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**ORIGINAL ARTICLE** ***Reproductive genetics***

# New mutations in non-syndromic primary ovarian insufficiency patients identified via whole-exome sequencing

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**STUDY QUESTION:** Is it possible to identify new mutations potentially associated with non-syndromic primary ovarian insufficiency (POI) via whole-exome sequencing (WES)?

**SUMMARY ANSWER:** WES is an efficient tool to study genetic causes of POI as we have identified new mutations, some of which lead to protein destablization potentially contributing to the disease etiology.

**WHAT IS KNOWN ALREADY:** POI is a frequently occurring complex pathology leading to infertility. Mutations in only few candidate genes, mainly identified by Sanger sequencing, have been definitively related to the pathogenesis of the disease.

**STUDY DESIGN, SIZE, DURATION:** This is a retrospective cohort study performed on 69 women affected by POI.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** WES and an innovative bioinformatics analysis were used on non-synonymous sequence variants in a subset of 420 selected POI candidate genes. Mutations in BMPR1B and GREM1 were modeled by using fragment molecular orbital analysis.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Fifty-five coding variants in 49 genes potentially related to POI were identified in 33 out of 69 patients (48%). These genes participate in key biological processes in the ovary, such as meiosis, follicular development, granulosa cell differentiation/proliferation and ovulation. The presence of at least two mutations in distinct genes in 42% of the patients argued in favor of a polygenic nature of POI.

**LIMITATIONS, REASONS FOR CAUTION:** It is possible that regulatory regions, not analyzed in the present study, carry further variants related to POI.

**WIDER IMPLICATIONS OF THE FINDINGS:** WES and the *in silico* analyses presented here represent an efficient approach for mapping variants associated with POI etiology. Sequence variants presented here represents potential future genetic biomarkers.

**STUDY FUNDING/COMPETING INTEREST(S):** This study was supported by the Universidad del Rosario and Colciencias (Grants CS/CIGGUR-ABN062-2016 and 672-2014). Colciencias supported Liliana Catherine Patiño´s work (Fellowship: 617, 2013). The authors declare no conflict of interest.

**Key words:** whole-exome sequencing / primary ovarian insufficiency / female infertility / molecular etiology / polygenic disease

# Introduction

Primary ovarian insufficiency (POI), is a frequently occurring complex pathology affecting 1% of women under 40 years old (Conway, 2000). Clinically, it is characterized by amenorrhea, hypoestrogenism, and high gonadotropin levels reflecting precocious ovarian depletion of the follicular reserve (Nelson, 2009; De Vos *et al.*, 2010). POI has been proposed as a progressive condition describing ovarian dysfunction (e.g. ovarian function impairment and irregular ovulation) leading to infertility (premature ovarian failure, POF) (Welt, 2008). Although most POI cases are considered idiopathic, genetic anomalies have been described in syndromic and non-syndromic forms of the disease, such as chromosomal abnormalities and point mutations in POI genes' coding regions (autosomes and X-linked genes) (Laissue, 2015; Qin *et al.*, 2015). Mutations in only a few candidate genes have been definitively related to pathogenesis of the disease, despite numerous attempts at identifying sequence variants via Sanger sequencing (Laissue, 2015; Qin *et al.*, 2015 and references therein). This might have been due to the fact that female reproduction requires numerous steps, from sex determination/gametogenesis to ovulation, to guarantee oocyte health for normal fecundation.

It has been shown that several transcription factors (e.g. NR5A1, NOBOX, FIGLA and FOXL2) play key roles during female gonadal development and their mutations lead to POI (Laissue, 2015). Transforming growth factor (TGF-β) molecules and their downstream molecular pathways have also demonstrated to be essential for ovary physiology in distinct mammalian species. BPM15 and GDF9 are especially interesting as they participate as major regulators of mammalian ovulation rate. Furthermore, their mutations have been related to POI origin (Laissue, 2015). Meiotic genes as *MCM8*, *MCM9*, *STAG3*, *SYCE1*, *MSH3*, *MSH4* and *MLH3* have been considered as important molecules for determining the oocyte pool. To date, more than 60 mouse models presenting a well-defined phenotype of ovarian failure have been described (Barnett, 2006; Roy and Matzuk, 2006; Edson *et al.*, 2009; Jagarlamudi *et al.*, 2010; Sullivan and Castrillon, 2011; Monget *et al.*, 2012 and www.jax.org). Such a scenario, in which hundreds of genes are involved in complex dynamic regulatory networks, has hampered selecting relevant candidates to be screened by Sanger sequencing. This constraint, as well as the rarity of families affected by the disease (theoretically facilitating classical genetic mapping), has made research concerning POI genetic causes particularly challenging. Very recently, some studies based on next generation sequencing (NGS) have been successfully undertaken as they have led to new genes being proposed, as well as mutations associated with POI etiology (Caburet *et al.*, 2014; de Vries *et al.*, 2014; Wood-Trageser *et al.*, 2014; Fonseca *et al.*, 2015; Bouilly *et al.*, 2016; Bramble *et al.*, 2016; Fauchereau *et al.*, 2016). However, experiments have not been performed on large genomic regions in unrelated POI individuals.

