FEMALE INFERTILITY: GENETICS OF REPRODUCTIVE AGEING, MENOPAUSE AND PRIMARY OVARIAN INSUFFICIENCY

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FEMALE INFERTILITY: GENETICS OF REPRODUCTIVE AGEING, MENOPAUSE AND PRIMARY OVARIAN INSUFFICIENCY

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frontiers in Genetics

EDITORIAL
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Editorial: Female Infertility: Genetics of Reproductive Ageing, Menopause and Primary Ovarian Insufficiency

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Editorial on the Research Topic

Female Infertility: Genetics of Reproductive Ageing, Menopause and Primary Ovarian Insufficiency

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INTRODUCTION

Worldwide, infertility affects between 8 and 12% of reproductive-aged couples (Vander Borght and Wyns, 2018). Female infertility represents a growing health problem, especially in industrialized countries, where the ongoing trends of delaying pregnancy beyond 35 years of age significantly reduce fertility rates.

The age-related decline in fertility is characterized by a gradual decrease in the number of primordial follicles. Menopause ensues around 51 years of age and is exemplified by the cessation of ovarian functions. However, fertility begins to decline around 30 years of age in women. Therefore, understanding the molecular mechanisms underlying the age-related decline in fertility and ovarian function and associated conditions, including primary ovarian insufficiency (POI), reproductive aging, and menopause, is critical to find ways to preserve fertility and manage comorbidities related to the premature onset of estradiol deficiency.

With this research topic (RT), we aimed to tackle the issue of female infertility from different perspectives related to age, endocrine imbalances, and genetic disorders, particularly the early decline in ovarian function such as in POI.

Articles Contributions

This RT includes 13 papers: five original research articles, one brief research report, five reviews, one case report, and one clinical trial.

Early decline in ovarian function occurs in around 10% of women and is described by a markedly reduced follicular reserve and ovarian response. POI, previously referred to as "premature ovarian failure," is characterized by impairment of ovarian function before 40 years and affects approximately 1% of women under 40 and 0.1% of women under 30. POI is a heterogeneous condition due to genetic and non-genetic factors, such as autoimmunity (e.g., oophoritis), environmental toxins, and chemicals. Attrition in the number of residual ovarian follicles and ensuing deficiency in ovarian sex hormones are hallmarks of POI, thus rendering a woman subfertile and estrogen-deficient several years (even decades) before the average age of menopause. Clinical presentations of POI can also be due to FSH/LH receptor mutations leading to "resistant ovaries" retaining normal follicles in otherwise normal ovaries.

In contrast, POI cases with no detectable FSH/LH receptor mutations show a deficit in ovarian follicles. These vary from complete failure of germ-cell development, resulting in primary amenorrhea, to an accelerated decline in germ cell numbers, leading to the cessation of ovarian function before age 40 (Crisponi et al., 2001). Several genetic factors have been reported in such cases, including *FOXL2, STAG3, FOXO3a*, and X-linked genes, such as *FMR1* and *BMP15*. However, POI remains poorly understood to date, with 90% of cases still with unknown causes (ESHRE Guideline, 2016).

Piedade et al. provided an excellent review of genetic disorders associated with POI and evaluated the clinical phenotypes and molecular mechanisms of POI. The authors also addressed the quality of life, emotional health, and quality of care of women with Overt POI. Importantly, they suggested methods to optimize fertility in these women. They also described a case report of a successful pregnancy and birth achieved by follicle monitoring in a woman with Overt POI. The method is based on the hypothesis that employing the NIH P-HRT regimen to suppress luteinizing hormone (LH) levels could prevent follicle luteinization, restore follicle function and ovulation, and increase the chance of pregnancy in women with Overt POI.

Jiao et al. characterized ovarian response indicators in women with POI. The authors focused on AMH and AFC and concluded that changes in their levels could predict pre-POI and facilitate early diagnosis.

Rossetti et al. used target next-generation sequencing with a panel of 295 known and novel candidates to study the genetics of 64 POI patients. They identified 34 novels and 9 already known variants, suggesting an oligogenic nature of POI.

Louwers and Visser described genetic determinants in common between age at menopause, early menopause, POI, and other traits, highlighting that a critical analysis of genetic variants and methods used is essential and that multidisciplinary research teams are necessary for proper study design and result interpretation.

In certain rare and specific cases, defects in biological systems important in maintaining DNA integrity appear to be involved in the age-related decline of ovarian function.

Homer reviewed data from the Senataxin knockout mouse model ($Setx^{-/-}$), pointing to the importance of SETX in delaying the age-related decline in ovarian function and discussing the implications for understanding this phenomenon in humans. SETX is an RNA/DNA helicase involved in repairing oxidative stress-induced DNA damage. It is well known for its role in preventing neurodegenerative disease, and it has recently been found involved in male fertility by maintaining genomic integrity during spermatogenesis. Although dispensable for oogenesis, mouse SETX is critical for protecting oocyte DNA integrity and exhibits a unique role in slowing the age-related decline in ovarian function.

The age-related decline in fertility is not necessarily of ovarian origin. For instance, there is a distinct possibility of the central nervous system changes playing a role.

Nicola et al. arose an additional link between aging and fertility involving circadian clocks. The capacity of circadian rhythm regulator genes seems reduced during the senescence period, and previous studies showed the importance of the suprachiasmatic nucleus (SCN) in regulating the circadian system in aged organisms (Satinoff et al., 1993; Cai et al., 1997; Li and Satinoff, 1998). The involvement of the SCN and the activity of vasopressinergic neurons in maintaining the rhythmicity of the female reproductive system depends on the mRNA transcription-translation feedback loops. Therefore, circadian clock function is involved in the events determining age-related decline in fertility and ovarian function like most physiological processes. Nicola et al. demonstrated that the feedback loops of clock genes on the hypothalamus-pituitary-gonadal axis (HPG) modulate cyclicity in female rodents and that the desynchronization between the central and peripheral circadian clocks contributes to the irregularity of reproductive events.

Guo et al. analyzed the ubiquitously expressed miR-29 family in mice. The authors report that female miR-29a/b1 knockout mice exhibit severe fertility problems, possibly due to disrupted secretion of the luteinizing hormone leading to ovulation failure and subfertility.

Some of the age-related fertility declines are related to embryonic events.

Zhang et al., Gu et al., and Sun et al. analyzed different aspects of infertility related to preimplantation embryo lethality (PEL), pregnancy loss (<11 gestational weeks), and spontaneous abortions (<20 weeks). Zhang et al. described a novel biallelic transducin-like enhancer of the split 6 (TLE6) variant in a cohort of patients with PEL. TLE6 is a transcriptional co-repressors component of the subcortical maternal complex (SCMC). Recent evidence from ART and embryo research suggests that PEL may be a rare cause of primary female infertility (Yatsenko and Rajkovic, 2019).

Gu et al. conducted a retrospective study on 1,102 women who experienced singleton pregnancy loss and underwent chromosomal microarray analysis (CMA). Their study demonstrated that pregnancy loss in women over 35 is associated with a higher chromosomal aneuploidy rate and increased autosomal trisomy. Chromosome trisomy mainly results from un-separated chromosomes in oogenesis, related to advanced maternal age. The strengths of this study include its population-based setting and relatively large sample size.

This RT also includes manuscripts on PCOS, the most common endocrine-metabolic disorder causing infertility due to anovulation. Worldwide its prevalence ranges from 4 to 21% depending on the diagnostic criteria (Lizneva et al., 2016).

Sun et al. aimed to assess the impact of relevant risk factors on spontaneous abortion in patients with polycystic ovary syndrome (PCOS). The authors examined the correlation of spontaneous abortions in patients undergoing assisted reproductive treatment (ART) with risk factors including body mass index (BMI), age, hyperandrogenism, insulin resistance (IR), and chromosome aberrations. Their analysis concluded that high BMI and insulin resistance are two risk factors for increased spontaneous abortion in PCOS patients undergoing ART.

In an independent study, Qorbani et al. analyzed the effects of oligopin supplementation on hormonal and metabolic profiles in PCOS by a randomized controlled trial (RCT). Oligopin is a plant extract with strong antioxidant and anti-inflammatory activity (Sedighiyan et al., 2018). However, according to the authors, oligopin supplementation does not benefit women's hormonal and metabolic parameters.

The involvement of FOXO1 in PCOS has been reviewed by Xu et al. FOXO1, a member of the forkhead transcription factor family (FoxO), plays a vital role during glycolipid metabolism, IR, and oxidative stress. Since PCOS has been associated with IR and low-grade inflammatory response, the authors support the need to clarify the role of FOXO1.

Hong Zhang et al. detailed the function of another FoxO, FOXO3, in the physiological regulation of ovarian follicular development. Their contribution provides an important reference for further studies of ovarian biology, including valuable insights for the modulation of the age-related decline in ovarian function, reproductive lifespan, and ovarian disease.

CONCLUSION

The results presented in this RT summarize some of the latest findings in infertility research and the regulation of ovarian biology, underlying the role of genes affecting the age-related decline in fertility (i.e., the well-known FOXO factors and SETX) and embryonic lethality (i.e., TLE6), and highlighting emerging mechanisms including the regulation of ovarian functions by circadian rhythms and miRNAs.

These reports highlight the critical role of aging in female infertility through several mechanisms affecting ovarian function, including the HPG axis, the role of hormones, integrity of meiotic and mitotic divisions, thus affecting follicle dynamics (changes in follicle numbers due to processes including oocyte awakening, primordial follicle activation, follicular growth and maturation), preimplantation embryo lethality, pregnancy loss, and aneuploidy of conception products.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Expression Regulation and Physiological Role of Transcription Factor FOXO3a During Ovarian Follicular Development

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In mammals, developing ovarian follicles transform from primordial follicles to primary follicles, secondary follicles, and mature follicles, accompanied by changes in follicular secretory functions. FoxO3a is a member of the forkhead transcription factor family (FoxO), which plays an important role in the cell cycle, DNA damage repair, apoptosis, oxidative stress, and energy metabolism. Recent studies have shown that FOXO3a is involved in the physiological regulation of follicular development and pathological progression of related ovarian diseases, which will provide useful concepts and strategies for retarding ovarian aging, prolonging the ovarian life span, and treating ovarian diseases. Therefore, the regulation of FOXO3a expression, as well as the physiological contribution during ovarian follicular development are detailed in this paper, presenting an important reference for the further study of ovarian biology.

Keywords: transcription factor FOXO3a, cell cycle, apoptosis, oxidative stress, energy metabolism, follicular development

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INTRODUCTION

Follicular development is a complex reproduction-related physiological process characterized by cell proliferation, differentiation, and apoptosis. Typically, based on morphology and function, follicular development can be artificially divided into different stages, including primordial follicles, primary follicles, secondary follicles, and mature follicles (Wei et al., 2012, 2019; Huang et al., 2016; Wu et al., 2019). Notably, various diseases could be induced by follicular dysplasia, including premature ovarian failure, polycystic follicular syndrome, and infertility (Yang et al., 2010; Thanatsis et al., 2019). Forkhead box (Fox) proteins are highly conserved transcription factors structurally, currently attracting a great deal of attention. Among them, FOXO3a is an important member, and its discovery originates from its homologous protein DAF-16, which is also a well-studied transcription factor (Liu et al., 2018). Ogg et al. (1997) revealed that the FOXO3a homologous protein, DAF-16, is negatively regulated by the insulin signaling pathway. Furthermore, it participates in the regulation of the cell cycle and life expectancy, which is closely related to the lifespan, metabolism, and reproduction of worms (Ogg et al., 1997). Thus, FOXO3a may be closely related to the development, metabolism, and other functions of organisms. Recent studies have shown that FOXO3a is involved in follicular development, thus presenting a valuable

target for the study of follicular development, and displays important theoretical and practical significance for better understanding the mammalian reproductive mechanism.

THE DEVELOPMENT OF OVARIAN FOLLICLES

In mammals, the primordial follicle is the basic female reproductive unit and the only form of ovarian cell reserve (Wei et al., 2012, 2019; Huang et al., 2016; Wu et al., 2019). Primitive follicular pools are formed during early life such as the late embryonic stage in humans and the fourth day after birth in rats (Tang et al., 2017). Furthermore, once primordial follicles are formed, their total number remains fixed and is no longer increased. Usually, after the formation of primordial follicles, there will be a continuous batch of developing primordial follicles, and then forming follicles at different developmental stages, finally becoming dominant follicles triggering ovulation to commence a new life journey (Zhang Z. et al., 2019). During follicular development, most follicles die a programmed death or degeneration, which is termed follicular apoptosis or atresia (Tang et al., 2017). There are two types of follicular atresia from the morphological standpoint, starting from oocytes or granulosa cells, respectively (Manabe et al., 2004; Shimizu et al., 2009).

TRANSCRIPTION FACTOR FOXO3a

The first forkhead protein was discovered in *Drosophila melanogaster* by Weigel et al. (1989). To date, more than 100 family members have been verified, from FOXA to FOXS (Anderson et al., 1998; Lee and Dong, 2017; Murtaza et al., 2017). FOXO belongs to the "O" class of the FOX superfamily. In mammals, this group contains four members: FOXO1/FKHR/FOXO1a, FOXO3/FKHRL1/FOXO3a, FOXO4/AFX, and FOXO6 (Murtaza et al., 2017). All FoxO proteins share a highly conserved DNA-binding domain, presenting 110 amino acids folded into three α-helixes and two wing-like large loops. In addition, the structure includes a nuclear localization signal, a nuclear export signal motif, and a C-terminal transcriptional active region (Obsil and Obsilova, 2008). These proteins are ubiquitously expressed in various tissues throughout the body, except for FOXO6, which currently has been reported only in the adult brain tissue (Jacobs et al., 2003). Notably, the Human Protein Atlas[1] indicates that the expression of FOXO3 in human ovarian stromal and follicular cells is abundant.

EXPRESSION REGULATION OF FOXO3a DURING FOLLICULAR DEVELOPMENT

During recent years, several studies have investigated the regulation of FOXO3a expression. The activity of FOXO3a can be improved at multiple levels, in which post-translational modification is the main approach, including phosphorylation, acetylation, and ubiquitination (**Figure 1**).

Phosphorylation and Dephosphorylation

FOXO3a can be phosphorylated by kinases such as protein kinase B (PKB), extracellular-regulated kinase, serum and glucocorticoid-induced kinase, and inhibitor kappa B kinase β (Brunet et al., 2001). The transcriptional regulation of FOXO3a is closely related to phosphoinositide-3 kinase (PI-3K)/protein kinase B (PKB) signaling, which was first proposed by Brunet et al. (1999). In mammals, FOXO3a can be phosphorylated by PKB in the nucleus and then transported from the nucleus to the cytoplasm, utilizing the 14-3-3 molecular chaperone after the activation of insulin signaling. FOXO3a is translocated into the cytoplasm and could bind with the polyubiquitination system, to be subsequently degraded by proteasomes (Plas and Thompson, 2003; Wang et al., 2015), which accompanies the transcriptional activity loss of FOXO3a, which no longer plays a regulatory role during cell development and metabolism (Datta et al., 1999; Brunet et al., 2002; Plas and Thompson, 2003, 2005). Therefore, the localization of FOXO3a in the cytoplasm not only inactivates its function but is also extremely crucial for the degradation of FOXO3a.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a key negative regulator for PI3K/PKB signaling, which can improve the suppression of FOXO3a through dephosphorylation (Ding et al., 2010; Jang et al., 2016; Li J. et al., 2020). Additionally, endogenous PKB and FOXO3a can form a complex. Furthermore, FOXO3a can negatively regulate PKB and its downstream molecules (Takaishi et al., 1999; Lin et al., 2001; Junger et al., 2003; Puig et al., 2003). Simultaneously, elevated 14-3-3 can increase FOXO3a expression and phosphorylation, maintaining the phosphorylated FOXO3a protein stability (Cahill et al., 2001).

Reddy et al. (2005) reported the activation of PKB and suppression of FOXO3a in mouse and rat oocytes using stem cell factor (SCF) during follicular development. Meng et al. (2007) showed the stage/cell-specific expression patterns of FOXO3a and PKB, suggesting that these proteins might play potential roles in the follicular development of the mini-pig. Furthermore, consistent results were observed in fetal and neonatal pig ovaries (Ding et al., 2010). These results indicate the important role of PKB/FOXO3a and the impact of PKB regulation on FOXO3a phosphorylation during follicular development.

Acetylation and Deacetylation

In addition to the regulation of PKB, FOXO3a is also mediated via acetylation and deacetylation (Kim et al., 2010; Xiang et al., 2012; Zhang et al., 2013; Wang et al., 2014; Zhou et al., 2014; Liu et al., 2015; Long et al., 2019). CREB binding protein, p300, and other nuclear proteins can acetylate lysine on the DNA-binding region of FOXO3a protein, resulting in reduced FOXO3a transcriptional activation (Watroba et al., 2012). Sirtuin 1 (SIRT1) is a NAD-dependent histone deacetylase (Long et al., 2019). Typically, SIRT1 in the nucleus may activate the transcriptional activity of FOXO3a, regulating cellular functions

[1] http://www.proteinatlas.org

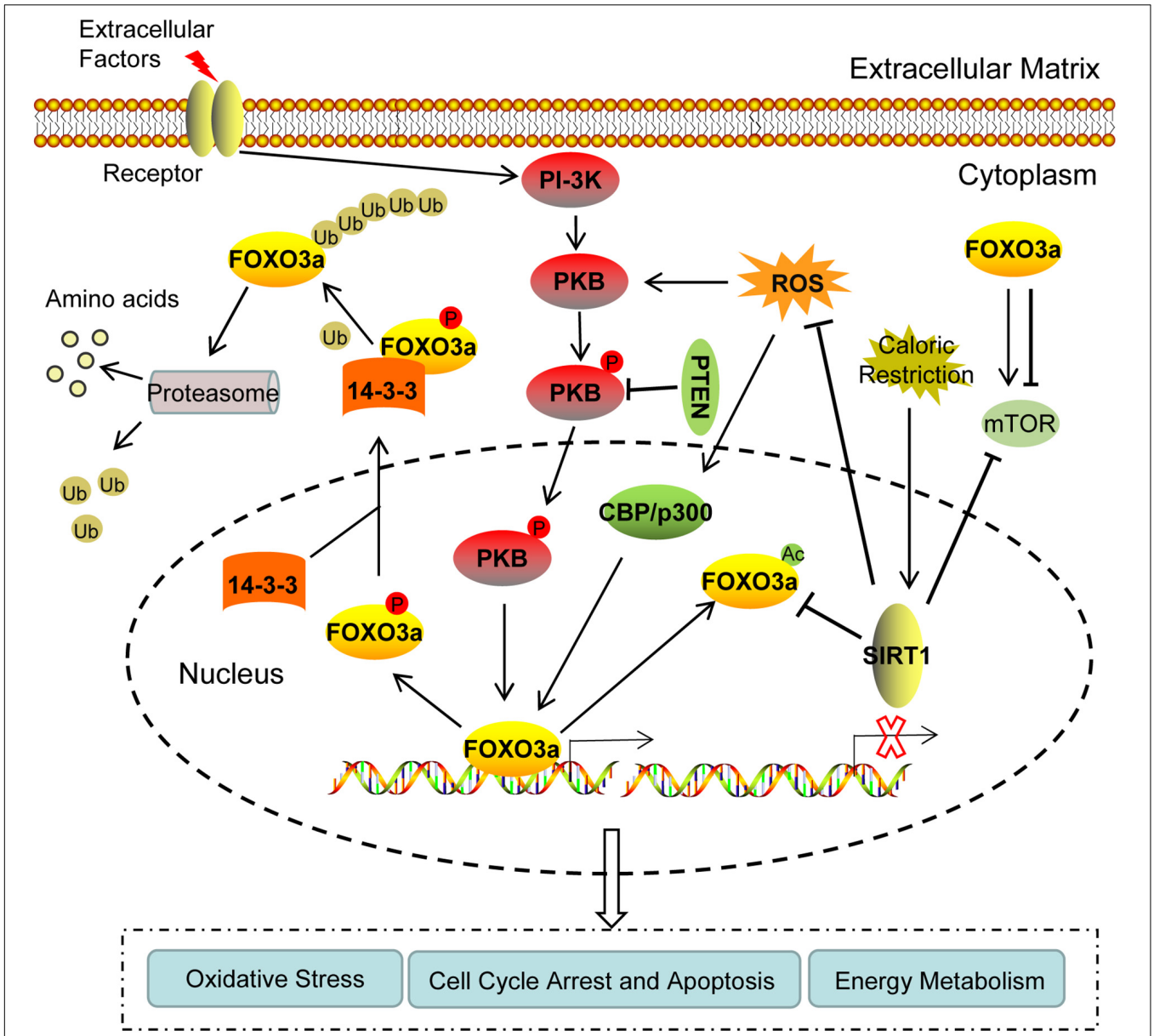

FIGURE 1 | The regulation of FOXO3a expression and its physiological role of during follicular development. (1) FOXO3a can be phosphorylated by PKB, and then transported from the nucleus to the cytoplasm by 14-3-3 molecular chaperone, accompanied by the loss of transcriptional activity. PTEN can inhibit the inactivation of FOXO3a by dephosphorylating p-PKB. (2) In the cytoplasm, the polyubiquitination of FOXO3a results in its degradation by proteasomes. (3) Acetylase CBP, p300, and other nuclear proteins can acetylate FOXO3a protein. In the nucleus, SIRT1 may activate FOXO3a by deacetylating Ac-FOXO3a. (4) Under oxidative stress, the accumulated ROS can increase post-translational modifications of FOXO3a, whereas SIRT1 can downregulate ROS. (5) FOXO3a is involved in autophagy by regulating mTOR. Caloric restriction may activate SIRT1 signaling and suppress mTOR. Collectively, the regulation of FOXO3a can mediate cell cycle arrest and apoptosis by inducing the transcription of downstream target genes, as well as by participating in oxidative stress and energy metabolism through communication with PKB, SIRT1, ROS, and mTOR, thereby affecting the activation of primordial follicles, oocyte and granulosa cell apoptosis, and regulating the growth and development of follicles. PKB, protein kinase B; PTEN, phosphatase and tensin homolog deleted on chromosome ten; SIRT1, sirtuin 1; ROS, reactive oxygen species; mTOR, mammalian target of rapamycin.

by deacetylating Ac-FOXO3a (Glauser and Schlegel, 2007). Gorczyca et al. (2019) demonstrated the presence of SIRT1 and SIRT6 in ovarian cells, and their involvement in the control of follicular atresia. Recent studies revealed that energy restriction can increase the expression of SIRT1 and activate SIRT1-related signaling pathways in adult mice (Xiang et al., 2012; Zhang et al., 2013; Liu et al., 2015; Long et al., 2019). Additionally, Kim et al. (2010) reported that the high expression of FOXO3a can upregulate SITR6 activity, whereas the inhibition of FOXO3a expression could downregulate SIRT6 activity. Simultaneously, the downregulation of FOXO3a can prevent the effect of SIRT6 on energy limitation, as well as on SIRT1 (Kim et al., 2010).

PHYSIOLOGICAL ROLES OF FOXO3a DURING FOLLICULAR DEVELOPMENT

Currently, numerous studies have presented that FOXO3a is associated with follicular development (Brenkman and Burgering, 2003; Adhikari and Liu, 2009; Monniaux et al., 2016). Experimental studies have reported that female FOXO3a knockout mice exhibit global follicular activation at an early stage of follicular growth, leading to oocyte death, early depletion of functional ovarian follicles, and secondary infertility (Castrillon et al., 2003). Conversely, FOXO3 overexpression can delay the development of primordial follicles, increase the follicular reserve, and ovarian reproductive capacity in mice. Compared with wild-type littermates, increased follicle numbers and decreased gonadotropin levels were documented in aging FOXO3-transgenic mice (Pelosi et al., 2013). Thus, FOXO3a may play an important role in maintaining the pool number of primordial follicles and the physiological functions of the ovarian reserve, as well as female fertility. Furthermore, some researchers have reported that the FOXO3 protein regulates follicle growth and atresia by promoting apoptosis of granulosa cells and oocytes in mammalian ovaries (Liu et al., 2009; Matsuda et al., 2011).

Although the function of FOXO3a in ovarian follicle development has been relatively known, its mechanism remains unclear. It is generally accepted that FOXO3a is widely involved in the cell cycle, DNA damage repair, apoptosis, oxidative stress, and metabolism. Hence, we presented evidence postulating that the role of FOXO3a in follicular development is related to these processes (**Figure 1**).

Cell Cycle Arrest and Apoptosis

FOXO3a activity impacts the expression of downstream target genes, resulting in cell cycle and apoptotic disturbances (Medema et al., 2000). FoxO3a can increase the expression of the cyclin-dependent kinase inhibitor protein, p27kip, and decrease the expression of cyclin D in the nucleus, maintaining cells in a stationary phase and inhibiting follicular development (Schmidt et al., 2002). Liu et al. (2009) suggested that FOXO3a is involved in oocyte apoptosis in the neonatal rat ovary, and the SCF-PI3K/PKB-FOXO3a signaling pathway mediates primordial follicle formation and oocyte apoptosis by regulating the expression of p27kip1 and proapoptotic factors such as Bim, Bad, and Bax. Moreover, research on chicken primary ovarian granulosa cells indicated that in the absence of FOXO3, mRNA levels of proapoptotic factors BNIP3 and BCL2L11 decreased, along with poly [ADP-ribose] polymerase 1 (PARP-1) and cleaved caspase3 protein levels. After treatment with a recombinant FOXO3 protein, mRNA levels of BNIP3 and BCL2L11, as well as protein levels of PARP-1 and caspase3, were reportedly increased (Cui et al., 2019). Experiments in human ovarian granulosa-like tumor cells (KGN) have shown that expression of the proapoptotic factors FASLG and BCL2L11 is upregulated and cell death is induced by transfection of FOXO3 expression vectors (Matsuda et al., 2011). Collectively, these studies have consistently demonstrated that FOXO3 is expressed in reproductive tissues, including ovarian oocytes and granulosa cells, and promotes apoptosis.

