



Identification of new variants and candidate genes in women with familial premature ovarian insufficiency using whole-exome sequencing

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Abstract

Purpose To identify candidate variants in genes possibly associated with premature ovarian insufficiency (POI).

Methods Fourteen women, from 7 families, affected by idiopathic POI were included. Additionally, 98 oocyte donors of the same ethnicity were enrolled as a control group. Whole-exome sequencing (WES) was performed in 14 women with POI to identify possibly pathogenic variants in genes potentially associated with the ovarian function. The candidate genes selected in POI patients were analysed within the exome results of oocyte donors.

Results After the variant filtering in the WES analysis of 7 POI families, 23 possibly damaging genetic variants were identified in 22 genes related to POI or linked to ovarian physiology. All variants were heterozygous and five of the seven families carried two or more variants in different genes. We have described genes that have never been associated to POI pathology; however, they are involved in important biological processes for ovarian function. In the 98 oocyte donors of the control group, we found no potentially pathogenic variants among the 22 candidate genes.

Conclusion WES has previously shown as an efficient tool to identify causative genes for ovarian failure. Although some studies have focused on it, and many genes are identified, this study proposes new candidate genes and variants, having potentially moderate/strong functional effects, associated with POI, and argues for a polygenic etiology of POI in some cases.

Keywords Premature ovarian insufficiency · Whole-exome sequencing · Candidate variants · Ovarian function · Polygenic origin

Introduction

Premature ovarian insufficiency (POI) is defined as the loss of ovarian function before the age of 40 and it is considered as a common cause of female infertility affecting 2–4% of women [1]. The main characteristics of POI are absence of regular menstrual cycles, increased follicle-stimulating hormone (FSH) levels and low serum oestrogen levels. Anti-Müllerian hormone (AMH) is another biochemical marker that can be used for POI diagnosis since it is correlated with the number of developing follicles in the ovary and declines

to very low values at menopause independently of hypothalamic-gonadal axis. The POI condition has a heterogeneous etiology, including iatrogenic, autoimmune or genetic causes. Chromosomal and genetic alterations could explain approximately 20% of cases. Total or mosaic Turner syndrome, X chromosome deletions or X,autosomal translocations are the main causes of POI, followed by premutations in fragile X mental retardation I (*FMR1*) gene [2]. In the past decades, many genes associated with ovarian function have been discovered, especially since the incorporation of next-generation sequencing (NGS) [3–5]. Moreover, through exome sequencing studies, new variants in several candidate genes related with premature ovarian insufficiency (POI) are being identified, not only in genes associated with folliculogenesis, but also involved in processes such as metabolism, DNA repair, apoptosis, meiosis and others [6–8]. Therefore, genetic causes of POI seem to be more common than previously thought. Initially, premature ovarian insufficiency was considered as a monogenic condition; however, recent

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studies have showed a digenic or polygenic origin [9–11]. In the study performed by Bouilly et al. [9], 36% of patients carried mutations in two different genes, while Patiño et al. [10] and Tang et al. [11] found at least two possible candidate variants in distinct genes in a 42% and 85% of the POI patients, respectively, suggesting a synergistic effect of different mutations in several genes. Despite intense research in this field, many genes involved in this pathology remain to be discovered and more studies are necessary. The aim of this study was to identify candidate variants in genes possibly associated with the ovarian failure in a cohort of 7 families affected by POI.

Materials and methods

Study design and population

Fourteen women, from 7 families, affected by idiopathic POI were included in the study from October 2019 to September 2020. Seven POI patients were recruited when they came to our clinic to undergo assisted reproductive treatment. In the anamnesis, it was found that they had at least one first-degree relative with a diagnosis of POI, who were also recruited for the study (with the exception of one family, where the patient's maternal aunt was included). All of them were of Caucasian ethnicity. The inclusion criteria were amenorrhea before 38 years old and ultrasound/analytical signs of ovarian insufficiency ($\text{FSH} \geq 25 \text{ IU/L}$ and/or $\text{AMH} \leq 0.1 \text{ ng/ml}$). Additionally, 46,XX peripheral blood karyotype and normal *FMRI* premutation study. The exclusion criteria were iatrogenic or autoimmune causes of POI, endometriosis and endocrine pathologies. The clinical parameters recorded were menarche age, age at POI diagnosis and serum FSH and/or AMH levels (the latter were only available in index cases, not in relatives).

Moreover, we selected 100 women from our oocyte donation program as an initial control group of the same ethnicity. All women with a 46,XX karyotype and no *FMRI* premutations had an apparently normal ovarian reserve by ultrasound and no family history of POI. The oocyte donors had performed at least one controlled ovarian stimulation (COS) following the standard protocol in our clinic, varying the dose of gonadotropins according to their characteristics (antral follicle count and weight). Ninety-eight of these donors had an adequate response to ovarian stimulation treatments and two of them had a suboptimal response, so they were excluded from the control group. Therefore, in the end, the control group consisted of 98 donors with a normal ovarian reserve and no history of POI.

