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# The molecular complexity of primary ovarian insufficiency aetiology and the use of massively parallel sequencing

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ABSTRACT

Primary ovarian insufficiency (POI) is a frequently occurring pathology, leading to infertility. Genetic anomalies have been described in POI and mutations in numerous genes have been definitively related to the pathogenesis of the disease. Some studies based on next generation sequencing (NGS) have been successfully undertaken as they have led to identify new mutations associated with POI aetiology.

The purpose of this review is to present the most relevant molecules involved in diverse complex pathways, which may contribute towards POI. The main genes participating in bipotential gonad formation, sex determination, meiosis, folliculogenesis and ovulation are described to enable understanding how they may be considered putative candidates involved in POI. Considerations regarding NGS technical aspects such as design and data interpretation are mentioned. Successful NGS initiatives used for POI studying and future challenges are also discussed.



**Contents**



## 1. Introduction

The World Health Organization has defined human infertility as a disorder characterised by the failure to become pregnant after at least 12 months of regular and unprotected intercourse. It can be considered a public health concern since it affects more than 50 million couples worldwide (Datta et al., 2016; Mascarenhas et al., 2012). Although precise infertility prevalence has been difficult to establish, especially due to differences regarding its definition and because study populations vary depending on the pertinent inclusion/exclusion factors, 5%–20% has been reported (Datta et al., 2016; Mascarenhas et al., 2012).

Abnormalities explaining infertility in 70% of couples can be detected after standard clinical testing; however, despite considerable advances in human infertility diagnosis the aetiology remains unexplained in 30% of cases. Exclusively female factors

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(accounting for a third of all causes of infertility) include dysfunction leading to ovulation disorders (e.g. oligomenorrhoea, amenorrhoea), tubal disease (e.g. obstruction) and endometrial pathology (endometriosis, miscarriage) (Smith et al., 2003). The correct functioning of each step (e.g. sex determination, meiosis, gametogenesis, folliculogenesis/oocyte maturation, ovulation) leading to healthy competent oocyte ovulation is certainly crucial for ensuring reproductive success in mammals. Dysregulation regarding any of these steps may lead to primary ovarian insufficiency (POI), currently affecting ~1.5% of women worldwide (Luborsky et al., 2003). POI is defined as the cessation of menses or spaniomenorrhea over 4 months and plasmatic levels of FSH over 25 IU/l, reflecting ovarian impairment thereby closing a feed-back loop in the pituitary gland (European Society for Human Reproduction and Embryology (ESHRE) Guideline Group on POI, 2016). It can result from disturbances during development (formation and recruitment) during follicles' stock establishment during embryo life or by its premature depletion due to abnormally high levels of atresia (Persani et al., 2011). Several POI aetiologies have been described, but most cases are still classified as idiopathic, thereby enhancing genetic factor research. Chromosomal abnormalities have been described, especially those involving the X chromosome, as deletions (Turner's syndrome) and translocations (Elsheikh et al., 2002; Lacombe et al., 2006). Point mutations have also been identified in some POI candidate genes, some of which have been formerly validated by functional tests as being aetiological (see below) (Laissue, 2015) (and references therein).

From a molecular view, it has been shown that hundreds of genes belonging to overlapping complex molecular cascades in ovarian tissue are subtly regulated during the numerous steps involved (i.e. from sex determination to ovulation) (Biason-Lauber and Chaboissier, 2015; Eggers et al., 2014; Hunter, 2015; Koopman, 2016; Matzuk and Burns, 2012; Matzuk and Lamb, 2008, 2002; Suzuki et al., 2015; Wood and Rajkovic, 2013) (and references therein). These genes are particularly interesting as their mutations might contribute towards POI aetiology.

Numerous genes have been analysed during the past two decades (especially by Sanger sequencing) to identify sequence variants related to POI. However, this task has been particularly challenging due to the high number of candidate genes and because of the limited genomic coverage of Sanger sequencing. Indeed, analyzing large genomic regions via direct sequencing involves numerous time-consuming PCR/sequencing experimental setup steps.

In an attempt to overcome this drawback, recent next generation sequencing (NGS) genomic screening approaches to family-based and non-related cases have identified new genes and mutations associated with specific female infertility disorders, such as POI (AlAsiri et al., 2015; Bouilly et al., 2016; Caburet et al., 2014, 2012; Carlosama et al., 2017; de Vries et al., 2014; Desai et al., 2017; Fauchereau et al., 2016; Fonseca et al., 2015; Guo et al., 2017; Patiño et al., 2017a; Qin et al., 2015a; Wood-Trageser et al., 2014; Bramble et al., 2016; França et al., 2017). NGS has certainly revolutionised medical genomics research as it has led to hundreds of monogenic and complex disease-related genes being described (McCarthy et al., 2013). NGS is currently being widely used for research and clinical purposes due to a concomitant decrease in its costs. However, data filtering and analysis is still challenging since it implies (especially for complex frequently-occurring pathologies) an in-depth knowledge of the molecular cascades regulating numerous physiological steps, as well as expertise in interpreting the potential pathogenicity conferred by particular mutations.

This review has thus been aimed at targeting the most relevant molecules involved in the diverse pathways playing key roles regarding ovarian tissue which may contribute towards POI. The main genes and proteins participating in bipotential gonad formation, sex determination, meiosis, folliculogenesis and ovulation are described to enable understanding how they may be considered putative candidates involved in POI. Considerations regarding NGS technical aspects such as design and data interpretation are mentioned. Successful NGS initiatives and future challenges are also discussed.