Oxidative Stress

Reportedly, accumulated evidence suggests that oxidative stress is associated with disrupted follicular development, which may result in increased follicular atresia (Yan et al., 2020). Under oxidative stress, accumulated reactive oxygen species (ROS) leads to post-translational modifications of FOXO3a, thereby regulating the activity and function of FOXO3a. Park et al. (2020) demonstrated that SIRT1 can downregulate ROS and form a complex with FOXO3a in cells, which can improve the ability of FOXO3a to induce cell cycle arrest and promote cell survival. Recent findings have indicated that resveratrol, a plant polyphenolic compound, can enhance SIRT1 and decrease ovarian oxidative stress as well as inhibit phosphorylation of p66Shc, both *in vivo* and *in vitro* (Wang et al., 2020). Thus, in terms of follicular development, there undoubtedly exists an interactive relationship between ROS, SIRT1, and FOXO3a. However, the specific mechanism needs to be elucidated.

Energy Metabolism

Mammalian target of rapamycin (mTOR) is a major negative regulatory factor of autophagy (Choi et al., 2011). It has been previously reported that PKB-mediated activation of mTOR inhibits granulosa cell autophagy during follicular development (Choi et al., 2014). Growing evidence strongly indicates that FOXO3a is involved in autophagy. If abundant energy is available, the modification of FoxO3 inhibits its activity, thereby decreasing the transcription of autophagy genes and downregulating autophagy. However, PI3K-PKB-FOXO3 can promote autophagy by mediating mTOR inhibition (van der Vos et al., 2012). Long et al. (2019) reported that oocyte-specific SIRT1-overexpressing mice demonstrated an improved follicle reserve and a prolonged ovarian lifespan by continuously activating FOXO3a and suppressing mTOR. Furthermore, SIRT1 can facilitate primordial follicle recruitment through directly modulating PKB and mTOR transcription, independent of deacetylase activity (Zhang T. et al., 2019). High-fat diet-induced obesity may accelerate ovarian follicle development and the rate of follicle loss by activating mTOR and suppressing SIRT1 signaling. Caloric restriction may improve the adverse effects of high-fat diet-induced obesity on ovarian follicles (Xiang et al., 2012; Wang et al., 2014; Li et al., 2015; Liu et al., 2015). Thus, FOXO3a, mTOR, PKB, and SIRT1 may be implicated in autophagy and energy metabolism during follicular development.

FOXO3a AND OVARIAN DISEASE

Reportedly, the deletion of FOXO3a, FOXL2, PTEN, and p27 leads to early exhaustion of the primordial follicle pool and premature ovarian insufficiency in transgenic mice (Thanatsis et al., 2019). Melatonin prevents cisplatin-induced primordial follicle loss by suppressing the PTEN/AKT/FOXO3a pathway in the mouse ovary (Jang et al., 2016). Li Y. et al. (2020) observed that

oral oyster polypeptide can protect the ovaries from D-galactose-induced premature ovarian failure, mediated via anti-oxidative stress activity. Meanwhile, growing data demonstrate that excess androgen may be the primary cause of polycystic ovary syndrome (PCOS). During the early stage of mouse folliculogenesis, testosterone induces the redistribution of FOXO3a, suggesting the involvement of FOXO3a in the pathogenesis of PCOS (Yang et al., 2010).

It has been well established that ovarian cancer presents the highest mortality rate among gynecological malignancies. Reportedly, FOXO3a expression can be increased by *LSD1* knockdown, thereby inhibiting the proliferation and metastasis of ovarian cancer HO8910 cells (Liu et al., 2020). Kaplan-Meier survival analysis suggested that the low expression of FOXO3a was significantly related to poor prognosis in ovarian cancer patients (Fei et al., 2009). Recently, Xia et al. (2020) revealed that microRNA-506-3p inhibits proliferation and promotes apoptosis in ovarian cancer cells by targeting the AKT/FOXO3a signaling pathway. O'Neill et al. (2013) suggested that blocking the epidermal growth factor receptor (EGFR) results in PI3K-PKB inhibition and increases FOXO3a activation, which provides a new and valuable treatment strategy for breast cancer, prostate cancer, and ovarian cancer.

SUMMARY AND CONCLUSION

Based on the studies investigating the regulation of FOXO3a expression, it is currently established that FOXO3a can enhance the transcriptional regulation of its target genes, thereby enhancing its physiological contribution during the cell cycle and apoptosis regulation, resistance to oxidative stress, and prolongation of life span in organisms (**Figure 1**). Furthermore, FOXO3a signaling can induce oocyte and granulosa cell apoptosis, inhibit the activation of primordial follicles, and regulate the growth and development of follicles. The activation of FOXO3a signaling can inhibit the developmental initiation of primordial follicles, maintain the initial state of primordial follicles, reduce the number of primordial follicles transformed into mature follicles, thus preserving the follicular reserves, delaying the depletion of follicles, and delaying the aging of ovaries. It is important to further explore the mechanism concerning the regulation of FOXO3a expression on its target genes, the physiological contribution of FOXO3a during ovarian follicular development, and its future clinical applications, further advancing the field of reproductive biology.

AUTHOR CONTRIBUTIONS

HZ, FL, and JZ wrote the manuscript and ZW revised it. All authors read and approved the final version of the manuscript for publication.

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High BMI and Insulin Resistance Are Risk Factors for Spontaneous Abortion in Patients With Polycystic Ovary Syndrome Undergoing Assisted Reproductive Treatment: A Systematic Review and Meta-Analysis

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Background: The risk of spontaneous abortion in patients with polycystic ovary syndrome (PCOS) undergoing assisted reproductive treatment (ART) is higher than that in patients without PCOS, however, no definitive risk factors have been confirmed to associate with the high spontaneous abortion rate in PCOS patients undergoing ART. This study was performed to assess the impact of relevant risk factors on spontaneous abortion in patients with PCOS. Clinical questions were formulated and organized according to the PICOS principle.

Methods: A systematic review and meta-analysis were conducted on all published studies on PCOS and spontaneous abortion in Embase, PubMed, Web of Science and Cochrane Library. Related risk factors included body mass index (BMI), age, insulin resistance (IR), hyperandrogenism, and chromosome aberrations. All patients were diagnosed as PCOS using the Rotterdam criteria. The primary endpoint was miscarriage and live birth rate. Fixed-effect models were used to analyze homogeneous data, and subgroup and sensitivity analyses were performed on heterogeneous data. The source of heterogeneity was evaluated, and the random effect model was used to summarize the heterogeneity.

Results: Among 1836 retrieved articles, 22 were eligible and included in the analysis with 11182 patients. High BMI (OR = 1.48, 95% CI [1.32, 1.67], MD = 1.35, 95% CI [0.58,2.12]) and insulin resistance (MD = 0.32, 95% CI [0.15, 0.49]) were associated with an increased risk of spontaneous abortion in PCOS patients undergoing ART. Older age (OR = 0.29, 95% CI [0.29, 0.44], MD = 2.01, 95% CI [0.04, 4.18]), embryonic chromosomal aberrations (OR = 0.75, 95%CI [0.31,1.77]), and hyperandrogenism (MD = 0.10, 95% CI [- 0.02, 0.22]) were not associated with the high spontaneous abortion rate

in patients with PCOS. A subgroup analysis of BMI showed that there was no statistically significant difference in the effect between overweight and obesity on spontaneous abortion in PCOS patients undergoing ART (OR = 1.34, 95% [0.97, 1.85]).

Conclusion: High BMI and insulin resistance are two risk factors for an increased risk of spontaneous abortion in PCOS patients undergoing ART, and losing weight and mitigating insulin resistance may decrease the spontaneous abortion rate in these patients undergoing ART.

Keywords: polycystic ovary syndrome, spontaneous abortion, body mass index, insulin resistance, assisted reproductive treatment

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex endocrinopathy. According to the Rotterdam criteria, two of the three criteria had to be met to fit the definition of PCOS: chronic anovulation, clinical and/or biochemical evidence of hyperandrogenism, and polycystic ovaries (1). After the Endocrine Society Clinical Practice Guideline has suggested use of the Rotterdam criteria for diagnosing PCOS (2), the Rotterdam criteria has become the most widely used PCOS diagnostic standard in the world.

The characteristics of PCOS are follicular dysplasia, insulin resistance, and hyperandrogenism, affecting 5–10% women in childbearing age (3, 4). Due to abnormal endocrine changes, it is often associated with obesity, amenorrhea, hairiness, infertility, and most importantly, miscarriage. Previous meta-analyses (5, 6) have reported that women with PCOS have an increased risk of miscarriage compared to those without PCOS. In addition, patients with PCOS usually have a high abortion rate of 30–50% in the first 3 months of pregnancy, a high incidence of recurrent early abortion of 36–82%, and a high incidence of habitual abortion of 58% (7). Recurrent miscarriage was defined as two or more consecutive abortions with the same sexual partner (8). At this time, the patient has suffered at least 2 pregnancy losses. The related risk factors of recurrent abortion include chromosomal abnormalities, uterine abnormalities, antiphospholipid syndrome, obesity (9), and high risk of thrombosis (10). Among them, the recurrent abortion caused by abnormal chromosome can reach 60.6% (9). Spontaneous abortion is defined as pregnancy loss before 20 weeks of pregnancy (11). It has been found that the frequency and distribution of chromosomal abnormalities in the spontaneous abortion group are different from those in the recurrent abortion group (12). Moreover, abnormalities in the endocrinology, immunology and anatomy also play different roles in both groups (13). The pregnancy loss rate in natural conception was reported to be 10–16% (14, 15). High rates of early pregnancy loss, ranging from 12 to 48%, have been reported in assisted reproductive treatment (ART) (16, 17). Maternal age, controlled ovarian hyperstimulation protocol, cycle type, and PCOS status (18) may have an impact on the miscarriage rate (19). However, ART is a choice that some PCOS patients have to face in order to get pregnant. If the systematic risk factors can be found for spontaneous abortion in patients with PCOS undergoing ART, the pregnancy conditions can be improved before the first pregnancy so as to prevent pregnancy loss and economic loss in PCOS patients.

However, no specific meta-analyses have been conducted to analyze the risk factors for an increased risk of spontaneous abortion in patients with PCOS. These risk factors may include body mass index (BMI), age, hyperandrogenism, insulin resistance, and chromosome aberrations, which can be detected by observational studies on patients with or without these factors (20–42). Nevertheless, it is still unclear whether the above mentioned risk factors were comprehensive and whether they could cause an increase in the spontaneous abortion rate in PCOS patients undergoing ART. For instance, controversies exist regarding the role of obesity or overweight in adverse pregnancy outcomes in PCOS patients, with some authors (20, 36) considering obesity as a risk factor for adverse pregnancy outcomes while others (35) finding that BMI had no adverse effects on the pregnancy outcome. In view of these controversies, we believed that a meta-analysis was necessary to evaluate the risk factors of spontaneous abortion in PCOS patients undergoing ART in order to provide recommendations for clinical treatment of PCOS. In order to display all the viewpoints in a comprehensive and balanced manner, we decided to objectively search all documents related to PCOS and abortion. We selected the literature that reported spontaneous abortion after ART and studied possible risk factors for analysis.

METHODS

This study was performed in accordance with the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement (43). The PRISMA Checklist is shown in **Supplementary Table 1**. The protocol had been submitted to the International System Evaluation Expected Register (PROSPERO) with the registration number of CRD42020171499. The clinical reasoning was broken down and organized according to the PICOS principle.

Search Strategy and Selection Criteria

We selected the search keywords PCOS and abortion, covering all the subject terms and free words under the classification of

PCOS and abortion. The search database included Embase, PubMed, Web of Science and Cochrane Library (including Cochrane Database of Systematic Reviews, Database of Abstracts of reviews of effects, Cochrane Central Register of Controlled Trials, Cochrane Methodology Register, Health Technology Assessments database, NHS Economic Evaluation Database and About the Cochrane collaboration). Citation retrieval and manual retrieval were also performed to ensure that the largest number of relevant documents could be retrieved. Unpublished articles such as conference proceedings were also included. Relevant observational studies published from January, 1970 to March, 2020 were searched with no language restrictions. The search strategy is shown in **Supplementary Table 2**. The electronic search and the eligibility of the studies were independently assessed by two of the authors (Y-FS and Z-YC),

Study Selection and Data Extraction

Observational studies with either a cohort study or case-control design in women with PCOS were selected. The inclusion criteria were articles evaluating spontaneous abortion in women with PCOS diagnosed according to the Rotterdam criteria (Revised 2003 criteria1: chronic anovulation, clinical and/or biochemical signs of hyperandrogenism, polycystic ovaries and exclusion of other etiologies (congenital adrenal hyperplasia, androgen-secreting tumors, and Cushing's syndrome), and parameters which were associated with abortion in PCOS patients including age, BMI, and hyperandrogenism (1). The exclusion criteria were articles that did not use the Rotterdam criteria for diagnosis of PCOS, that included research subjects who had a history of recurrent abortion, that involved drug administration (including metformin) or intervention for purposes other than assisted reproductive technology (ART), and that were case reports, case series, or reviews (**Supplementary Figure 1**). Two authors independently studied the titles, abstracts, and full text for inclusion. In disagreement, a third physician was involved to reach an agreement. The following data were extracted from each selected study: author name, year of publication, study design, study location, participants' characteristics (such as race, age, and BMI), and number of spontaneous abortion or ongoing pregnancy in patients with PCOS. All information was entered into a researcher-developed data extraction form.

All included studies were assessed for risk of bias using the Newcastle-Ottawa Scale (NOS) for non-randomized studies (**Supplementary Table 3–4**). The NOS checklist contains three parameters of quality: (i) selected population, (ii) comparability of groups, and (iii) assessment of either the exposure or outcome of interest for case-control or cohort studies (44). Individual items assessed according to the NOS included representativeness of miscarriage and ongoing pregnancy groups in PCOS patients undergoing ART, ascertainment of diagnostic criteria for PCOS, pregnancy and delivery outcomes, cohort comparability on the basis of design or analysis, reliability of the results obtained, and adequate follow-up time. The quality of studies was assessed with the maximal stars of nine, and the studies were ranked as poor if there were less than five stars and good if there were five or more than five stars (45).

Statistical Analysis

The Review Manger (version 5.3, The Cochrane Collaboration, Copenhagen, Denmark) was used for all statistical analyses. According to the results of literature search, the associations of the spontaneous abortion rate in PCOS patients undergoing ART were evaluated with the risk factors of BMI, age, hyperandrogenism, insulin resistance, and chromosome aberrations (6). Hyperandrogenism, BMI, age, and insulin resistance were treated as continuous variables and presented as mean difference (MD) and 95% confidence interval (CI) between exposed and control groups. If the 95% CI does not include 0, the study is statistically significant, indicating that this indicator is an influencing factor affecting the spontaneous abortion rate in patients with PCOS. If the MD is >0, this factor is a risk factor; otherwise the factor is a protective factor. The age, BMI, and chromosome aberrations were also analyzed as dichotomous variables and presented with odds ratio (OR) and 95% CI between exposed and control groups. If the 95% CI does not include 1, the study is statistically significant, indicating that this indicator is an influencing factor affecting the spontaneous abortion rate in patients with PCOS. If the OR is >1, this factor is a risk factor; otherwise the factor is a protective factor. The homogeneity of effect size across studies was tested by Q statistics, and the I^2 statistics was used to measure the inconsistency of risk factors' effects across studies, with I^2 of 0–24% indicating slight heterogeneity, 25-49% moderate heterogeneity, 50–75% substantial heterogeneity, and over 75% considerable heterogeneity (46). If the article was homogeneous ($P<0.1$ or $I^2>25\%$), the fixed effect model (Mantel-Haenszel method) was chosen to test the additional uncertainty associated with the risk factors of different abortion rates in patients with and without PCOS. Otherwise, the random-effect model (DerSimonian–Laird method) was preferred. The possibility of publication bias was assessed by constructing a funnel plot.

RESULTS

Search Results

A total of 1,843 studies were retrieved according to the search strategy. A total of 54 articles were excluded at the full text stage, including 8 articles which did not use the Rotterdam criteria for the diagnosis of PCOS, 13 reviews, 11 non-clinical trials, 6 about recurrent spontaneous abortion, 12 which were not about miscarriage related risk factors, and 4 which did not have complete data. Finally, 22 studies were chosen for the analysis (**Supplementary Figure 1**). These 22 studies were published between 2006 and 2019 (14 of which were published in the last three years) (**Supplementary Table 5**).

Characteristics of Included Studies

Outcomes of interest were reported in 11182 patients in 17 retrospective cohort studies (20–26, 28, 30–36, 40, 41), three prospective cohort studies (38, 39, 42) and two case-control studies (27, 37). Fourteen studies were conducted in China (22,

23, 25, 28, 30–33, 35, 36, 38, 39, 41, 42), five in the USA (20, 21, 24, 26, 27), and the remaining three in Japan (37), Turkey (34), and Australia (40). Seventeen studies measured BMI (20–26, 30–32, 34–38, 41, 42), four assessed age (28, 30, 37, 40), three evaluated insulin resistance (30, 37, 42), five investigated chromosome aberrations (26, 30, 31, 33, 39), and the other four assessed hyperandrogenism (27, 30, 37, 42).

BMI

Fifteen articles with a total of 7499 patients evaluated the relationship between spontaneous abortion and BMI in PCOS patients undergoing ART (20–26, 32, 34–38, 41, 42), including 13 articles with categorical variables (20–26, 32, 34–36, 38, 41) and two with continuous variables (37, 42). Assessment of the risk of bias demonstrated a symmetrical funnel chart, indicating no publication bias (**Supplementary Figure 2**). The BMI was defined as weight in kilograms divided by the square of height in meters. In order to better show the homogeneity in heterogeneity analysis, patients were divided into overweight and obesity based on the BMI of different races. Among 13 articles with two categorical variables, eight were from China (22, 23, 25, 32, 35, 36, 38, 41), five of which used the Chinese BMI standard (47) of BMI ≥ 24 kg/m^2 as overweight (22, 23, 25, 35, 38), while the remaining three used the WHO BMI standard (48) of BMI ≥ 25 kg/m^2 as overweight (32, 36, 41). All the others studies applied the standard of BMI ≥ 25 kg/m^2 as overweight (20, 21, 24, 26, 34). All these included articles showed a good homogeneity (I^2 = 8% for categorical variables and I^2 = 13% for continuous variables). The fixed effect model analysis showed that PCOS patients who had a high BMI were associated with a high rate of spontaneous abortion (OR = 1.48, 95% CI [1.32, 1.67], MD = 1.35, 95% CI [0.58, 2.12]) (**Figures 1** and **2**), However, different ethnic BMI standards may lead to selection bias and inaccurate results. In order to solve the problems of possible heterogeneity and selection bias, we conducted a subgroup analysis of the included literature: first, subgroup analysis was further performed according to the BMI standard (OR = 1.53, 95% CI [1.02, 2.30] for Chinese BMI standard, and OR = 1.49, 95% CI [1.32, 1.69] for WHO BMI standard) (**Figures 3** and **4**). The results of the two-subgroup analysis were consistent with the overall results, that is, high BMI was a risk factor for spontaneous abortion in patients with PCOS, indicating that different BMI standards did not affect the final results. Secondly, subgroup analysis was further performed according to the race (OR = 1.52, 95% CI [1.26, 1.82] for Chinese, and OR = 1.48, 95% CI [1.27, 1.73] for non-Chinese) (**Figures 5** and **6**). The subgroup analysis results of different races were consistent with the overall results, indicating that the BMI standard was applicable to the selected races and did not affect the final results.

In addition, six articles further divided PCOS patients with high BMI into overweight and obese subgroups (20–22, 32, 35, 36), two of which used the Chinese standard (47) of BMI ≥ 28 kg/m^2 as obese and BMI 24–28 kg/m^2 as overweight (22, 35), while the remaining four used the WHO standard (48) of BMI ≥ 30 kg/m^2 as obese and BMI 25–30 kg/m^2 as overweight (20, 21, 32, 36). Analysis of these two subgroups did not show any significant differences in the spontaneous abortion rate caused by obesity compared with overweight (OR = 1.34 95% [0.97,

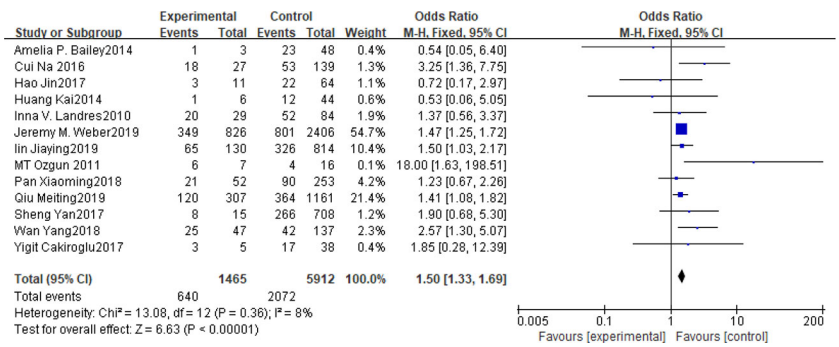

FIGURE 1 | Meta-analyses for BMI in categorical data.

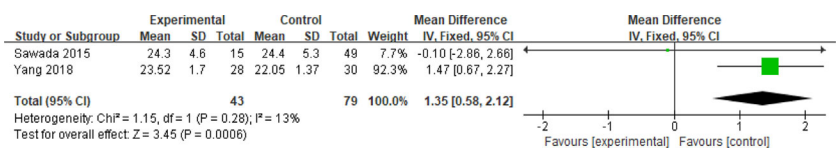

FIGURE 2 | Meta-analysis for BMI in continuous data.

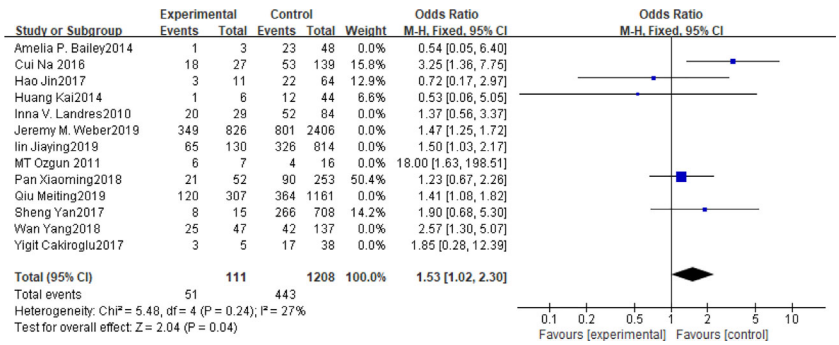

FIGURE 3 | Meta-analyses for BMI with Chinese BMI standard in categorical data.

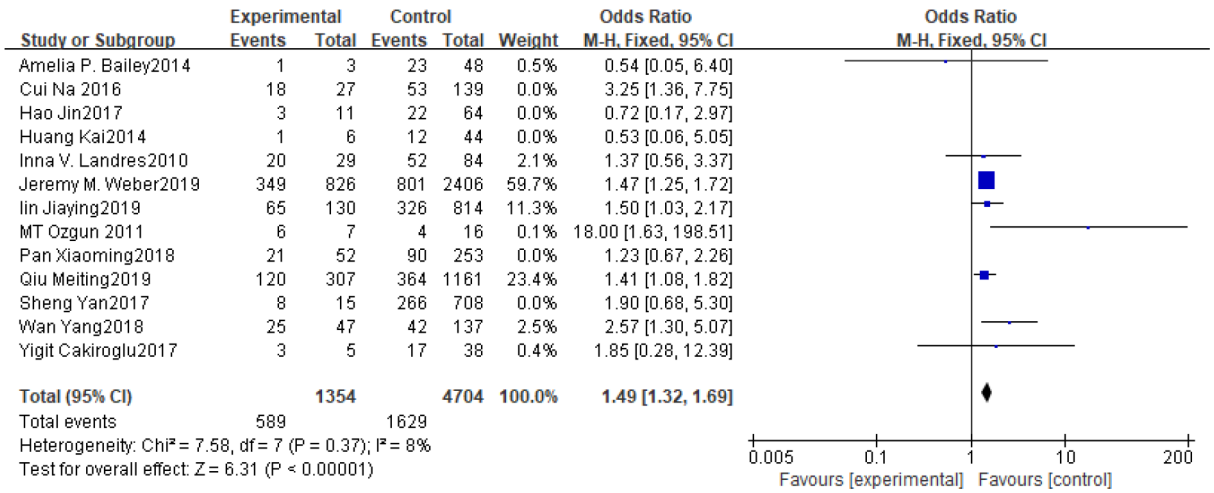

FIGURE 4 | Meta-analyses for BMI with WHO BMI standard in categorical data.