All work was conducted with the formal approval of the Instituto Bernabeu Institutional Review Board and it follows

the principles of the Declaration of Helsinki. Informed consent was obtained from all participants prior to the study.

Whole-exome sequencing

Peripheral blood sample was obtained from all participants. Genomic DNA was extracted using the MagMAX DNA Multi-Sample Ultra 2.0 kit (Thermo Fisher Scientific, Colchester, UK) on a KingFisher™ Duo Prime system (Thermo Fisher Scientific, Colchester, UK), following the manufacturer's instructions. DNA was quantified using Qubit™ dsDNA HS Assay Kit with the Qubit Fluorometer (Thermo Fisher Scientific, Colchester, UK). Whole-exome sequencing (WES) of the genomic DNA was performed using Trusight One Sequencing Panel (Illumina®) with 150 paired-end reads on NextSeq 550 (Illumina®), according to the manufacturer's protocol. Sequenced data were aligned to the human genome 19 (*hg19*) through Burrows-Wheeler Alignment tool (BWA) and GATK algorithm was used for single nucleotide variations (SNVs)/InDel identification. Variant Call Format files (VCF) were annotated using Variant Interpreter software. In the POI families, only the variants shared by each family were extracted for analysis, and the following criteria were used for filtering and annotation of candidate variants: (1) minor allele frequency (MAF) < 0.05 in the gnomAD and 1000 genomes project for European population, (2) exonic/splicing variants in genes previously associated with POI, genes involved in biological ovarian functions or involved in DNA repair, transcription, meiosis and cell division processes, (3) variants having potentially strong/moderate functional effects on the protein (nonsense, frameshift, inframe deletion, splice region and missense variants). Within the variants with a potentially moderate effect, the missense variants were evaluated using three in silico prediction algorithms (SIFT, PolyPhen-2 and MutationTaster). Other moderate variants, as inframe deletions or variants in splice regions were considered separately. Moreover, the variants were assessed with the guidelines from the American College of Medical Genetics and Genomics (ACMG) [12], and only pathogenic/likely pathogenic variants, or variants of uncertain significance (VUS) were included. According to ACMG score: extremely low frequency in gnomAD population databases was recognized moderate evidence of pathogenicity (PM2). Null variant in a gene (as stop gain or frameshift) where loss of function is a known mechanism of disease was considered as pathogenic (PVS1). Protein length changes resulting from in-frame deletions/insertions in a non-repeat region or a stop-loss variant was recognized pathogenic moderate (PM4). Missense or splicing region variant was assigned as pathogenic, if computational prediction tools unanimously support a deleterious effect on the gene (PP3). Missense variant in a gene with low rate of benign missense mutations and for which

missense mutation is a common mechanism of a disease was included within the possibly pathogenic variants (PP2). Allele frequency is greater than expected for disorder (BS1). Variants observed in a homozygous state in population databases more than expected for disease (BS2). For a missense or a splice region variant, computational prediction tools unanimously support a benign effect on the gene (BP4).

Whole-exome sequencing was performed in oocyte donors using the same protocol. However, in donors, only the candidate genes possibly related with the POI phenotype found in POI families were analysed within the exome sequencing data. The filtering process for variant calling and annotation to identify possibly pathogenic variants was the same as the one detailed above for POI families. The candidate variants identified by WES, in the POI patients/relatives and oocyte donors, were confirmed by Sanger sequencing using BigDye™ Terminator v3.1 Cycle Sequencing Kit and SeqStudio Genetic Analyzer (Applied Biosystems).

Results

A total of 14 women, from 7 families (POI 1–7), affected by idiopathic POI were included, all of them with secondary amenorrhea. The mean menarche age was 13.1 ± 1.0 years and the mean age at POI diagnosis was 34.2 ± 2.3 years, being similar within each family. AMH and FSH levels were available only in the index cases and the mean was 0.04 ± 0.03 ng/ml for AMH and 33.8 ± 6.7 IU/L for FSH (Table 1). After samples sequencing, the mean read depths were 100–180× (varying for each sample), and over 98% of bases were covered at a

minimum of 10× depth in all samples. 32,413 variants with a familial segregation were identified in the WES results of the 14 women, and 3,829 of these, with a minor allele frequency (MAF) < 0.05. Exonic/splicing variants in genes potentially related to the POI phenotype were annotated manually obtaining 76 variants in the seven families (synonymous and non-coding variant were excluded). Among these candidate variants, 2 were nonsense, 1 frameshift, 1 inframe deletion, 66 missense and 6 splice region. Within the missense variants 16 were predicted to have deleterious effects by minimum two of the three in silico algorithms used (SIFT, PolyPhen-2 and MutationTaster), and according to the ACMG classification, we discarded 3 splice region with likely benign annotation based on computational prediction tools. After all filtering process, the final number of candidate variants were 23 (in 22 genes) (Fig. 1), within them, three variants were likely pathogenic and 20 of unknown significance (ACMG criteria). All variants were heterozygous, and five families carried two or more candidate variants. Figure 2 shows the family pedigrees with the genes identified in each family. The variants details are shown in Table 2.