The present study involved whole-exome sequencing of 69 unrelated Caucasian women affected by POI. Innovative bioinformatics analysis was used on non-synonymous sequence variants in a subset of 420 selected POI candidate genes. Fifty-five coding variants in 49 genes potentially related to the phenotype were identified in 33 out of 69 patients (48%). These genes participate in key biological processes in the ovary, such as meiosis, follicular development, granulosa cell differentiation/proliferation and ovulation. The presence of at least two mutations in distinct genes in 42% of the patients argued in favor of a polygenic nature of POI. Computational 3D modeling, via fragment molecular orbital method, of three mutations (two in BMPR1B and one in GREM1) argued strongly in favor of pathogenic effects. The novel genes and mutations described here represent potential future genetic biomarkers for POI.

# Materials and Methods

## Women affected by POI

Sixty-nine women (Pt-1 through Pt-69) affected by idiopathic POI were included in the study.

These patients were Caucasians living in France who were referred for evaluation to the Reproductive Endocrinology Department at Bicêtre Teaching Hospital and the Endocrinology Department at Reims Robert Debré Hospital, both in France. All patients exhibited at least 6 months of amenorrhea before age 40 with FSH values >20 IU/L measured in two samples at least one month apart and had a normal 46,XX karyotype. Turner syndrome, X-chromosome karyotypic abnormalities and *FMR1* premutations were excluded and none of the patients had circulating ovarian antibodies. Women having antecedents of pelvic surgery, ovarian infections, chemotherapy and/or autoimmune disease were also excluded from the study. Twelve and 57 displayed primary or secondary amenorrhea, respectively.

## NGS, Sanger sequencing and bioinformatics analysis

Total DNA from patients was extracted from blood leukocytes by conventional salting-out procedure. Experimental details of NGS experiments, Sanger sequencing and bioinformatics analysis have been included as Supplementary data.

## Structure preparation, modeling and fragment molecular orbital calculations

Details on the *in silico* approaches for modeling BMPR1B-p.Arg254His, BMPR1B-p.Phe272Leu and p.GREM1-p.Arg169Thr mutations have been included as Supplementary data.

## Ethical approval

All clinical and experimental steps of this study were approved by Institutional Review Board (reference PHRC No. A0R03 052) and by Bicêtre Ethical committee (CPP # PP 16-024 Ile-de-France VII). The clinical investigation was performed according to Helsinki Declaration guidelines (1975, as revised in 1996). All the women had given their informed consent to participate.

# Results

The percentage of reads on target (coverage) ranged from 80% to 95%. Coverage was defined as the percentage of target bases that are sequenced a given number of times. More than 85% of the target was covered at 40× depth. Exome data was uploaded at the Open Science Framework (Patiño, L. 2016, December 16, http://doi.org/10.17605/OSF.IO/EY9ME). Forty three thousand three hundred and thirty seven sequence variants were identified in the POI-420 subset (Fig. 1). 2544 variants having minor allele frequency (MAF) <0.05 were present in the POI-420 group while 137 996 were found throughout the exome (all exome data, *All-ex*). Among POI-420, 524 induced a protein change: seven nonsense, four splice site, 53 frameshift and 460 missense variants. Among these 460 missense variants, 119 had scores compatible with deleterious effects by using PolyPhen-2 and SIFT bioinformatics tools. Fifty-five sequence variants were definitely confirmed by Sanger sequencing (Table I, Fig. 1). All variants were found at heterozygous state. In this series of 69 POI patients, 33 presented one or more confirmed variant (Table I). The frequency of each variation in the ExAC database was indicated. Four genes displayed at least two mutations: *NOTCH2* ($n = 3$), *ADAMTS16* ($n = 2$), *BMPR1A* ($n = 2$), *BMPR1B* ($n = 2$) and *C3ORF77* ($n = 2$).

Clinical characteristics of patients having candidate mutations are shown in Table I. Four patients presented with primary amenorrhea with varying pubertal development. The other patients presented with normal puberty and secondary amenorrhea. Symptoms appeared between 15 and 39 years of age. Hormonal characteristics included markedly elevated FSH (in average 73,6 IU/L), LH (in average 36,5 IU/L) and low levels of estradiol (in average 14,7 ng/L). In sum, among the 33 patients, 19, 9, 2 and 3 patients were found to carry 1, 2, 3 and 4 mutations, respectively. Interestingly 43% of these patients had at least two mutations in different genes arguing in favor of a polygenic origin for POI.

