signalling cascade), adding to the knowledge of potential phatomechanisms of OF. The results presented herein will help generate new hypotheses and insights to elucidate the underlying genetic aetiologies of idiopathic OF, setting a foundation for the development of predictive and diagnostic OF-related biomarkers which could be implemented to provide a timely diagnosis and improve fertility counselling of affected patients.

Abbreviations

AFC	Antral follicle count
AMH	Anti-Müllerian hormone
ESHRE	European Society of Human Reproduction and Embryology
FSH	Follicle stimulating hormone
IGSR	The International Genome Sample Resource
KEGG	Kyoto Encyclopaedia of Genes and Genomes
MAF	Minor allele frequency
OF	Ovarian failure
RNAi	RNA interference
SNVs	Single nucleotide variants
UAS	Upstream Activating Sequence
WES	Whole-exome sequencing

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions

The research idea was conceived by P.D.-G., who coordinated and supervised all the study. The study was designed by P.D.-G. and J.R. with the help of N.P. for the functional validation with ***D. melanogaster*** and I.M. for the in-silico methodology. The bioinformatics pipeline, including variant prioritization, burden testing, and functional analysis were designed and implemented by I.H.-C., I.M., P.S.-L., supervised by I.M., P.S.-L., and P.D.-G. Genes were selected for experimental validation by I.H.-C., F.S., and C.S.-M. with supervision from P.D.-G. and N.P. Experimental validation was performed by C.S.-M. and F.S. with the supervision and coordination of N.P. Results were interpreted by I.H.-C., C.S.-M, F.S., N.P., and P.D.-G. Tables and figures were designed by I.H.-C., F.S., and P.D.-G with the help of P.S.-L. and supervised by P.D.-G and N.P. The manuscript was written by I.H.-C., F.S, N.P. and P.D.-G. and the final draft was reviewed by all co-authors.

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Data availability

The data underlying this article are available in PRJNA1010150, the manuscript and its online supplementary materials.

Declarations

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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