In 2 families (POI 1 and 3), we found variants in genes previously associated with POI phenotype (*CSMD1* and *PIWIL3*) [13, 14]. The remaining candidate genes (*CASP2*, *XPC*, *RXFP2*, *ROBO1*, *HOXA1*, *VWF*, *TPM1*, *TTI2*, *NFKB2*, *VDR*, *MTNRIA*, *EFNA5*, *DLL1*, *JAG1*, *PTHIR*, *HSD3B1*, *SLC6A9*, *GLO1*, *PCMI* and *DDX11*) had never been reported in POI patients; however, they are involved in biological ovarian functions or in cell division, apoptosis, DNA repair or transcription processes, and most of them are expressed in ovary, oocytes or granulosa cells. In

Table 1 Clinical characteristics of POI patients and their relatives

Family ID	Patient-relative ID	Menarche age (y)	Age at POI diagnosis (y)	FSH (IU/L)	AMH (ng/mL)
POI 1	P1	12	30	35	0.11
	R1 (mother)	11	33	-	-
POI 2	P2	14	37	40	0.02
	R2 (sister)	14	35	-	-
POI 3	P3	13	30	31	0.01
	R3 (sister)	14	34	-	-
POI 4	P4	13	34	30	0.05
	R4 (sister)	13	33	-	-
POI 5	P5	12	37	38	0.02
	R5 (sister)	13	36	-	-
POI 6	P6	15	35	-	0.02
	R6 (maternal aunt)	14	37	-	-
POI 7	P7	13	35	39	0.07
	R7 (sister)	13	33	-	-

Menarche age and age at POI diagnosis of all POI patients/relatives are specified. FSH (follicle-stimulating hormone) and AMH (Anti-Mullerian Hormone) were available only in index cases

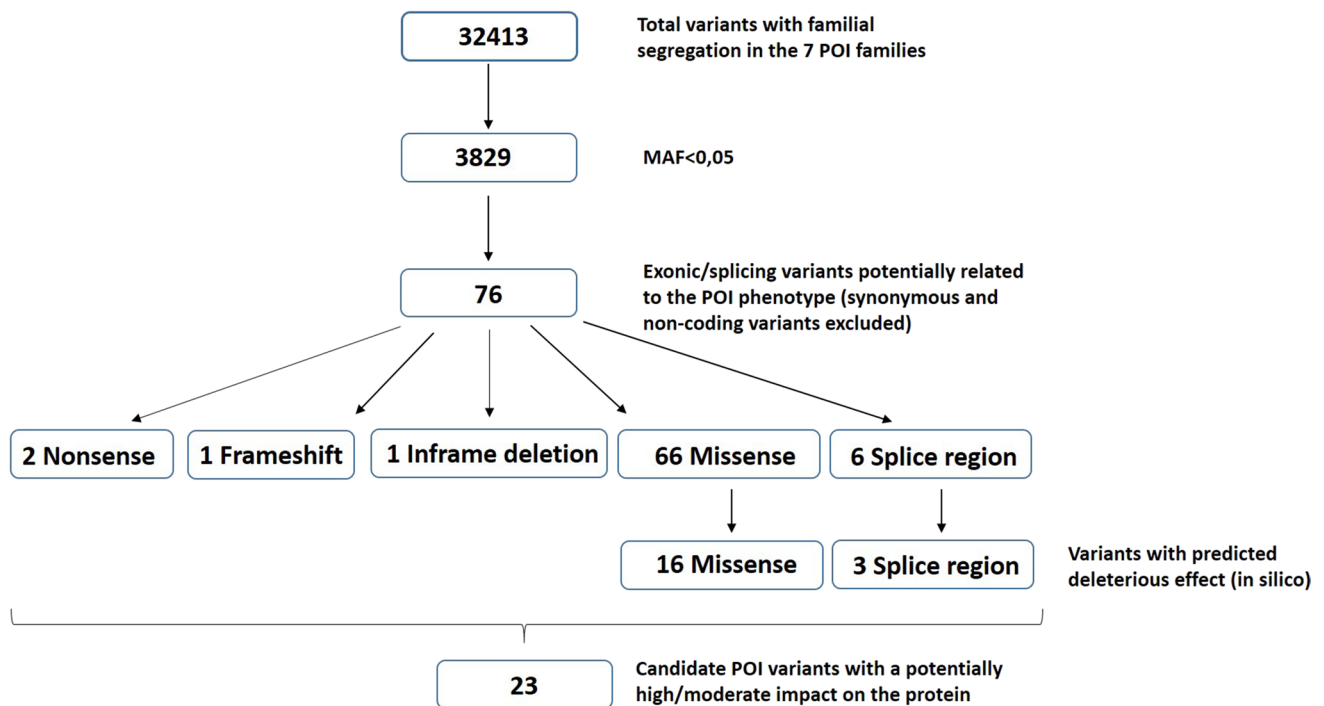


Fig. 1 Flowchart describing variant filtering process. Only the variants with familial segregation were selected (32,413), and filtered according to their minor allele frequency (MAF) < 0.05 (3,829 variants). Exonic/splicing variants potentially related to the POI pheno-

