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Genetics of Primary Ovarian Insufficiency in the Next-Generation Sequencing Era

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Primary ovarian insufficiency (POI) is characterized by amenorrhea, increased follicle-stimulating hormone (FSH) levels, and hypoestrogenism, leading to infertility before the age of 40 years. Elucidating the cause of POI is a key point for diagnosing and treating affected women. Here, we review the genetic etiology of POI, highlighting new genes identified in the last few years using next-generation sequencing (NGS) approaches. We searched the MEDLINE/PubMed, Cochrane, and Web of Science databases for articles published in or translated to English. Several genes were found to be associated with POI genetic etiology in humans and animal models (*SPIDR, BMPR2, MSH4, MSH5, GJA4, FANCM, POLR2C, MRPS22, KHDRBS1, BNC1, WDR62, ATG7/ATG9, BRCA2, NOTCH2, POLR3H, and TP63).* The heterogeneity of POI etiology has been revealed to be remarkable in the NGS era, and discoveries have indicated that meiosis and DNA repair play key roles in POI development.

Freeform/Key Words: primary ovarian insufficiency, premature ovarian insufficiency, ovary, genetics, infertility, next-generation sequencing, NGS

1. Definition

Primary ovarian insufficiency (POI) is defined by the depletion of ovary follicles, leading to infertility before the age of 40 years [1]. This condition is characterized by the cessation of menses (amenorrhea or oligomenorrhea) for at least 4 months, increased gonadotropin levels (FSH > LH), and hypoestrogenism [2].

In 1942, Albright and colleagues [3] reported the first case of primary ovarian insufficiency. There is no consensus regarding the name of this disorder. On the one hand, the European Society of Human Reproduction and Embryology guidelines [2] recommend the term "premature ovarian insufficiency" for describing this disorder in research and clinical practice [2]. On the other hand, the American College of Obstetricians and Gynecologists (ACOG) committee is in favor of "primary ovarian insufficiency" [1]. According to the National Institutes of Health, this term is appropriate because some women with POI can present with spontaneous pregnancy; therefore, POI can be distinguished from natural menopause, and the term can be used to describe ovarian deficiency with amenorrhea manifestation. Some authors have chosen the term "ovarian dysgenesis" for POI, which is inadequate in the absence of anatomopathological data.

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This review adopts primary ovarian insufficiency as the best term for referring to this condition.

2. POI Phenotype and Prevalence

POI patients show a wide range of clinical phenotypes, and the disease can occur in women from puberty up to 40 years old. The patients can present with primary amenorrhea, which is usually diagnosed at a young age in individuals with delayed puberty and an absence of breast development and menarche, whereas secondary amenorrhea is diagnosed at an age from < 20 to 40 years and is characterized by normal pubertal development and an irregular menstrual cycle followed by amenorrhea. Secondary amenorrhea is the most frequent POI phenotype [1, 2].

The broad clinical manifestations of POI have been demonstrated in different cohorts. A large Australian POI cohort comprising 675 women showed that secondary amenorrhea occurred in more women (84%) than did primary amenorrhea (16%). Delayed puberty was characterized in patients presenting with primary amenorrhea as well as absent or incomplete breast development (70%) as a consequence of hypoestrogenism at such an early age [4]. In contrast, in our Brazilian cohort of 74 women, we evaluated 51 with primary amenorrhea and 23 with secondary amenorrhea [5]. The higher prevalence of primary amenorrhea in our cohort might have been due to the severe phenotype, as primary amenorrhea is primarily evaluated in the Endocrinology Department, whereas the mild phenotype, presenting as secondary amenorrhea, tends to be managed by the Gynecology Department.

Although the prevalence of POI is related to ethnicity, there is a lack of epidemiological data. However, the prevalence appears to increase with age (1:10 000 by age 20, 1:1000 by age 30, and 1:100 by age 40) [2, 6].

A national retrospective study from Sweden reported a higher prevalence of POI (1.9%) than previously demonstrated in the general population. Of the 1 036 918 women, 1.7% presented spontaneous POI, and 0.2% of the cohort was diagnosed with iatrogenic POI [7]. Moreover, a cross-sectional survey of women aged 40–55 years was conducted at 7 sites in the USA (the Study of Women Across the Nation [SWAN]) and identified the prevalence of self-reported POI in 11 652 women, with no discernible cause by ethnic group [8]. Indeed, POI was reported in 1.1% of women, of which 1.0% were Caucasian, 1.4% were African American, 1.4% were Hispanic, 0.5% were Chinese, and 0.1% were Japanese [8]. The prevalence of POI remains unclear in Brazil.

3. Diagnosis

Based on current American and European guidelines, POI diagnosis is performed through elevated gonadotropin measurement on two consecutive occasions at least 1 month apart (elevated FSH levels in the menopausal range are usually greater than 20 IU/ml) and amenorrhea for at least 3 or 4 months [1, 2].