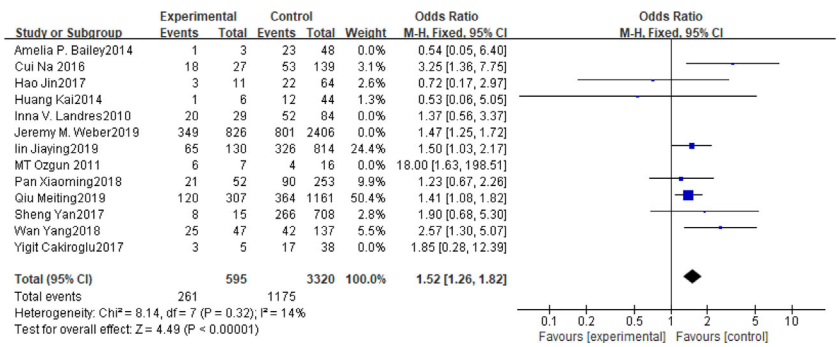

FIGURE 5 | Meta-analyses for BMI in Chinese in categorical data.

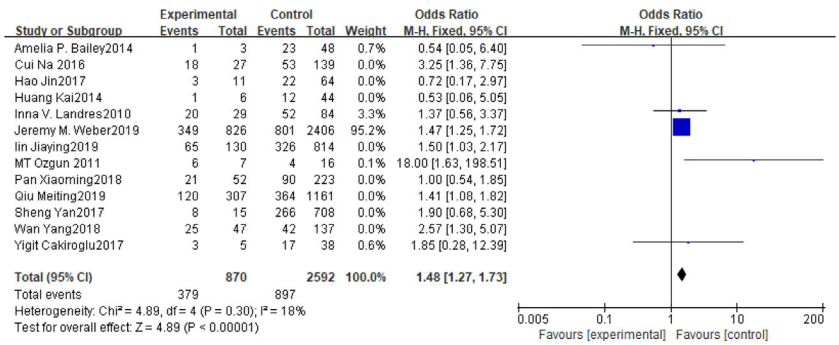

FIGURE 6 | Meta-analyses for BMI in non-Chinese in categorical data.

1.85]) even though these articles were homogeneous (I^2 = 0%) (**Figure 7**).

Age

Four articles with a total of 2585 patients focused on the relationship between age and spontaneous abortions in PCOS patients undergoing ART (28, 30, 37, 40). Among them, two articles reported dichotomous data (28, 40), while the other two reported continuous variables (30, 37). People over 35 years old were considered senior in one study (28), and 38 years of age was the line for senior in another (40). Age was demonstrated to be a protective factor for spontaneous abortions in patients with PCOS (OR = 0.29, 95% CI [0.29, 0.44]) in the two articles with dichotomous variables which had good homogeneity (I^2 = 0%) (**Figure 8**). However, age was not related to the spontaneous abortions rate in PCOS patients undergoing ART (MD = 2.01, 95% CI [0.04, 4.18]) in the articles with continuous variables (**Figure 9**).

Insulin Resistance

The relationship between insulin resistance and spontaneous abortion in PCOS patients undergoing ART were examined in a total of 1393 patients in three reports (30, 37, 42). The homeostasis model assessment-insulin resistance (HOMA - IR) was obtained from the following equation: HOMA - IR = fasting plasma insulin [mIU/L] × fasting plasma glucose [mmol/L]/22.5 (49) or HOMA - IR = fasting glucose level (mg/dL) ×fasting insulin level (μU/mL)/405 (37). The effects of HOMA-IR on parameters and outcomes were analyzed, and it was shown that the IR increased the risk of spontaneous abortions in patients with PCOS (MD = 0.32, 95% CI [0.15, 0.49]) (**Figure 10**).

Chromosome Aberrations

Five articles with a total of 952 patients with dichotomous variables reported the relationship between embryo chromosomal abnormalities and spontaneous abortion in patients with PCOS undergoing ART (26, 30, 31, 33, 39). The

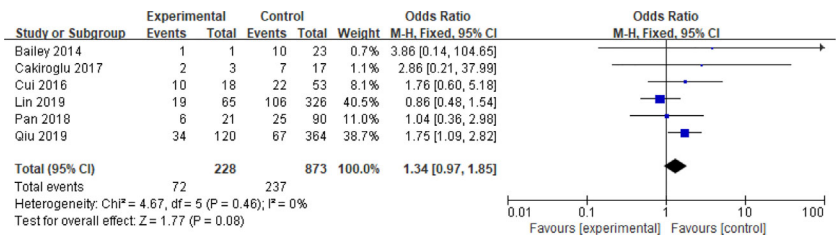

FIGURE 7 | Meta-analysis for overweight and obesity in categorical data.

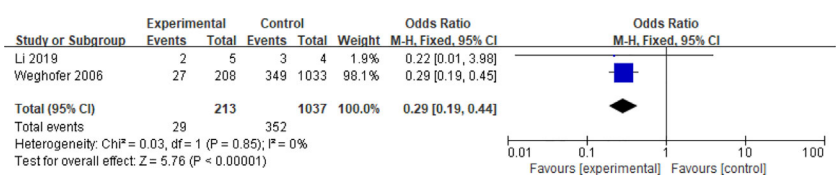

FIGURE 8 | Meta-analysis for age in categorical data.

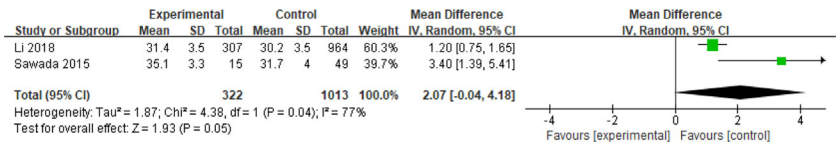

FIGURE 9 | Meta-analysis for age for continuous data.

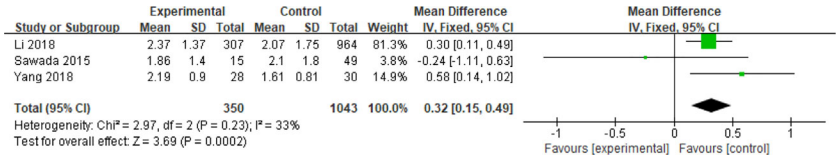

FIGURE 10 | Meta-analysis for insulin resistance.

probability of embryonic chromosome aberrations in abortion was not significantly increased in these PCOS patients (OR = 0.75, 95% CI [0.31, 1.77]) (**Figure 11**), indicating that the embryo chromosomal abnormalities was not a risk factor for spontaneous abortion in patients with PCOS. Further analysis of these articles revealed that the two studies by Landres et al. (26) and Li et al. (30) used G-banded chromosome karyotype analysis to detect chromosomal abnormalities, whereas the two studies by Li et al. (31) and Lu et al. (33) used SNP-array analysis. The remaining one article applied the traditional karyotyping combined with MLPA subtelomere assay, FISH analysis or ArrayCGH analysis to diagnose chromosome aneuploidy (39). Analysis of the above two pairs of studies found that these articles were homogeneous (both I^2 = 0%) (**Figures 12** and **13**). The G-banded chromosome karyotype analysis did not show a significant increase in the probability of chromosome aberrations in embryos of patients with PCOS (OR = 0.54, 95% CI [0.35, 0.85]) (26, 30), whereas the SNP array analysis demonstrated the embryo chromosomal abnormality to be a risk factor for increased abortions in patients with PCOS (OR = 1.86, 95% CI [1.23, 2.82]) (31, 33).

Hyperandrogenism

Four articles including a total of 1,452 patients with continuous data assessed the effect of hyperandrogenism on spontaneous abortions in PCOS patients undergoing ART (27, 30, 37, 42). The 2003 Rotterdam criteria did not clearly specify the standard range of androgen values in hypersexual hormones. A study according to the Rotterdam criteria defined hyperandrogenemia as the finding of elevated androgens, and the specific cutoff values for testosterone, dehydroepiandrosterone sulfate (DHEAS) and Δ4 were 65 ng/dL, 2,800 μg/L and 2.5 ng/mL, respectively (50). Due to the differences in measurement methods in the included articles, the Standard Mean Difference (SMD) was chosen as the effect size indicator for analysis. The SMD could eliminate not only the influence of the absolute value but also the influence of the unit on the result. Analysis of these articles which had moderate heterogeneity (I^2 = 25%) revealed that hyperandrogenism did not increase the risk of miscarriage in patients with PCOS (MD = 0.10, 95% CI [- 0.02, 0.22]) (**Figure 14**).

Basal Luteinizing Hormone

Two articles enrolling a total of 1329 patients with continuous data assessed the effect of basal luteinizing hormone (bLH) on spontaneous abortions in patients with PCOS (30, 42). Analysis of these articles which had low heterogeneity (I^2 = 23%) revealed that bLH did not increase the risk of miscarriage in patients with PCOS (MD = -0.15, 95% CI [- 0.89, 0.60]) (**Figure 15**).

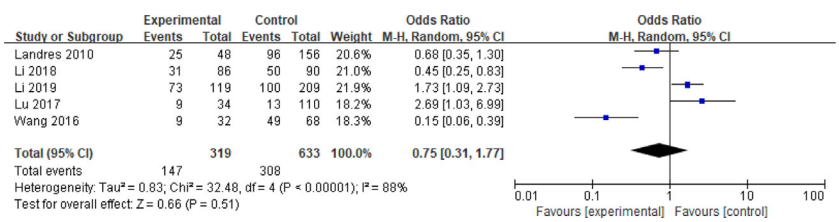

FIGURE 11 | Meta-analysis for Chromosome Aberrations.

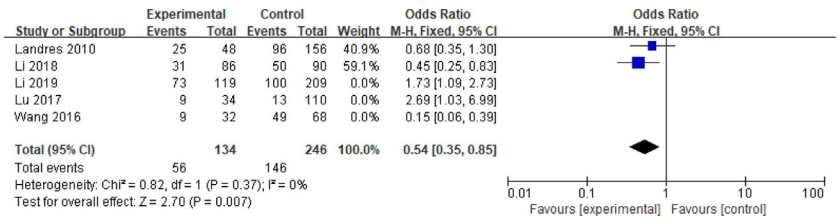

FIGURE 12 | Meta-analysis for G-banded chromosome karyotype analysis.

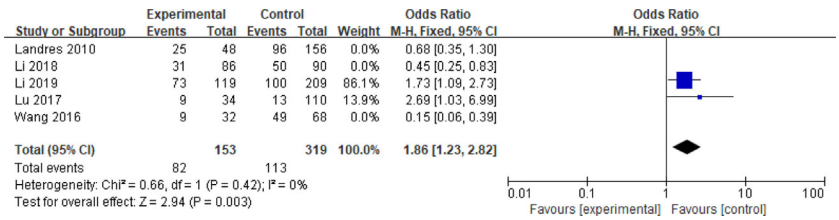

FIGURE 13 | Meta-analysis for SNP-array analysis.

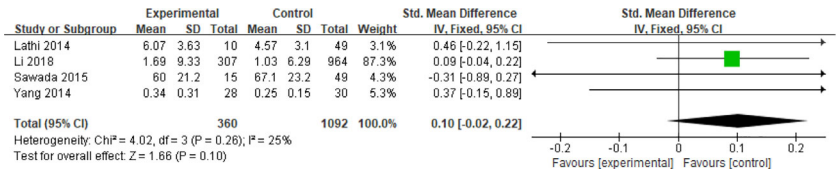

FIGURE 14 | Meta-analysis for hyperandrogenism.

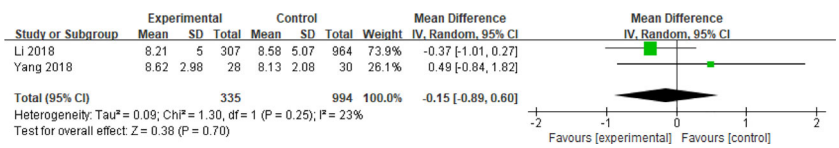

FIGURE 15 | Meta-analysis for basal luteinizing hormone.

Adjustment of Confounding Factors

The above data were from the original data of the included literature, and the risk factors had not been adjusted. However, there were mutual influences between risk factors. In order to eliminate the influence of possible confounding factors, we analyzed the extracted adjusted OR value. Unfortunately, only two articles (30, 42) had adjusted the confounding effects of BMI, age, bLH, basal testosterone (bT) levels, and number of oocytes retrieved on spontaneous abortion in PCOS patients. The results showed that BMI (OR = 1.27, 95% CI [1.17,1.38]) was a risk factor for spontaneous abortion in PCOS patients undergoing ART, whereas age (OR = 1.06, 95% CI [0.95, 1.19]), bLH (OR = 1.01, 95% CI [0.95, 1.08]), and bT (OR = 1.10, 95% CI [0.70, 1.73]) had no effect on spontaneous abortion in PCOS patients. This was consistent with the conclusion obtained with the original data (**Supplementary Figure 3**).

DISCUSSION

In this systematic review and meta-analysis, it was found that high BMI (OR = 1.48, 95% CI [1.32, 1.67], MD = 1.35, 95% CI

[0.58, 2.12]) and insulin resistance (MD = 0.32, 95% CI [0.15, 0.49]) were associated with an increased risk of spontaneous abortion in patients with PCOS undergoing ART. Older age (OR = 0.29,95% CI [0.29, 0.44], MD = 2.01, 95% CI [0.04, 4.18]), embryonic chromosomal aberrations (OR = 0.75,95%CI [0.31,1.77]) and hyperandrogenism (MD = 0.10, 95% CI [- 0.02, 0.22]) were not associated with higher spontaneous abortion rates in PCOS patients with undergoing ART.

This meta-analysis found that PCOS patients with spontaneous abortion history showed higher BMI than those with ongoing pregnancy (OR: 1.71, 95% CI [1.34, 2.19]). The results of subgroup analysis of overweight and obesity under different standards (i.e., under the Chinese BMI standard with the BMI ≥ 28 kg/m2 as obese and BMI 24–28 kg/m2 as overweight; under the WHO standard with the BMI ≥ 30 kg/m2 as obese and BMI 25–30 kg/m2 as overweight) showed that there was no difference in the spontaneous abortion rate between overweight and obese groups. This is also the advantage of our analysis, because although researchers currently believe that high BMI will lead to a higher miscarriage rate in PCOS patients, it is still unclear whether higher BMI values will have worse impact on miscarriage. Our research shows that after reaching the limit of overweight (i.e., BMI ≥ 24 kg/m2 under the Chinese BMI standard or BMI ≥ 25 kg/m2 under the WHO standard), the spontaneous abortion rate of patients with PCOS has increased. However, after reaching the limit of obesity (i.e., BMI ≥ 28 kg/m2 under the Chinese BMI standard or BMI ≥ 30 kg/m2 under the WHO standard), the spontaneous abortion rate of PCOS patients is not statistically different from that of PCOS patients who only reach the standard of overweight (i.e., BMI 24–28 kg/m2 under Chinese BMI standards or BMI 25– 30 kg/m2 under WHO standards). It shows that the effect of high BMI on spontaneous abortion rate may be a process of qualitative change. This also provides clinicians with suggestions that for obese patients, the standard of BMI after weight loss should be as close as possible to the normal range in order to obtain ideal pregnancy results. Marquard et al. (51) found that PCOS women with high BMIs tended to have smaller oocytes than the control, but whether this can cause adverse pregnancy outcomes in PCOS patients remains to be determined. Khomami et al. (52). suggested that the combination of hyperandrogenism and IR and/or hyperinsulinemia may lead to adverse pregnancy outcomes in women with PCOS. High BMI may have a profound effect on the secretion and metabolism of sex hormones, resulting in changes in the bioavailability of estrogen and androgens and thereby affecting the normal development of follicles (53). As the degree of obesity increases, peripheral aromatization of androgens to estrogens improves, while liver synthesis of sex hormone-binding globulin (SHBG) decreases, which will result in increased levels of free estradiol and testosterone. This condition can be exacerbated by hyperinsulinemia, leading to further reduction and stimulation of ovarian androgen production by SHBG, excessive secretion of luteinizing hormone, and increased ratios of androgen to estrogen. These changes may disturb systemic endocrine environment, resulting in impaired follicle production and follicular atresia. Notably, the definitive mechanism of interactions between follicle generation and endometrial receptivity remains to be fully elucidated. Some authors (29) found that excessive administration of gonadotrophin during controlled ovarian stimulation due to a high body mass may also adversely affect embryo quality and endometrial receptivity, thereby leading to adverse pregnancy outcomes in overweight patients with PCOS.

Studies on gene expression in PCOS patients have found that the expression of multiple genes related to endometrial receptivity is decreased (54, 55). After investigating the endometrial transcriptome during the implantation window, Bellver et al. found that obese women have altered gene expression which is worsened in patients with PCOS (56). Furthermore, glucose metabolism plays an important role in the decidualization of endometrium during embryo implantation (57, 58). The uptake of glucose by cells in endometrium is mediated by glucose transporters (GLUT) - SLC2A family (59). It has been proven that SLC2A4 exists in human endometrium, and immunohistochemical studies suggest that it exists only in endometrial epithelial cells (60). As SLC2A4 is an insulin-dependent glutand obesity and PCOS are in a state of insulin resistance, Zhao et al. hypothesized that the decrease of SLC2A4 in endometrium would lead to endometrial insulin resistance and may damage endometrial metabolism (61). After further studying whether metformin could improve endometrial insulin resistance, they found that the expression of SLC2A4 mRNA and protein in obese PCOS patients was significantly lower than that in obese non-PCOS patients (61). After treatment with metformin for 3 months, the expression of SLC2A4 mRNA and protein was significantly improved (61). Other studies also found that the expression of SLC2A4 in obese PCOS patients was significantly lower than that in non-obese PCOS patients (60). As mentioned above, insulin resistance is also a metabolic feature of PCOS patients. It has been recognized that insulin can inhibit the production of IGFBP-1, a biomarker of decidualization (62). The study by Chang et al. also showed that embryonic development was not affected in insulin-resistant patients, but the endometrial receptivity was impaired (63). These mechanisms provide a basis for the treatment of metformin in PCOS patients. Several clinical studies have demonstrated that metformin can improve the status of hypergonadism and insulin resistance in PCOS patients with endometrial cancer and protect their fertility (64, 65). Compared with placebo, metformin significantly increased the ovulation rate (66). It is worth noting that the use of metformin before pregnancy may reduce the abortion rate (67, 68), but the evidence is insufficient. Other studies seemed to believe that the use of metformin has no significant impact on the abortion rate (69–72). Regarding the role of metformin in ovulation in infertile PCOS patients in the guidelines of the Practice Committee of the American Society for Reproductive Medicine, it has been pointed out that there is fair evidence that metformin used while attempting pregnancy and stopped at the initiation of pregnancy does not affect the rate of miscarriage (Grade B) and that there is insufficient evidence to recommend

metformin during pregnancy to reduce the chance of miscarriage (Grade C) (73).

The effect of age was analyzed on the risk of spontaneous abortion in patients with PCOS undergoing ART. Although fewer articles were related to this issue with less representative results, it was interesting to find that patients with PCOS may have better pregnancy outcomes than those without PCOS at the same age. Patients without PCOS usually have fertility declined sharply when older than 35, but the IVF (*in vitro* fertilization) -related fertility declines moderately in patients with PCOS (28). This may be related to the number of oocytes and embryos available in patients with PCOS.

Subgroup analysis on chromosomal abnormalities revealed contradictory outcomes. Li et al. (31) considered that traditional G-banded chromosome karyotype analysis and/or fluorescence *in situ* hybridization had lower sensitivity than genome hybridization array test. The G-banded chromosome karyotype analysis is a morphological test based on naked eye recognition, with limited resolution and 5 to 10 Mb changes required (74), whereas the SNP array can detect a large area of changes from 1.89 to 16.00 Mb deletions or duplications and make up for the low resolution of the karyotype analysis (75). Chang et al. (76) compared the roles of single nucleotide polymorphism array (SNP array) and karyotype analysis in high-risk pregnant women prenatal diagnosis and found that the performance of the SNP array (11.3%) is significantly (p=0.039) better than that of the karyotype analysis (6.4%). However, more evidence is needed to prove whether minor chromosomal abnormalities could lead to different results.

In addition to analyses of the above factors, we tried to assess dyslipidemia because it might affect the spontaneous abortion rate of PCOS patients (29). However, the related articles were too limited for a meta-analysis. Dyslipidemia was defined as any one of the following criteria: serum cholesterol (TC)> 6.20 mmol/L, triacylglycerol (TG)> 2.25 mmol/L, low density lipoprotein (LDL)> 4.10 mmol/L, and high density lipoprotein (HDL) < I.03 mmol/L (29). Abnormal lipid metabolism may cause adverse pregnancy outcomes in patients with PCOS. Glueck et al. (77) found that the 4G polymorphism of the PAI-1 gene was more common in PCOS women than in the normal counterparts and was associated with high BMI and hyperinsulinemia complicated with hypertriglyceridemia. Sun et al. (78) proposed that homozygosity of ACE D or PAI-1 4G genes and complex carrier status were related to early pregnancy abortion. It was speculated that the 4G polymorphism of the PAI-1 gene may cause dyslipidemia to promote pathological changes and subsequently increase the abortion rate in patients with PCOS. However, Li et al. (29) found that patients with dyslipidemia, especially increased triacylglycerol levels, had higher BMI and increased gonadotropin during assisted reproduction dosage, resulting in an increase in the early abortion rate. Dyslipidemia can promote the development of IR, hyperandrogenism, oxidative stress, and anovulation in PCOS, increasing the risk of cardiovascular disease (79).

Dyslipidemia promotes pathological changes in patients with PCOS (80–87). It has been found that high visceral fat levels in patients with PCOS increased the fatty acid levels in circulating blood, which leads to increased lipolysis of circulating fatty acids in obese patients with PCOS, and this in turn impairs the role of insulin in adipose tissue and causes IR (80–83). In addition, dyslipidemia plays an important role in PCOS-associated inflammation (84), and high levels of free fatty acids may activate mononuclear cells to regulate the expression of chemokines and the release of cellular inflammatory factors in adipocytes. Apo-lipoprotein AI (Apo-AI) levels are reduced in the serum of patients with PCOS, and the Apo-AI content in granulosa cells proportionally changes the expression of steroidogenic enzymes, including CYP11A, 17-hydroxysteroid dehydrogenation Catalase (HSD), 3-HSD, and CYP19 (85). Decreased expression of CYP19 due to reduced Apo-AI may hinder subsequent conversion of testosterone to estradiol, leading to occurrence of hyperandrogenemia. Moreover, anovulation in PCOS patients was associated with abnormal blood lipid metabolism (86). High levels of triglycerides, free fatty acids, and oxidized LDLs (oxLDLs) in the serum can cause mitochondrial dysfunction while promoting release of reactive oxygen species, which ultimately contributes to ovarian damage and follicular atresia (87). Lectin-like oxLDL receptor-1 (LOX-1), toll-like receptor 4 (TLR4), and cluster of differentiation 36 (CD36) are oxLDL receptors. Activations of these oxLDL-dependent receptors can cause human granulosa cell (GC) apoptosis and ovulatory disorders. However, definitive relationship between dyslipidemia and adverse pregnancy outcomes in patients with PCOS remains to be elucidated because of insufficient research data.

It needs to be explained that there are two situations about pregnancy complicated with diabetes. In one condition, the patient's glucose metabolism is normal before pregnancy, and diabetes only appears during pregnancy, which is called gestational diabetes mellitus (GDM). In the other condition, pregnancy is combined with pre-existing diabetes, which is also known as diabetes mellitus complicated with pregnancy. Hyperglycemia can cause abnormal embryonic development and even death, increasing the incidence of miscarriage (88). Patients with PCOS may have an increased risk of gestational diabetes (89). If the relationship between abnormal glucose metabolism status and miscarriage in patients with PCOS can be found, it will be of great help to clinicians for administration of preventive treatment.

Hyperglycemia first detected during pregnancy is classified as GDM. Although it can occur anytime during pregnancy, GDM generally affects pregnant women during the second and third trimesters. The oral glucose tolerance test (OGTT) for pregnant women is usually completed 24 weeks after pregnancy. However, many abortions in PCOS patients occur in the first trimester before 12 weeks, and few studies on abortion in PCOS patients would involve markers of glucose metabolism, such as fasting blood glucose (FBG) and oral glucose tolerance test 2h blood glucose (OGTT 2hBG). We did not find enough data to analyze in this study. Therefore, insulin, as a hormone that regulates human blood sugar levels, is often used to assess the level of glucose metabolism in patients, and a lot of studies had

investigated insulin. That is why we chose homeostasis model assessment-insulin resistance (HOMA-IR) as the metabolic markers of patients with PCOS.

In addition, our exclusion criteria involved drug administration (including metformin) or intervention for purposes other than assisted reproductive technology, and many patients with diabetes mellitus complicated with pregnancy have used medications. This is why we did not collect enough data to analyze this point of view, which is also one of the limitations of this article.