Taken together, the information presented here should enable a better understanding of the molecular origin of a frequently-occurring female disorder for clinicians and basic science researchers. It should also facilitate designing new diagnostic approaches and interpreting the molecular tests used in clinical environments.

## 2. NGS: design and data interpretation

During the past 10 years NGS has substantially contributed to the understanding of the molecular origin of rare and frequent diseases. This technique, which has evolved technically leading to improved levels of sensitivity, is available nowadays at affordable costs. All NGS platforms need DNA pre-processing to create a library for sequencing. Various steps are required: DNA fragmentation into specific sizes, generating blunt-ended DNA fragments, adapter ligation, PCR to increase library concentration and enrichment with specific capture probes (e.g. exons for exome sequencing). Varied NGS sequencing technologies are currently being offered by commercial brands, such as Illumina, Ion Torrent, Pacific Biosciences, Solid and Roche 454, each having advantages and pitfalls regarding genomic coverage of specific regions (repeated and GC rich, read sensitivity and costs) (Buermans and den Dunnen, 2014; Goodwin et al., 2016). Distinct NGS formats, mainly defined by the length of the genomic region which is to be sequenced, are available for research and diagnostic purposes. Three main formats are currently available: target sequencing microarrays (TSM) as panels of specifically selected regions, whole-exome sequencing (WES) which includes the entire genome's coding regions and whole-genome sequencing (WGS). TSM and WES sequencing are used for research and diagnosis while WES is reserved for investigative purposes due to the inclusion of extensive intron regions for which the functional nature remains largely unknown. One technically important NGS variable (named sequencing depth) is associated with the number of times that each nucleotide is read during a specific experiment. An appropriate read depth is essential for obtaining reliable results due to NGS' inherent nature which is based on resequencing DNA fragments susceptible to carrying unexpected variations caused by PCR amplification (Sims et al., 2014) (and references therein). For instance, it has been estimated that a 40X to 80X average depth is necessary to cover >90% of target genes for WES experiments and for high sensitivity genotyping of heterozygous SNPs (Clark et al., 2011; Meynert et al., 2014, 2013; Sims et al., 2014). Regarding diagnostic purposes, it has been proposed that a 120X depth in WES is suitable for identifying most non-synonymous variants causing disease (Kim et al., 2015). However, most research reports use PCR/direct sequencing to validate candidate sequence variants identified by NGS screenings as the average depth read ranges between 10X-60X. After confirmation by Sanger sequencing, variants are used to establish links with normal or pathogenic traits. Distinct pipelines and variables have been used to select variants underlying potential pathogenic effects, depending on a disease's genetic nature (mono/polygenic), type of inheritance (dominant, recessive) and disease presentation (familial/non-familial). Several bioinformatics methods, some of them involving automatic tools for directly analysing NGS data, have been used for predicting missense variants' potential pathogenic effect. Some of them (e.g.

SIFT, Polyphen) take conservation into consideration during interchanged residues' evolution for estimating a variant's probability of being deleterious. However, as databases still contain incomplete (not curated) sequences and because of structural/functional proteomics' inherent complexity, such predictors are not completely reliable. Manual inter-specific sequence alignments are also used, assuming that amino acid conservation at specific positions during evolution must have been related to functional characteristics. These features, as well as the fact that heterozygous variants are very numerous in the human genome, hamper selecting candidate variants which may potentially be responsible for frequently occurring oligogenic/polygenic diseases (in some cases).

Advantages of NGS on Sanger sequencing include the possibility to analyse numerous large genomic regions simultaneously at competitive costs. However, although NGS technology has rapidly evolved it still has disadvantages, as some genomic regions are not suitable covered and false positive/negative findings result from low read depth.

Regarding POI, several NGS studies, accompanied by genetic linkage analysis via microarray genotyping in some cases, have been performed in familial and isolated (non-related women) cases (Table 1) (AlAsiri et al., 2015; Bouilly et al., 2016; Caburet et al., 2014, 2012; Carlosama et al., 2017; de Vries et al., 2014; Desai et al., 2017; Fauchereau et al., 2016; Fonseca et al., 2015; Qin et al., 2015b; Wood-Trageser et al., 2014). These studies will be reviewed in next sections.

## 3. Bipotential gonad formation molecules: an overview

Molecular factors and mechanisms, especially the subtle regulation of hundreds of genes belonging to numerous pathways, are major determinants of reproductive success in mammals. Sex determination is a fundamental early developmental event for establishing sexual dimorphism as well as a prerequisite for normal fertility and species' maintenance. It involves numerous molecular pathways enabling ovary and testis differentiation from a single undifferentiated gonad. Some genes have complex functioning as they are expressed during distinct phases of bipotential gonad formation/sex determination and their mutations can lead to sex reversal and infertility phenotypes.

An undifferentiated gonad becomes evident before embryonic day (E) 10.5 in mice, the genital ridges arise from the coelomic epithelium located in mesonephric tissue (Svingen and Koopman, 2013; Wainwright et al., 2014). Important roles have been described for at least six transcription factor-coding genes during bipotential gonad determination: Wilms' tumor suppressor 1 (*WT1*), nuclear receptor subfamily 5, group A, member 1 (*NR5A1*), LIM homeobox gene 9 (*LHX9*), GATA-binding protein 4 (*GATA4*), chromobox homolog 2 (*CBX2*) and empty-spiracles homeobox gene 2 (*EMX2*) (Biason-Lauber and Chaboissier, 2015). Although mutations in these genes have been mainly related to sex reversal phenotypes, they have also been linked to infertility conditions in some cases; this has been due to them being able to be expressed during other development stages.