type were annotated manually (excluding synonymous and non-coding variants), resulting in 76 variants. Finally, we retained 23 variants with a potentially deleterious effect on the protein

the case of *VWF* gene, different variants were observed in two families. The gene information is specified in Table 3.

When we analysed the 22 candidate genes in the 98 oocyte donors who formed the control group, we did not find potentially pathogenic variants. The ovarian stimulation of these donors was optimal according to our results within the oocyte donation program with an average of 14.96 oocytes and 11.57 MII retrieved in the oocyte pick-up. However, in the two donors with suboptimal results in the COS cycles who were excluded from the control group, we found one candidate variant in *PIWIL3* gene in the first donor (D1) and two variants in *VWF* and *CSMD1* genes in the second donor (D2) (Table 2). In the first donor, carrying a variant in *PIWIL3* gene, the COS was cancelled on day 7 due to low follicular development after daily administration of 300 IU of rFSH. In the second one (with variants in *VWF* and *CSMD1* genes), only 5 MII were retrieved, after 8 days of stimulation with 300 IU of rFSH.

Discussion

In the current study, we report 23 probably damaging genetic variants in 22 genes expressed, mostly in ovary, oocyte or granulosa cells and related to POI or possibly linked to ovarian physiology. The variants are not yet classified as

pathogenic, but as likely pathogenic or VUS. However, as in previous studies [11, 15], they have been selected because they are very rare in the general population and in silico predictions confer a deleterious effect on protein function. Functional studies are required to confirm the expected pathogenic effect of the variants. In five of the seven POI families (71.4%), we identified two or more variants in distinct genes, suggesting a polygenic aetiology of POI in some cases, according to previous studies [9–11]. For each family, we will discuss the function of the different genes and their possible relationship to the POI phenotype or the ovarian function.

Family POI 1: *CSMD1* and *CASP2*

CSMD1 is a complement regulatory protein that is enriched at the germ-cell/somatic-cell interface in both male and female gonads. Knockout females show significant reduction in ovarian quality and breeding success [16]. In humans, Jaillard et al. [13] identified a copy number variation (CNV) including *CSMD1* gene in a patient younger than 40 years with ovarian failure, and propose this gene as candidate potentially implicated in reproductive function. Caspase-2 (***CASP2***) plays a central role in the execution-phase of cell apoptosis. Several caspases, including caspase-2, have been implicated in granulosa cell death during atresia of maturing