This is the first systematic review and meta-analysis of the risk factors that increase the spontaneous abortion rate in patients with PCOS diagnosed by using the Rotterdam criteria with ART. Other criteria like the National Institute of Health (NIH) criteria and Abdrigen Excess PCOS Society criteria are also used in the diagnosis of PCOS (2). The NIH definition uses the following two criteria to make the diagnosis: Chronic anovulation, Clinical and/or biochemical signs of hyperandrogenism (90). However, the NIH definition has the following limitations: difficulty to objectively measure the ovulatory dysfunction and to quantify both the clinical hyperandrogenism which may vary with different ethnic groups and the hypersensitivity of PCO morphology to ovarian stimulation. The Abdrigen Excess PCOS Society criteria is more focused on hypersexual hyperandrogenemia. Compared to patients with PCOS diagnosed according to the NIH criteria which defines PCOS as clinical and/or biochemical hyperandrogenism associated with ovulatory dysfunction (91), the Rotterdam criteria has a lower prevalence of impaired glucose tolerance or hyperinsulinemia (92). Therefore, one of the inclusion criteria for this meta-analysis was use of the Rotterdam criteria for the diagnosis of PCOS, which is generally accepted worldwide. Bias resulted from inconsistent diagnostic criteria was eliminated. Moreover, studies involving medications for overweight, hyperinsulinemia, and hyperandrogenemia were also eliminated, and the results obtained in this meta-analysis were thus objective. Because this systematic review and meta-analysis excluded patients with PCOS diagnosed according to criteria other than the Rotterdam criteria, the sample size was thus possibly reduced. In addition, fewer studies may have been retrieved regarding certain risk factors and may thus affect the effectiveness of the outcome. The current outcome of analysis can only represent the currently available studies, and further investigation is needed to confirm the outcome. Another limitation of this study was no hierarchical analysis on the influence of assisted reproductive technology. Studies (93, 94) have shown that the spontaneous abortion rate of assisted reproductive technology is significantly higher than that of normal pregnancy. However, the records about the impact of assisted reproductive technology on spontaneous abortion rate in patients with PCOS were too limited to be analyzed. All included studies in this report were related to assisted reproductive technology. In addition, since the included data were the original data mentioned in the literature rather than the adjusted OR value, there may be interference between risk factors, even though we had done the analysis of adjusted OR value about BMI, age, bLH, and bT in patients with PCOS. Small ample size is another limitation of this study.

Given the results of this systematic review and meta-analysis, it is suggested that patients with PCOS may reduce the spontaneous abortion rate by losing weight before preparing for pregnancy, and the ideal target of weight control is within the normal BMI range. Obese patients may not benefit much if they have lost some weight but remain in the overweight status. Furthermore, mitigating insulin resistance may also reduce the spontaneous abortion rate in PCOS patients undergoing ART. Preimplantation genetic testing may reveal some chromosomal abnormalities that cause miscarriage, but the optimal method remains to be elucidated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

G-MH contributed to the study design and critical revision of the manuscript. Y-FS, Z-YC, and Y-MX selected studies for inclusion and abstracted data. Y-FS did the statistical analyses and wrote the first draft. JZ, Y-MX, Y-ZW, and B-LG contributed to the study and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.592495/full#supplementary-material

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The Effect of Oligopin Supplementation on Hormonal and Metabolic Profiles in the Polycystic Ovary Syndrome: A Randomized Controlled Trial

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Background: A double blind clinical trial was performed to evaluate whether the polycystic ovary syndrome (PCOS)-specific serum markers and metabolic parameters would change in the women with PCOS during the three-month administration of oligopin.

Methods: In this double-blind multicenter trial, we randomly assigned 80 PCOS women, based on a 1:1 ratio, to receive oligopin (n= 40) or maltodextrin as placebo (n = 40) for up to 3 months. As PCOS-specific outcomes, we investigated the changes in testosterone, sex hormone binding globulin (SHBG), free androgen index (FAI), dehydroepiandrosterone (DHEA), *follicle-stimulating* hormone (*FSH*) and *luteinizing hormone* (*LH*). Secondary end points were metabolic (fasting glycaemia, hemoglobin A1c (HbA1c), lipids, insulin resistance (HOMA-IR)), anthropometrics parameters and blood pressure from the baseline to the end of treatment. We investigated serum transaminase, alkaline phosphatase (ALP), creatinine (Cr) and blood urea nitrogen (BUN) levels as hepatic and kidney outcomes, respectively.

Results: The first participant was enrolled on April 18, 2018, and the last study visit took place on May 14, 2019. PCOS-specific serum parameters did not change during the three-month administration of oligopin ($p > 0.05$), except for a small increase in the FSH levels ($p=0.03$). Oligopin neither changed the metabolic profile nor the anthropometric parameters or blood pressure. ALP levels was significantly increased in placebo group, as compared with oligopin ($p=0.01$).

Conclusion: Oligopin supplementation does not seem to be exerting a beneficial effect on both hormonal and metabolic parameters in the women with PCOS.

Clinical Trial Registration: www.irct.ir, identifier IRCT20140406017139N3.

Keywords: oligopin, polycystic ovary syndrome, PCOS, endocrine, metabolic profile

BACKGROUND

Polycystic ovary syndrome (PCOS) is the most common endocrine-metabolic disorder causing the infertility, affecting 5%–20% of women in their reproductive lifespan (1). PCOS can often be characterized by elevated circulating androgen levels, hirsutism, acne, oligomenorrhea or amenorrhea, and/or polycystic ovarian morphology (PCOM), as determined by ultrasound. Endocrine and metabolic derangements and cardiovascular disorders may also coexist (2). Hyperinsulinemia, a consequence of insulin resistance, can cause hyperandrogenism, leading to the inappropriate gonadotropin secretion (reduction of *follicle-stimulating* hormone (*FSH*) and the increase of *luteinizing hormone* (*LH*) levels) in *PCOS* (3).

Oxidative stress has been implicated in mediating the insulin resistance and excessive ovarian androgen levels seen in these patients (4, 5). Based on the large evidence showing the relationship between oxidative stress and impaired insulin action (6) and hyperandrogenism, an independent correlation between antioxidant decline and PCOS has been reported (7). In the recent years, several studies have demonstrated the efficacy of antioxidants such as bioflovonids in reducing PCOS-associated hyperinsulimemia and correcting common endocrine and metabolic dysfunctions found in the women with PCOS (8). A lower circulation level of LH/FSH has been reported in isoflavone consumption, as compared to the controls in the meta analysis (9). Moreover, flavonoids, as a potent antioxidant, have been demonstrated to reduce the clinical and biochemical markers of hyperandrogenism in the PCOS patients (10), as well as in the animal models affected by PCOS (11, 12). Such findings have encouraged the development of other flavonoids-rich medicinal plants for the treatment of PCOS

Among the available compounds, oligopin (a pine bark extract of the French maritime pine), a plant extract containing procyanidins (catechin and epicatechin), protects tissues from oxidative stress and the inflammation-related damage due to its strong antioxidant and anti-inflammatory activity (13, 14). Furthermore, animal studies have demonstrated that the pine bark extract and derivatives may affect the ROS levels and lipid accumulation by the inhibition of ROS production through the mechanism associated with pro -oxidant and antioxidant enzyme responses and the suppression of the adipogenic gene expression in adipocytes (15). Administration of the pine bark extract was also shown to reduce fasting glucose levels and HbA1c levels, as compared to the control, through several mechanisms (16, 17). Pycnogenol may stimulate the glucose uptake *via* the *PI3K/AKT* signaling *pathway* (18). In addition, it is suggested that the reduced blood glucose, by the inhibition of α-glucosidase activity, may reduce glucose absorption in the intestinal cells (19). A very recent meta -analysis aimed to investigate the effect of pycnogenol on the cardiometabolic factors in healthy and affected individuals (16); notably, there has been no study assessing the effects of this extract on the PCOS women. Therefore, the aim of the present study was to assess the efficacy and safety of the 3-month oligopin administration on the hormonal and metabolic features of the women affected by PCOS.

Abbreviations: ALT, Alanine Transaminase; ALP, Alkaline Phosphatase; AST, Aspartate Transaminase; BUN, Blood Urea Nitrogen; BIA, Body Impedance Analyzer; BMI, Body Mass Index; Cr, Creatinine; DHEA, Dehydroepiandrosterone; DBS, Diastolic Blood Pressure; FEM, Fat Free Mass; FM, Fat Mass; *FSH, Follicle-Stimulating* Hormone; FAI, Free Androgen Index; GGT, Gamma–Glutamyl Transferase; HbA1c, Hemoglobin A1c; HDL, High-Density Lipoprotein Cholesterol; hs-CRP, High Sensitive- C Reactive Protein; HOMA, Homeostasis Model Assessment; LH, Increase *Luteinizing Hormone;* LDL, Low-Density Lipoprotein Cholesterol; NO, Nitric Oxide; PCOM, Polycystic Ovarian Morphology; *PCOS, Polycystic Ovary Syndrome*; SHBG, Sex Hormone Binding Globulin; SBP, Systolic Blood Pressure; TSH, Thyroid Stimulating Hormone; TC, Total Cholesterol; TG, Triglycerides.

METHODS

Study Design

This trial (IRCT.IR identifier) was a 3-month randomized placebo-controlled double blind trial performed at three university hospitals in Tehran, Iran. Patients were included if they were in the age range of 18-40 years and provided their written informed consent. Further, PCOS was documented according to the Rotterdam criteria if two out of the three following conditions were met: a) oligomenorrhea (menstrual cycle>35 days) or amenorrhea, b) clinical and/or biochemical signs of hyperandrogenism, and c) polycystic ovaries (≥12 follicles of 2–9 mm diameter on at least one ovary and/ or *ovarian volume ≥10* ml) on the abdominal ultrasound (20). Key exclusion criteria included pregnancy, history of diseases that could cause menstrual disturbances (e.g., elevated prolactin and thyroid disease), or the use of drugs known to influence metabolism (like metformin or *glucocorticoid)* and ovarian functions (such as oral contraceptives) for at least 30 days or more before screening; also, evidence of diabetes, significant liver and renal impairment, Cushing's syndrome and acromegaly were the other exclusion criteria.

Ethics approval was obtained from the Ethics Committee of Endocrinology Metabolism Research Institute, Tehran University of Medical Sciences (REC.1396.00163). This study adhered to the CONSORT guidelines.

Randomization

Patients were randomized, based on a 1:1 ratio, according to the method of block randomization; sample size was estimated 40 patients per groups using two mean comparison sample size formula, with power 80% and test of significance at 5% level (a standard deviation of 24 mIU /L for the FSH of either group was assumed) (21). Placebo was provided in identical intakes; the recommended intake was oligopin 50 mg/day or placebo (maltodextrin), for the whole duration of the study (3 months).

Procedures

The trial was undertaken in the early follicular phase (cycle days 3–7) in regularly menstruating women or random days for women with oligo/amenorrhea. The height was measured with a wall-mounted meter with an approximation of 0.5 cm. Body weight and composition were measured without footwear and with light clothing, using the body impedance analyzer (BIA) (Tanita, Japan). The circumferences of waist were measured as the value between the iliac crest and the lateral costal margin. Body mass index (BMI) = weight (kg)/ height (m^2) was calculated as well. The grade of hirsutism was established using the Ferriman-Gallwey score (22). Acne was evaluated in four grades, as described previously (23).

A trans-abdominal ultrasound was performed by one of two well-trained radiologists using a 3 MHz to 5.5 MHz curvilinear probe (acuson s2000, Siemens Medical Solutions, USA). Ovarian volume was calculated for each ovary using the prolate ellipsoid formula: π/6 *maximum diameter in transverse*anteroposterior * longitudinal axes (24). The total number of antral follicles (2–10 mm in diameter) was counted (25).

The blood samples were collected from 8:00 to 9:00 AM after following overnight fasting, frozen and stored at 20°C until analysis. The serum levels of sex hormone binding globulin (SHBG) were assessed using ELISA kits (Demeditec, Germany). The free androgen index (FAI) was calculated (FAI) = testosterone (ng/ml) × 3.47/SHBG (nmol/L) (26). All remnant hormonal (dehydroepiandrosterone (DHEA), testosterone, FSH, LH, prolactin, C-peptide, insulin and thyroid stimulating hormone (TSH)) assays were performed with the ELISA kits (Monobind Inc. Lake Forest, California, USA). Fasting blood glucose levels were measured using the glucose oxidase method on an autoanalyzer (Cobas c 311, Roche Dignostics, Risch-Rotkreuz, Switzerland). Hemoglobin A1c (HbA1c) level was determined on a daily basis using a high performance liquid chromatography analyzer (Tosoh, Tokyo, Japan). Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald formula. The insulin resistance was calculated based on the Homeostasis Model Assessment (HOMA): HOMA-IR= (fasting glucose (mg/dl)) × (fasting insulin (μIu/ml))/405 (27). Concentrations of the total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), liver function tests (alanine transaminase (ALT), aspartate transaminase (AST) and Alkaline phosphatase (ALP)), kidney function tests (blood urea nitrogen (BUN), creatinine (Cr)) and high sensitive- C reactive protein (hs-CRP) were measured by applying the ELISA kit (Roche, Germany).

Statistical Analysis

Statistical analysis was carried out using the SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA) on an intention-to-treat basis. Continuous variables were evaluated for normality by applying the Shapiro-Wilk test. Non-normally distributed variables were transformed using an appropriate transformation method. Continuous and categorical variables were reported as the mean [standard deviation (SD)] and number (%), respectively. Comparison of the continuous variables in the oligopin and placebo groups at the baseline was done using independent-samples t-tests. Two-way repeated-measures of ANOVA were also used to assess the effect of intervention on the continuous outcomes. Chi-squared test was also employed to assess the categorical variables at the baseline in the groups. All statistical tests were two-tailed; $P< 0.05$ was considered as the threshold significant level.

RESULTS

The first participant was enrolled on April 18, 2018, while the last patient visit was on May 14, 2019. Of 239 patients assessed for eligibility, 80 were enrolled and randomly assigned to treatment with once-daily 50 mg oligopin (n=40) or placebo (n=40) (**Figure 1**). The treatment schedule was completed by 31 (77.5%) participants in the oligopin group and 30 (75%) in the placebo group. Baseline characteristics are shown in **Table 1**. The mean (SD) age was 27.99 ± (6.28) years, the mean disease duration was 6.17 years, and the majority were non-smokers (98.8%); they had irregular menses (80%). Hirsutisim degree was different in the two groups at the baseline (p=0.008) (**Table 1**). As the safety, no serious oligopin-related adverse events occurred during the study.

Regarding the primary endpoints, changes in the androgen levels such as testosterone, SHBG, FAI and DHEA levels, from the baseline to 3 months, did not differ significantly in the two study groups (p>0.05), except for the increase of the FSH levels in the oligopin group (the mean difference of 0.62 mIU/ml [95%CI, 0.04 to 1.19]), as compared with the placebo (the mean difference of −0.41 mIU/ml [95%CI, −1.13 to −0.33]), p=0.03 (**Table 2**). Similarly, none of the indicators of metabolic control (fasting blood glucose, HbA1c, insulin levels, lipid profile), hs-CRP levels and anthropometric parameters [BMI, fat free mass (FFM), fat mass (FM), waist circumference] showed any significant changes in the oligopin and placebo groups after 3 months (**Tables 2**, **3**). Changes in ALP at the end of the trial differed in the oligopin (the mean difference of 4.66 U/L [95%CI, −1.14 to 10.45]) and placebo groups (the mean difference of 25.15 U/L [95%CI, 11.08 to 39.21]), (p=.0.01). However, changes in other transaminases levels (ALT and AST) and kidney function factors (Cr, BUN) and TSH levels were not significantly different in treatment and placebo groups (P>0.05) (**Table 2**). Parameters related to blood pressure (systolic and diastolic) were also similar in the PCOS patients after oligopin treatment (**Table 3**).

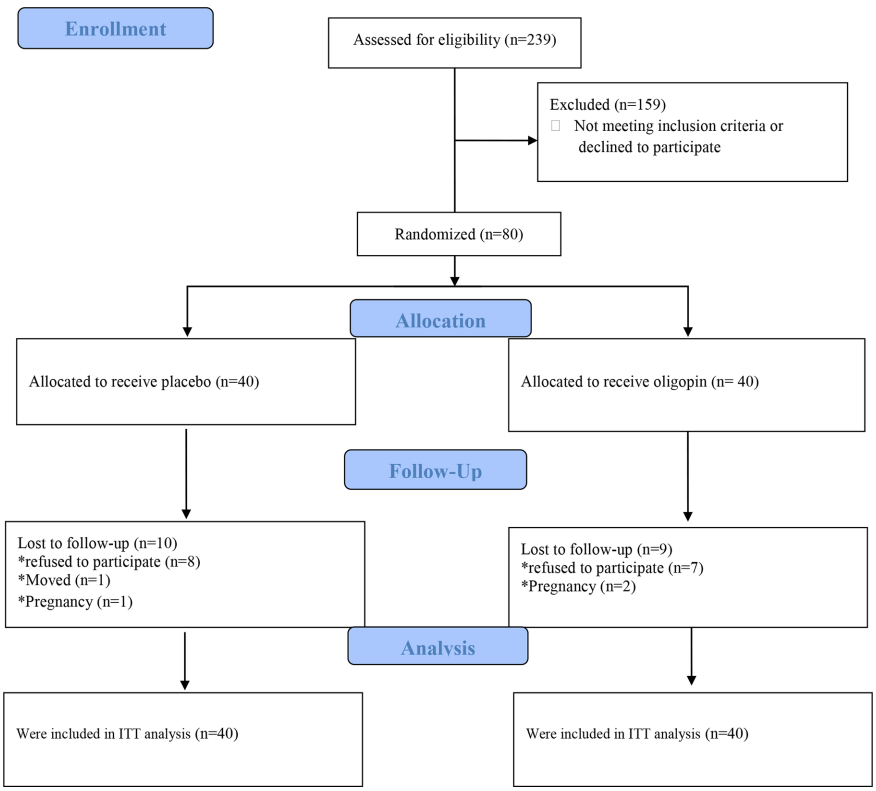

FIGURE 1 | Trial profile.

TABLE 1 | Baseline characteristics of patients according to study group.

	Placebo (n= 40)	Oligopin (n=40)	P value
Age (year)	27.6±6	28.38±6.57	0.58[a]
Smoking (yes) n(%)	0	1(2.5)	0.31[b]
Height(meters)	160.05±5.91	159.72±5.67	0.8[a]
Disease duration (year)	4.83±5.69	7.51±7.28	0.07[a]
Menes n(%)			
Regular	8(20)	8(20)	0.99[b]
irregular	32(80)	32(80)	
Hair losses (yes) n(%)	22(64.7)	32(80)	0.14[b]
Hirsutism	17.28±5.54	21±6.58	0.008[a]
Acne score	1.5±1.48	1.55±1.39	0.87[a]

[a]*Independent samples t-test.*
[b]*chi-square.*
Each value represents mean± SD except for smoking n(%), menes n(%), hair losses n(%).

DISCUSSION

There is not much scientific literature on the commercially available pine bark extract (oligopin or pycnogenol). To the best of our knowledge, this is the first randomized, double-blind, placebo-controlled trial of oligopin supplementation in the women with PCOS. We hypothesize that including oligopin in our subjects would induce benefits; however, with the exception of change in the FSH level, our intervention failed to change the levels of androgens and metabolic profile. These results were not accompanied by any unexpected safety finding.

This trial showed that an antioxidant intervention based on the oligopin supplementation had no effect on the serum androgen levels, except for a small increase in the FSH level. The changes in the FSH levels observed in this trial over the 3-month period were difficult to explain, even though the mean FSH was increased following oligopin treatment; there were no significant changes in insulin or androgen levels. On the other hand, although the mean FSH levels were lower for the women with PCOS, as compared to those with normal ovaries (28), this small rise in the FSH concentrations was not clinically important in the patients with PCOS. Whether the effect FSH on raising oligopin was partly due to catechin is unclear. It has been reported that there is a significant dose-response relationship between catechein supplementation and FSH levels in the PCOS rats (29).

No differences could be demonstrated in any of metabolic profiles and anthropometrics parameters, blood pressure and hs-CRP levels, except for the increase in the ALP levels in the placebo group, as compared to the oligopin one. Our findings showed that oligopin failed to significantly influence cardiovascular disease risk factors (insulin, fasting glucose, lipid profiles and hs-CRP); this is an observation previously recorded in the case of overweight and obese adults (30). The meta-analysis by Malekamadi et al. and collaborators also

TABLE 2 | Hormonal and metabolic parameters at baseline and after 3 months of treatment with oligopin and placebo in the study population.

	Placebo (n=40) means (95%CI)	Oligopin (n=40) means (95%CI)	P value
Testosterone (ng/ml)			
Baseline	0.41(0.34 to 0.48)	0.44(0.38 to 0.51)	
12 weeks	0.41(0.34 to 0.50)	0.46(0.38 to 0.55)	0.84[a]
DHEA (ng/dl)			
Baseline	160.02(138.53 to 183.06)	155.07(133.81 to 177.87)	
12 weeks	131.14(106.91 to 157.65)	151.16(125.19 to 179.56)	0.13[a]
SHBG (nmol/l)			
Baseline	44.77(35.16 to 57.02)	33.11(26 to 42.17)	
12 weeks	40.83(33.8 to 49.2)	31.77(26.3 to38.28)	0.74[a]
FAI			
Baseline	2.98(2.20 to 4.03)	4.33(3.20 to 5.87)	
12 weeks	3.19(2.47 to 4.11)	4.84(3.75 to 6.24)	0.84[a]
LH (mIU/ml)			
Baseline	10.44(7.57 to 14.42)	8.95(6.48 to 12.33)	
12 weeks	9.88(7.83 to 12.47)	10.66(8.45 to 13.45)	0.34[a]
FSH (mIU/ml)			
Baseline	5.76 (5.13 to 6.4)	5.16(4.52 to 5.8)	
12 weeks	5.35(4.91 to 5.79)	5.79(5.35 to 6.23)	0.03[a]
LH/FSH			
Baseline	1.96(1.51 to 2.54)	1.88(1.45 to 2.43)	
12 weeks	1.9(1.52 to 2.38)	1.91(1.52 to 2.38)	0.8[a]
Prolactin (ng/ml)			
Baseline	14.69(12.79 to 16.86)	13.49(11.75 to 15.50)	
12 weeks	15.27(13.40 to17.42)	14.38(12.62 to 16.40)	0.73[a]
TSH (mIU/ml)			
Baseline	2.28(1.95 to 2.63)	1.98(1.67 to 2.31)	
12 weeks	2.69(2.34 to 3.1)	2(1.69 to 2.34)	0.15[a]
Insulin (µIU/ml)			
Baseline	12.98(11.41 to 14.55)	13(11.43 to 14.57)	
12 weeks	13.41(11.95 to 14.86)	12.31 (10.85 to 13.76)	0.41
C-peptide (ng/ml)			
Baseline	1.17(1 to 1.35)	1.32(1.15 to 1.5)	
12 weeks	1.25(1.12 to 1.38)	1.32(1.19 to 1.46)	0.44[a]
Fasting blood sugar (mg/dl)			
Baseline	88.3(85.29 to 91.31)	89.32(86.32 to 92.33)	
12 weeks	88.62(86.27 to 90.97)	90.486(88.14 to 92.83)	0.69[a]
HOMA-IR			
Baseline	2.58(2.22 to 3)	2.63(2.26 to 3.06)	
12 weeks	2.73(2.42 to 3.1)	2.58 (2.28 to 2.92)	0.55[a]
HbA1C			
Baseline	5.24(5.14 to 5.33)	5.29(5.19 to 5.39)	
12 weeks	4.82(4.6 to 5.05)	4.93(4.7 to 5.16)	0.76[a]
Triglyceride (mg/dl)			
Baseline	98.17(86.09 to 112.20)	109.14(95.5 to 124.45)	
12 weeks	98.17(85.9 0to 111.94)	105.20(92.04 to 119.94)	0.56[a]
Cholesterol (mg/dl)			
Baseline	168.72(158.24 to 179.20)	177.45(166.97 to 187.93)	
12 weeks	165.55(155.73 to 175.37)	174(164.18 to 183.82)	0.94[a]
HDL-C (mg/dl)			
Baseline	41.57(38.6 to 44.54)	46.27(43.3 to 49.24)	
12 weeks	42.03(39.35 to 44.7)	46.09(43.41 to 48.75)	0.65[a]
LDL-C (mg/dl)			
Baseline	96.38(89.33 to 103.99)	98.85(91.83 to 106.65)	
12 weeks	95.49(88.92 to 102.32)	98.85(92.26 to 105.92)	0.76[a]
AST (U/L)			
Baseline	19.01(17.74 to 20.41)	18.7(17.39 to 20.23)	
12 weeks	16.18(15.06 to 17.33)	16.14(14.92 to 17.45)	0.7[a]
ALT (U/L)			
Baseline	10.52(9.43 to 10.53)	9.61(8.69 to 10.75)	
12 weeks	10.64(9.26 to 12.5)	8.47(7.57to 9.61)	0.21[a]
ALP (U/L)			
Baseline	122.37(104.77 to 139.97)	149.52(131.93 to 167.12)	
12 weeks	147.52(129.32 to 165.74)	154.19(135.98 to 172.39)	0.01[a]
hs-CRP (mg/L)			
Baseline	1.03(0.72 to 1.47)	1.61(1.12 to 2.3)	
12 weeks	1.32(0.92 to 1.89)	1.77(1.24 to 2.54)	0.38[a]
Urea (mg/dl)			
Baseline	22.37(20.48 to 24.27)	22.97(21.08 to 24.86)	
12 weeks	23.35(21.25 to 25.43)	23.57(21.48 to 25.66)	0.75[a]
Creatinine (mg/dl)			
Baseline	0.78(0.75 to 0.82)	0.86 (0.82 to 0.89)	
12 weeks	0.8(0.77 to 0.84)	0.87(0.83 to 0.91)	0.63[a]

[a]*Time to treatment interaction according to two-way repeated measure ANOVA. Data are not conforming to a normal distribution were log or square root or inverse transformed. DHEA, dehydroepiandrosterone; SHBG, sex hormone-binding globulin; AFI, free androgen index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid stimulating hormone; HOMA-IR, homeostasis model assessment insulin resistance; HbA1c, hemoglobinA1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; hs-CRP, high-sensitivity C-reactive protein.*

TABLE 3 | Anthropometrics, blood pressure and physical activity at baseline and after 3 months of treatment with oligopin and placebo in the study population.