After confirmation of POI diagnosis, chromosomal analysis, fragile-X premutation (FMR1) analysis, adrenal (21-hydroxylase) and thyroid antibody assessment, and pelvic ultrasonography should be performed [1]. This screening might be helpful for the identification of POI etiology; however, it has been well established that most POI cases remain without a clarified etiology.

4. POI Etiology

Primary ovarian insufficiency can be caused by genetic defects, autoimmune diseases, iatrogenic factors (chemotherapy or radiation therapy), viral infections, or toxins, or it can remain idiopathic despite exhaustive investigation [6]. Regarding genetic defects, chromosomal abnormalities and monogenic defects can lead to POI. Recently, an oligogenic etiology

for this disorder has been proposed [5, 9, 10]. In this review, our goal is to provide an overview of the genetic basis of POI etiology, mainly with regard to monogenic genes identified by NGS approaches.

A. Chromosomal Abnormalities and Syndromic POI

Chromosomal abnormalities are a well-established cause of POI, and their frequency is approximately 10–13% [11]. Numerical defects have been described as X monosomy (45,X; Turner syndrome), mosaic forms (45,X/46,XX and 45,X/47,XXX), Trisomy X (47,XXX), X-deletions, X-autosomal translocations, and small or large rearrangements [6]. Evaluation of karyotypes for numerical changes can be performed by cytogenetic analysis, and the NGS approach has recently become a powerful tool with which to evaluate copy number variations (CNVs) for diagnosis of POI and other endocrine disorders [12, 13]. Moreover, syndromic POI may also be caused by the expansion of a CGG repeat in the 5' regulatory region of the *FMR1* gene, which causes Fragile-X syndrome. In affected women, the number of CGG repeats in *FMR1* is greater than 200, and the mutation is called a complete mutation due to methylation and silencing of this gene. In the premutation stage, the number of CGG repeats is between 55 and 199. The presence of premutation of *FMR1* should be investigated in women with POI, since this premutation is associated with POI in approximately 20% of carrier women [14]. In addition, the X chromosome region from Xq13.3 to Xq27 has been shown to be a critical region for normal ovarian function (POI1 [Xq23-Xq27] and POI2 [Xq13-Xq21]). Moreover, genes interrupted by breakpoints in balanced X-autosome translocations or harboring point mutations in the X chromosome have been associated with POI etiology, including *COL4A6, DACH2, DIAPH2*, *NXF5, PGRMC1*, *POF1B*, and *XPNPEP2* [12, 15–25].

B. Nonsyndromic POI: Well-known and Novel POI Genes in the NGS Era

B-1. Well-known POI genes

Ovarian development- and function-related genes. During the NGS era, information on the molecular basis of idiopathic POI has rapidly increased. Large-scale sequencing techniques have identified several novel pathogenic variants of well-known genes in recent years (*FSHR, GDF9, BMP15, FIGLA,* and *NOBOX*) (Table 1) [26–42]. These genes were first implicated in POI etiology because of their roles in development and/or ovarian function. They can be functionally classified into genes associated with (1) germ cell development, (2) oogenesis and folliculogenesis, (3) steroidogenesis, and (4) hormone signaling. During embryonic development, a large number of germ cells are eliminated through the process of apoptosis, and mutations in genes involved in this process, such as *NANOS3* [43] and *EIF4ENIF1* [44], may lead to the POI phenotype. Moreover, many factors are involved in the recruitment, development, and maturation of follicles and oocytes. Indeed, mutations in genes encoding hormone receptors, such as *FSHR* and *LHCGR*, are obvious causes of ovarian function impairment and may elicit variable clinical phenotypes [39, 45]. Another essential step for appropriate ovarian function is steroidogenesis, through which estrogen is synthesized. Any alteration in the estrogen synthesis pathway may lead to amenorrhea and high FSH levels; however, Anti-Mullerian Hormone should be normal [4]. Women with mutations in genes involved in the steroidogenic pathway, such as *NR5A1* and *STAR*, may present syndromic or isolated POI phenotypes [46, 47]. In addition, growth factors such as TGFβ family members (*BMP15* and *GDF9*) play crucial roles in ovarian functions [48]. Thus, defects in these genes are associated with the POI phenotype. *BMP15* promotes ovarian growth and maturation, and mutations in this gene lead to a POI phenotype in cases of both autosomal dominant [28] and recessive inheritance (Table 1) [5, 30]. In addition, the GDF9 protein is also essential for ovarian folliculogenesis [49], and mutations in POI patients presenting secondary amenorrhea were first described to follow autosomal

Table 1. Novel Pathogenic Variants Associated with Well-known POI Genes Identified by NGS