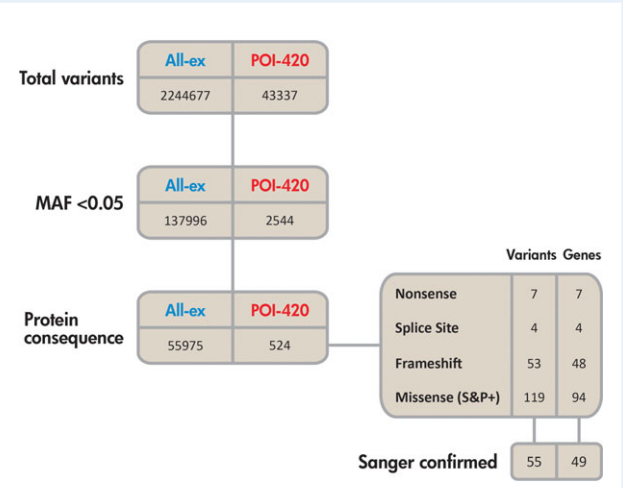


**Figure 1** POI gene subset included 420 candidate genes. Among 2 244 677 total variants, 55 were selected and confirmed by Sanger sequencing. All ex: variants found throughout the exome; MAF: minor allele frequency; Missense S&P+: missense mutations displaying potential deleterious effects by both SIFT and PolyPhen2 bioinformatic tools.

The BMPR1B modeled mutations by fragment molecular orbital (FMO) analysis involved changes in stabilizing interactions (Supplementary Fig. S1). The mutations highlighted a major change in total interaction energy from −54.75 (WT) to −29.54 (MT) kcal/mol (in position Arg254) and −44.69 (WT) to −33.38 (MT) kcal/mol (in position Phe272). Replacing a charged amino acid by a neutral amino acid and the loss of a non-classical H-bond (CH–π interactions) contributed to BMPR1B-MT protein destablization. Similarly, changes of one order of magnitude were found (−239.86 kcal/mol WT versus −27.86 kcal/mol MT) concerning stabilizing interactions between GREM1-WT (wild type) and GREM1-MT (mutant, in position Arg169) (Supplementary Fig. S2). Detailed information on results from FMO analysis has been included as Supplementary Results.

# Discussion

The present work describes whole-exome sequencing in 69 patients who were affected by classical clinical signs of POI. Primary analysis of data was focused on 420 POI candidate genes which had been systematically selected from public databases. Stringent filters (e.g. low MAF, non-synonymous mutations, SIFT and PolyPhen2 software screening) were used to facilitate the selection of rare mutations having (theoretically) moderate/strong pathogenic functional effects. These mutations affected genes involved in several key biological processes, such as meiosis, follicular development, granulosa cell differentiation/proliferation, ovulation, cell metabolism and extracellular matrix regulation (Table I). Although all the 55 filtered variants (and genes) may have contributed to the POI phenotype (some of them probably in an additive/epistatic fashion), several of them belonging to distinct molecular cascades are especially interesting because of their previously described roles in ovary physiology.

*GDF9, BMPR1B, GREM1*, which participate in the TGF-β signaling pathway, have been clearly linked to specific ovary biological functions, such as granulosa cell proliferation, ovulation and/or follicular development regulation (Fig. 2).

*GDF9* (as well as its close homolog *BMP15*) is a soluble oocyte-secreted factor which binds to specific serine/threonine kinase types I and II receptors located on granulosa cell surface (Weiss and Attisano, 2013; Laissue, 2015). Several mutations in humans, most located in the protein's pro-region, have been identified in POI patients and women displaying twinning (Montgomery *et al.*, 2004; Palmer *et al.*, 2006; Laissue *et al.*, 2008; Persani *et al.*, 2014). Functional tests of mutant GDF9 have been seen to have deleterious effects, such as the synthesis of defective mature products, the reduction of mature protein expression/secretion and the inhibition of granulosa cell proliferation (Inagaki and Shimasaki, 2010; Wang *et al.*, 2013; Persani *et al.*, 2014; Simpson *et al.*, 2014). Some mutations, especially those located at the end (C-ter) of the pro-domain, have been related to an increase in granulosa cell proliferation (Simpson *et al.*, 2014). The GDF9 p.Ser83Cys mutation identified in Pt-34 was located in the protein's pro-region which is important for proper protein folding, dimerization, secretion and stability. Similar to other GDF9 mutations located in the pro-region, GDF9-p.Ser83Cys might lead to mature peptide dysfunction and granulosa cell proliferation inhibition.