	Placebo (n=40) means (95%CI)	Oligopin (n=40) means (95%CI)	P value
Body mass index (kg/m^2)			
Baseline	26.3(24.55 to 27.54)	27.54(25.7 to 29.51)	
12 weeks	26.3(24.54 to 28.18)	28.18(26.66 to 30.06)	0.5[a]
Fat mass(kg)			
Baseline	22.85(20.09 to 26)	27.41(24.10 to 31.19)	
12 weeks	23.5(20.84 to 26.48)	28.38(25.18 to 31.99)	0.87[a]
Fat free mass (kg)			
Baseline	43.35(41.97 to 44.67)	42.36(41.02 to 43.65)	
12 weeks	43.35(42.17 to 44.46)	43.05(41.88 to 44.15)	0.14[a]
Waist circumference (cm)			
Baseline	91.62(87.7 to 95.94)	93.32(89.12 to 97.5)	
12 weeks	92.47(88.71 to 96.38)	93.32(89.53 to 97.27)	0.59[a]
Systolic blood pressure (mmHg)			
Baseline	111.15(106.34 to 115.96)	104.3(99.54 to 109.05)	
12 weeks	107.36(102.56 to 112.16)	101.83(97.09 to 106.57)	0.67[a]
Diastolic blood pressure (mmHg)			
Baseline	76.28(72.54 to 80.02)	73.25(69 to 76.95)	
12 weeks	73.99(70.44 to 77.54)	69.46(65.95 to 72.96)	0.49[a]
Physical activity (METs/h)			
Baseline	29.62(28.38 to 30.87)	29.03(27.8 to 30.26)	
12 weeks	30.59(29.38 to 31.8)	29.34(28.15 to 30.54)	0.48[a]

[a]*Time to treatment interaction according to two-way repeated measure ANOVA. Data are not conforming to a normal distribution were log and square root transformed.*

showed several biological effects of this extract, such as decreased glycemia and lipid profile, reduced weight and blood pressure and declined hs-CRP level, which could be due to pooling low quality and high heterogeneity studies (16). Notably, the meta-analysis of randomized trials indicated that pycnogenol significantly raised the AST levels and decreased the Gamma–Glutamyl Transferase (GGT) concentration by 1.53 U/I (16). In the present study, we did not observe any significant changes in the ALT and AST levels in the oligopin group. However, there was an increase in the ALP levels in both treatment groups, which was greater in the placebo group, as compared to oligopin. Overall, based on the results and the previous study, oligopin did not show any toxic effects on the liver function (16). The hypoglycemic effects of the pine bark extract may be related to the inhibition of the alpha glycosidase activity in the small intestinal brush border due to procyanidins (flavonoids), independent of the effect on insulin secretion (19, 31). We speculate that the efficiency of oligopin on glycemia and HbA1c depends on the baseline glycemia. We enrolled the patients with normal glycemia (glycated hemoglobin levels less than 6% and FBS less than 126 mg/dl).

Pycnogenol (the pine bark extract) has been explored as a potential natural antihypertensive agent. While the results have not been consistent, pycnogenol supplementation has been shown to reduce the systolic and diastolic blood pressure (32). The effect is mediated *via* nitric oxide (NO) production (32) or angiotensin converting enzyme (ACE) inhibition (33) and /or reduction of endothelin -1 (17). In our study, blood pressure was reduced after oligopin treatment for 3 months, although the difference was not significant. As most of our study participants displayed well-controlled blood pressure levels (97.5% SBP<140 mmHg, 85% DBS <90 mmHg) at the baseline, we postulate that oligopin supplementation could exert favorable effects on blood pressure only among hypertensive patients (16, 33, 34). On the other hand, the subgroup analysis in the recent meta-analysis indicated that the effect of this extract on blood pressure was more prominent in the trails with a longer intervention duration (>12 weeks) (32). As a result, a longer period is required to obtain results.

Supplementation with oligopin has been suggested to decrease the levels of CRP, with an anti-inflammatory effect (35). However, our data failed to show any significant change in the hs-CRP levels with oligopin administration. We have not measured the circulation of other inflammatory factors in this study; so, we cannot conclude that oligopin is ineffective on inflammation.

There are some possible explanations for the apparent lack of a positive finding in our study. First, the dose of oligopin might have been inadequate. One study involved the use of oligopin in type 2 diabetes, showing that a daily consumption in the amount of 100 mg/d to 200 mg/d was required to achieve a protective effect (36). It is uncertain whether the dose required to achieve androgen reduction is similar, or the response to oligopin may be different in different subjects; we included the PCOS women. Moreover, the recent meta-analysis suggested a possible benefit for pine bark extract supplementation when adding other treatments and no benefit when consuming pine bark extracts as a solitary therapy (32).

Furthermore, the impact of oligopin on hormonal and cadiometabolic profiles may be different for the two sexes; sex may be a modifier of the effect of the pine bark extract on the cardiometabolic profile, or the link between oligopin and PCOS may be appreciated only in the sub-population of the PCOS patients; so, insulin resistance vs. noninsulin resistance and also, between lean and obese should be noted. This can be partially explained by the fact that a large number of cases in the population in our trial were not insulin-resistant (mean HOMA <3.8) and they were relatively lean (overweight on average).

CONCLUSION

This study demonstrated, for the first time, that a nutraceutical intervention based on a 3-month 50 mg oligopin (pine bark extract) administration among PCOS women was safe, but it did not improve androgen or metabolic/anthropometric parameters.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics approval was obtained from the Ethics Committee of Endocrinology Metabolism Research Institute, Tehran University of Medical Sciences (REC.1396.00163). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM: study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript, critical revision. MiS: acquisition of data; MM-T: critical revision; AM-G: critical revision; SK: acquisition of data; HG: acquisition of data, NS: critical revision; MaS: acquisition of data; FB: critical revision; MQ: analysis and interpretation of data; SH: study conception and design. All authors contributed to the article and approved the submitted version.

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Involvement of Transcription Factor FoxO1 in the Pathogenesis of Polycystic Ovary Syndrome

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FoxO1 is a member of the forkhead transcription factor family subgroup O (FoxO), which is expressed in many cell types, and participates in various pathophysiological processes, including cell proliferation, apoptosis, autophagy, metabolism, inflammatory response, cytokine expression, immune differentiation, and oxidative stress resistance. Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in the women of childbearing age, which is regulated *via* a variety of signaling pathways. Currently, the specific mechanism underlying the pathogenesis of PCOS is still unclear. As an important transcription factor, FoxO1 activity might be involved in the pathophysiology of PCOS. PCOS has been associated with insulin resistance and low-grade inflammatory response. Therefore, the studies regarding the role of FoxO1 in the incidence and associated complications of PCOS will help provide novel ideas for establishing the treatment strategy of PCOS.

Keywords: forkhead transcription factor FoxO1, polycystic ovary syndrome, low-grade inflammatory response, insulin resistance, tumor necrosis factor alpha

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INTRODUCTION

At present, the incidence rate of polycystic ovary syndrome (PCOS) is about 5.6% among women of reproductive age (19–45 years) in Chinese Han population based on a large community-based study (Li et al., 2013), but the specific mechanism underlying the pathogenesis of PCOS is still unclear. Apart from polycystic ovaries, hyperandrogenism, and ovulatory disorders, PCOS is often accompanied by insulin resistance (IR), low-grade chronic inflammatory response, obesity, abnormal lipid metabolism, and long-term complications, such as hypertension, type 2 diabetes, and endometrial cancer (Li et al., 2013; Barthelmess and Naz, 2014; Nandi et al., 2014; Wang et al., 2015, 2017a,b; Wang and Wang, 2017; Lin et al., 2019; Zhang et al., 2019).

Forkhead transcription factor subfamily O (FoxO) widely exists in various mammalian tissues and plays an important role in metabolism, cell proliferation, apoptosis, and stress resistance, while FoxO1, a member of FoxO, has been shown to play a vital role during glycolipid metabolism, IR, and oxidative stress (Wang et al., 2016; Lee and Dong, 2017; Murtaza et al., 2017). Previous studies indicate that hepatic IR involves ceramide-induced activation of atypical protein kinase C, which selectively impairs protein kinase B (PKB/Akt)-dependent FoxO1 phosphorylation (Sajan et al., 2014, 2015). In granulosa cells (GCs) derived from PCOS patients and the ovarian

tissues of PCOS rats, the expression levels of insulin growth factor 1 (IGF-1R) and Wnt family member 1 (Wnt1) were found to be decreased and PKB/Akt$^{Ser473/Thr308}$ phosphorylation was lowered (Mao et al., 2018). Recent research demonstrated that Cangfudaotan decoction alleviated IR and improved follicular development in rats with PCOS via IGF-1-PI3K/Akt-Bax/Bcl-2 pathway (Wang et al., 2020). Advanced glycation end product-induced apoptosis involves the formation of reactive oxygen species, nitric oxide, and ceramide, and further leads to p38 and JNK mitogen-activated protein kinase (MAPK) activation, which in turn induces FoxO1 and caspase-3 (Alikhani et al., 2007). MicroRNA-145 (miR-145) mimics inhibit the activation of p38 MAPK and extracellular signal-regulated kinase through targeting insulin receptor substrate 1 (IRS1), and overexpressed IRS1 abrogated this suppressive effect in the GCs derived from PCOS patients (Cai et al., 2017). *Klotho* gene knockdown blocked the effects of insulin on apoptosis/proliferation in the GCs derived from PCOS patients, and inhibited caspase-3 activity in the ovarian tissues of PCOS rats (Mao et al., 2018). Interleukin-1β (IL-1β)-dependent regulation of FoxO1 protein content and its localization in a novel ceramide-dependent manner through IL-1β stimulation of primary rat hepatocytes and in HEK293 cells overexpressing IL-1β receptor have been demonstrated previously (Dobierzewska et al., 2012). Yang et al. (2020) found that cryptotanshinone (CRY) significantly alleviated the changes in the body and ovary weight, and the levels of hormone and inflammatory factor in PCOS rats through regulating the HMGB1/TLR4/nuclear factor-kappa B (NF-κB) signaling pathway. Furthermore, the upregulation of miR-204 improved IR of PCOS *via* the inhibition of HMGB1 and inactivation of the TLR4/NF-κB pathway (Jiang et al., 2020), while increased HMGB1 and reduced FOXO1 were found to be dependent on the loss of cystic fibrosis (CF) transmembrane conductance regulator (CFTR) function in case of CF (Smerieri et al., 2014; Montanini et al., 2016b; Cirillo et al., 2019b).

At present, the investigation on the contribution of FoxO1 in the pathogenesis of PCOS is being conducted. Therefore, exploring the underlying mechanism of FoxO1 activity in the pathogenesis of PCOS will help provide a novel target for establishing the treatment of PCOS and associated complications (**Figure 1**).

PCOS PATHOGENESIS

The origin of PCOS is multifactorial with individual differences, such as abnormal ovarian steroid secretion, hyperinsulinemia, increased luteinizing hormone, and other aspects, which lead to complementary or synergistic effects, and affect the development of the disease (Wang and Wang, 2017) and, therefore, the exact cause of PCOS is still unclear. Given the polymorphism of PCOS phenotype, it is considered to be a multi-gene-mediated disease (Li et al., 2013; Barthelmess and Naz, 2014; Nandi et al., 2014; Wang and Wang, 2017). For example, PCOS has previously been related to insulin receptor (INSR) gene with racial differences (Stewart et al., 2006; Wang et al., 2017a; Lin et al., 2019), and the family history might be a potential risk factor for the incidence of PCOS (Azziz and Kashar-Miller, 2000; Wang and Wang, 2017). It has been also reported that visceral obesity, proinflammatory factors, hyperinsulinemia, and IR are likely associated with the occurrence of PCOS (Wang et al., 2015, 2017b; Hughan et al., 2016; Zhang et al., 2019).

Polycystic ovary syndrome patients with hyperinsulinemia or IR are not dependent on obesity, body fat distribution, and androgen levels, and the risk of impaired glucose tolerance and type 2 diabetes mellitus is higher in these patients than the normal individuals (Nandi et al., 2014). The high androgen level and occurrence of IR in PCOS patients might be related to the continuous release of inflammatory factors from adipose tissue (Barthelmess and Naz, 2014; Wang and Wang, 2017; Cirillo et al., 2019a; Barber and Franks, 2021). A large number of studies have demonstrated a role of inflammation in the pathogenesis of PCOS (Alikhani et al., 2007), and the association of increased inflammatory markers, such as C reactive protein (CRP), ferritin, tumor necrosis factor (TNF) alpha, interleukin-6 (IL-6), and interleukin-18 (IL-18) with the occurrence of PCOS (González et al., 2006, 2007, 2012). Increased levels of plasminogen activator inhibitor-1 (PAI-1) and free fatty acid affect the phosphorylation of serine residue, leading to IR. PCOS patients exhibit high levels of ferritin and transferrin of hemoglobin along with a decrease in the levels of anti-inflammatory cytokines and anti-oxidant factors, thereby leading to chronic inflammation (Escobar-Morreale and Luque-Ramírez, 2011; Escobar-Morreale, 2012; Yang et al., 2015). Therefore, obesity may increase the level of oxidative stress in adipose tissue, activate the inflammatory signaling, and finally aggravate the chronic inflammatory state and IR in PCOS patients (Furukawa et al., 2004).

STRUCTURE AND FUNCTION OF FoxO1

The forkhead protein family was discovered in a study regarding the abnormal head mutations of Drosophila in 1989, which contained a highly conserved DNA binding domain, which corresponds to the forkhead conserved region composed of 110 amino acid residues and the domains of three helixes, three folds, and two wing-like structures (Weigel et al., 1989). At present, more than 100 forkhead (FOX) genes have been identified, belonging to 19 subfamilies, namely FOXA~S (Genin et al., 2014). The subgroup O of FoxO is the earliest discovered and widely distributed subgroup, which comprises FoxO1, FoxO3, FoxO4, and FoxO6 (van der Vos and Coffer, 2011). The first two are expressed in almost all human tissues, while FoxO4 is mainly expressed in muscles, kidney, and colon tissues, and FoxO6 is expressed in the brain and liver (van der Vos and Coffer, 2011).

The function of transcription factor FoxO1 is complex, which is mainly through the activation or inhibition of the transcription of its downstream target genes (Xu et al., 2017; Xing et al., 2018). FoxO1 in the endometrium has been shown to play an important role in the transformation of endometrium during menstruation, and in the protection of fetal mothers from oxidative damage during pregnancy (Kajihara et al., 2013). Moreover, FoxO1 knockout leads to embryo death due to vascular dysplasia (Hosaka et al., 2004). It has been reported that mice

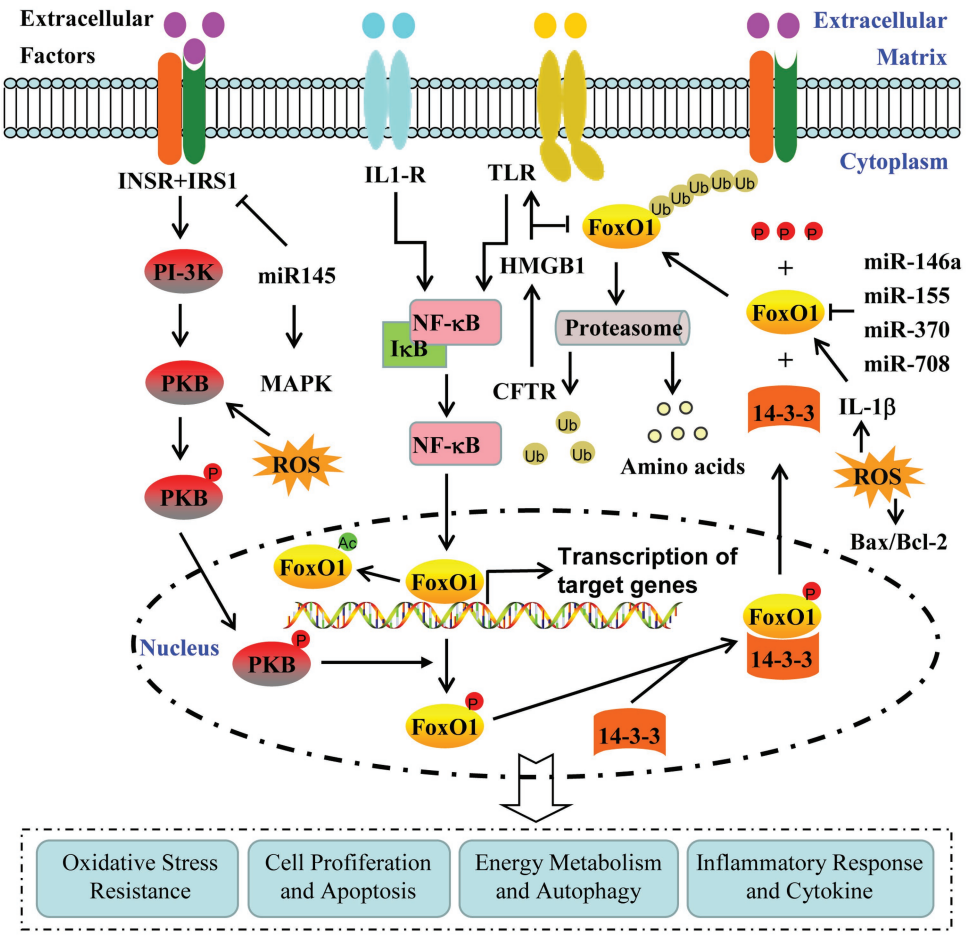

FIGURE 1 | Regulation and contribution of FoxO1 activity in the pathogenesis of polycystic ovary syndrome (PCOS). FoxO1 activity is mainly regulated by the post-translational modifications, including phosphorylation, acetylation, and ubiquitination. FoxO1 is involved in the pathogenesis of PCOS through various signaling pathways, including phosphoinositide 3-kinase (PI-3K)/protein kinase B (PKB), mitogen-activated protein kinase (MAPK), high-mobility group box 1(HMGB1)/Toll-like receptor 4(TLR4)/nuclear factor-kappa B (NF-κB), and Interleukin-1β (IL-1β).

with specific loss of FoxO1 in liver can resist IR induced by high-fat diet, while those with specific over-expression of FoxO1 in liver can increase IR (Kim et al., 2009; Balakumar et al., 2016; Pandey et al., 2017; Zeng et al., 2019). The acute inflammation process is related to the increase in glucocorticoid production activated *via* the FoxO1 pathway, and then, glucocorticoid reduces insulin-like growth factor 1 (IGF-1) production and increases TNF alpha/NF-kB signaling during the induction of protein hydrolysis system (Kim et al., 2009; Schakman et al., 2012).

REGULATION OF FoxO1 ACTIVITY

The transcriptional activity of FoxO1 is mainly accomplished through complex post-translational modifications, including phosphorylation, acetylation, and ubiquitination. These modifications can be activating or inactivating. The activity of specific targets can be altered through four functional sequences, thereby resulting in different biological effects (Tsai et al., 2007).

Phosphorylation of FoxO1 is directly by several protein kinases, which can modify different sites of this transcription factors through changing their subcellular location, DNA binding affinity, and transcriptional activity (Zhao et al., 2004; Tikhanovich et al., 2013). FoxO1 is phosphorylated through the activation of the serine-threonine kinases, including PKB/AKT and serum glucocorticoid inducible kinase (SGK), by phosphatidylinositol-3 kinase (PI-3K) to associate FoxO1 with 14-3-3 couple protein binding for translocating from the nucleus to the cytoplasm, thereby resulting in its transcription inactivation (Wang et al., 2016). Furthermore, growth factor-activated protein kinases, such as extracellular signal-regulated kinase and cyclin-dependent kinase-2, also induce FoxO1 phosphorylation and its transport to the cytoplasm through different pathways, thereby resulting in a decrease in FoxO1 transcriptional activity (Zhao et al., 2004).

Acetylation of FoxO1 promotes and decreases the transcriptional activity of FoxO1, which is mediated through

histone acetyltransferase and deacetylase, thereby regulating different biological functions (Lalmansingh et al., 2012). FoxO1 regulates the affinity and sensitivity of DNA binding regions through the acetylation of K262, K265, K274, and K294, thereby altering downstream PKB/AKT phosphorylation (Calnan and Brunet, 2008; Lalmansingh et al., 2012). Additionally, FoxO1 also reduces its own activity through acetylating two basic residues, Lys242, and Lys245, in the carboxyl terminal of DNA-binding region of cAMP responsive element binding protein (Daitoku et al., 2004).

Unlike the reversibility of phosphorylation/dephosphorylation and acetylation/deacetylation of FoxO, ubiquitination of FoxO1 is irreversible, and thus, is responsible for the degradation of FoxO1 (Huang and Tindall, 2011). Ubiquitin-protein ligating enzyme (E3) is a key enzyme for recognizing ubiquitin and degrading protein substrates. The degradation of FoxO1 is achieved *via* the multi-ubiquitination of multiple E3 complexes (Huang and Tindall, 2011).

FoxO1 AND PCOS

Polycystic ovary syndrome is a disease with an endocrine disorder and the development of PCOS may be caused due to the imbalance in the levels of sex hormones, inflammatory factors, and insulin. Notably, FoxO1 expression was found to be increased significantly in cumulus cells of PCOS women with BMI 21.5 ± 2.5 kg/m^2 than that in non-PCOS patients with BMI 20.7 ± 2.1 kg/m^2 (Shi et al., 2015).

FoxO1 and Insulin Resistance

Polycystic ovary syndrome patients with hyperinsulinemia or IR were reported to be about 44–77% (Vigil et al., 2007). IR is a state of pathological metabolism with decreased ability to use glucose, and thus, insulin secretion is increased to compensate and maintain the normal blood glucose level, thereby leading to hyperinsulinemia. Interestingly, hyperinsulinemia not only increases androgen secretion through selectively increasing the sensitivity of theca cells to luteinizing hormone, but also increases the level of free androgen through inhibiting the synthesis of sex hormone binding globulin in the liver, thereby promoting the occurrence of PCOS (Bremer and Miller, 2008; Wang et al., 2015, 2017a,b; Lin et al., 2019; Zhang et al., 2019).

FoxO1 is a key downstream molecule of the INS/IGF-1 signaling pathway, regulating the circulatory metabolism and hormone levels in liver, pancreas, hypothalamus-pituitary axis, and adipose tissue through increasing the level of circulating glucose (Wang et al., 2015, 2017a,b; Kamagate and Dong, 2018; Lin et al., 2019; Zhang et al., 2019). For example, FoxO1 elevates the blood glucose levels through acting on the key enzymes, such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase during the process of gluconeogenesis, and it also affects the apoptosis of beta cells and development of type 2 diabetes mellitus through INS/IGF-1 signaling (Wang et al., 2015, 2017a,b; Kamagate and Dong, 2018; Lin et al., 2019; Zhang et al., 2019). Rosas et al. (2010) found that the expression of glucose transporter 4 (GLUT4) related molecules in endometrium during secretory phase of normal menstrual cycle was beneficial for glucose uptake, while some molecules in PCOS patients related with hyperandrogenism decreased, and the exposure of GLUT4 and absorption of glucose reduced, thereby resulting in IR. Kohan et al. (2010) found that the decrease in GLUT4 expression in endometrium of PCOS patients with IR was related to FoxO1 phosphorylation, indicating that FoxO1 phosphorylation inhibited the expression of GLUT4 gene, and thus, affected the function of endometrium and caused IR.

FoxO1 and Chronic Inflammation

The expression of several chronic inflammatory factors was found to be increased in PCOS patients, including CRP, IL, and TNF alpha. These inflammatory factors reduce the sensitivity of tissue cells to insulin through endocrine, paracrine, and autocrine mechanisms, thereby leading to IR (González et al., 2006, 2007, 2012; Escobar-Morreale et al., 2011). Conversely, there is a common pathway between the signal transduction of inflammatory factors and INSR. Inflammatory factors can directly interfere with the phosphorylation of INSR, thereby changing the downstream pathway, leading to IR. Conversely, some inflammatory factors can increase the expression of rate-limiting enzymes that catalyze steroid production in theca cells and increase androgen levels in PCOS patients.