Gene	Phenotype	Mutation	Inheritance	First Report	Mechanism	References in NGS Era
GDF9	PA	c.783delC:p.Ser262Hisfs*2	AR	[26]	Ovarian development and function	[27]
BMP15	SA	c.581T>C:p.Phe194Ser[a,b]	AD	[28]	Ovarian development and function	[29]
	SA	c.986G>A:p.Arg329His	AD			
	SA	c.1070G>A:p.Cys357Tyr	AR			[30]
	SA	[c.151_152delGA:p.Glu51Ilefs*27] and [c.189-198delAGGGCATTCAinsTG:p.Glu64Alafs*12][b]	AR			[31]
NOBOX	PA	c.567delG:p.Thr190Hfs*13	AR	[32]	Ovarian development and function	[33]
	PA	c.1489delT:p.Cys497Valfs*53	AR			[34]
FIGLA	PA	c.2T>C:p.Met1Thr	AR	[35]	Ovarian development and function	[36, 37]
FSHR	PA	c.1298C>A:p.Ala433Asp	AR	[38]	Ovarian development and function	[39]
	PA	c.175C>T:p.Arg59*[b]	AR			[40]
	PA	c.419delA:p.Lys140Argfs*16	AR			[41]
	PA	c.1510C>T:p.Pro504Ser	AR			[41]
	SA	c.44G>A:p.Gly15Asp	AD			[41]
	PA	c.1789C>A:p.Leu597Ile[b]	AD			[42]
	SA	c.793A>G:p.Met265Val[b]	AD			[42]
MCM8	PA	c.482A>C:.His161Pro	AR	[61]	Meiosis/DNA repair	[62]
MCM9	PA	c.1651C>T; p.Gln551*	AR	[63]	Meiosis/DNA repair	[64]
	PA	[c.905-1G>T] and [c.1784C>G:p.Thr595Arg]	AR			[64]
STAG3	PA	c.677C>G:p.Ser227*	AR	[65]	Meiosis/DNA repair	[66]
	PA	c.1573 + 5G>A:p.Leu490Thrfs*10	AR			[67]
	PA	[c.291dupC:p.Asn98Glnfs*2] and [c.1950C>A:p.Tyr650*]	AR			[68]
PSMC3IP	PA	c.489C>G:p.Tyr163*	AR	[69]	Meiosis/DNA repair	[70]
	PA	[c.496_497delCT:p.Arg166Alafs] and [c.430_431insGA:p.Leu144*]	AR			[71]
HFM1	SA	c.3479G>A:p.Cys1157Ser	AD	[72]	Meiosis/DNA repair	[73]
NUP107	PA	c.1063C>T:p.Arg355Cys	AR	[74]	Meiosis/DNA repair	[75]

[a]Previously reported.
[b]This variant was identified by the Sanger method.
Abbreviations: AD, autosomal dominant; AR, autosomal recessive; PA, primary amenorrhea; SA, secondary amenorrhea.

dominant inheritance [26, 50, 51]; however, heterozygous *Gdf9*$^{+/-}$ female mice are fertile, and only *Gdf9*-null female mice are infertile due to a block at the primary follicle stage [52]. In contrast with previously described heterozygous missense mutations, our group described the first homozygous 1-bp deletion (c.783delC) in the *GDF9* gene in one Brazilian patient with primary amenorrhea, a more severe phenotype (Table 1) [27]. Some transcription factors associated with postnatal oocyte differentiation in human and animal models have been described in the past two decades, such as *NOBOX* [32, 53], *SOHLH1* [54, 55], *SOHLH2* [56, 57], *FIGLA* [35, 58], and *LHX8* [59, 60]. *NOBOX* is able to regulate several ovarian genes, including *GDF9* and *BMP15*. In mice, the absence of the NOBOX protein leads to the progressive loss of primordial follicles and, consequently, the absence of mature follicles [53]. Initially, heterozygous variants with dominant negative effects were described [32], and a familial case with a homozygous variant has been described in two Brazilian sisters [34]; one Chinese patient has also presented with primary amenorrhea (Table 1) [33]. *SOHLH1* is involved in the maintenance of germ cells and, therefore, in the initial phase of folliculogenesis [54]. In humans, biallelic variants in *SOHLH1* were identified in two Turkish families with isolated POI [55]. Nonsyndromic POI is also associated with heterozygous deletions in the *FIGLA* gene, a transcription factor of the helix-loop-helix family [35]. This transcription factor regulates the expression of genes in the zona pellucida and other genes expressed only in the ovaries; therefore, its absence or defect may promote ovarian failure in humans and mice [35, 58].