BMP15:GDF9 heterodimers (which have greater biological activity than either BMP15 or GDF9 homodimers alone) act in human and mouse species via a receptor complex constituted by the BMPR2

**Table I** Clinical and molecular findings of POI patients studied via whole-exome sequencing.

| Patient ID | Phenotype | Age at diagnosis | Hormone values | | | Gene | Locus (position) | Accession number | Mutation | | ExAC allele frecuency | Biological process |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FSH (IU/L) | LH (IU/L) | E2 (ng/L) | | | | Sequence variation | Protein position | | |
| Pt-2 | Primary | 29 | 50 | 16 | 17 | *HK3* | 5;176318162 | NM_002115.2 | c.290G>A | p.Gly97Glu | 0.001034 | Cell metabolism |
| | | | | | | *NOTCH2* | 1;120458122 | NM_024408.3 | c.7223T>A | p.Leu2408His | 0.001788 | Granulosa cell differentiation and proliferation |
| Pt-3 | Secondary | 17 | 91 | 34 | 9 | *GATA4* | 8;11615928 | NM_002052.3 | c.1273G>A | p.Asp425Asn | 0.002117 | Granulosa cell proliferation and differentiation |
| | | | | | | *INHBC* | 12;57843255 | NM_005538.3 | c.509T>A | p.Leu170Gln | 0.002969 | Meiosis |
| | | | | | | *MLH3* | 14;75515926 | NM_001040108.1 | c.433A>G | p.Thr145Ala | ND | Meiosis |
| | | | | | | *PCSK5* | 9;78796345 | NM_001190482.1 | c.2035T>C | p.Tyr679His | 0.000008274 | Ovulation |
| Pt-6 | Secondary | 21 | 64 | 28 | 55 | *TSC1* | 9;135781014 | NM_000368.4 | c.1951A>G | p.Arg651Gly | ND | Follicular development |
| Pt-7 | Secondary | 37 | 83 | 17 | 1 | *ATG7* | 3;11389434 | NM_006395.2 | c.1209T>A | p.Phe403Leu | 0.000008238 | Ovarian reserve |
| Pt-11 | Secondary | 35 | 44 | 23 | 6 | *UMODL1* | 21;43547856 | NM_173568.3 | c.3989T>A | p.Ile1330Asn | 0.0002650 | Granulosa cell differentiation and proliferation |
| Pt-14 | Secondary | 39 | 64 | 27 | 11 | *HTRA3* | 4;8295883 | NM_053044.3 | c.1006C>T | p.Arg336Cys | 0.00004640 | Granulosa cell differentiation and proliferation |
| | | | | | | *NBL1* | 1;19981530 | NM_182744.3 | c.112C>T | p.Leu38Phe | 0.0005640 | Follicular development |
| Pt-16 | Secondary | 39 | 141 | 58 | 7 | *UBR2* | 6;42571438 | NM_015255.2 | c.644C>T | p.Pro215Leu | 0.0001484 | Meiosis |
| Pt-17 | Secondary | 35 | 22 | 14 | 31 | *PCSK1* | 5;95730629 | NM_000439.4 | c.1823C>T | p.Thr608Met | 0.00002471 | Other |
| | | | | | | *BMP6* | 6;7862681 | NM_001718.4 | c.1154G>A | p.Arg385His | 0.00004124 | Follicular development |
| Pt-22 | Secondary | 20 | 101 | 28 | 2 | *CXCR4* | 2;136873083 | NM_003467.2 | c.415G>A | p.Val139Ile | ND | Ovulation |
| Pt-23 | Secondary | 32 | 37 | 5 | 8 | *FGFR2* | 10;123353268 | NM_022970.3 | c.64C>T | p.Arg22Trp | 0.00009078 | Follicular development |
| Pt-24 | Secondary | 37 | 58 | 7 | 12 | *GREM1* | 15;33023397 | NM_013372.6 | c.506G>C | p.Arg169Thr | ND | Follicular development |
| Pt-25 | Primary | 29 | 54 | 25 | 9 | *MEI1* | 22;42095664 | NM_152513.3 | c.122C>A | p.Pro41His | 0.00006178 | Meiosis |
| | | | | | | *GJA4* | 1;35260779 | NM_002060.2 | c.965G>A | p.Arg322His | 0.00009366 | Meiosis |
| | | | | | | *IPO4* | 14;24649689 | NM_024658.3 | c.3205G>C | p.Asp1069His | 0.000008760 | Meiosis |
| | | | | | | *ADAMTS16* | 5;5239880 | NM_139056.2 | c.2365C>T | p.Arg789Cys | 0.001292 | Regulation of the extracellular matrix |
| Pt-34 | Secondary | 16 | 18 | 19 | 21 | *GDF9* | 5;132199978 | NM_005260.4 | c.248C>G | p.Ser83Cys | ND | Granulosa cell differentiation and proliferation |
| | | | | | | *PDE3A* | 12;20769270 | NM_000921.4 | c.1376G>A | p.Arg459Gln | 0.001566 | Meiosis |
| Pt-35 | Secondary | 39 | 72 | 38 | 60 | *PTCH1* | 9;98215817 | NM_000264.3 | c.3392T>C | p.Val1131Ala | ND | |