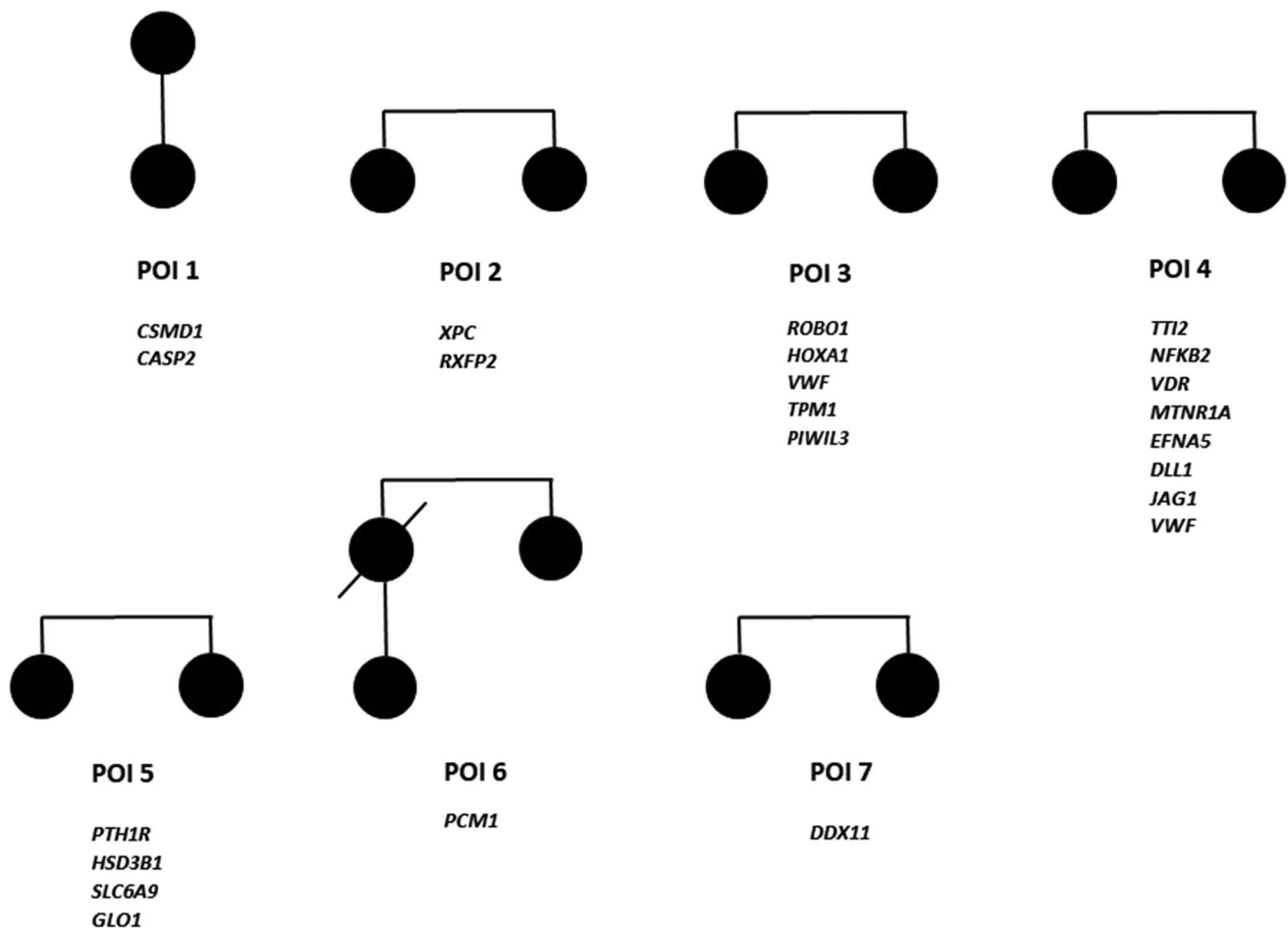


Fig. 2 Pedigrees in POI families (1–7). Filled shapes represent affected individuals. Candidate genes identified in each family are listed

antral follicles in the rat [17], and in oocyte apoptosis occurring developmentally [18, 19].

Family POI 2: *XPC* and *RXFP2*

The protein encoded by *XPC* gene plays an important role in the early steps of genome nucleotide excision repair (NER). It is expressed in the ovary and oocytes where it performs a tissue-specific DNA repair activity [20, 21]. On the other hand, *RXFP2* gene encodes a member of the GPCR (G protein-coupled, 7-transmembrane receptor) family. The receptors for glycoprotein hormones such as follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) are also GPCRs. Kawamura et al. [22] reported paracrine regulation of mammalian oocyte maturation, demonstrating the importance of the *RXFP2* receptor in mediating gonadotropin actions. Moreover, several studies have reported the presence of *RXFP2* receptor and its ligand *INSL3* in the ovary of different animals during the development of antral follicles, which implies a potential

role of the ligand-receptor pair in female reproduction in mammals [23, 24].

Family POI 3: *ROBO1*, *HOXA1*, *VWF*, *TPM1* and *PIWIL3*

Previous studies have showed that the SLIT/Roundabout (**ROBO**) pathway is involved in follicle formation during foetal ovary development [25, 26]. **HOXA1** is a transcription factor that is preferentially expressed in the oocytes and is essential for folliculogenesis and the regulation of oocyte-specific gene expression in the mouse [27]. **VWF** has been suggested to be associated with follicular atresia in mammals [28] and, recently, Wang et al. [29] have proposed **VWF** (an ovarian endothelial cell marker) as a new diagnostic biomarker in age-related human ovarian disorders. **TPM1** is a protein located in the cytoskeleton of granulosa cells and is involved in the regulation of follicular growth [30, 31]. Finally, **PIWIL3** has an important role in maintaining genome integrity in mammalian oocytes [32], and in a

Table 2 Candidate variants identified in POI families (POI 1–7) and oocyte donors (D1 and D2)