WT1 encodes a zinc finger transcription factor having many alternative transcripts and isoforms. Two main isoforms having (KTS) or lacking (-KTS) three amino-acids (lysine, threonine, serine) between the third and the fourth zing fingers have mainly been studied. The protein's −KTS form has been shown to have classical DNA-binding transcription factor properties and greater DNA affinity than WT1-KTS which has higher binding affinity for RNA molecules (Bickmore et al., 1992; Laity et al., 2000; Rauscher et al., 1990). The homozygous deletion of *Wt1* in mice has led to a lack of gonads and kidneys in both genders, thereby demonstrating its crucial role during bipotential gonad formation (Kreidberg et al., 1993). Furthermore, the complete absence of *Sf1* expression in $Wt1^{-/-}$ embryos has led to establishing that Wt1 participates in *Sf1* regulation (Wilhelm, 2002). WT1 expression has also been observed during sex determination and testicular formation, underlining its crucial role during gonadal development (see

**Table 1**
Studies using NGS for identifying sequence variants related to POI aetiology.

| POI presentation | NGS format | Gene | Zygocity | In vitro testing | Reference |
|---|---|---|---|---|---|
| Familial | WES | *STAG3* | HH | Yes | Caburet et al., 2014 |
| Familal | WES | *SYCE1* | HH | No | de Vries et al., 2014 |
| Familial | WES | *MCM9* | HH | Yes | Wood-Trageser et al., 2014 |
| Familial | WES | *MCM9* | HH | No | Fauchereau et al., 2016 |
| Familial | WES | *MCM8* | HH | Yes | AlAsiri et al., 2015 |
| Familial | WES | *CSB-PGBD3* | HT | Yes | Qin et al., 2015a,b |
| Familial | WES | *FSHR* | HH | Yes | Bramble et al., 2016 |
| Familial | WES | *FSHR* | HH | No | França et al., 2017 |
| Familial | WES | *MSH5* | HH | Yes | Guo et al., 2017 |
| Familial | WES | *MSH4* | HH | Yes | Carlosama et al., 2017 |
| Non-familial | TSM | *LHCGR, BMPR2, ADAMTS19* | HT | No | Fonseca et al., 2015 |
| Non-familial | WES | *MCM8/MCM9* | HH/HT/CH | No | Desai et al., 2017 |
| Non-familial | TSM | *LHX8, SOHLH1, SMC1β* | HT | Yes | Bouilly et al., 2016 |
| Non-familial | WES | *HK3, NOTCH2, GATA4, INHBC, MLH3, PCSK5, TSC1, ATG7, UMODL1, HTRA3, NBL1, UBR2, PCSK1, BMP6, CXCR4, FGFR2, GREM1, MEI1, GJA4, IPO4, ADAMTS16, GDF9, PDE3A, PTCH1, BMPR1B, TSC2, BMPR1A, LAMC1, PTX3, FANCG, MCM9, SEBOX, FANCL, ZP1, BMPER, CYP26B1, PRDM1, STAG3, PADI6, KIT, THBS1, MTHFR, BRD2, SOX15, LEPR, PCSK6, SAPCD1, BMP5, C3orf77* | HT | No | Patiño et al., 2017a |

**WES:** whole-exome sequencing; **TSM:** target sequencing microarrays; HH: homozygous; HT: heterozygous; **CH**: compound heterozygous.

below). In humans, mutations in *WT1* have been liked to various phenotypes and diseases: Wilms tumor, WAGR (Wilms tumor, aniridia, genitourinary anomalies and mental retardation) syndrome, Frasier syndrome, Denys-Dash syndrome, Meacham syndrome and isolated diffuse mesangial sclerosis (Miller-Hodges and Hohenstein, 2012; Ezaki et al., 2015; Bahrami et al., 2017).

*NR5A1* encodes an orphan nuclear receptor which is expressed from E9.0 in mice. It performs essential actions during hypothalamic-pituitary-adrenal-and gonadal axis development (Luo et al., 1994; Wong et al., 1996). Structurally, SF1 has a DNA-binding domain (DBD) with two zinc-chelating modules modulating receptor interaction with response elements. SF1 has a ligand-binding domain (LBD) and two activation domains, named AF-1 and AF-2. *Nr5a1* homozygous XY null mice have been affected by gonadal dysgenesis and male-to female sex reversal while *NR5A1* mutations in humans have been linked to sex determination disturbances and infertility, including POI (El-Khairi and Achermann, 2012).

*LHX9* encodes a LIM protein family transcription factor consisting of a DNA-binding homeobox domain and two N-terminal domains expressed in the urogenital ridge's medial region (E9.5) (Birk et al., 2000). Lhx9 regulates *Sf1* during early gonad development and its homozygous ablation in mice has displayed male-to-female XY sex reversal secondary to anti-Müllerian hormone (AMH) and testosterone deficiency (Birk et al., 2000; Wilhelm, 2002). GATA4 is a member of the GATA transcription factor family, having two conserved type IV finger motifs which are involved in DNA-recognition, binding stability and interaction with co-factors (Tevosian, 2014) (and references therein). It has been shown that *Gata4* is necessary for genital ridge development before the expression of other key genes, such as *Emx2*, *Wt1*, *Nr5a1* and *Lhx9* (Hu et al., 2013). Although, *Gata4* knock-out (KO) mice die before birth secondary to drastic abnormalities in heart-tube development, animals carrying a target mutation located at the N-terminal zinc finger domain (*Gata4*$^{Ki}$ model) have been affected by testis differentiation disturbances (Kuo et al., 1997; Molkentin et al., 1997).