*Continued*

**Table I** *Continued*

| Patient ID | Phenotype | Age at diagnosis | Hormone values | | | Gene | Locus (position) | Accession number | Mutation | | ExAC allele frecuency | Biological process |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FSH (IU/L) | LH (IU/L) | E2 (ng/L) | | | | Sequence variation | Protein position | | |
| | | | | | | | | | | | | Granulosa cell differentiation and proliferation |
| Pt-36 | Secondary | 27 | 102 | 64 | 5 | *BMPR1B* | 4;96051153 | NM_001256793.1 | c.816C>G | p.Phe272Leu | 0.000008247 | Ovulation |
| | | | | | | *TSC2* | 16;2138096 | NM_000548.3 | c.5116C>T | p.Arg1706Cys | 0.0002665 | Follicular development |
| Pt-37 | Primary | 17 | 56 | 26 | 8 | *BMPR1A* | 10;88681384 | NM_004329.2 | c.1274A>G | p.Tyr425Cys | ND | Follicular development |
| Pt-38 | Secondary | 34 | 105 | 69 | 20 | *LAMC1* | 1;183079729 | NM_002293.3 | c.961C>T | p.Pro321Ser | 0.0005848 | Regulation of the extracellular matrix |
| Pt-39 | Secondary | 28 | 86 | 84 | 7 | *ADAMTS16* | 5;5146365 | NM_139056.2 | c.298C>T | p.Arg100Trp | 0.0008860 | Regulation of the extracellular matrix |
| Pt-41 | Primary | 17 | 42 | 17 | 45 | *PTX3* | 3;157160530 | NM_002852.3 | c.908C>G | p.Pro303Arg | 0.0005518 | Ovulation |
| Pt-42 | Secondary | 32 | 96 | 82 | 20 | *FANCG* | 9;35078733 | NM_004629.1 | c.176G>A | p.Gly59Glu | 0.00003301 | Meiosis |
| Pt-43 | Secondary | 35 | 77 | 52 | 9 | *NOTCH2* | 1;120462920 | NM_024408.3 | c.5411C>T | p.Ser1804Leu | 0.00002472 | Granulosa cell differentiation and proliferation |
| Pt-45 | Secondary | 34 | 136 | 46 | 2 | *MCM9* | 6;119234579 | NM_017696.2 | c.911A>G | p.Asn304Ser | 0.003325 | Meiosis |
| | | | | | | *BMPR1B* | 4;96051098 | NM_001256793.1 | c.761G>A | p.Arg254His | 0.001081 | Ovulation |
| Pt-47 | Secondary | 35 | 12 | 24 | 7 | *SEBOX* | 17;26691490 | NM_001080837.2 | c.362_371delGCACCTCAGT | p.Ser116Ala*fs7 | ND | Meiosis |
| Pt-49 | Secondary | 24 | 136 | 37 | 1 | *FANCL* | 2;58386928 | NM_004629.1 | c.1114_1115insATTA | p.Thr372Asnfs*11 | ND | Meiosis |
| | | | | | | *ZP1* | 11;60637010 | NM_207341.3 | c.319G>A | p.Asp107Asn | 0.002254 | Follicular development |
| | | | | | | *BMPER* | 7;34086005 | NM_133468.4 | c.664C>T | p.Pro222Ser | 0.0002637 | Follicular development |
| Pt-51 | Secondary | 38 | 137 | 78 | 1 | *NOTCH2* | 1;120462898 | NM_024408.3 | c.5433G>C | p.Gln1811His | ND | Granulosa cell differentiation and proliferation |
| | | | | | | *CYP26B1* | 2;72362437 | NM_019885.3 | c.541G>A | p.Val181Met | 0.00009900 | Granulosa cell differentiation and proliferation |
| | | | | | | *PRDM1* | 6;106554919 | NM_001198.3 | c.2036G>A | p.Arg679His | 0.00004120 | Meiosis |
| | | | | | | *STAG3* | 7;99797247 | NM_012447.3 | c.1657G>A | p.Gly553Ser | ND | Meiosis |
| Pt-54 | Secondary | 16 | 65 | 22 | 12 | *PADI6* | 1;17698849 | NM_207421.3 | c.109C>T | p.Leu37Phe | ND | Follicular development |
| | | | | | | *KIT* | 4;55524204 | NM_000222.2 | c.23G>C | p.Trp8Ser | ND | Regulation of follicular development |
| | | | | | | *THBS1* | 15;39874613 | NM_003246.2 | c.287A>G | p.Gln96Arg | ND | Regulation of follicular development |
| Pt-55 | Secondary | 23 | 96 | 43 | 10 | *MTHFR* | 1;11850895 | NM_005957.4 | c.1813T>C | p.Ser605Pro | 0.000008245 | Cell metabolism |
| Pt-56 | Secondary | 31 | 75 | 68 | 10 | *BRD2* | 6;32942354 | NM_001199456.1 | c.4G>T; c.5C>G | p.Ala2Cys | ND | Meiosis |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pt-58 | Secondary | 15 | 60 | 29 | 15 | *SOX15* | 17;7492861 | NM_006942.1 | c.134C>T | p.Pro45Leu | ND | Other |
| | | | | | | *BMPR1A* | 10;88681435 | NM_004329.2 | c.1325G>A | p.Arg442His | ND | Follicular development |
| Pt-59 | Secondary | 23 | 23 | 20 | 19 | *LEPR* | 1;66064368 | NM_002303.5 | c.875C>A | p.Ser292Tyr | 0.0001735 | Ovulation |
| Pt-64 | Secondary | 37 | 114 | 33 | 10 | *PCSK6* | 15;101845484 | NM_002570.3 | c.2891C>T | p.Thr964Met | 0.003727 | Granulosa cell differentiation and proliferation |
| | | | | | | *SAPCD1* | 6;31731303 | NM_001039651.1 | c.226C>T | p.Gln76Ter | 0.0009166 | Other |
| Pt-67 | Secondary | 35 | 38 | 15 | 8 | *BMP5* | 6;55739432 | NM_021073.2 | c.232C>T | p.Pro78Ser | ND | Granulosa cell differentiation and proliferation |
| Pt-68 | Secondary | 39 | 76 | 59 | 30 | *C3orf77* | 3;44284349 | NM_001145030.1 | c.351G>T | p.Lys117Asn | ND | Meiosis |
| | | | | | | *C3orf77* | 3;44284351 | NM_001145030.1 | c.353A>T | p.Glu118Val | ND | Meiosis |