González et al. demonstrated that the expression of NF-kB increased in PCOS patients with high blood glucose, and the increased activity of NF-kB resulted in the secretion of pro-inflammatory cytokine TNF alpha (González et al., 2006, 2012; Escobar-Morreale et al., 2011). TNF alpha induces lipolysis of visceral fat, releases free fatty acids, and eventually leads to IR and hyperandrogenism (González et al., 2006, 2007, 2012; Escobar-Morreale et al., 2011). Elevated androgen may change the local expression of androgen receptor in the ovaries, and then increase the occurrence of PCOS (González et al., 2006, 2012; Escobar-Morreale et al., 2011). Ibfelt et al. (2014) found that TNF alpha induces IR through inhibiting the tyrosine phosphorylation of insulin receptor substrates, and also affects the intracellular glucose transport through downregulating the expression of GLUT4. Miao et al. (2012) found that TNF alpha is positively correlated with FoxO1 expression and FoxO1 might increase the production of pro-inflammatory factors in diabetic hepatocytes with IR. Li et al. (2017) found the association of FoxO1 signaling with the aggravation of inflammation and occurrence of IR in PCOS macrophages.

FoxO1 and Obesity

During the last four decades, obesity has driven the rise in obesity-related co-morbidities, including PCOS (Barber and Franks, 2021). PCOS is associated with IR, which is independent of (but amplified by) obesity (Barber and Franks, 2021). Multiple factors contribute to the severity of IR in PCOS, including most notably, weight gain (Barber and Franks, 2021). In the study conducted by Šimková et al. (2020), the authors demonstrated that there were no differences in hormonal, but in metabolic parameters, between normal-weight and obese PCOS women.

Obese PCOS women exhibited significantly higher IR, fatty-liver index, triglycerides, and cytokines (IL-2, IL-13, and IFN-gamma; Šimková et al., 2020). Ni et al. (2015) found that the expression level of high-mobility group box 1 (HMGB1) was increased in the serum from PCOS women with IR/hyperinsulinemia. Further investigation discovered that the high concentration of insulin not only mimicked IR model, but also promoted apoptosis of ovarian GCs through HMGB1 (Ni et al., 2015). Montanini et al. (2016a) also found that HMGB1 expression was increased in CF patients with deranging glucose metabolism. The increase in HMGB1 was related to the loss of CFTR function, and insulin lowered HMGB1 (Montanini et al., 2016a). CFTR inhibitor and siRNA experiments demonstrated that the changes in FoxO1 were also related to CFTR loss of function in CF (Smerieri et al., 2014), and reduced FoxO1 is correlated with reduced gluconeogenesis and increased adipogenesis, which are the characteristic features of insulin insensitivity (Smerieri et al., 2014). In PCOS women with BMI 25.92 ± 0.99 kg/m^2, CFTR and FoxO1 expression levels reduced in GCs (Cirillo et al., 2019b), and HMGB1 expression increased in follicular fluids and serum of PCOS women (Cirillo et al., 2019b). Additionally, miRNAs analyzed by Cirillo et al. (2019a) demonstrated the changes in PCOS ovaries and their relationships with inflammation and insulin sensitivity. Montanini et al. also found that significant changes in the expression of these four miRNAs (miR-146a, miR-155, miR-370, and miR-708) were dependent on the genotype and glucose tolerance state in CF patients (Cirillo et al., 2019a), which were selected as the potential FoxO1 regulators (Cirillo et al., 2019a). Cai et al. (2017) found that IRS1 gene is a direct target of miR-145, which was downregulated in GCs derived from PCOS patients. Further analysis demonstrated that miR-145 mimics inhibited cell proliferation and promoted apoptosis in GCs derived from PCOS women (Cai et al., 2017).

SUMMARY AND CONCLUSION

In conclusion, FoxO1, as a crucial transcription factor, plays an important role in regulating the gene expression, participating in gluconeogenesis, low-density lipoprotein production, oxidative stress, and cell apoptosis (Weigel et al., 1989; Furukawa et al., 2004; Escobar-Morreale and Luque-Ramírez, 2011; Escobar-Morreale, 2012; Kajihara et al., 2013; Genin et al., 2014; van der Vos and Coffer, 2011; Yang et al., 2015; Wang et al., 2016; Lee and Dong, 2017; Murtaza et al., 2017; Xu et al., 2017; Xing et al., 2018). Additionally, many studies have demonstrated that FoxO1 plays an important role in the pathogenesis of PCOS. The changes in the levels of hormones, TNF alpha, and GLUT4 in PCOS patients may affect the regulation of FoxO1 signaling on glucose transport, thereby leading to IR (Huang and Tindall, 2011). Moreover, the changes in FoxO1-mediated signaling may further induce the occurrence of low-grade chronic inflammation in the body, thereby leading to the hyperandrogenism of PCOS (Bremer and Miller, 2008; Rosas et al., 2010; Barthelmess and Naz, 2014; Nandi et al., 2014; Kamagate and Dong, 2018). Therefore, the study regarding the association of FoxO1 with the pathogenesis of PCOS can provide a basis for the etiology of PCOS, and a novel theoretical support for establishing the treatment of PCOS.

AUTHOR CONTRIBUTIONS

The manuscript was written by RX and revised by ZW. Both authors reviewed and approved the final version of the manuscript.

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Ovarian Reserve Markers in Premature Ovarian Insufficiency: Within Different Clinical Stages and Different Etiologies

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Objective: To characterize the ovarian reserve indicators for premature ovarian insufficiency (POI) at different disease stages and with various etiologies.

Methods: According to different FSH levels and menstrual conditions, patients with normal ovarian reserve (NOR with 5 IU/L<FSH<10 IU/L, n=987), precursor stage of POI (pre-POI with 10 IU/L<FSH ≤ 25 IU/L, n=410), early POI (25 IU/L<FSH ≤ 40 IU/L n=147), and premature ovarian failure (POF with FSH>40 IU/L, n=454) were retrospectively screened and their records were abstracted from Reproductive Hospital Affiliated to Shandong University between 2014 and 2019. Based on the known etiologies, POI patients were subdivided into genetic, iatrogenic, autoimmune and idiopathic subsets according to the known etiologies. The phenotypic features were compared within different subgroups, and the predictive value of ovarian reserve markers was analyzed.

Results: The ovarian reserve indicators consecutively deteriorated with the progress of ovarian insufficiency, indicated as an increase of FSH and LH but decrease of AMH, inhibin B, AFC, E_2 and T (P<0.01). Most of them changed significantly from NOR to pre-POI while remained relatively stable at a low level or even undetectable at early POI and POF stage. AMH showed the highest predictive value for pre-POI (AUC 0.932, 95% CI 0.918-0.945) and POI (AUC 0.944, 95% CI 0.933-0.954), and the combination of AMH and AFC was highly promising for early prediction. Additionally, significant differences existed in AMH, inhibin B and AFC among women with different etiologies of POI (P<0.05), and the genetic POI presented the worst hormone status.

Conclusions: Our study indicated a high heterogeneity of POI in both endocrine hormones and etiological phenotypes. The quantitative changes and cutoff values of AMH and AFC could provide new insights in the prediction and early diagnosis of POI.

Keywords: premature ovarian insufficiency (POI), premature ovarian failure (POF), ovarian reserve, clinical staging, etiology

INTRODUCTION

Premature ovarian insufficiency (POI) is a common reproductive endocrine disorder defined by the cessation of ovarian function before the age of 40. POI is clinically characterized by oligomenorrhea or amenorrhea with increased gonadotrophins (FSH>25 IU/L) and decreased estradiol (E2) (1). Ovarian insufficiency is a continuum of impaired ovarian function or ovarian aging rather than a specific dichotomous state. This condition can be transient or progressive, and usually results in eventual premature menopause (2, 3). According to different FSH levels, fecundity, and menstrual status, POI has been subdivided into three consecutive but progressive stages: occult, biochemical, and overt ovarian insufficiency (3). Premature ovarian failure (POF) is thus considered as the end stage of POI with FSH>40 IU/L. However, the evidence-based progression of POI is still lacking.

Either a small primordial follicle pool or rapid follicle exhaustion is associated with ovarian dysfunction and fecundity decline and results in POI (2, 4). Currently, the main ovarian reserve indicators widely used in clinics include FSH, E_2, inhibin B, Anti-Müllerian hormone (AMH), and antral follicle count (AFC) (5). Among these factors, FSH is the single one used for POI diagnosis but limited by its high inter- or intra-cycle variability (6). AMH and AFC have recently been considered more promising for assessing ovarian reserve, given their high sensitivity and specificity in predicting ovarian response and good inter-cycle reliability (7–9). Secreted mainly by small antral follicles, inhibin B is the most commonly utilized marker for ovarian activity rather than ovarian reserve (10, 11). However, none of these markers has been proven to be optimal for predicting the residual follicle pool and reproductive lifespan. Their dynamics and correlations during the progressive ovarian insufficiency still remain unclear.

POI is highly heterogeneous in etiology. A wide spectrum of causes has been considered, including genetic, autoimmune, or iatrogenic. Irrespectively, the majority remains to be elucidated (12). POI patients with different etiologies presented distinct phenotypes and endocrine hormones. Patients with genetic etiologies had the most severe disease phenotype compared to those with other etiologies (13). Whereas, Falorni et al. found that patients with autoimmune POI showed significantly higher inhibin B and AMH than those with idiopathic POI (14, 15). Therefore, whether ovarian reserve markers could be potential indicators for etiology classification in women with POI needs further exploration.

In the current study, ovarian reserve indicators were characterized in POI patients with different etiologies at different stages, and their significance in predicting POI progress and classifying different etiologies were further evaluated.

MATERIALS AND METHODS

Patients

A total of 1998 women less than 40 years old with different ovarian reserve were retrospectively screened, and their records were abstracted from the Reproductive Hospital Affiliated to Shandong University between July 2014 and July 2019. The study was approved by the Ethics Committee of Reproductive Medicine of Shandong University. Written informed consents were obtained from all participants.

According to the FSH level and menstrual conditions, all participants were sub-grouped into normal ovarian reserve (NOR, n=987), pre-POI (n=410), early POI (n=147), and POF (n=454) group. Women with regular menstrual cycles and normal endocrine hormones (5 IU/L<FSH<10 IU/L and 1.2 ng/mL<AMH<4.7 ng/mL), who sought infertility treatment due to tubal or male factors, were included as NOR. Women with regular or irregular menses and high FSH level (10 IU/L<FSH ≤ 25 IU/L, on two occasions >4 weeks apart) was considered as the precursor stage of POI and defined as pre-POI in our study. The diagnostic criteria of POI included oligo/amenorrhea for at least 4 months, and elevated FSH level >25 IU/L (on two occasions >4 weeks apart), among which women with 25 IU/L<FSH ≤ 40 IU/L were defined as early POI and FSH>40 IU/L as POF. Women with polycystic ovarian syndrome and hyperprolactinemia were excluded. According to known etiologies, patients with POI were subdivided into four groups: genetic POI, iatrogenic POI, autoimmune POI and idiopathic POI, as previously reported (13). Patients with chromosomal abnormalities (61/601, 10.15%) were included into genetic group, including 23 X-structural abnormalities, 22 X-numerical abnormalities, 9 X-autosomal translocations, 3 autosomal abnormalities and 4 (45,X/46,XY) mosaicism. Patients with ovarian surgery (31 ovarian cystectomy, 2 oophorectomy) and chemo-radiotherapy (2 cases) were included into iatrogenic group. Patients with autoimmune disease, including hypothyroidism or Hashimoto thyroiditis (n=25), psoriasis (n=1), rheumatoid arthritis (n=2), multiple sclerosis (n=1), dermatomyositis (n=1) or positive for thyroid antibodies (n=72) as immune POI. Ultimately, patients with unknown causes were classified as idiopathic POI (n=403).

Hormone Measurement and Ultrasonography

Peripheral blood was sampled on day 2-4 of the menstrual cycle or randomly (for women not menstruating frequently). Endocrine hormones FSH, LH, prolactin (PRL), E_2, and testosterone (T) were detected through chemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). AMH and inhibin B were detected by enzyme-linked immunosorbent assay (Kangrun Biotech, Guangzhou, China). The intra- and inter-assay coefficients of variation were <10% and <15%, respectively. Transvaginal ultrasonography was routinely conducted. AFC was defined as the number of bilateral follicles (2-10 mm in diameter) in early follicular phase.

Karyotype Analysis

Karyotype analysis was performed on GTG-banded metaphase chromosomes prepared from peripheral lymphocyte cultures, using a standard protocol that generated 400-450 band resolutions. Chromosome polymorphisms were recorded but classified as normal (16).

Statistical Analysis

SPSS 23.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. The single-sample Kolmogorov-Smirnov test was used for normality of distribution. Continuous data in normality distribution were expressed as mean ± standard deviation and compared by Student t-test or one-way analysis of variance. Continuous variables that were not normally distributed were presented as median (quartile interval) and compared by nonparametric test. Kruskal-Wallis ANOVA and multiple logistic regression were used for multiple comparisons, and P value was corrected by Bonferroni adjustment. The independent and combined predictive analyses were performed by binary logistic regression analysis and receiver-operator characteristic (ROC) curve. P<0.05 was considered statistically significant.

RESULTS

Baseline and Reproductive Characteristics

The records of 1998 women aged before 40 years old were retrospectively abstracted, including 987 women with NOR, 410 pre-POI, 147 early POI and 454 POF. The age at diagnosis significantly varied among pre-POI, early POI and POF groups (32.38 ± 4.07 y vs. 30.22 ± 3.72 y vs. 29.55 ± 4.19 y, P<0.001), indicating both women with early POI and POF presented with much earlier onset of ovarian insufficiency (P<0.001). Age at menarche was comparable among women with NOR, pre-POI and early POI (14.07 ± 1.46 y vs. 14.08 ± 1.84 y vs. 14.25 ± 2.05 y, all P>0.999), except that women with POF (14.92 ± 2.57 y) experienced significantly later menarche (all P<0.05). In addition, there were 131 cases (31.95%) with irregular menses in pre-POI group. Women with early POI and POF showed an earlier age of irregular menstruation (~ 2 years) compared with women in pre-POI (21.52 ± 5.71 y vs. 21.70 ± 5.89 y vs. 23.65 ± 7.38 y, P=0.007). There was no difference of age at amenorrhea among women with early POI and POF (22.59 ± 5.67 y vs. 23.24 ± 5.82 y, P=0.417). Women among four groups also showed different body mass index (BMI) (P=0.040), with difference mainly existing between patients with early POI and POF (23.30 ± 3.50 y vs. 22.44 ± 3.20 y, P=0.044). The baseline and reproductive characteristics of the participants were summarized in **Table 1** and **Supplemental Table 1**.

Variation of Ovarian Reserve Markers at Different Stages of Ovarian Insufficiency

As expected, the markers of ovarian reserve consecutively deteriorated as the progress of ovarian insufficiency, indicated as an increase of FSH and LH but a decrease of E_2, T, AMH, inhibin B and AFC, even after correcting for age and BMI through multiple logistic regression (P<0.01) (**Table 1** and **Figure 1**). No difference of PRL was found among different groups (P=0.268). Intriguingly, FSH and LH showed different change patterns. FSH showed a 2-fold increase steadily in each stage along with ovarian function decline (6.81 ± 1.17 IU/L vs. 14.90 ± 4.23 IU/L vs. 31.73 ± 4.35 IU/L vs. 70.77 ± 23.11 IU/L, P<0.001), whereas LH remained normal at pre-POI stage but presented a 2.5-fold increase later in early POI and POF (4.63 ± 1.70 IU/L vs. 6.53 ± 3.23 IU/L vs. 16.37 ± 8.28 IU/L vs. 35.65 ± 13.90 IU/L, P<0.001). The FSH/LH ratio thus increased significantly from NOR to pre-POI (1.64 ± 0.66 vs. 2.49 ± 1.22, P<0.001), and later maintained high constantly. Similar to LH,

TABLE 1 | Characteristics of 1998 women.

Characteristics	NOR	pre-POI	Early POI	POF	*P*	*P-adj*[i]
N	987	410	147	454	–	–
Age at diagnosis (y)[d,e]	–	32.38 ± 4.07	30.22 ± 3.72	29.55 ± 4.19	<0.001[g]	–
BMI (kg/m^2)[f]	22.81 ± 3.24	22.84 ± 3.43	23.30 ± 3.50	22.44 ± 3.20	0.040[g]	–
Age at menarche (y)[c,e,f]	14.07 ± 1.46	14.08 ± 1.84	14.25 ± 2.05	14.92 ± 2.57	<0.001[g]	–
Age at irregularity (y)[e]	–	23.65 ± 7.38	21.52 ± 5.71	21.70 ± 5.89	0.007[g]	–
Age at amenorrhea (y)	–	–	22.59 ± 5.67	23.24 ± 5.82	0.417[g]	–
FSH (IU/L)[a,b,c,d,e,f]	6.81 ± 1.17	14.90 ± 4.23	31.73 ± 4.35	70.77 ± 23.11	<0.001[g]	<0.001
LH (IU/L)[a,b,c,d,e,f]	4.63 ± 1.70	6.53 ± 3.23	16.37 ± 8.28	35.65 ± 13.90	<0.001[g]	<0.001
FSH/LH Ratio[a,b,c,e,f]	1.64 ± 0.66	2.49 ± 1.22	2.37 ± 1.14	2.14 ± 0.84	<0.001[g]	<0.001
PRL (ng/mL)	14.32 (10.15, 20.00)	13.59 (9.90, 19.50)	11.41 (7.75, 15.90)	9.95 (7.39, 14.52)	<0.001[h]	0.268
E_2 (pg/mL)[b,c,d,e,f]	34.30 (25.30, 44.90)	30.20 (19.50, 45.28)	15.65 (5.23, 31.60)	7.15 (5.00, 20.54)	<0.001[h]	<0.001
T (ng/dL)[b,c,e]	21.18 ± 9.67	20.55 ± 11.38	18.39 ± 12.16	18.14 ± 13.00	<0.001[g]	0.002
AMH (ng/mL)[a,b,c,d,e]	2.513 (1.817, 3.491)	0.424 (0.091, 1.024)	0.078 (0.071, 0.091)	0.078 (0.071, 0.087)	<0.001[h]	<0.001
Inhibin B (pg/mL)[a,b,c,d,e]	65.18 (45.04, 87.89)	37.05 (14.30, 65.07)	13.00 (10.00, 15.50)	13.30 (10.00, 16.00)	<0.001[h]	<0.001
AFC [a,b,c,d,e]	8 (6, 14)	4 (1, 6)	0 (0, 2)	0 (0, 1)	<0.001[h]	<0.001

Data are expressed as the mean ± standard deviation or median (interquartile range).

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; PRL, prolactin; T, testosterone; AMH, anti-müllerian hormone; AFC, antral follicle count.

[a]*P<0.05 for the comparison between NOR and pre-POI.*

[b]*P<0.05 for the comparison between NOR and early POI.*

[c]*P<0.05 for the comparison between NOR and POF.*

[d]*P<0.05 for the comparison between pre-POI and early POI.*

[e]*P<0.05 for the comparison between pre-POI and POF.*

[f]*P<0.05 for the comparison between early POI and POF.*

[g]*One-way analysis of variance.*

[h]*Kruskal-Wallis ANOVA test.*

[i]*Adjusted P-value after correcting for age and BMI through multiple logistic regression.*

no significant difference of E_2 existed between patients with NOR and pre-POI (34.30 [25.30, 44.90] pg/mL vs. 30.20 [19.50, 45.28] pg/mL, P>0.999), and it showed a 2-fold decrease thereafter (15.65 [5.23, 31.60] pg/mL vs. 7.15 [5.00, 20.54], P<0.01). The level of T declined slowly along with POI progress (21.18 ± 9.67 ng/dL vs. 20.55 ± 11.38 ng/dL vs. 18.39 ± 12.16 ng/dL vs. 18.14 ± 13.00 ng/dL, P=0.002), and no significant difference existed between any two adjacent stages (all P>0.05).

The decrease pattern of AMH, inhibin B and AFC was quite similar, with significance among NOR, pre-POI and early POI stages (P<0.001) but comparable between early POI and POF (P>0.05). From NOR to pre-POI, AMH showed the most significant decline and relatively high sensitivity with approximately 6 times of decrease (from 2.513 ng/mL to 0.424 ng/mL), compared to an approximately 2-fold decline of inhibin B (from 65.18 pg/mL to 37.05 pg/mL) and AFC (from 8 to 4). Similarly, the three indicators decreased at least twice from pre-POI to POI. While in 601 patients with early POI and POF, AMH was undetectable in 75.04% (451/601), inhibin B in 70.38% (423/601) and AFC was invisible in 65.72% (395/601) of cases.

The Predictive Value of Ovarian Reserve Markers on pre-POI and POI

To explore the predictive value of ovarian reserve indicators for pre-POI and POI, AMH, inhibin B, AFC and FSH/LH ratio were further analyzed given their significant difference from NOR to pre-POI. The specificity and sensitivity of these markers were analyzed by ROC curves (**Tables 2**, **3** and **Figure 2**). In terms of predicting pre-POI, the cutoff values of AMH, inhibin B, AFC and FSH/LH ratio were 1.211 ng/mL, 31.74 pg/mL, 5 and 2.11, respectively. AMH showed the best predictive value (AUC 0.932, 95% CI 0.918-0.945) both in sensitivity and in specificity, followed by AFC (AUC 0.868, 95% CI 0.848-0.885) and FSH/LH ratio (AUC 0.749, 95% CI 0.726-0.772), whereas inhibin B with the most unsatisfactory accuracy (AUC 0.704, 95% CI 0.679-0.727) (**Figure 2A**). To determine whether a combination of markers is more promising for pre-POI prediction, we included these four markers for multivariable prediction models. Among the dual-indicator models, AMH plus AFC showed the highest predictive accuracy, with 84.08% in sensitivity and 95.68% in specificity (95% CI 0.935-0.959,

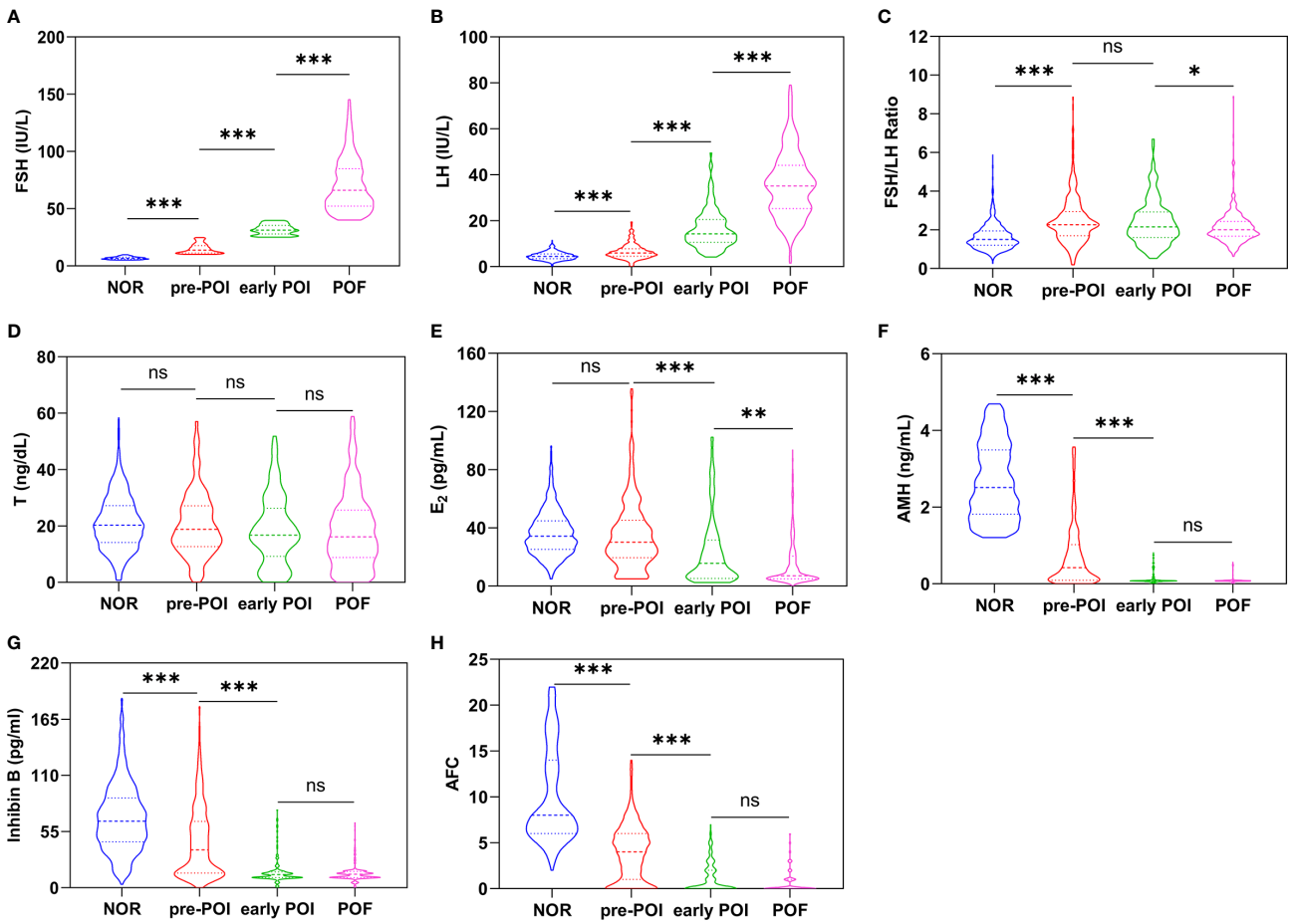

FIGURE 1 | **(A–H)** show the variations of FSH, LH, FSH/LH Ratio, T, E2, AMH, inhibin B and AFC in different stages of ovarian insufficiently respectively. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

TABLE 2 | The ROC curve analysis for pre-POI prediction.