Meiosis and DNA repair genes. High-throughput techniques have been crucial for revealing new genes that mainly play roles in cell division and/or DNA repair as new causes of POI (*MCM8, MCM9, STAG3, PSMC3IP, HFM1, NUP107,* and *SYCE1*) (Table 1) [61–75]. Oocytes begin the first stage of meiotic division before birth and remain in prophase I during fetal life, restarting cell division when the woman reaches puberty; the secondary oocytes are then released at ovulation. Due to the resting state of oocytes, alterations in genes involved in meiosis and DNA repair may induce different phenotypes of ovarian insufficiency, as demonstrated in various animal models [76]. Some coenzymes, such as *STAG3* and *SYCE1*, are essential for proper formation of the synaptonemal complex during cell division, and mutations in these genes lead to infertility in both humans and animal models [65, 77]. In addition, the helicases of the mini-chromosome maintenance proteins (MCM8 and MCM9) are crucial for the homologous recombination step during meiotic division [78]. Absence of the MCM8 and MCM9 proteins promotes errors during the process of meiosis in mice, such as arrest in meiotic prophase I, arrest of primary follicles, and frequent development of ovarian tumors in *Mcm8*$^{-/-}$ mice, and a complete absence of oocytes in *Mcm9*$^{-/-}$ mice [79]. In the past few years, homozygous mutations leading to loss of protein function in *MCM8* and *MCM9* have been described as causes of POI identified by NGS approaches (Table 1) [61, 63].

B-2. Novel genes revealed by NGS

In addition, at least 15 genes have recently been reported as novel causes of POI in human and animal models related to ovarian development and meiosis, as discussed below (Table 2) [10,80–95]. These genes are classified following the same pattern described above for the well-known genes associated with POI.

Ovarian development- and function-related genes:

BMP receptor 2 (BMPR2). BMPR2, a serine-threonine kinase type II receptor, seems to bind BMP factors to affect the downstream signaling of its ligands, compromising folliculogenesis [96]. Patiño and collaborators [10] reported in vitro evidence that a p.Ser987Phe mutation in *BMPR2* increases subcellular aggregation patterns at the endoplasmic reticulum, showing a potential association of this gene with isolated POI.

Table 2. Novel Genes Associated with Primary Ovarian Insufficiency Etiology

Gene	Phenotype	Mutation	Inheritance	Function Study	Mechanism	References
SPIDR	PA/associate	p.Trp280*	AR	In vitro	Meiosis/DNA repair	[80]
BMPR2	SA/isolated	p.Ser987Phe	AD	In vitro	Ovarian development and function	[10]
MSH4	SA/isolated	p.Ile743_Lys785del	AR	-	Meiosis/DNA repair	[81]
MSH5	SA/isolated	p.Asp487Tyr	AR	In vitro and in vivo (mouse)	Meiosis/DNA repair	[82]
GJA4	SA/isolated	p.Gly316Ser	AD	In vitro	Ovarian development and function	[83]
FANCM	SA/isolated	p.Gln1701*	AR	In vitro	Meiosis/DNA repair	[84]
POLR2C	SA/autoimmune	p.Lys152*	AD	In vitro	Metabolism/protein synthesis	[85]
MRPS22	PA/isolated	p.Arg135Gln; p.Arg202His	AR	In vitro and in vivo (fruit fly)	Metabolism/protein synthesis	[86]
KHDRBS1	SA/isolated	p.Met154Val p.Pro88Leu	AD	In vitro	Ovarian development and function	[87]
BNC1	SA/isolated	p.Arg356Valfs*6 p.Leu532Pro	AD	In vitro and in vivo (mouse)	Meiosis/DNA repair	[88]
WDR62	PA/isolated	p.Thr1068fs p.Cys599Tyr	AD	In vitro and in vivo (mouse)	Meiosis/DNA repair	[89]
ATG7	SA/isolated	p.Phe403Leu	AD	In vitro	Ovarian development and function	[10, 90]
ATG9A	PA/isolated	p.Arg758Cys	AD	In vitro	Ovarian development and function	[90]
BRCA2	PA/syndromic	[p.Val2527*];[p.Ser3231fs16*]	AR	In vitro and in vivo (fruit fly)	Meiosis/DNA repair	[91]
	PA/isolated	[c.68-1G>C];[p.Tyr1480*]	AR			[92]
	PA/isolated	[p.Asp2723Val];[Cys3233Trpfs*15]	AR			
POLR3H	PA/isolated	p.Asp50Gly	AR	In vivo (mouse)	Ovarian development and function	[93]
NOTCH2	PA/isolated	[p.Leu2408His]; [p.Ala2316Val]	AR	In vitro	Ovarian development and function	[94]
	SA/isolated	p.Ser1804Leu p.Pro2359Ala p.Gln1811His	AD			
TP63	PA/isolated	p.Trp598*	AD		Meiosis/DNA repair	[95]

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; PA, primary amenorrhea; SA, secondary amenorrhea.