*GATA4* mutations in humans have been described in individuals affected by congenital heart disease and in a family displaying testicular developmental defects (Gly221Arg mutation) (Lourenço et al., 2011).

CBX2 belongs to the Polycomb family of transcriptional regulators which function by regulating chromatin structure (Schuettengruber et al., 2007). These proteins have been seen to play multiple roles during cell differentiation and molecular development, such as cell fate decisions, X-chromosome inactivation and sexual differentiation (Biason-Lauber and Chaboissier, 2015) (and references therein). *Cbx2* homozygous XX KO mice have been seen to have small ovaries and infertility secondary to germ cell loss while XY individuals have been affected by male-to-female sex reversal (Katoh-Fukui et al., 2012, 1998). Although a loss of function mutations in humans has been identified in individuals displaying male-to-female sex reversal, other kinds of mutations may have led to ovarian pathology, such as POI (Biason-Lauber et al., 2009). EMX2, which encodes a homeodomain transcription factor, has been seen to play a relevant role during central nervous and urogenital system development as KO animals lack gonads and genital tract (Miyamoto et al., 1997). However, little is known concerning its regulation on downstream gene targets.

## 4. Sex determination: ovary genes

Classically, it has been accepted that a particular genomic region located on the Y-chromosome (the testis-determining locus-*Tdy* or testis-determining factor-*TDF*) is necessary for activating the male sex determination programme (Burgoyne et al., 1988; Koopman et al., 1989). Conversely, a lack of this region, as well as anti-testis and pro-ovarian gene expression, has led to female gonadal phenotype formation. *Sry* (the Y chromosome sex-determining region), located into the *Tdy*, has been defined as the key molecular actor which initiates testicular development (11.5 days post coitum (dpc) in mice) (Eggers et al., 2014; Koopman, 2016; Windley and Wilhelm, 2015) (and references therein). Thereafter, numerous genes such as *SOX9*, *AMH*, *DHH*, *FGF9* and *DMRT1* participate in a fine regulated network during male gonadal development (Koopman, 2016).

Regarding the female sex determination programme, wingless-type MMTV integration site family, member4 (*Wnt4*) and R-spondin 1 (*Rspo1*), initially expressed in early XX and XY structures, are specifically expressed in a developing ovary 11.5–12.5dpc (Nef et al., 2005; Parma et al., 2006; Vainio et al., 1999). Wnt4 is a member of the Wnt family of secreted factors acting in a paracrine manner during development. Wnt and Rspo1 activate and stabilise β-catenin (CTNNB1) thereby contributing to the regulation of downstream genes having key functions during ovary development. Molecular signalling involving WNT4, RSPO1 and CTNNB1 act downstream SRY expression by antagonising pro-testicular factors (Jameson et al., 2012; Kim et al., 2006; Nicol and Yao, 2015). *WNT4* heterozygous mutations (exerting a dominant negative effect) in humans have been linked to Mullerian aplasia and hyperandrogenism, while homozygous changes have led to sex reversion, kidneys, adrenal and lung dysgenesis (SERKAL syndrome) (Biason-Lauber et al., 2004; Biason-Lauber and Chaboissier, 2015; Mandel et al., 2008). Due to *WNT4*'s key role during female gonadal development, its mutations might also be related to specific ovary diseases lacking sex-reversal phenotypes (e.g. POI). However, although *WNT4* has been genotyped in women affected by POI, functional mutations have not been described (Chen et al., 2011; Lakhal et al., 2012). Concerning *RSPO1* and human disease, homozygous drastic mutations have been reported in patients affected by XX sex reversal, skin cancer and, palmoplantar hyperkeratosis (Parma et al., 2006; Tomaselli et al., 2008). However, other kinds of mutation may contribute to ovary dysfunction and infertility.

Forkhead box L2 (*FOXL2*) encodes a forkhead box transcription factor family member involved in several development-related biological processes. *FOXL2* has been described as being one of the earliest markers of ovarian development and its expression throughout female fertile life (in granulosa cells) contributes towards preserving ovarian function. *Foxl2* is also expressed in the mesenchyme of developing eyelids as well as in pituitary thyrotroph and gonadotroph cells (Crisponi et al., 2001; Ellsworth et al., 2006). It has been proposed that FOXL2 may directly and indirectly regulate many target genes (e.g. *SOX9*, *FST*, *CYP19A1*, *CDKN1B*, *PTGS2*, *BCL2A1*, *FSHB*) playing relevant roles during sex determination and ovarian function (Georges et al., 2013) (and references therein). *Foxl2* gene invalidation in XX mice has led to gonadal abnormalities without sex-reversal (Schmidt et al., 2004; Uda et al., 2004) whereas FOXL2 mutations in humans have been associated with blepharophimosis, ptosis, epicanthus inversus syndrome (BPES), a rare dominant disease, displaying POI (BPES type I) (Crisponi et al., 2001). Interestingly, although FOXL2 mutations have not been related to XX sex-reversal, three cases have been linked to POI without BPES (Elzaiat et al., 2017). These findings illustrate that sex determination factors can be coherent candidates related to gonadal-related female infertility disorders.