Family/Donor ID	Gene	Transcript	dsSNP	Nucleotide variation	Amino acid change	Effect	GnomAD	S/P/M	ACMG score	ACMG classification
POI 1	CSMD1	NM_033225.5	rs1435513451	c.5844 + 3A > G	-	Splice region	0.000072	-	PM2/PP3	VUS
	CASP2	NM_032982.3	rs1026228070	c.1144C > T	p.Arg382Ter	Stop gain	0.000029	D/-/D	PM2/PVS1	Likely pathogenic
	XPC	NM_004628.4	rs1346001152	c.2306 T > C	p.Ile769Thr	Missense	0	D/B/D	PM2	VUS
POI 2	RXFP2	NM_130806.3	rs150652160	c.1826A > G	p.Tyr609Cys	Missense	0.001	D/D/D	PM2/BS2	VUS
	ROBO1	NM_002941.3	rs375897144	c.703G > A	p.Ala235Thr	Missense	0.0001	T/D/D	PM2	VUS
	HOXA1	NM_005522.4	-	c.62G > A	p.Gly21Glu	Missense	0	D/D/D	PM2	VUS
POI 3	VWF	NM_000552.4	rs376285757	c.8324C > T	p.Ser2775Phe	Missense	0.00002	D/B/D	PM2/PP2	VUS
	TPM1	NM_001018005.1	rs397516376	c.548C > T	p.Ala183Val	Missense	0.00004	D/D/D	PM2/PM1/PP2/PP3/PP5	Likely pathogenic
	PIWIL3	NM_001008496.3	rs142590557	c.727C > T	p.Arg243Cys	Missense	0.00072	D/D/-	PM2	VUS
POI 4	TTI2	NM_025115.3	rs140977711	c.1135C > T	p.Arg379Trp	Missense	0.0001	D/D/D	PM2	VUS
	NFKB2	NM_001077494.3	rs200139098	c.1993A > T	p.Thr665Ser	Missense	0.00023	D/D/D	PM2/PP2	VUS
	VDR	NM_001017536.1	rs756858031	c.479G > A	p.Arg160Gln	Missense	0.00011	T/D/D	PM2	VUS
POI 5	MTNR1A	NM_005958.4	rs138837121	c.1002A > T	p.Lys334Asn	Missense	0.0022	D/D/D	PM2/BS2	VUS
	EFNA5	NM_001962.2	-	c.168C > A	p.Asp56Glu	Missense	0	D/D/D	PM2/PP3	VUS
	DLL1	NM_005618.3	rs776343632	c.2167-3del	-	Splice region	0.0018	-	BS1	VUS
POI 6	JAG1	NM_000214.2	rs375017114	c.1627C > T	p.Arg543Cys	Missense	0.00007	D/D/D	PM2/PP2	VUS
	VWF	NM_000552.4	rs368366214	c.3101_3103del	p.Thr1034del	Inframe deletion	0.0002	-	PM4/BS1	VUS
	PTH1R	NM_000316.2	-	c.822_831del	p.Ala275ThrfsTer8	Frameshift	0	-	PVS1/PM2	Likely pathogenic
POI 7	HSD3B1	NM_000862.2	rs775120619	c.258 T > G	p.Ile86Met	Missense	0.00008	D/D/D	PM2	VUS
	SLC6A9	NM_201649.3	rs757420753	c.577G > A	p.(Gly193Ser)	Missense	0.00002	T/D/D	PM2	VUS
	GLO1	NM_006708.2	rs141465532	c.383 T > C	p.(Ile128Thr)	Missense	0.0003	D/D/D	PM2	VUS
D1	PCMI	NM_006197.3	rs148806955	c.4057G > T	p.Glu1353Ter	Stop gain	0.003	-	PM2	VUS
D2	DDX11	NM_152438.1	rs2111769	c.1242 + 4 T > C	-	Splice region	0.000008	-	BP4	VUS
D2	PIWIL3	NM_001008496.3	rs747731185	c.106del	p.Gln36ArgfsTer41	Frameshift	0.0016	-	PM2	VUS
	VWF	NM_000552.4	rs1800386	c.4751A > G	p.Tyr1584Cys	Missense	0.004	D/D/D	PM2/PP2/PP5	Likely pathogenic
	CSMD1	NM_033225.5	rs763163101	c.8926G > A	p.Val2976Met	Missense	0.00002	D/D/D	PM2	VUS

GnomAD, the variant frequency in the *gnomAD* database for non-Finish European population; *S/P/M*, The prediction of SIFT/Polyphe2/MutationTaster bioinformatics tools (SIFT *D*, deleterious; *T*, tolerated/Polyphe2; *D*, probably damaging; *B*, benign/MutationTaster; *D*, disease causing). *ACMG*, American College of Medical Genetics

Table 3 Candidate genes identified in 14 women from 7 families (POI 1–7) with premature ovarian insufficiency