Gap junction protein alpha 4 (GJA4)/Connexin-37 (CX37). *GJA4* plays a role in ovarian follicle development, and disruption of this gene in mice results in ovarian folliculogenesis arrest at the preantral stage and, therefore, female infertility [97]. A heterozygous missense variant (c.946G>A:p.Gly316Ser) in *GJA4* was found in two POI patients presenting secondary amenorrhea. Although this mutation has not been reported in Caucasian controls, it is commonly observed in African individuals. In vitro studies have shown that p.Gly316Ser is able to decrease cell surface gap junction plaque expression at the cell surface in a dominant negative manner. The mechanism may involve increased gap junction endocytosis and lysosomal degradation [83]. Indeed, a candidate gene approach was performed in this French cohort; therefore, no other POI candidate genes were ruled out as causes of POI.

KH domain-containing RNA-binding signal transduction-associated protein 1 (KHDRBS1). KHDRBS1 appears to play a role in a variety of cellular processes, such as alternative splicing, cell cycle regulation, RNA 3′ end formation, tumorigenesis, and regulation of the human immune system. The role of *KHDRBS1*, also named *Sam68*, has been investigated in the ovaries of knockout female mice. *Sam68*$^{-/-}$ female mice show subfertility due to the delay of first pregnancy, small littermates, and reduced numbers of secondary and preantral follicles in the ovaries [98]. Using whole-exome sequencing (WES), one heterozygous variant (c.460A>G:p.Met154Val) in *KHDRBS1* was found in a Chinese mother and the oldest daughter affected by POI. A second monoallelic mutation was also identified (c.263C>T:p.Pro88Leu) in another patient. In vitro assays have shown the effect of a *KHDRBS1* mutation (c.460A>G) on alternative splicing; however, no in vivo studies have been performed [98]. Another heterozygous variant in *KHDRBS1* (c.887C>T:p.Pro296Leu) was also found in one POI patient harboring an *FGFR2* variant (c.64C>T:p.Arg22Trp) [99]. However, further functional studies are needed to validate its pathogenicity.

Autophagy-related protein 7 (ATG7) and autophagy-related protein 9 (ATG9A). Autophagy is an adaptive process that occurs in response to different forms of stress, such as nutrient deprivation, growth factor depletion, infection, and hypoxia. Autophagic processes modulate many pathologies, including neurodegenerative disorders, cancer, and infectious diseases [100]. Autophagic factors, such as autophagy-related proteins (ATGs) and their regulators, are essential for autophagic processes, including initiation, phagophore nucleation and expansion (ATG7 and ATG9), cargo sequestration, membrane sealing, autophagosome maturation, and autophagosome fusion with lysosomes [100]. Lack of *Atg7* in mice leads to impaired central nervous system function, resulting in behavioral defects and lethality at 28 weeks after birth. Knockout mice also have massive neuronal loss in the cerebral and cerebellar cortices [101]. Moreover, germ cell-specific knockout of *Atg7* promotes female subfertility in mice due to reduced primordial follicles in the ovary as a consequence of defects in the autophagic machinery [102]. Disruption of *Atg7* in male mice causes aberrant acrosome formation and the development of abnormal round-headed spermatozoa [103], leading to subfertility. *Atg9* conditional knockout mice display neurologic defects, including progressive degeneration in axons and their terminals but not in neuronal cell bodies, and these mice die within 4 weeks of birth [104]. In humans, two monoallelic mutations in *ATG7* (c.1209T>A:p.Phe403Leu) and *ATG9* (c.2272C>T:p.Arg758Cys) have been reported in two patients diagnosed with secondary and primary amenorrhea, respectively [10, 90]. These mutations have been found to impair the autophagy process in in vitro studies in a haploinsufficient manner by reducing the ability to produce autophagosomes [90].

RNA polymerase III subunit H (POLR3H). RNA polymerase III synthesizes several untranslated RNAs and plays key roles in cell growth, differentiation, and the innate immune response [105]. No mutations in this subunit have been reported to occur in the context of human disorders, although subunits A and B (*POLR3A* and *POLR3B*) are associated with the recessive 4H syndrome, which includes hypomyelination, hypodontia, hypogonadotropic hypogonadism, and leukodystrophy syndrome [106–108], or even with

isolated hypogonadotropic hypogonadism [109]. We previously reported a novel biallelic missense mutation (c.149A>G:p.Asp50Gly) in *POLR3H* in two unrelated families with POI and generated two mouse lines using the CRISPR/Cas9 method to evaluate the intrinsic mechanisms of *POLR3H*-p.Asp50Gly mutation [93]. Early embryonic lethality was observed in mice harboring a loss-of-function $Polr3h^{D50G}$ mutation [93]. Mice harboring the homozygous point mutation $Polr3h^{D50G}$ displayed pubertal delays, as observed in all 4 described patients. Small litter sizes and increased time to pregnancy or time to impregnate a female were observed in the $Polr3h^{D50G}$ female and male mice. Indeed, $Polr3h^{D50G}$ mice showed reduced expression of ovarian *Foxo3a* and fewer numbers of primary follicles than wild-type mice [93]. This was the first evidence of POI caused by pathogenic mutations in *POLR3H* leading to human infertility.