## 5. Meiosis genes

Human primordial follicles (formed by an oocyte and a single

layer of pre-granulosa cells) arise from a pool of primordial germ cells (PGCs) during the first steps of ovary development. Meiosis I is initiated after a series of mitotic PGC divisions but becomes transitorily arrested until puberty when mature follicles are activated secondary to LH secretion. LH effects at this stage lead to nuclear maturation, germinal vesicle breakdown and the termination of meiosis I. Oocytes then initiate meiosis II which is arrested during metaphase II before ovulation.

Several factors playing distinct roles for ensuring normal female fertility are involved in meiosis I. Retinoic acid (RA), derived from vitamin A metabolism, has been demonstrated to be one of the first molecules inducing the initiation of meiosis (Bowles et al., 2006; Koubova et al., 2006). A lack of vitamin A in rats has led to germ cell dysfunction as they were unable to enter meiosis (Li and Clagett-Dame, 2009). RA binding to its receptor (RAR) acts as a transcription factor for regulating *STRA8* expression, a key gene involved in meiosis initiation (Koubova et al., 2006). Female *Stra8* KO mice have been seen to have small ovaries (a large depletion of germ cells was observed at birth) and suffer infertility related to an arrest of germ cell development during the premeiotic stage. Indeed, these cells were unable to perform chromosome condensation (Baltus et al., 2006). It has been shown that *Stra8* expression is inhibited by *Nanos2* while *Nanos2* $^{-/-}$ mice males have been affected by an early loss of germ cells leading to infertility (Suzuki and Saga, 2008; Tsuda et al., 2003). It has been shown that *Nanos3* play an important role in mice aimed at maintaining the amount of PGC while a non-synonymous mutation in humans has been functionally linked to POI (Wu et al., 2013).

*STAG3*, *REC8*, *SMC3*, *SMC1B* and *RAD21L* encode cohesins during the pre-leptotene stage, thereby ensuring chromatid binding (Herrán et al., 2011; Hodges et al., 2005; Prieto et al., 2004). Caburet et al. (2014) have described exome sequencing experiments in POI-affected women belonging to a family from a Middle Eastern region having a background of consanguinity. This approach, which complemented previous mapping assays carried out via genetic linkage analysis, led to identifying the homozygous c.968delC (p-Phe187fs*7) in the *STAG3* gene (Caburet et al., 2014, 2012). Female *Stag3* KO mice were sterile secondary to very early ovarian dysgenesis and complete lack of follicles/oocytes in postnatal animals (Caburet et al., 2014). Synaptonemal complex axial elements were short in mutant foetal oocytes and the Sypc3 protein (a key transverse filament component) was absent. These findings, as well as low (or absent) Stag3 colocalisation levels with other cohesins (Rec8, Smc3, Smc1β, Rad21l) in *Stag3*$^{-/-}$ ovaries, underlined this protein's importance for synaptonemal complex and cohesion structure formation during meiosis.

Specific germ cell proteins, such as SYCP1, SYCP2, SYCP3, SYCE1, SYCE2, SYCE3 and HORMAD1, participate in synaptonemal complex formation during the leptotene phase, thereby enabling homologous chromosome pairing (Cahoon and Hawley, 2016; Lu et al., 2014) (and references therein). *Sycp1* has aroused particular interest since mouse *Sycp1*$^{-/-}$ ovaries have been seen to be small and lacking growing follicles and oocytes (de Vries et al., 2005). Such findings have suggested a key role for *Sycp1* during meiosis/oocyte development and fertility. Recently, WES has enabled detecting a drastic homozygous nonsense mutation (p. Gln205X) in the meiotic *SYCE1* gene in two daughters of consanguineous Muslim-Arab parents (de Vries et al., 2014). Syce1 is expressed in the synaptonemal complex of meiotic chromosomes and *Syce1*$^{-/-}$ KO female mice have been seen to be affected by infertility secondary to an almost complete absence of oocytes (Bolcun-Filas et al., 2009; Costa et al., 2005). Thus, it was highly probable that SYCE1-p. Gln205X was related to POI.

The *SPO11* gene, which is homologous to a type II DNA topoisomerase catalytic subunit, is also expressed during the leptotene stage, contributing to DNA double-strand break (DSB) formation and marking the initiation of recombination (de Massy, 2013). *Spo11* genetic invalidation in mice has led to infertility secondary to a drastic reduction in the number of follicles at adult age (Romanienko and Camerini-Otero, 2000).

A complex becomes formed with NBS1/MRE11/RAD50 proteins after DSB formation and H2AX histone binding to the breaks. DMC1, RAD51, SYCP3 and TEX15 interact at this stage, leading to chromosome synapsis and homologous recombination. Genetically modified mouse models for several of these genes have shown infertility-related ovary dysfunction. *Mre11* hypomorphic mutant alleles (due to a nonsense mutation truncating the protein's C-ter end) have led to an ataxia–telangiectasia-like disorder. Females suffering dysfunction during DSB formation, chromosome synapsis and recombination has led to oocyte elimination during folliculogenesis (Inagaki et al., 2016). *Rad50* modified genetic animals have produced fewer and smaller litters than their wild counterparts and ovarian atrophy has been recorded (Roset et al., 2014). *Dmc1* KO female embryos have ceased normal oogenesis, thereby leading to germ cell depletion in adult ovaries (Pittman et al., 1998; Yoshida et al., 1998). Interestingly, it has been proposed that the DMC1-p.Met200Val mutation might have led to infertility as it has been discovered at homozygous state in a woman affected by POI (Mandon-Pepin et al., 2008). *In vitro* functional tests have shown that the mutant protein has reduced stability and restricted capability for catalysing recombination reactions (Hikiba et al., 2008).