Family ID	Gene	Full name (HGNC)	OMIM	Biological process related to ovarian function
POI 1	CSMD1	CUB and Sushi multiple domains 1	608397	Reproductive structures development
	CASP2	Caspase 2	600639	Cellular apoptosis
POI 2	XPC	XPC complex subunit, DNA damage recognition and repair factor	613208	DNA damage repair
POI 3	RXFP2	Relaxin family peptide receptor 2	606655	Follicle formation and development
	ROBO1	Roundabout guidance receptor 1	602430	Follicle formation and development
	HOXA1	Homeobox A1	142955	Transcription
	VWF	Von Willebrand factor	613160	Follicle atresia
	TPM1	Tropomyosin 1	191010	Follicle formation and development
POI 4	PIWIL3	Piwi like RNA-mediated gene silencing 3	610314	Maintaining genome integrity in mammalian oocytes
	TTI2	TELO2 interacting protein 2	614426	DNA damage repair
	NFKB2	Nuclear factor kappa B subunit 2	164012	Transcription
	VDR	Vitamin D receptor	601769	Follicle formation and development
	MTNR1A	Melatonin receptor 1A	600665	Follicle formation and development
	EFNA5	Ephrin A5	601535	Follicle formation and development
	DLL1	Delta like canonical Notch ligand 1	606582	Follicle formation and development
	JAG1	Jagged canonical Notch ligand 1	601920	Follicle formation and development
	VWF	Von Willebrand factor	613160	Follicle atresia
	POI 5	PTH1R	Parathyroid hormone 1 receptor	168468
HSD3B1		3 beta- and steroid delta-isomerase 1	109715	Follicle formation and development
SLC6A9		Solute carrier family 6 member 9	601019	Oocyte growing
POI 6	GLO1	Glyoxalase I	138750	Follicle atresia
	PCM1	Pericentriolar material 1	600299	Cell division
POI 7	DDX11	DEAD/H-box helicase 11	601150	Cell division and DNA repair

HGNC, HUGO Gene Nomenclature Committee

recent study, *PIWIL3* has been proposed as causal and candidate gene for primary ovarian insufficiency [14].

Family POI 4: *TTI2*, *NFKB2*, *VDR*, *MTNR1A*, *EFNA5*, *JAG1*, *DL11* and *VWF*

The *TTI2* protein is a component of the Triple T complex (TTT) which also includes telomere length regulation protein and TELO2 interacting protein 1. The TTT complex is involved in cellular resistance to DNA damage. On the other hand, *NFKB2* gene encodes a subunit of the transcription factor complex, and may have a role in ovarian function [33]. As for the *VDR* gene, Vitamin D receptor-knockout mice fail to produce mature oocytes, indicating that vitamin D is crucial for folliculogenesis in mice. The presence of vitamin D receptor (VDR) in gonadal tissues in humans, as well as the existence of several animal models are showing adverse reproductive outcomes related to the lack of VDR signalling. Different studies have demonstrated that vitamin D and its receptor play important roles in female reproduction [34, 35]. *MTNR1A* gene encodes one of two high affinity forms of a receptor for melatonin, the primary hormone secreted by the pineal gland. Talpur et al. [36] showed that *MTNR1*

suppression interferes in the normal physiological function of the ovary by enhancing follicular apoptosis, inhibiting proliferation, and influencing hormonal signalling, suggesting that Melatonin receptor 1 performs a critical role in the regulation of the animal reproductive system, particularly in follicular growth. EphrinA5 (*EFNA5*) is a neurogenic factor; however, recent findings suggest that it has a novel role in female mouse fertility. Worku et al. [37] demonstrated in an in vitro study with mouse granulosa cells that *EFNA5* is a pro-apoptotic agent and plays an important role in folliculogenesis by mediating apoptosis, proliferation, and steroidogenesis in female mouse. Human jagged1 (*JAG1*) is the ligand for the receptor notch 1, which is involved in signalling processes. In the mammalian ovary, Notch signalling pathway plays an important role in the mammalian folliculogenesis, regulating primordial follicle formation and coordinating follicular growth [38, 39]. In a mouse model with conditional deletion of Jagged1 within germ cells (J1KO), the histological study showed changes in follicle dynamics, including perturbations in the primordial follicle pool and antral follicle development, and consequently, J1KO female mice were subfertile [40]. On the other hand, Hubbard et al. [41] generated a transgenic Notch Reporter

(TNR) mice, displaying that *JAG1* is necessary for activation of Notch signalling in the developing ovary. Related to this gene, **DLL1** is a human homolog of the Notch Delta ligand and is a member of the delta/serrate/jagged family. It plays a role in cell-to-cell communication. Li et al. [42] demonstrated, using in vitro ovary culture system, that Notch signalling-related genes including *DLL1* were involved in early ovarian follicle development. Finally, **VWF** gene has been suggested to be associated with follicular atresia in a previous study [28].

Family POI 5: **PTH1R, HSD3B1, SLC6A9, GLO1**

Guo et al. [43] in 2019 showed that parathyroid hormone-like hormone (PTH1R) and its receptor (**PTH1R**) play an important role in chicken follicle selection by stimulating cell proliferation and steroidogenesis. In the same way, it could have a similar function in humans. **HSD3B1** enzyme plays a crucial role in the biosynthesis of all classes of hormonal steroids and steroid signalling is required for ovarian primordial follicle formation. Fowler et al. [44] studied the steroid signalling pathways during primordial follicle formation in the human foetal ovary, showing that it is required for this process. GLYT1-mediated glycine transport (**SLC6A9**) is the main cell volume-homeostatic mechanism in mouse eggs and early preimplantation embryos. The *SLC6A9* protein that is responsible for GLYT1 activity and *SLC6A9* transcripts are present in growing oocytes and increased over the course of oogenesis. Furthermore, *SLC6A9* becomes localized to the oocyte plasma membrane as the oocyte grows. Thus, oocytes acquire the ability to regulate their cell volume by releasing adhesion to the ZP and activating GLYT1 as they approach the end of oogenesis [45, 46]. On the other hand, Zhang et al. [47] investigated proteins differentially expressed in the ovaries of menopausal women in comparison to childbearing women, showing that glyoxalase I (**GLO1**) displayed an altered expression pattern, with higher expression in the atresic follicles of menopausal women. These data suggest that this protein may play a role in the regulation of follicle atresia in menopausal women.