Notch receptor 2 (NOTCH2). The NOTCH pathway is involved in cell fate decisions and differentiation processes during fetal and postnatal life [110]. The related proteins, including four NOTCH receptors (NOTCH 1–4) and five NOTCH ligands (Jagged 1–2 and DELTA-LIKE 1, 3, and 4), are associated with organogenesis during embryonic development and with the maintenance of homeostasis of self-renewing systems in invertebrates (*Drosophila*, *Caenorhabditis elegans*) and mammals [110]. A functional role of NOTCH signaling in the regulation of primordial follicle formation has been demonstrated in mice [111]. In the presence of NOTCH signaling inhibitors, reductions in primordial follicles are observed in newborn ovaries. Studies have also shown that Jagged-1, NOTCH2, and *HES1* are the most abundantly expressed ligand, receptor, and target genes, respectively. Moreover, NOTCH2 is expressed in pregranulosa cells of primordial follicles [111]. In humans, NOTCH2 is associated with Alagille syndrome (ALGS), an autosomal dominant multisystem disorder clinically defined by hepatic bile duct paucity and cholestasis in association with cardiac, skeletal, and ophthalmologic manifestations (MIM-118450). In addition, Hajdu-Cheney syndrome (HJCYS), also associated with *NOTCH2*, is a rare autosomal dominant skeletal disorder characterized by short stature, coarse and dysmorphic facies, bowing of the long bones, and vertebral anomalies (MIM-102500). The NOTCH2 mutations associated with POI have been recently reported. Four patients harboring different NOTCH2 variants have been identified: one patient presented primary amenorrhea and carried a compound heterozygous mutation (c.[7223T>A:p.Leu2408His]; [6947C>T:p.Ala2316Val]), while 3 patients presented secondary amenorrhea and each carried a monoallelic variant (c.5411C>T:p.Ser1804Leu, c.7075C>G:p.Pro2359Ala, or c.5433G>C:p.Gln1811His). Transcriptional activity has been demonstrated for 3 of the abovementioned NOTCH2 mutations (p.Ser1804Leu, p.Ala2316Val, and p.Pro2359Ala), although no protein level differences have been revealed between controls and individuals with all described mutants [94].

Meiosis and DNA repair genes:

Scaffolding protein involved in DNA repair (SPIDR/KIAA0146). SPIDR is a protein that links helicase and the homologous recombination (HR) machinery. Depletion of SPIDR promotes increased rates of sister chromatid defects, genomic instability, and hypersensitivity to DNA damaging effects [112]. A nonsense homozygous mutation (c.839G>A:p.Trp280*) in *SPIDR* was found in 2 sisters with POI born from consanguineous parents of Israeli–Muslim–Arab ancestry. The sisters presented with delay of puberty, raised gonadotropin levels, and some differences in clinical presentation, which included hypoplastic ovaries and café au lait spots (younger sister) or absent ovaries (oldest sister). A normal 46,XX karyotype and no dysmorphic features were identified in both sisters. The p.Trp280* mutation revealed that SPIDR activity was impaired during homologous recombination, resulting in 53BP1-labeled double-strand breaks postionizing radiation and gH2AX-labeled damage during unperturbed growth [80].

MutS homolog 4 (MSH4) and MutS homolog 5 (MSH5). MSH4 and MSH5 are meiosis-specific proteins that are required for the recombination and proper segregation of homologous chromosomes. Male and female mice harboring *Msh4* or *Msh5* defects have infertility due to meiotic failure [113, 114], and both genes can contribute to POI pathogenesis. Two sisters diagnosed with secondary amenorrhea were found to harbor a homozygous donor splice-site mutation in *MSH4* (c.2355 + 1G>A:p.Ile743_Lys785del) [81]. In a Chinese cohort, a novel homozygous missense mutation (c.1459G>T:p.Asp487Tyr) in *MSH5* was identified in 2 sisters with isolated POI. Functional evaluation using knock-in mice ($Msh5^{D486Y/D486Y}$) showed atrophic ovaries, and MSH5 disruption impaired DNA homologous recombination repair in an in vitro study [82].