MCM8 and MCM9 are additional important molecules involved in DNA damage repair a key process occurring during homologous recombination. A study of two Turkish families affected by POI and short stature, analysed by 180K comparative genomic hybridisation (CGH), SNP oligonucleotide arrays and WES, led to identifying two homozygous mutations in *MCM9* (c.1732 +2T > C and c. 394C > T) leading to the synthesis of truncated proteins lacking important functional regions (Wood-Trageser et al., 2014). Similar to the phenotype observed in infertile Mcm9-deficient mice, these mutations were related to an increased amount of chromosomal breaks due to an impairment regarding DNA damage repair and, *in fine*, to oocyte death and POI (Lutzmann et al., 2012; Wood-Trageser et al., 2014). Fauchereau et al. (2016) identified the *MCM9* c.1483G > T mutation (p.Glu495X) by linkage analysis and WES sequencing experiments in a familial case of POI. The MCM8 c.446C > G (p.Pro149Arg) homozygous mutation, identified via homozygote mapping and WES in three sisters born from consanguineous parents, had a similar functional effect to that produced by MCM9 homozygous variants (AlAsiri et al., 2015).

In a familial case of POI (4 affected women) with no history of consanguinity, WES led to finding the heterozygous p. Gly746Asp mutation in the *CSB-PGBD3* fusion gene (Qin et al., 2015b). Functional tests have shown that the mutant protein was unable to normally respond to DNA damage, leading to oocyte depletion. Sanger sequencing on >400 POI unrelated women has revealed additional mutations (p. Val1056Ile, p. Glu215X) also displaying functional disturbances (Qin et al., 2015b).

Another group of proteins which is necessary for meiosis progression consists of MSH4, MSH5, MLH1, MLH3, TEX11 and PMSD2. These factors act during genetic recombination regulation, genome stability conservation and repeat sequence expansion (Manhart and Alani, 2016) (and references therein). *Msh4* and *Msh5* knock out (KO) mice (males and females) have been seen to be sterile secondary to defective chromosome synapsis during meiosis (de Vries et al., 1999; Edelmann et al., 1999; Kneitz et al., 2000). Similarly, germ cell loss and sterility have been recorded in models of mice lacking *Mlh1* and *Mlh3* (Baker et al., 1996; Edelmann et al., 1996; Lipkin et al., 2002).

WES screening of a Han Chinese family identified the

homozygous c.1495G > T (p.Asp487Tyr) mutation in the *MSH5* gene which led, in the Msh5 $^{D486Y/D486Y}$ KI mouse model, to a drastic infertility phenotype (Guo et al., 2017). When WES was recently performed in a familial POI case, it led to the identification of the homozygous *MSH4* c.2355+1G > A splice-site mutation which was associated with the generation of aberrant transcripts and MSH4 inactivation (Carlosama et al., 2017).

Oocytes arrest and begin early folliculogenesis following chromosome recombination, at the meiosis I of diplotene stage. Meiosis then becomes resumed during puberty (diakinesis stage).

## 6. Folliculogenesis and ovulation genes

Folliculogenesis encompasses all stages of ovarian follicle maturation (primordial, primary, secondary and tertiary follicles), involving the fine-tuned expression of hundreds of genes. Some of them attract particular interest as their genetic invalidation in mice and mutations in humans have been linked to female infertility phenotypes. Human primordial follicles are formed during gestation compared to their development after birth in rodents. The number of primordial follicles and oocytes at this stage constitutes one of the main variables responsible for ovarian reserve and time of fertility.

The factor in the germline α (FIGLA), belonging to the bHLH transcription factor family, has been described as having key functions during primordial follicle formation.

This factor (specifically synthesised by oocytes) regulates the expression of genes that encode glypoproteins of the zona pellucida (*ZP1*, *ZP2* and *ZP3*) (Liang et al., 1997). Human and mouse primordial follicles express *FIGLA*. *Figla*$^{-/-}$ mice are sterile secondary to primordial follicle loss after birth (Bayne et al., 2004; Soyal et al., 2000). It has been shown that Figla activates specific downstream oocyte-specific genes while testis genes become simultaneously inhibited. *FIGLA* mutations in humans were formerly associated with non-syndromic POI by functional tests (Zhao et al., 2008). Other genes have been shown to play important roles during primordial follicle stock establishment and ovarian reserve. This is true for *Ccn2*, *Foxl2*, *Pdk1*, *Atg7*, *Birc1*, *Ntrk1/2/3*, *Bdnf*, *Ngf*, *Mcm8* and *Fst* which have mainly been studied in genetically-modified models of mice.

Primordial to primary follicle activation is independent of the effect of hormones and implies several genes belonging to different molecular cascades. FOXO3, which is expressed by primordial follicle oocytes, acts downstream of the PI3K/AKT signalling pathway to participate in various molecular pathways having relevant roles in ovarian biology (e.g. galactose-1-phosphate uridyltransferase-GALT and prolactin). Mouse *Foxo3*$^{-/-}$ become massively activated leading to premature ovarian reserve depletion (Castrillon et al., 2003). Some mutations in *FOXO3* coding regions have been linked to POI but not to functional tests performed to date (Vinci et al., 2008; Wang et al., 2010).