Family POI 6: **PCMI**

PCMI is a component of centriolar satellites, essential for the correct localization of different centrosomal proteins, and for joining microtubules to the centrosome. It shows high level of expression in testis and ovaries.

Family POI 7. **DDX11**

DDX11 protein is a RNA helicase implicated in a number of cellular processes involving alteration of RNA secondary

structure and may function at the interface of replication-coupled DNA repair and sister chromatid cohesion. Based on their distribution patterns, some members of this family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division.

The results of this study support the hypothesis that POI is a genetically heterogeneous condition, with a polygenic origin in many cases and still poorly studied. Candidate variants were found in known POI-associated genes (*CSMD1* and *PIWIL3*). In addition, we have identified variants in 20 genes that have never been described in POI patients, but which are expressed in the ovary, and are involved in processes previously associated with POI. Most of these novel genes can be classified into the following categories: apoptosis (*CASP2* and *EFNA5*), DNA damage repair (*XPC*, *TTI2* and *DDX11*), transcription (*NFKB2* and *HOXA1*), cell division (*PCMI* and *DDX11*), follicle formation and development (*RXFP2*, *ROBO1*, *TPM1*, *VDR*, *MTNRIA*, *EFNA5*, *JAG1*, *DL11*, *PTH1R* and *HSD3B1*) and follicle atresia (*VWF* and *GLO1*).

Initially, premature ovarian insufficiency has been considered as a monogenic condition and most papers describe homozygous or compound heterozygous mutations in a gene. The fact that only one rare heterozygous variant has been detected in families POI 6 and 7 could mean that in these families, *PCMI* and *DDX11* genes, both of which are involved in cell division, may be causing POI in the autosomal dominant mode of inheritance. There are a few descriptions of POI associated with monoallelic pathogenic variants in normally autosomal recessively inherited genes, also with an important role in cell division processes, e.g. *FANCA*, *FANCL* and *MCM9*. Heterozygous variants of these genes cause haploinsufficiency and contribute to the pathogenesis of POI, especially secondary amenorrhea [5, 48–51].

Moreover, the presence of two or more heterozygous possibly damaging variants in different genes, in a high percentage of families, means that in some patients ovarian failure could be caused by mutations in genes involved in different processes, as previous studies have shown [9–11]. The phenomenon, in which different genetic variants influence a biological function, as could be ovarian function, is known as synergistic heterozygosity. This is where heterozygous variants of recessive inheritance in different genes partially affect the function of the respective genes and their combination affects intersecting biological routes leading to the phenotype. Synergistic heterozygosity is a special case of multigenic condition where variations in two or more genes are necessary for phenotypic manifestation [52]. However, to test the contribution of these multiple heterozygous variants to ovarian function, a functional validation with RNA and protein expression assays are required.

The finding that no potentially pathogenic variants in the POI candidate genes in this study were found in the control group donors, and that candidate variants were found in two donors who had poor COS cycle outcomes, argues in favour that these genes could be important for ovarian function. Although the phenotype of these two donors is completely different from the POI phenotype of the patients, the low response to ovarian stimulation in these young women could mean a diminished ovarian reserve (DOR) and could be a precondition for a future ovarian failure. In fact, previous studies suggest that POI and DOR are different stages of the same process that may have a common genetic cause [11, 53].

This study has identified potentially pathogenic variants in candidate genes associated to the ovarian function in 14 women affected by POI, from seven families, and two young women with a very low response to the ovarian stimulation cycle. These findings may help future research in this complex pathology. However, a limitation of this study is that in addition to the proband and one POI-affected relative in each family, there were no other affected or non-affected relatives available for analysis. Further studies identifying these genes in other women or families with ovarian failure are necessary to establish a definitive correlation between them and the POI phenotype, especially in case of the polygenic origin.

In summary, it is well demonstrated that the WES is a very useful tool to identify candidate causative mutations in POI patients. This is not only important at the level of diagnosis, but also at the level of prevention in risk patients who can be counselled for oocyte cryopreservation before the ovarian reserve decreases. Moreover, the discovering of new genes potentially involved in ovarian decline will supply better understanding of the ovarian function and the pathophysiological mechanisms of POI.

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Declarations

Conflict of interest None.

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