receptor, the ALK4/5/7 type I receptor and the BMPR1B (ALK6) co-receptor (Peng *et al.*, 2013). ALK6 has been shown to be essential for downstream intracellular signaling by triggering SMAD1/5/8 phosphorylation. *Alk6* knockout females have been shown to suffer infertility secondary to cumulus expansion impairment while the p.Gln249Arg mutation in sheep (located in the protein's highly conserved intracellular kinase signaling domain) has been linked to hyperfertility, due to an increase in ovulation rate (Souza *et al.*, 2001; Yi *et al.*, 2001; Davis, 2004). Overexpression of BMPR1B has been described in women having a reduced ovarian reserve (Regan *et al.*, 2016). Both mutations identified in BMPR1B (p.Arg254His and p.Phe272Leu) in the present study were located in the functional intracellular kinase domain, suggesting that they might be associated with POI pathogenesis. In addition, results from FMO analysis suggested a significant change in protein stability secondary to these mutations, which might related to and impairment of the TGF-β signaling between oocytes and granulosa cells (Supplementary Fig. S1).

Regarding TGF-β signaling regulation, *GREM1* (Gremlin1), a member of the DAN family of BMP inhibitors, binds to BMP proteins, preventing them from activating specific receptors (Kattamuri *et al.*, 2012). Although the mechanism used by DAN proteins during BMP ligand inhibition is not well understood, it has been shown that GREM1 regulates important factors having roles during folliculogenesis, such as BMP2, BMP4 and BMP15 (Hsu *et al.*, 1998; Pangas *et al.*, 2004; Nilsson *et al.*, 2014; Church *et al.*, 2015; Bayne *et al.*, 2016) (Fig. 2). *Grem1* knockout mice have displayed delayed meiotic progression, defects regarding primordial follicle assembly dysfunction and a reduced amount of oocytes (Myers *et al.*, 2011). GREM1 is expressed in humans during early and until late stages of follicular development, and has been linked to granulosa cell development (Kristensen *et al.*, 2014; Bayne *et al.*, 2016). Furthermore, a significant decrease in its expression has been reported in women having reduced ovarian reserve (Jindal *et al.*, 2012).