Fanconi anemia complementation group M (FANCM). FANCM is involved in the repair of DNA replication and homologous recombination. Monoallelic mutations in this gene are associated with a predisposition to breast and ovarian cancer. Moreover, *FANCM* is no longer listed as a Fanconi anemia gene due to the lack of genetic data or other functional evidence of a causative role of a biallelic mutation in the disorder [115]. However, a homozygous nonsense mutation in *FANCM* (c.5101C>T:p.Gln1701*) was found in two Finnish siblings diagnosed with nonsyndromic POI. Lymphocyte analyses of the sisters showed increased levels of chromosomal breakage and hypersensitivity to mitomycin C [84]. Furthermore, a biallelic mutation in *FANCM* (c.5791C>T:p.Arg1931*) was revealed in a Portuguese man diagnosed with azoospermia [116]. *FANCM* mutations have been shown to be associated with meiotic defects and infertility in females and males.

Basonuclin 1 (BNC1). BNC1 is a zinc finger protein that is highly expressed in the germ cells of the testes and ovaries, in keratinocytes, and in hair follicles. Knockdown of *BNC1* in mouse oocytes reduces the levels of RNA polymerase transcription and leads to small and irregular follicle morphology. Indeed, knockout ovaries reveal corpora lutea presenting normal ovulation, although female subfertility occurs [117]. A Chinese family with 7 POI-affected women was screened by WES, and a heterozygous 5-bp deletion was found in *BNC1* (c.1065_1069del:p.Arg356Valfs*6). Additionally, a heterozygous missense variant in *BNC1* (c.1595T>C:p.Leu532Pro) was identified in 4 unrelated POI patients [88]. *BNC1* haploinsufficiency was demonstrated in in vitro and in vivo assays. Transfected cells with the deletion and missense mutations exhibited abnormal nuclear localization and impaired meiosis in the ovaries. Heterozygous ($Bnc1^{+/-}$) and homozygous ($Bnc1^{-/-}$) mice harboring the 5-bp deletion showed female infertility due to diminished ovarian reserves (ie, elevated FSH, decreased ovary size, and follicle size) [88].

WD repeat-containing protein 62 (WDR62). WDR62 is a ubiquitously expressed scaffold JNK-binding protein. This protein plays a role in mediating mRNA homeostasis after stress, with JNK as its partner [118]. Bilguvar and collaborators [119] first identified recessive missense and loss-of-function mutations in *WDR62* in 10 patients and found that these mutations caused a wide spectrum of cerebral cortical malformations, including microcephaly, pachygyria with cortical thickening, and hypoplasia of the corpus callosum. Later, disruption of *Wdr62* in mice led to microcephaly by reducing the proliferation of neocortical progenitors during neurogenesis due to mitotic defects, neuronal migration delay, and altered neuronal differentiation. These mice were also infertile and had smaller body sizes at early postnatal stages than normal mice [120]. Furthermore, *Wdr62* knockout mice exhibited female meiotic initiation defects that were rescued by JNK1 overexpression in germ cells, presenting infertility with reduced ovaries and absent follicles [89]. Using WES, the researchers also assessed two sporadic POI cases diagnosed with primary amenorrhea, each of which harbored one missense (c.1796G>A:p.Cys599Tyr) or one frameshift mutation (c.3203_3206del:p.Thr1068fs) in *WDR62*. Although in vitro studies have shown that the dominant negative effects of these mutations are regulated by *Stra8* expression and that the mouse phenotype correlates with

the primary amenorrhea phenotype, the patient carrying the p.Cys599Tyr mutation also had 3 additional variants in 2 distinct genes associated with female infertility (*BRCA2* and *SPTB*); hence, the genetic etiology of this patient remains unclear [89].