Similarly, the oocyte-specific deletion (conditional KO) of *Pten* (another molecule involved in the PI3K/AKT/FOXO3A pathway) has led to infertility in mice due to premature activation of primordial follicles (Reddy et al., 2008). Although screened for mutations, women affected by POI no causative mutations have been identified to date (Shimizu et al., 2009).

SOHLH1 and SOHLH2, LHX8 and NOBOX (participating in the same molecular cascade) are relevant transcription factors involved in primordial to primary follicle transition. *SOHLH1* and *SOHLH2* are expressed in germ cell cysts, primordial and primary follicles but not in secondary follicles (Choi et al., 2008b; Pangas et al., 2006). Mice lacking these genes have displayed premature infertility secondary to a blockade of primordial follicle development. *SOHLH2* sequence variants in humans have been identified in women suffering POI, though functional tests have not been performed (Qin et al., 2014).

LHX8, directly regulated by SOHLH1, is a LIM homeodomain protein having functions from the embryonic ovary throughout all the stages of folliculogenesis (Choi et al., 2008a; Pangas et al., 2006). This protein participates in regulating various downstream key genes involved in ovary physiology and development, such as *Zp1*, *Zp2*, *Zp3*, *Kit*, *Kitl*, *Pou5f1*, *Casp2*, *Casp3* and *Nlrp* members (Wood and Rajkovic, 2013) (and references therein). *Lhx8*$^{-/-}$ KO female mice have been seen to become infertile due to premature oocyte loss and impaired follicular development (primordial to primary transition) (Choi et al., 2008a; Pangas et al., 2006).

The newborn ovary homeobox gene (*NOBOX*) is another gene coding a transcription factor involved in the transition to primary follicles. *Nobox* is expressed in mice in oogonia from E15.5 and thereafter throughout folliculogenesis (Rajkovic et al., 2004; Suzumori et al., 2002; Wood and Rajkovic, 2013). Numerous downstream genes involved in follicular development such as *Oosp1*, *Gdf9*, *Ast1* and *Jag1* are directly or indirectly regulated by Nobox (Choi et al., 2007). Similarly to *Sohlh1*$^{-/-}$ animals, newborn female mice lacking *Nobox* have displayed a normal number of germ cells and primary follicles but follicles were not able to progress to the primary stage of development during early postnatal stage, leading to their premature depletion and infertility (Rajkovic et al., 2004). Regarding humans, numerous coding mutations have been identified in POI-affected women, some of whom have displayed functional *in vitro* pathogenic affects (Bouali et al., 2016; Bouilly et al., 2015, 2011; Ferrari et al., 2016; Li et al., 2017; Qin et al., 2007). Interestingly, it has been shown that deleterious protein aggregates formed by some NOBOX variants colocalize with FOXL2 which argues in favor of an oligogenic/polygenic nature of POI (Ferrari et al., 2016).

BMP15 and GDF9 are other factors involved in regulating folliculogenesis and ovulation, constituting key actors in ovarian biology and fertility in mammals. These proteins (belonging to the TGF-β growth factor family) are expressed by oocytes throughout development and participate in various functions, such as follicle development, granulosa cell mitosis, ovulation rate physiology and modulating granulosa cell sensitivity to FSH (Laissue, 2015; Persani et al., 2011). BMP15 and GDF9 (forming homodimers and heterodimers) bind to specific serine/threonine kinase receptors located on granulosa cell surface to activate intracellular signalling (e.g. via SMAD molecules) and regulate target genes.

BMP15 and GDF9 biological functional behaviour has been seen to have particular complexity since mutations in different mammalian species have displayed distinct phenotypes. Homozygous and heterozygous missense mutations in sheep have led to infertility and hyperfertility phenotypes due to ovulation rate dysfunction (Galloway et al., 2000; Hanrahan et al., 2004; Laissue et al., 2008). Homozygous *Gdf9* and *Bmp15* KO female mice have displayed complete infertility and hypofertility, respectively (Dong et al., 1996; Yan et al., 2001). Concerning humans, *BMP15* genotyping of more than 1000 women to date has led to identifying numerous coding variants potentially related to the phenotype, several of which have been tested by different *in vitro* functional tests (Di Pasquale et al., 2004; Laissue, 2015; Laissue et al., 2008; Patiño et al., 2017b; Persani et al., 2014; Rossetti et al., 2009). These variants' functional impact has been associated with cellular and molecular deleterious effects/mechanisms, such as reduced granulosa cell growth, mature proteins' defective secretion and activity, a dominant heterozygous mutation effect and significantly reduced ability to synergise with GDF9 (Di Pasquale et al., 2004; Patiño et al., 2017b; Rossetti et al., 2009). Interestingly, the *BMP15* c.-9C > G sequence variant has been linked to POI as it has led to promoter transactivation disturbances secondary to potential

PITX1 transcription factor binding dysfunction (Fonseca et al., 2014). Regarding *GDF9*, mutations have been described in POI-affected patients and in women with twinning, though no functional tests have been undertaken (Laissue et al., 2008; Persani et al., 2014).

Apart from the aforementioned genes, dozens of others are involved in follicle formation and maturation (Roy and Matzuk, 2006). These molecules will be not reviewed in depth in the present manuscript but may constitute relevant candidates for POI aetiology.