The GREM1-p.Arg169Thr mutation found in Pt-24 strongly suggests a functional role since it is located in a critical region ($Pro^{145}$ to $Gln^{174}$ residues) of the DAN domain which directly interacts with BMP4 (Sun *et al.*, 2006). Furthermore, the GREM1-$Arg^{169}$ residue is conserved in other DAN-family members and among numerous vertebrate species (Sun *et al.*, 2006; Veverka *et al.*, 2009). Indeed, abnormal folding of the β2/β3 (finger 2) sheet could modify the protein's local chemical properties which might then lead to interaction disturbances with BMP4 (or other BMP factors). As for BMPR1B mutations, the FMO analysis showed that the GREM1-p.Arg169Thr mutation led to changes in protein stability which might contribute to the phenotype (Supplementary Fig. S2). These findings strongly suggest a relevant role for TGF-β proteins, especially those involved in oocyte-to-granulosa cell signaling, during POI pathogenesis.

Concerning molecules involved in meiosis, the present study was able to identify 16 mutations potentially contributing to the phenotype. Functional protein association networks of some meiotic proteins have been included as supplemental material (Supplementary Fig. S3). STAG3 and MCM9 are especially interesting due to their well-established role during female fertility and POI. To date, all mutations in meiotic genes linked to POI etiology have been found in biallelic state (homozygous or compound heterozygous) thereby underlining meiosis' key role in reproduction and species maintenance (Caburet *et al.*, 2014; de Vries *et al.*, 2014; Wang *et al.*, 2014; Wood-Trageser *et al.*, 2014; AlAsiri *et al.*, 2015; Fauchereau *et al.*, 2016). Mutations in meiotic genes were present at heterozygous state in our present