DNA repair-associated / breast cancer type 2 susceptibility protein / fanconi anemia group D1 protein (BRCA2). BRCA2 is involved in the maintenance of genome stability, specifically in the signaling of the homologous recombination pathway for double-stranded DNA repair [121]. Davies and collaborators [122] showed that BRCA2 plays a dual role in regulating the actions of RAD51, a protein essential for homologous recombination and DNA repair. Therefore, loss of control of these processes following BRCA2 inactivation may lead to genomic instability and tumorigenesis [122]. Germline monoallelic mutations in *BRCA2* (and *BRCA1*) increase lifetime cancer risk; they were first described as causing breast and ovarian cancer in familial cases, followed by sporadic cases and later male breast cancer and prostatic cancer cases [123]. Moreover, Fanconi anemia type D1 is caused by homozygous mutations in *BRCA2*. Male and female patients have multiple congenital abnormalities, bone marrow failure, and expected susceptibility to cancer. These patients often present an infertility phenotype that includes premature menopause in females and altered sperm production in males [123]. Two sisters born to nonconsanguineous Ethiopian parents were diagnosed with POI, presenting primary amenorrhea, pubertal delay, short stature, café au lait spots, microcephaly, and, in one sister, long-term remission from acute myelocytic leukemia [91]. These siblings carried compound heterozygous truncating mutations in *BRCA2* (c.[7579delG:p.Val2527*] and [9693delA:p.Ser3231fs16*]). Interestingly, segregation analysis revealed a monoallelic *BRCA2* mutation (c.7579delG) in their mother, who was diagnosed with ovarian cancer stage III. An impaired response to DNA damage was demonstrated by chromosomal breakage observed in peripheral lymphocytes obtained from the proband and by the failure of RAD51 to be recruited to double-stranded DNA breaks. Moreover, disruption of the *BRCA2* orthologue in *Drosophila* leads to sterility and gonadal dysgenesis in males and females [91]. In addition, two Chinese women, one with familial and one with sporadic POI, were reported to harbor compound heterozygous variants in *BRCA2* (c.[68-1G>C];[4440T>G:p.Tyr1480*] and c.[8168A>T:p.Asp2723Val];[9697_9700del:p.Cys3233Trpfs*15]), respectively) [92]. These patients presented primary amenorrhea; however, no hematologic abnormalities or tumors were identified in these cases. Moreover, 2 sisters presenting primary amenorrhea and microcephaly were diagnosed with early-onset colorectal cancer and breast cancer. Two variants in *BRCA2* (c.[6468_6469delTC];[c.8471G>C]) were revealed in both siblings and subsequently confirmed by long-range PCR to be in trans [92]. Although these last 2 cases may expand the spectrum of the *BRCA2* phenotype, the pathogenicity of the variants needs further functional validation.

Tumor protein p63 (TP63). TP63, a member of the p53 family, is a transcription factor that is implicated in cancer, development, and reproduction [124]. Combined loss of *p63* and *p73* impairs the induction of *p53*-dependent apoptosis in response to DNA damage in mouse embryo fibroblast cells and in *in vivo* approaches [125]. Furthermore, *p63*, specifically the TAp63 isoform, suppresses tumorigenesis and metastasis by regulating DICER and miR130b [126]. In the ovaries, *p63* is required to maintain the integrity of the female germ line during meiotic arrest. In addition, *p63* plays a key role in the process of DNA damage-induced primary oocyte death not involving *p53* [126]. The oocytes of *p63*-null mice show resistance to the same dose of radiation that kills all oocytes of WT and *p53*-null mice [126]. *TP63* has been implicated in complex syndromes that affect several organs through autosomal dominant inheritance (MIM 603273); however, 1 monoallelic nonsense pathogenic variant (c.1794G>A:p.Arg594*) in *TP63* was recently identified in an isolated POI patient presenting with primary amenorrhea [95]. Further functional studies are warranted to evaluate the pathogenicity of this variant.

Metabolism- and protein synthesis-related genes:

RNA polymerase II subunit C (POLR2C). POLR2C encodes the largest subunit of RNA polymerase II, which synthesizes messenger RNA in eukaryotes [127]. A heterozygous nonsense mutation in *POLR2C* (c.454A>T:p.Lys152*) was identified in a woman with familial POI who was also diagnosed with immune thrombocytopenia, pernicious anemia, and hypothyroidism. An *in vitro* study with p.Lys152* knockdown showed decreased POLR2C levels and impaired cell proliferation [85].

Mitochondrial ribosomal protein S22 (MRPS22). MRPS22 is implicated in protein synthesis within the mitochondria. Mutations in some genes, such as *HARS2 and LARS2*, are related to mitochondrial translation and Perrault syndrome, which includes ovarian failure and hearing loss [128, 129]. Two homozygous missense mutations (c.404G>A:p.Arg135Gln and c.605G>A:p.Arg202His) in the *MRPS22* gene were found in two consanguineous familial cases presenting delayed puberty and elevated gonadotropin levels. No mitochondrial defects in oxidative phosphorylation were found in the fibroblasts of these POI patients. Although $Mrps22^{-/-}$ knockout mice show embryonic lethality, while $Mrps22^{+/-}$ mice are fertile, knockdown of *mRpS22*, an orthologous gene in *Drosophila*, results in female sterility due to the absence of germ cells [86], which occurs through a mechanism mainly related to meiosis and DNA repair.

5. Conclusion

POI is a highly heterogeneous disorder associated with mutations in more than 75 genes that are mainly related to meiosis and DNA repair, each of which affects only a few women. Some of the genes have not yet been proven to be associated with POI etiology, and functional studies or additional reports on affected women are warranted to confirm their associations with POI etiology. Although the genetic etiology of POI has been studied by several groups, and although NGS techniques have increased the numbers of known genes identified to play roles in POI etiology and have allowed the discovery of new players in POI etiology, most cases remain without a clear genetic diagnosis. In the next few years, new genetic etiologies will be identified for POI phenotypes, considering the strong genetic background of this disorder and the widespread use of low-cost, high-throughput parallel sequencing techniques.

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