Variable	AUC	95% CI[b]	cutoff	Sensitivity	Specificity	P value
AMH	0.932	0.918-0.945	1.211 (ng/mL)	79.74%	99.80%	<0.001
AFC	0.868	0.848-0.885	5	69.53%	85.91%	<0.001
FSH/LH ratio	0.749	0.726-0.772	2.11	62.44%	81.05%	<0.001
Inhibin B	0.704	0.679-0.727	31.74 (pg/mL)	46.59%	89.06%	<0.001
AMH+AFC	0.948	0.935-0.959	–	84.08%	95.68%	<0.001
AMH + FSH/LH ratio	0.932	0.918-0.945	–	79.95%	99.90%	<0.001
AMH + Inhibin B	0.932	0.917-0.945	–	81.58%	97.67%	<0.001
AFC + FSH/LH ratio	0.884	0.866-0.900	–	77.83%	83.85%	<0.001
AFC + Inhibin B	0.880	0.862-0.897	–	77.40%	82.41%	<0.001
FSH/LH + Inhibin B	0.715	0.691-0.739	–	49.88%	86.83%	<0.001
AMH + AFC + FSH/LH ratio	0.948	0.935-0.959	–	84.04%	95.58%	<0.001
AMH + AFC + Inhibin B	0.948	0.935-0.959	–	84.88%	95.16%	<0.001

AMH, anti-müllerian hormone; AFC, antral follicle count; ROC, receiver-operator characteristic curve; AUC, the area under the curve.
[b]Binomial exact test.

TABLE 3 | The ROC curve analysis for POI prediction.

Variable	AUC	95% CI[b]	cutoff	Sensitivity	Specificity	P value
AMH	0.944	0.933-0.954	0.250 (ng/mL)	92.46%	90%	<0.001
AFC	0.927	0.915-0.938	3	92.51%	85.21%	<0.001
Inhibin B	0.902	0.889-0.915	19.08 (pg/mL)	87.52%	86.90%	<0.001
FSH/LH ratio	0.627	0.605-0.648	1.54	81.70%	42.41%	<0.001

AMH, anti-müllerian hormone; AFC, antral follicle count; ROC, receiver-operator characteristic curve; AUC, the area under the curve.
[b]Binomial exact test.

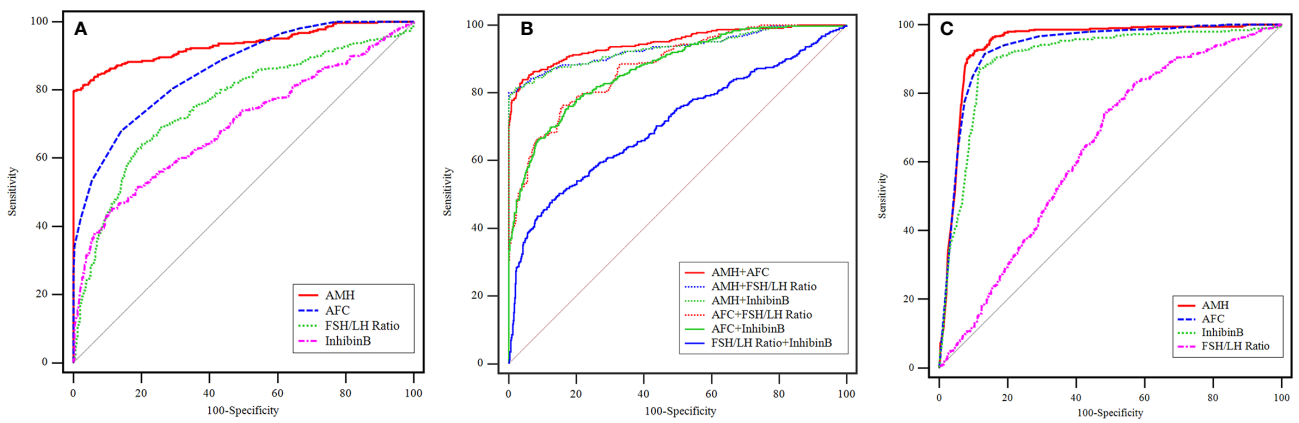

FIGURE 2 | The ROC curves of AMH, AFC, inhibin B and FSH/LH ratio for pre-POI and POI prediction. **(A)** Single variable for pre-POI prediction. **(B)** Multivariable models for pre-POI prediction. **(C)** Single variable for POI prediction.

P<0.001). When making pairwise comparison for all prediction models, we found that the combination of inhibin B or FSH/LH ratio with AMH single model or AMH + AFC dual model made no significant difference for predictive accuracy (P>0.05) (**Table 2**, **Supplemental Table 2** and **Figure 2B**).

For predicting POI, AMH also showed the highest accuracy (AUC 0.944, P<0.001), with the sensitivity and specificity of 92.46% and 90% respectively. AFC (AUC 0.927, P<0.001) and inhibin B (AUC 0.902, P<0.001) had slightly lower but comparable performance, but FSH/LH ratio showed the most unsatisfactory predictive value on POI (AUC 0.627, P<0.001). The cutoff value for POI diagnosis was 0.250 ng/mL for AMH, 3 for AFC, 19.08pg/mL for inhibin B and 1.54 for FSH/LH ratio, respectively (**Table 3** and **Figure 2C**).

Ovarian Reserve Markers in POI Patients Within Different Etiologies

The causes for 601 POI patients included were assessed and consisted of genetic (n=61, 10.15%), iatrogenic (n=35, 5.82%), autoimmune (n=102, 16.97%) and idiopathic (n=403, 67.05%) (**Table 4** and **Supplemental Table 3**). No differences existed in age at diagnosis, BMI, and age at amenorrhea among four etiological subgroups (P>0.05). However, patients with genetic anomalies experienced later menarche and a trend toward earlier

TABLE 4 | Characteristics of POI patients within different etiologies.

Characteristics	Genetic (n=61)	Iatrogenic (n=35)	Autoimmune (n=102)	Idiopathic (n=403)	*P*
Age at diagnosis (y)	29.16 ± 4.22	30.11 ± 3.79	29.90 ± 4.30	29.71 ± 4.05	0.648[f]
BMI (kg/m^2)	22.30 ± 2.98	21.88 ± 2.65	22.52 ± 2.98	22.80 ± 3.46	0.327[f]
Age at menarche (y)[a,c,d,e]	15.95 ± 3.34	13.40 ± 1.56	14.81 ± 2.84	14.68 ± 2.19	<0.001[f]
Age at irregularity (y)	21.18 ± 5.28	24.41 ± 5.24	21.63 ± 6.30	21.54 ± 5.83	0.056[f]
Age at amenorrhea (y)	22.14 ± 5.08	25.11 ± 5.35	23.02 ± 6.23	23.12 ± 5.80	0.186[f]
FSH (IU/L)	63.14 ± 26.66	59.82 ± 32.99	59.43 ± 23.35	62.21 ± 27.71	0.756[f]
LH (IU/L)	30.28 ± 15.91	33.53 ± 23.43	30.59 ± 15.52	31.99 ± 16.22	0.762[f]
PRL (ng/mL)	11.26 (7.94, 16.54)	11.23 (7.86, 18.30)	10.84 (7.07, 15.12)	9.90 (7.56, 14.16)	0.240[g]
E_2 (pg/mL)	9.08 (5.00, 23.90)	12.35 (6.53, 24.78)	11.50 (5.00, 28.25)	9.00 (5.00, 23.15)	0.419[g]
T (ng/dL)	18.76 ± 14.50	17.35 ± 13.55	17.53 ± 13.11	20.36 ± 16.03	0.353[f]
AMH (ng/mL)[a,d]	0.076 (0.072, 0.083)	0.080 (0.076, 0.33)	0.078 (0.069, 0.083)	0.079 (0.071, 0.090)	0.011[g]
Inhibin B (pg/mL)[a,b,c]	10 (10, 13.69)	14 (10, 16.6)	14 (10, 16.20)	13.5 (10, 16)	0.003[g]
AFC[a,c]	0 (0, 0)	1 (0, 2)	0 (0, 1)	0 (0, 1)	<0.001[g]

Data are expressed as the mean ± standard deviation or median (interquartile range).

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; PRL, prolactin; T, testosterone; AMH, anti-müllerian. Hormone; AFC, antral follicle count.

[a]P < 0.05 for the comparison between genetic POI and iatrogenic POI.

[b]P < 0.05 for the comparison between genetic POI and autoimmune POI.

[c]P < 0.05 for the comparison between genetic POI and idiopathic POI.

[d]P < 0.05 for the comparison between iatrogenic POI and autoimmune POI.

[e]P < 0.05 for the comparison between iatrogenic POI and idiopathic POI.

[f]One-way analysis of variance.

[g]Kruskal-Wallis ANOVA test.

irregular menstruation. Interestingly, of all ovarian reserve markers, there were significant differences of AMH (P=0.011), inhibin B (P=0.003) and AFC (P<0.001) among different etiological groups. AMH level in iatrogenic POI (0.080 [0.076, 0.33] ng/mL) was significantly higher than that of genetic (0.076 [0.072, 0.083] ng/mL, P=0.022) and autoimmune POI (0.078 [0.069, 0.083] ng/mL, P=0.016) but comparable with that of idiopathic POI (0.079 [0.071, 0.090] ng/mL, P=0.108). The concentration of inhibin B was significantly lower in patients of genetic POI than that of others (P<0.05). AFC had the same distribution pattern with inhibin B except for no difference between genetic and autoimmune POI.

DISCUSSION

POI imposes a great challenge on women's fertility and lifelong health. However, it is highly heterogeneous both in phenotype and in etiology. Currently, it remains controversial on its nomenclature, recruitment criteria, clinical staging and early indicators for prediction. Here we have comprehensively characterized different ovarian markers at different stages of ovarian insufficiency and within different etiologies in a large cohort of POI patients. Our results indicated that ovarian function decline was a continuum and progressive progress, in analogy to a shortened chronological aging-associated menopausal transition. When ovarian dysfunction started, the ovarian reserve indicators have begun deteriorating, especially the highly sensitive markers, such as AMH, AFC, inhibin B and FSH/LH ratio; once entered the POI stage, these indicators remained stable at low levels or even undetectable. AMH *per se* showed high predictive values for both pre-POI and POI, and a combination of AMH and AFC was highly promising to predict ovarian dysfunction in advance. More interestingly, POI patients with different etiologies showed distinct characteristics of endocrine hormones, and genetic POI showed much smaller AFC and lower level of inhibin B.

Ovarian reserve mainly encompasses the quantity and quality of oocytes. It determines a woman's reproductive potential and, subsequently, her reproductive lifespan and age of menopause onset (17). Normal ovarian function demands integrative functioning and interactive feedback of the hypothalamic-pituitary-ovarian (HPO) axis (18). Due to the decreased quantity or quality of follicles, the insufficient secretion of ovarian hormones contributed to a preferential rise in FSH over LH through negative feedback. In pre-POI stage, FSH increased much earlier and more sharply than LH, and the FSH/LH ratio thus significantly increased. Previous evidence have shown that the FSH/LH ratio was an independent factor to predict poor ovarian response and associated with poor outcomes *in vitro* fertilization (IVF) treatment (19, 20). Here our results also demonstrated its importance in predicting pre-POI and early ovarian decline.

Within the stage of early rise of FSH, AFC, inhibin B and AMH showed similar decrease pattern before the decline of E_2, enabling them as sensitive markers in early clinical staging of ovarian decline. AFC evaluates immediate quantity of antral follicles with good inter-cycle reliability and has been reported to be positively correlated with the number of primordial follicles (6, 21, 22). While representing the gonadotrophin-responsive antral follicle pool, inhibin B selectively inhibits pituitary FSH over LH, potentiates FSH withdrawal from non-dominant follicles and facilitates the development of a single dominant ovulatory follicle (23, 24). Both indicators showed ~ 2 folds decrease from NOR to pre-POI and provided acceptable accuracy for pre-POI prediction. The early FSH rise was probably attributed to decreased negative feedback of inhibin B decline in early follicular phase from a smaller pool of the pre-antral and early antral follicles remaining in the ovaries.

Interestingly in our cohort of pre-POI, AMH declined by 5-6 folds compared to NOR and showed the highest single predictive value for pre-POI, suggesting a high sensitivity and specificity in the assessment of early ovarian dysfunction. Produced by preantral and small antral follicles, AMH can reflect more completely the size of primordial follicle pool and the number of remaining follicles. It can restrain the initial resting follicle recruitment and decreases the FSH-responsiveness of growing follicles, thus retarding the rate of follicles depletion (25, 26). Serum AMH, along with AFC, has a high sensitivity and specificity to detect the quantitative aspects of ovarian reserve, and is the most reliable contemporary ovarian reserve tests (ORT) employed today in clinical practice (27). Consistent with our data, Knauff et al. (11) found that compared with inhibin B and AFC, AMH was more consistently correlated with the clinical degree of follicle pool depletion in young women presenting with elevated FSH levels.

Currently, except for basal FSH, no standardized reference or cutoff value is available for pre-POI diagnosis (3). In our study, the ROC curve analysis revealed an area under the curve of 0.932, which implies a good discriminatory performance, and suggests that a threshold AMH value of 1.211 ng/mL would probably be a reasonable compromise for discriminating pre-POI from NOR women. Whereas both low AFC of 5 follicles and low inhibin B of 31.74 pg/mL had high specificity for predicting pre-POI, but their clinical significance was limited by its low sensitivity. Consistently, the 2016 POSEIDON criteria adopted the thresholds of AMH=1.2 ng/mL and AFC=5 for the grouping of poor ovarian response (POR) (28, 29). Although distinct concepts and diagnosis indicated, pre-POI and POR could contribute to each other due to diminished ovarian reserve, implying the clinical significance and applicability of the reference thresholds. For multiple prediction models, AMH along with AFC showed a better predictive value on pre-POI. While inhibin B and FSH/LH ratio had no additive or synergistic effects, which therefore further highlighted the importance of AMH and AFC in the very early stage of ovarian insufficiency. Although there is insufficient evidence to recommend any ovarian reserve test as a sole criterion for the use of ART (5), our quantitative changes and cutoff values in ovarian reserve markers provided a critical reference for early ovarian insufficiency, which would greatly facilitate to identify patients of high risks and timely guide family planning and fertility intervention in clinical practice. Unfortunately, the ovarian reserve markers normally change with chronological age. The age-specific cutoff values were not available and further prospective longitudinal study is warranted to confirm the predictive role of different indicators.

It was generally considered that E_2 played a critical role in the negative feedback for FSH secretion. However, at the initial phase of ovarian insufficiency, the monotropic rise in FSH cannot be merely explained by E_2 decrease. Estradiol levels remained unchanged or slightly elevated in early ovarian dysfunction (30–32). Consistently, no difference of E_2 levels between women with pre-POI and NOR was revealed in this study. The compensatory HPO-axis and intra-ovarian mechanisms are operative early in ovarian aging. Lower levels of AMH in conjunction with elevated levels of FSH drive increased recruitment of the resting follicles into the growing pool. Although it contributes to accelerated follicle depletion, the increased growing follicles and continued follicle development could also maintain both estradiol levels and reproductive cycles, and serve to extend fertility and reproductive competence (33, 34). Therefore, basal estradiol level may fluctuate for variable periods of time, and it alone should not be used to predict pre-POI.

With ongoing follicle loss, the above-mentioned compensatory hormonal mechanisms are no longer adequate; follicle development becomes unpredictable, serum inhibin B and estradiol levels continue to decrease, resulting in a dramatic increase of FSH, an accelerated follicle depletion occurs (34). At this stage, oligomenorrhea or amenorrhea occurred, signifying the onset of POF. As expected, all patients with early POI already exhibited typical endocrine profiles with continuously elevated FSH and decreased E_2. The concentrations of AMH and inhibin B were critically low or undetectable as reported previously (35), and the presence of growing follicles was found in only 34.28% of POI patients. Importantly compared with early POI, patients with POF only showed further increased LH and decreased E_2, whereas no difference in any sensitive ovarian markers, including AMH, Inhibin B, AFC, and FSH/LH ratio. Therefore, the sensitive ovarian reserve markers have achieved a plateau once POF occurred and their predictive advantage is reflected at the very early stage of ovarian decline.

Consistent with our previous study, women with POF experienced delayed menarche and thereafter established normal periods (13, 36). We also found that women experienced amenorrhea within two years, more than 65% within one year, after irregularity occurred, highlighting the rapid decline of ovarian function during POI progress (13). Of note, 31.95% of patients with pre-POI already exhibited irregular menstruation. Whether they are more likely to develop into POI needs long term follow-up, and corresponding intervention and fertility guidance are warranted. Another concern was the delayed diagnosis. It took approximately 6-7 years for a confirmed diagnosis of POI after amenorrhea. Although it has been reported that 5-10% of cases experienced intermittent and unpredictable resumption of ovarian activity, ovulation or spontaneous pregnancy, even years after diagnosis, occasionally occurs (37). The resumption activity is extremely subtle and hard to catch. Up to now, there are no effective treatments to restore ovarian function or improve fertility (38). A delay in diagnosis of POI, as evidenced by elevated FSH or amenorrhea, might place young women at increased risk of developing POF. Therefore, early evaluation and intervention on ovarian dysfunction according to early biochemical changes is of great significance. A standardized staging system with correct terminology for clinical assessment needs to be established based on longitudinal studies of women across the ovarian insufficiency spectrum.

POI is highly heterogeneous in etiologies, and the correlation of phenotypes and different causes currently remains poorly-uncovered. In this study, patients with genetic anomalies had the most severe defect in ovarian function, distinct from that of autoimmune or iatrogenic induced. In addition to menses abnormality, the genetic POI patients also had significantly lower inhibin B and AFC than those of other etiologies. A longitudinal study is needed to confirm the predictive value of inhibin B for the

diagnosis of genetic POI. The pathogenesis and progression of autoimmune POI was assumed distinct from those of other etiologies, with follicular theca cells selectively destructed while the function of granulosa cells preserved (39). Theca cell impairment resulted in decreased estradiol synthesis and subsequent increased FSH, which stimulated the viable granulosa cells to produce more inhibin B, and the preserved ovarian follicle pool contributed to the normal AMH range. Falorni *et al.* have found that both inhibin B and AMH were significantly higher in autoimmune POI than idiopathic POI (14, 15). On the contrary, Luborsky *et al.* found no difference in inhibin B between women with and without positive ovarian antibodies (40). Similarly, the level of inhibin B and AMH was comparable between autoimmune POI and idiopathic POI patients in our study. The different recruitment criteria for autoimmune POI might explain the discrepancy. The presence of steroid-cell autoantibodies (StCA) directed against steroidogenic cells or enzymes were defined as autoimmune POI in Falorni's studies (14, 15). While in the current study, patients with concomitant autoimmune disease, such as psoriasis, rheumatoid arthritis or thyroid autoimmunity (positive TPOAb or TGAb) were included as autoimmune POI. Given the lack of reliable and effective monitoring or diagnostic indicators for autoimmune POI currently (41), future researches to characterize the specific ovarian markers for autoimmune POI with more definite diagnostic criteria are needed.

Of note, although providing hints, our cross-sectional analysis in the current study could not exactly elucidate the progression of ovarian insufficiency. Further prospective longitudinal studies are warranted to confirm the predictive role of different indicators. Given that FSH is the single ovarian reserve marker currently used for POI defining and disease subgrouping in our study, our results, although not perfectly, could provide some information on their performance and corresponding cutoff values of other indicators inhibin B, AFC, FSH/LH and AMH, which will facilitate the identification of patients with high risks and benefit the timely fertility guidance during clinical practice.

CONCLUSION

Our study depicted the dynamic changes of ovarian reserve markers in POI patients with different progressive stages and various etiologies, which provides essential evidence to confirm the high heterogeneity of POI in phenotype and etiology. The quantitative changes and cutoff values of AMH and AFC in predicting pre-POI provide new insights into the standardized staging, prediction and early diagnosis of POI. Future prospective, longitudinal cohort studies are warranted to confirm predictors and to develop strategies for fertility improvement in POI.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Reproductive Medicine of Shandong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XJ, TM, YZ, LZ, WL, and PL recruited subjects, collected data, and conducted data analysis. XJ and TM drafted the manuscript. YQ revised the manuscript critically for intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.601752/full#supplementary-material

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Chromosomal Aneuploidy Associated With Clinical Characteristics of Pregnancy Loss

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Objective: Embryonic aneuploidy is found in about half of sporadic pregnancy losses and the associations between the chromosomal aneuploidy and clinical characteristics of pregnancy loss remain unclear. The aims of this study were to evaluate the associations between chromosomal aneuploidy of products of conception (POC) and clinical features of pregnancy loss.

Methods: We conducted a retrospective cohort study including 1,102 women experienced singleton pregnancy loss and underwent chromosomal microarray analysis (CMA) detection of POC in our hospital. The results of molecular karyotypes and clinical features including maternal age, history of pregnancy loss, gestational age, vaginal bleeding and ultrasonographic findings were extracted from the medical records. χ^2 test was used to compare categorical data between groups.

Results: 631 (57.26%) POC specimens were detected to be chromosomal aneuploidy. Aneuploid rates were significantly higher in women >35 years ($P < 0.001$) and pregnancy loss <11 gestational weeks ($P = 0.044$), but the rates of sex chromosome abnormalities and triploid were significantly higher in women ≤35 years ($P < 0.001$, $P = 0.002$) and the rates of viable autosomal trisomy and sex chromosome abnormalities were significantly high in those women with pregnancy loss ≥11 weeks ($P < 0.001$, $P < 0.001$). Aneuploid rate was overall similar between the sporadic and the recurrent pregnancy loss (RPL) ($P = 0.404$), but the rate of sex chromosome abnormalities was higher in women with sporadic pregnancy loss ($P = 0.03$). Aneuploid rates were higher in subjects with yolk sac or embryo than in those without ($P < 0.001$ and $P = 0.001$).

Conclusion: Advanced maternal age is mainly associated with autosomal trisomy, while sex chromosome abnormalities and triploid might be more likely to occur in younger women. Aneuploidy rates might be no association with previous pregnancy loss except for sex chromosome abnormalities. Pregnancy loss without yolk sac or embryo might be less related to embryonic aneuploidy, and other factors should be emphasized.

Keywords: chromosomal aneuploidy, products of conception, pregnancy loss, chromosomal microarray analysis, clinical characteristics

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INTRODUCTION

It is well known that 15–20% of clinically recognized pregnancies end in pregnancy loss, and approximately 1–2% of couples experience recurrent pregnancy loss (RPL) (Practice Committee of the American Society for Reproductive Medicine, 2012; The Eshre Guideline Group on RPL et al., 2018). Embryonic/fetal chromosomal abnormalities are found in about 50% of early sporadic pregnancy loss, and aneuploidy is the most frequently observed abnormality (Hassold et al., 1980; Sahoo et al., 2017). The chromosomal analysis of products of conception (POC) is not a routine practice for women who have pregnancy loss. However, accurate identification of the genetic characteristics of a pregnancy loss can provide important information for medical management, reproductive counseling, and supportive patient care (Menasha et al., 2005; Zhang et al., 2009).

Although G-banding karyotyping has been used for many years to evaluate samples of POC, the high rate of culture failure and maternal cell contamination are two primary limitations (Lomax et al., 2000; van den Berg et al., 2012). Evaluation of aneuploidy by fluorescence *in situ* hybridization (FISH) analysis avoids the above defects, but is significantly limited to a smaller number of targeted chromosomes (Shearer et al., 2011; Russo et al., 2016). Chromosomal microarray analysis (CMA) using genome-wide oligonucleotide or single-nucleotide polymorphism (SNP)-based arrays has replaced karyotyping in some prenatal diagnostic applications owing to its higher resolution and detection rates of chromosomal abnormalities (Hillman et al., 2011; Dhillon et al., 2014), but it can't be widely used because of high cost and technical requirements.

The advanced maternal age is the most convincing clinical factor of pregnancy loss because of age-related meiotic errors in oogenesis (Grande et al., 2012; Hardy et al., 2016). However, it is unclear whether all the aneuploid karyotypes of POC are more frequent in women of advanced age. Another possible factor is maternal history of RPL. However, a decreased chromosomal aneuploid rate of POC in women with RPL has been reported in some but not all studies (Ogasawara et al., 2000; Morikawa et al., 2004; Sullivan et al., 2004; Goldstein et al., 2017). In addition, pregnancy loss with different chromosomal karyotypes may have disparate developmental potentials (Andrews et al., 1984; Minelli et al., 1993). Ultrasonographic findings of pregnancy loss show a range of development arrest stages: an empty sac with or without yolk sac, having little evidence of an embryo or a proper crown-rump length (CRL). Several studies have reported that the presence of a fetal pole or fetal cardiac activity related to the frequency of chromosomal abnormalities in pregnancy loss (Munoz et al., 2010; Cheng et al., 2014; Zhang et al., 2014; Romero et al., 2015). But two other studies had not found a relation between ultrasound findings and karyotypes (Bessho et al., 1995; Coulam et al., 1997).