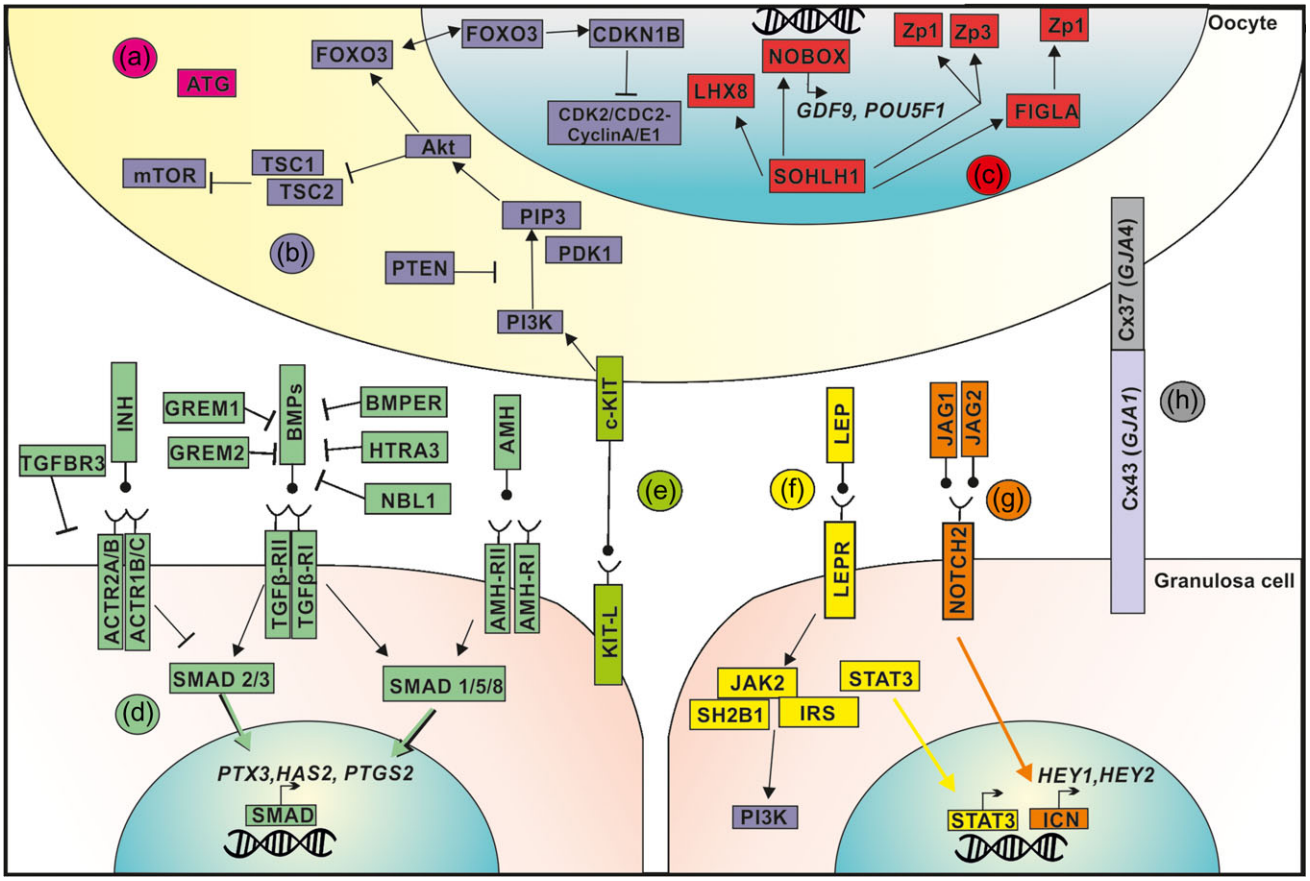


**Figure 2** Signaling pathways and proteins involved in follicular development. (**a**) Autophagy; (**b**) P13K/AKT pathway; (**c**) SOHLH1 pathway; (**d**) TGF-β's pathway; (**e**) KIT-L and c-Kit; (**f**) leptin pathway; (**g**) NOTCH pathway; and (**h**) connexins.

study, which might be associated with a background of POI predisposition. Further variants would be necessary to originate the phenotype in such hypothetically scenario. Interestingly, we found that 64% (7 out 11) of patients having a heterozygous mutation in a meiotic gene were carriers of at least one further variant in the same or a distinct gene.

Interestingly, we have found three different mutations in *NOTCH2*, a gene encoding one of the four NOTCH family single-pass Type I (SPTI) transmembrane receptors (Andersson *et al.*, 2011). The NOTCH2-p.Ser1804Leu, p.Gln1811His and p.Leu2408His mutations identified in the present study were located in the intracellular domain of the protein which translocates to the nucleus where it mediates transactivation/repression (Kopan and Ilagan, 2009). Thus, it would be possible that these mutant forms lead to expression disturbances of key target genes involved during oocyte development.

We consider that additional mutations in genes participating in follicular development, granulosa cell differentiation and proliferation, ovulation and extracellular matrix regulation could also contribute to the phenotype due to their molecular behavior during ovary development and physiology. For example, this is the case of ATG7-p.Phe403Leu, THBS1-p.Gln96Arg, PTCH1-p.Val1131Ala, PCSK6-p.Thr964Met, UMODL1-p.Ile1330Asn, ADAMTS16-p.Arg100Trp, p.Arg789Cys and PTX3-p.Pro303Arg.

To note, in clinical practice it has been observed that patients affected by POI report similar phenotypes in some women from their families which suggests a genetic origin of the disease.

In our case, although candidate mutations have not shown to be clustered in particular familial cases, incomplete penetrance cannot be excluded. Thus, it would be interesting to study potential segregation analysis of interesting variants but, unfortunately, although we did propose to most of our POI patients the idea of contacting their parents regarding their participation in our study they decided not to involve their families.

The genetic approach presented here revealed that 33 out of 69 (48%) patients were carriers of mutations potentially related to the phenotype. Interestingly, 42% of these patients had at least two mutations in different genes and 49 out 55 variants were identified in distinct genes, thereby arguing in favor of a polygenic origin for POI. Furthermore, our findings evoke the importance of rare variants in complex disease pathogenesis and contribute information for resolving genomic concerns such as 'missing hereditability' (Manolio *et al.*, 2009; Gibson, 2012; Lee *et al.*, 2014; Laissue, 2015).

Concerning our methodological approach it is clear that correct gene subset configuration depends on multiple variables, such as the availability of previous accurate data relating specific genes to ovarian biology

and the rigor (and method) used when investigating potential candidates. This approach may lose further candidates contributing to the phenotype. However, we consider that it represents interesting middle ground between a large amount of genomic data (e.g. All-ex variants) and the results obtained from other sequencing designs (custom array sequencing or single Sanger approaches). An advantage of the present design is that the availability of sequences from all encoding regions enables future reanalysing of data by including additional genes and/or by setting up alternative methods (e.g. interactome approaches).

We estimate that whole-exome sequencing and the *in silico* analysis presented here represent an efficient approach for mapping variants (having potentially moderate/strong functional effects) associated with POI etiology. Further NGS studies, performed in larger panels of women affected by POI, would be a valuable exercise to identify novel causative mutations. Taken together, our findings add valuable information regarding POI molecular etiology and ought to form the starting point for further functional *in vitro* and *in vivo* studies.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

## Authors' roles

Clinical work was performed by B.D., J.Y., N.B. and I.B. The experiments were performed by L.C.P., C.C., J.C.B. M.A.P., C.F.S. and R.G. performed the FMO analysis. All authors contributed to interpretation of findings. The study was designed and directed by P.L. The manuscript was draft by P.L. with contributions to revision and final version by all authors.

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## Conflict of interest

The authors declare no conflict of interest.

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