The transition from preantral to antral follicles represents the beginning of the extraovarian regulation of folliculogenesis which is mainly regulated by hormone signalling through the hypothalamic-pituitary-gonadal axis. FSH and LH (and their receptors, FSHR and LHCGR, respectively) are the most important proteins involved in this step which leads to ovulation. In rodents, folliculogenesis can proceed up to the preantral stage without *Fshr* (Dierich et al., 1998; Abel et al., 2000). Numerous steps and feedback loops are necessary to regulate the final steps of follicle development/maturation and ovulation, involving several key molecules, such as estradiol, follistatin (FST), inhibins and activins. Rare homozygous *FSHR* mutations in humans have been linked to POI, especially in Finnish population (e.g. the partial loss of function FSHR-p. Ala189Val mutation) (Aittomaki et al., 1995; Rannikko et al., 2002; Laissue, 2015). A complete loss of function mutation (Pro519Thr) in FSHR has been associated to a blockade of follicular growth after the primary stage (Meduri et al., 2008). Recently, two novel *FSHR* homozygous mutations (c.1222G > T, p. Asp408Tyr and c.1298C > A, p. Ala433Asp) have been identified by NGS in women belonging to consanguineous families affected by POI (Bramble et al., 2016; França et al., 2017). Functional tests showed that the p. Asp408Tyr mutation led to a significant reduction of protein in membrane and downstream FSH-stimulated cAMP production (Bramble et al., 2016).

The luteinising hormone receptor (LHCGR) is highly expressed in pre-ovulatory granulosa cells to respond to LH secretion, thereby contributing to cumulus expansion, follicle rupture and meiosis resumption. Lhcgr-KO mice have displayed small ovaries lacking pre-ovulatory follicles and thin uteri which have been related to infertility (Lei et al., 2001; Zhang et al., 2001). Several LHCGR mutations leading to POI have been described in humans, especially in families having distinct 46XY and 46XX fertility phenotypes. Conditional KO of the activin/inhibin-βA subunit in the ovary of mice has led to subfertility while inhibin-α subunit deletion has produced sex cord stromal tumours (Matzuk et al., 1992; Pangas et al., 2007). Some *INHBA* sequence variants have been identified in POI-affected women but no functional tests have been performed to formerly associate them with the disease's aetiology (Shelling, 2012).

Taken together, the genes described above as well as numerous additional molecules can be considered as candidates for POI which can be analysed at genomic level via massively parallel sequencing.

## 7. NGS in unrelated POI women

At least four studies, performed in non-related women affected by POI, have described using NGS for selecting candidate aetiological variants (Bouilly et al., 2016; Fonseca et al., 2015; Patiño et al., 2017a; Desai et al., 2017). TSM format has been used by Fonseca et al. (2015) for sequencing 70 genes and by Bouilly et al. (2016) for sequencing 19 genes (Bouilly et al., 2016; Fonseca et al., 2015). Nineteen percent of Fonseca's patients and 25% of Bouilly's patients were carriers of potential heterozygous deleterious variants, some of which (e.g. BMPR2-p. Ser987Phe, LHX8-p.Ala325Val, SOHLH1-p.Ser174Leu, SOHLH1-p.Pro218Thr, SOHLH1-p.Leu306Met, SMC1β-pIle221Thr) had functional effects (Bouilly et al., 2016; Patiño et al., 2017c). Interestingly, 36% of the patients (7 out 19) referred to in Bouilly et al.,'s paper had a digenic origin for the disease. Similarly, in a very recent report, a POI oligogenic/polygenic nature was also found by using NGS (Patiño et al., 2017a); a panel of 420 genes from WES experiments was analysed in this study which involved 69 unrelated POI women. Forty-eight percent of these patients had potentially deleterious sequence variants while 42% carried at least two mutations in two genes. Robust computational approaches suggested that GREM1 and BMPR1B mutations (BMPR1B-p.Arg254His, BMPR1B-p.Phe272Leu, GREM1-p.Arg169Thr) likely led to protein stability disturbances contributing to the disease's origin (Patiño et al., 2017b). Interestingly, 64% of patients having candidate aetiological heterozygous mutations in a meiotic gene have also carried a second mutation in a different one. This feature has led to proposing that some heterozygous mutations in meiotic genes might be associated with a background of POI predisposition. Sanger sequencing of *MCM8* and *MCM9* and WES in a panel of unrelated POI patients led to the description of novel variants in these genes which were potentially related to the disease's pathogenesis (Desai et al., 2017). WES sequencing (n = 19) was focused on *MCM8*/*MCM9* analysis in that study and did not explore further potential aetiological mutations.

## 8. Concluding remarks

Ovary physiology and molecular regulation in mammals involve hundreds of molecules, finely regulated in terms of gene expression, which have been strictly selected during evolution to guarantee reproductive fitness and species maintenance. Fruitful projects have demonstrated that NGS is an efficient tool for mapping new variants participating in POI aetiology which should be used in the near future for diagnosis/prognosis. However, several challenges still have to be overcome, such as the analysis of a considerable amount of data issuing from massive NGS experiments, the interpretation of human genomic variability in terms of interactome pathways and the participation of non-coding genomic regions regarding the disease's origin. Indeed, although NGS approaches have increased the amount of genes and mutations involved in POI, a relevant proportion of the “missing heritability” has yet to be resolved. WGS and epigenetic research therefore seem mandatory for a better understanding of POI pathogenesis as well for other frequently-occurring reproductive phenotypes. In such scenario it is essential that clinical practitioners and scientists reinforce their research interactions in the context of new translational medicine initiatives.

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