Given the conflicting results of existing studies, we conducted a retrospective cohort study to investigate the associations between the molecular karyotypes of POC detected by CMA with clinical features, including the maternal age, history of pregnancy loss and the ultrasound findings.

MATERIALS AND METHODS

We conducted a retrospective, hospital-based cohort study at Guangzhou Women and Children's Medical Center, a tertiary referral hospital in South China. The study protocol was approved by the ethics committee of the institute (2020-15001). In the cohort, women who were treated for pregnancy loss and underwent CMA test in our hospital between May 2016 and May 2020 were included. All patients provided a written informed consent for the tests and the inclusion of results in research. All women were clinically confirmed pregnancy by transvaginal ultrasound that detected an intrauterine gestational sac. Pregnancy loss was diagnosed by transvaginal ultrasound and blood β human chorionic gonadotropin according to the guideline (Doubilet et al., 2013). All patients underwent expectant management, or medical management (Mifepristone/Misoprostol) or dilation and curettage after the diagnosis of pregnancy loss. Fresh POC specimens were collected and villous tissue was carefully separated for the CMA detection. Maternal peripheral blood was obtained for the quantitative fluorescent-polymerase chain reaction (QF-PCR).

DNA was extracted using QIAamp DNA Blood and Tissue kits (Qiagen, Dusseldorf, Germany). All samples were tested for maternal cell contamination using QF-PCR based on short tandem repeat (STR) markers for chromosomes 13, 18, 21, and X, Y (Diego-Alvarez et al., 2005; Nagan et al., 2011). The CMA platform used Cyto Scan 750 K Array (Affymetrix Inc., Santa Clara, CA, United States) containing 550,000 oligonucleotide probes and 200,436 single nucleotides polymorphic (SNP) probes. Data were visualized and analyzed with the chromosome analysis Suite (ChAS) software (Affymetrix, Santa Clara, CA, United States) based on the GRCh37/hg19 assembly.

Demographic and baseline clinical data such as maternal age, history of pregnancy loss, gestational age of pregnancy loss and whether or not presenting vaginal bleeding was obtained from the clinical records. Ultrasonographic findings such as size of sac, with or without a yolk sac and CRL were also extracted from ultrasonic reports. Maternal age was classified into two groups: $\leq$35 and >35 years. With regard to history of previous pregnancy loss, the subjects were divided into two groups: sporadic (precious pregnancy loss < 2) and recurrent (precious pregnancy loss $\geq$2) pregnancy loss according to the guideline (The Eshre Guideline Group on RPL et al., 2018). According to gestational age while the embryo or fetus demising, the subjects were divided into two groups: <11 gestational weeks and $\geq$11 gestational weeks. The subjects were divided into two groups according to whether there was vaginal bleeding and whether yolk sac and embryo were found on ultrasound scan. On the basis of the embryonic/fetal size detected by ultrasound at pregnancy loss, the patients were divided into two groups: CRL $\leq$ 20 mm and >20 mm.

Clinical features were compared between the euploidy and aneuploidy groups by χ^2 test or adjusted χ^2 test. We further limited the analysis to subjects with aneuploidy to explore the associations that assumed to exist between specific aneuploidy karyotype with clinical features. P value < 0.05 was considered

statistically significant. All statistical analyses were performed using Statistical Package for the Social Sciences for Windows, version 12.0 (SPSS, Inc., Chicago, IL, United States).

RESULTS

A total of 1,102 single pregnant women with pregnancy loss were included in the cohort. CMA test of fresh POC samples was successful in all cases, with 471 (42.74%) chromosomal euploid and 631 (57.26%) chromosomal aneuploid detected. The mean age of the euploid and aneuploid pregnancy loss women were 31.30 years (range 20–48 years) and 32.51 years (range 18–45 years) ($P < 0.001$), respectively. The mean gestational ages of the euploid and aneuploid groups were 11.1 weeks (range 4–34 weeks) and 9.7 weeks (range 4–28 weeks) ($P < 0.001$), respectively.

Table 1 illustrates euploid and aneuploid rates according to maternal age, gestational age, history of pregnancy loss, vaginal bleeding, and ultrasonographic features. The aneuploid rate was significantly higher in the women >35 years than in those ≤ 35 years ($P < 0.001$). There was no statistical difference in the rate of aneuploid between the sporadic and RPL ($P = 0.404$). The aneuploid rate of the group of pregnancy loss < 11 gestational weeks was statistically higher than those ≥11 gestational weeks ($P = 0.044$). The chromosomal aneuploid rate was not statistically different between the group presenting or not vaginal bleeding ($P = 0.334$). With respect to ultrasonographic features, the results showed that the rates of chromosomal aneuploid were significantly higher in subjects with yolk sac or embryo than in those without ($P < 0.001$, $P = 0.001$). In addition, the chromosomal aneuploid rate was significant higher in the group of CRL ≤ 20 mm than in the group of CRL >20 mm ($P < 0.001$).

TABLE 1 | The comparison of clinical characteristics between euploid and aneuploid pregnancy loss.

		Euploid *n* = 471 (42.74%)	Aneuploid *n* = 631 (57.25%)	P
Maternal age (years)	≤35	391 (46.44)	451 (53.56)	<0.001
	>35	68 (27.76)	177 (72.24)	
Gestational age (week)	<11	287 (40.37)	424 (59.63)	0.044
	≥11	173 (46.76)	197 (53.24)	
History of pregnancy loss	Sporadic	397 (42.37)	540 (57.63)	0.404
	Recurrent	65 (46.1)	76 (53.9)	
Vaginal bleeding	Yes	153 (40.58)	224 (59.42)	0.334
	No	305 (43.63)	394 (56.37)	
Yolk sac	Yes	307 (39.51)	470 (60.49)	<0.001
	No	60 (64.52)	33 (35.48)	
Embryo	Yes	285 (37.95)	466 (62.05)	0.001
	No	72 (52.94)	64 (47.06)	
CRL (mm)	≤20	100 (32.05)	212 (67.95)	<0.001
	>20	42 (53.16)	37 (46.84)	

CRL, crown-rump length.

Figure 1 displays aneuploid spectrum detected in the cohort, the most common aneuploid karyotype was trisomy 16 following with 45, X and triploid. Autosomal trisomy accounted for 64.82% (409/631) in the all POC aneuploidy. It is noteworthy that almost all chromosome trisomy or monosomy was found except for chromosomes 1 and 19.

We then conducted subgroup analysis to explore the associations between the aneuploid karyotype and the clinical features (**Table 2**). The frequency of viable autosomal trisomy was significantly increased in the women >35 years old ($P = 0.019$), gestational age ≥11 weeks ($P < 0.001$), and CRL >20 mm

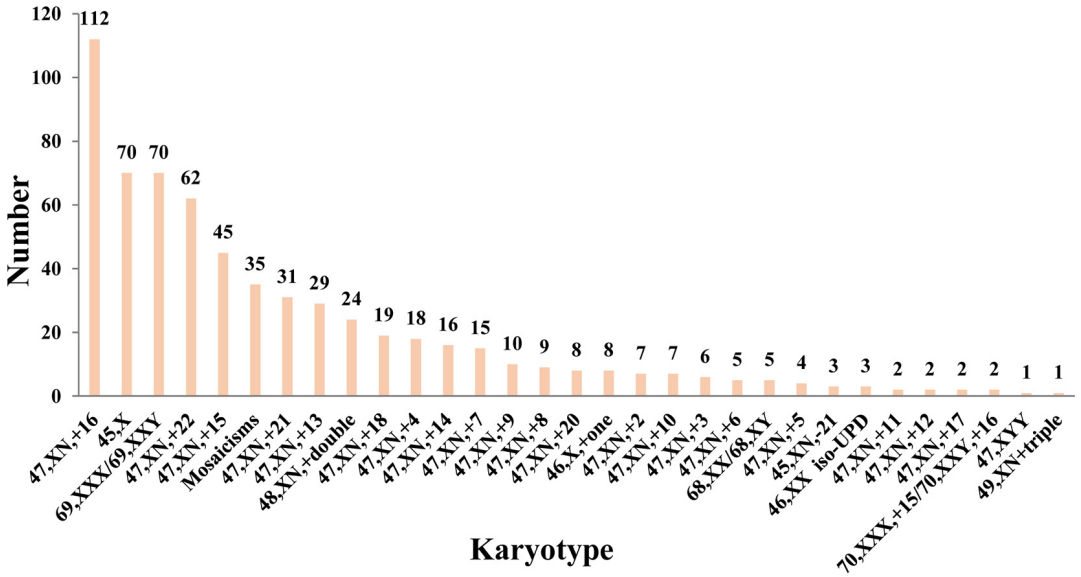

FIGURE 1 | Chromosomal aneuploid spectrum of POC in 631 pregnancy loss.

TABLE 2 | The association between the frequency of chromosomal aneuploidy with clinical characteristics.

		Maternal age			History			Gestational Age			CRL		
Karyotype	**Number**	**≤35 Y**	**>35 Y**	**P**	**Sporadic**	**Recurrent**	**P**	**<11 W**	**≥11 W**	**p**	**≤20 mm**	**>20 mm**	**p**
Viable autosomal trisomy[a]	79	48 (10.64)	31 (17.51)	**0.019**	71 (13.15)	6 (7.89)	0.195	26 (6.13)	53 (26.9)	**<0.001**	19 (8.96)	10 (27.03)	**0.002**
Sex chromosome abnormalies[b]	71	64 (14.19)	7 (3.95)	**<0.001**	67 (12.41)	3 (3.95)	**0.03**	18 (4.25)	53 (26.9)	**<0.001**	17 (8.02)	16 (43.24)	**<0.001**
Non-viable autosomal trisomy[c]	330	224 (49.67)	106 (59.89)	**0.021**	281 (52.04)	45 (59.21)	0.241	266 (62.74)	58 (29.44)	**<0.001**	133 (62.74)	2 (5.41)	**<0.001**
Two or more chromosome anomalies[d]	33	19 (4.21)	14 (7.91)	0.062	28 (5.19)	5 (6.58)	0.613	28 (6.84)	5 (2.54)	**0.028**	5 (2.36)	1 (2.7)	0.9
Triploid	77	67 (14.86)	10 (5.65)	**0.002**	62 (11.48)	12 (15.79)	0.279	55 (12.97)	20 (10.15)	0.316	27 (12.74)	8 (21.62)	0.151
Mosaicisms	35	26 (5.76)	9 (5.08)	0.738	28 (5.19)	5 (6.58)	0.613	27 (6.37)	8 (4.06)	0.246	11 (5.19)	0 (0)	0.156

CRL, crown-rump length.
[a] *Trisomy 13, 18, or 21.*
[b] *45, X; 47, XXY.*
[c] *Autosomal trisomy excepting for Trisomy 13, 18, or 21.*
[d] *48, XN, +double and 49, XN, +triple.*
The bold values mean statistically different results.

($P = 0.002$), but was not associated with the history of pregnancy loss ($P = 0.195$). The rate of sex chromosome abnormalities was statistically increased in the group of maternal age ≤ 35 years old ($P < 0.001$), sporadic pregnancy loss ($P = 0.003$), gestational age ≥ 11 weeks ($P < 0.001$), and CRL > 20 mm ($P < 0.001$). The frequency of non-viable autosomal trisomy, accounted for 52.3% (330/631) of the all aneuploid karyotypes, was significantly high in the group of maternal age >35 years ($P = 0.021$), gestational age < 11 weeks ($P < 0.001$), and CRL ≤ 20 mm ($P < 0.001$), and was not associated with the history of pregnancy loss ($P = 0.241$). The frequency of two or more chromosomal abnormalities was increased in the group of <11 gestational weeks ($P = 0.028$), but not associated with maternal age ($P = 0.062$), history of pregnancy loss ($P = 0.613$), and CRL ($P = 0.9$). The rate of triploid was statistically higher in maternal age ≤ 35 years ($P = 0.002$), but was not associated with other clinical features. The frequency of mosaicisms showed no statistical difference between all those subgroups.

DISCUSSION

An increased detection rate of chromosomal aneuploidy has been reported while using CMA to analyze the POC (Dhillon et al., 2014). In present study, aneuploid rate of the POC was 57.26% detected by CMA. In accordance with previous reports (Eiben et al., 1990; Ozawa et al., 2019), our results demonstrate that pregnancy loss in women over 35 years of age is associated with a higher chromosomal aneuploid rate. However, this characteristic only presents in autosomal trisomy, which accounted for 64.54% of the aneuploidy in our series, on the contrary, the triploid and the sex chromosome abnormalities, especially 45, X, are more likely to occur in women ≤ 35 years old, and the two or more chromosome abnormalities and mosaicisms might not be related to maternal age. It is well known that chromosome trisomy mainly results from un-separated chromosome in oogenesis, which is related to advanced maternal age[13,14]. Triploid is supposed to result from incorrect ploidy at fertilization, and it may be diandry (two paternal sets) or digyny (two maternal sets) (Marton et al., 1999). Monosomy X is thought more likely to be caused by meiotic error of the father rather than the mother (Hassold et al., 1988; Segawa et al., 2017). Further research including couples' age should be conducted to confirm whether triploid and monosomy X are related to younger maternal age.

Overall, our results showed that chromosomal aneuploidy was more likely to occur in women with pregnancy loss ≤ 11 gestational weeks and CRL < 20 mm. However, this feature was just demonstrated in non-viable autosomal trisomy and two or more chromosome abnormalities, contributed 57.5% (363/631) of the aneuploid karyotypes in this cohort, which resulting in earlier embryo demise is reasonable. Meanwhile, in those viable aneuploid such as trisomy 13, 18, 21, monosomy X and 47, XXY, pregnancy loss occurs in later gestational age. And the triploid and mosaicisms showed no difference between different gestational ages of pregnancy loss.

Three previous studies reported no difference of the aneuploid rate between sporadic and RPL (Coulam et al., 1996;

Stephenson et al., 2002; Grande et al., 2012), but Ogasawara et al. (2000) and Sullivan et al. (2004) described decreased rates of chromosomal abnormalities in RPL. From out data, in overall, the aneuploid rate is no difference between sporadic and RPL, but sex chromosomal abnormalities might occur less frequently in RPL.

With regard to the correlation between ultrasound embryonic pole and chromosomal karyotype, several previous studies have reported contradictory results (Lathi et al., 2007; Munoz et al., 2010; Cheng et al., 2014; Romero et al., 2015; Ouyang et al., 2016). The discrepancy may result from the difference in population, sample size, and laboratory method. Our study separately investigated the correlation between molecular karyotype with yolk sac and embryo in a larger cohort, and the results suggest that chromosomal aneuploidy is more likely to occur in pregnancy loss with yolk sac or embryo in ultrasound scan than in those without. Pregnancy loss is a complex condition caused by multi-etiological factors, and women with very early pregnancy loss, those without yolk sac or embryo in ultrasound scan, might need to consider more in other factors than chromosomal aneuploidy.

Another noteworthy result is that among all the 409 detected trisomy, chromosome 1 and 19 were not detected in trisomy or monosomy. Through literature review, we realize that trisomy 1 has only been reported in four pregnancy loss cases (Hanna et al., 1997; Dunn et al., 2001; Banzai et al., 2004; Vicic et al., 2008), and trisomy 9 has been reported in seven pregnancy loss cases (Hoshi et al., 1997; Choi et al., 2014; Hardy et al., 2016; Ouyang et al., 2016; Segawa et al., 2017). It is unclear that why trisomy 1 and trisomy 19 occur rarely, it may be related to the mechanism of gamete meiosis. Chromosome 1 is the biggest chromosome in human, which might not be prone to chromosome non-separation in the process of gamete meiosis. All those enigmas need more research in the future.

The strengths of this study include its population-based nature and relatively large sample size. The methods for both sample retrieving and CMA testing were uniform for all the study populations, in contrast to some series including cases in different laboratories. In addition, this is the first study to extensively investigate the associations between a wide spectrum of chromosomal aneuploids and clinical features of pregnancy loss. However, our study has two limitations. Firstly, we were unable to analyze the association between gender of POC and chromosomal euploidy since gender identification of embryo/fetus is not permitted by regulations in China. Secondly, we were unable to confirm the potential impact of *in vitro* fertilization (IVF) treatment on chromosomal euploidy as data on IVF were largely missing.

In conclusion, advanced maternal age mainly relates to increased autosomal trisomy, but sex chromosome abnormalities and triploid might more likely occur in younger women. The rate of chromosomal aneuploidy might not be different in history of previous pregnancy loss except for sex chromosome abnormalities, which might be less likely to occur in RPL. Pregnancy loss without yolk sac or embryo might be less relates to embryonic aneuploidy and other factors should be emphasized in the causes of the pregnancy loss. This study not only provides evidence for patient genetic counseling and management, but also provides hints for future research in exploring the mechanism of high aneuploidy rate in human embryo.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the Guangzhou Women and Children's Medical Center (2020-15001). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CG and YH drafted the manuscript. CG and KL contributed to the design of the research. LL and XL contributed to the development of the statistical plan and statistical analysis of the data. CG contributed to the conception of the study. XD and RL contributed to data management and prepared the retrospective data for analysis. All authors reviewed, read and approved the final manuscript.

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REVIEW
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Senataxin: A New Guardian of the Female Germline Important for Delaying Ovarian Aging

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Early decline in ovarian function known as premature ovarian aging (POA) occurs in around 10% of women and is characterized by a markedly reduced ovarian reserve. Premature ovarian insufficiency (POI) affects ~1% of women and refers to the severe end of the POA spectrum in which, accelerated ovarian aging leads to menopause before 40 years of age. Ovarian reserve refers to the total number of follicle-enclosed oocytes within both ovaries. Oocyte DNA integrity is a critical determinant of ovarian reserve since damage to DNA of oocytes within primordial-stage follicles triggers follicular apoptosis leading to accelerated follicle depletion. Despite the high prevalence of POA, very little is known regarding its genetic causation. Another little-investigated aspect of oocyte DNA damage involves low-grade damage that escapes apoptosis at the primordial follicle stage and persists throughout oocyte growth and later follicle development. Senataxin (SETX) is an RNA/DNA helicase involved in repair of oxidative stress-induced DNA damage and is well-known for its roles in preventing neurodegenerative disease. Recent findings uncover an important role for SETX in protecting oocyte DNA integrity against aging-induced increases in oxidative stress. Significantly, this newly identified SETX-mediated regulation of oocyte DNA integrity is critical for preventing POA and early-onset female infertility by preventing premature depletion of the ovarian follicular pool and reducing the burden of low-grade DNA damage both in primordial and fully-grown oocytes.

Keywords: premature ovarian aging, premature ovarian insufficiency, ovarian aging, ovarian reserve, oocyte, DNA damage, oocyte quality, oxidative stress

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INTRODUCTION

Senataxin (Setx) is an RNA/DNA helicase required for multiple DNA processes including transcriptional regulation, the resolution of RNA:DNA hybrids (or R-loops) arising at transcription pause sites and DNA repair (Lavin et al., 2013; Groh et al., 2017). Regarding DNA repair, Setx is especially critical for promoting the repair of DNA damage induced by increased oxidative stress (Suraweera et al., 2007).

In humans, *SETX* mutations cause the autosomal recessive neurodegenerative disorder, ataxia with oculomotor apraxia type-2 (AOA2). AOA2 belongs to a group of rare autosomal recessive cerebellar ataxias (ARCAs), which also include Friedreich ataxia and ataxia-telangiectasia (A-T; Palau and Espinós, 2006). AOA2 is characterized primarily by cerebellar atrophy with prominent

gait ataxia, a peripheral sensorimotor neuropathy, areflexia, and elevated levels of α-fetoprotein. Onset of symptoms is usually early, occurring between 10 and 20 years of age (Lavin et al., 2013). Interestingly, *SETX* mutations may also cause up to three other neurological conditions including the less common autosomal dominant neurodegenerative disorder, juvenile amyotrophic lateral sclerosis (ALS4), a form of juvenile ALS characterized by limb weakness, severe muscle wasting, normal sensation, and pyramidal tract signs (Lavin et al., 2013). Intriguingly, therefore, different mutations in the same *SETX* gene can give rise to several distinct disorders depending on their nature and localization.

Separate from its role in neurological disorders, more recent research has begun to shed light on a lesser-known role of Setx in reproduction (Becherel et al., 2013, 2019; Subramanian et al., 2020, 2021). Setx has been shown to be critical for male fertility through maintaining genomic integrity during spermatogenesis (Becherel et al., 2013). This arises because DNA double-strand breaks (DSB) induced during the process of reciprocal recombination remains unrepaired in the absence of Setx thereby activating a pachytene checkpoint that eliminates spermatocytes (Becherel et al., 2013). This function is conserved in humans as spermatogenesis in men with AOA2 is severely compromised and associated with persistent DNA breaks in spermatocytes (Becherel et al., 2019). Notably, there have been case reports of compromised reproductive function in females with AOA2 (Lynch et al., 2007; Gazulla et al., 2009) suggesting that *SETX* may also be important for ovarian function. Here, I review very recent data from the mouse model revealing that Setx is important for protecting DNA integrity in oocytes but unlike males, is dispensable for oogenesis and exhibits a unique role in slowing ovarian aging.

VULNERABILITY OF OOCYTES TO OXIDATIVE STRESS AND DNA DAMAGE DURING AGING

Females are born with a finite complement of germ cells, which diminish throughout postnatal life becoming almost completely exhausted by the time of the menopause (Oktem and Urman, 2010; Wallace and Kelsey, 2010). During fetal life, germ cells become surrounded by flattened somatic cells forming primordial follicles, which constitute the most abundant follicle-type in the ovary (Oktem and Urman, 2010). The ovarian reserve of oocytes is therefore defined by the primordial follicle reservoir. Cohorts of primordial follicles undergo spontaneous activation and thereafter progress over a period of 2–3 months in humans (2–3 weeks in mice) through primary, secondary, and antral stages of development concomitantly with growth of their contained oocytes (**Figure 1A**; Oktem and Urman, 2010; Greaney et al., 2018). Within follicles, oocytes are arrested at the dictyate stage of prophase of the first meiotic division (Oktem and Urman, 2010; Greaney et al., 2018). Since primordial follicles are laid down before birth, some oocytes remain arrested for up to 4–5 decades in humans before resuming development.

Cellular oxidative stress is harmful to key cellular components and levels increase with aging (Lim and Luderer, 2011). In keeping with this, oocytes from older females exhibit increased levels of reactive oxygen species (ROS; Subramanian et al., 2020). Increased oxidative stress is therefore considered a leading cause of the age-related decline in oocyte quality (Tarin, 1996; Kasapoğlu and Seli, 2020; Homer, 2021). Since mitochondrial oxidative phosphorylation is the major source of cellular ROS, mitochondrial dysfunction is a focal point of oocyte aging (May-Panloup et al., 2016; Homer, 2020, 2021; Kasapoğlu and Seli, 2020).

Recent *in vivo* findings reinforce the critical importance of oxidative stress in female reproductive aging. Levels of the essential cellular co-factor, nicotinamide adenine dinucleotide (NAD^+), were shown to decline with aging in oocytes and, importantly, restoring oocyte NAD^+ reserves by feeding of an NAD^+ precursor could rejuvenate oocytes and restore fertility in older females (Bertoldo et al., 2020). NAD^+ in turn regulates oxidative stress in oocytes at least in part *via* a family of NAD^+-dependent deacetylases and deacylases known as sirtuins (SIRT1-7); oocytes from mice over-expressing SIRT2 exhibit delayed aging associated with reduced ROS (Bertoldo et al., 2020), while deletion from oocytes of another sirtuin, SIRT1, results in increased oxidative stress that compromises embryonic development leading to early-onset female infertility (Iljas et al., 2020). Interestingly, the importance of NAD^+ for oocyte quality extends beyond sirtuin-support roles as it is also critical for enabling oocytes to retain maximum cytoplasmic reserves during meiotic division (Wei et al., 2020). It has also been found that expression of enzymes responsible for producing coenzyme Q10 (CoQ10), a major antioxidant and component of the mitochondrial electron transport chain, is reduced in oocytes from aged mouse and human oocytes (Ben-Meir et al., 2015). Moreover, supplementation of aging female mice with CoQ10 markedly improved oocyte developmental competence thereby boosting fertility (Ben-Meir et al., 2015).

Since oxidative stress is known to induce DNA damage (Suraweera et al., 2007; Subramanian et al., 2020), progressive mitochondrial deterioration and increasing ROS leaves oocytes uniquely vulnerable to DNA damage during their protracted dormancy in ovaries. In line with this, oocytes from older female mice and women exhibit increased levels of DNA DSBs (Titus et al., 2013).

TWO FATES FOR OOCYTES FOLLOWING *IN VIVO* ACQUIRED DNA DAMAGE

Extensive levels of DSBs in primordial-stage oocytes secondary to severe chemotherapy/irradiation-induced injury in pre-pubertal female mice activate TAp63 *via* ATM and Chk2 kinases (Suh et al., 2006; Livera et al., 2008; Gonfloni et al., 2009; Bolcun-Filas et al., 2014; Tuppi et al., 2018). This then activates Puma and Noxa thereby triggering primordial follicle apoptosis, depletion of the follicular pool and infertility (Kerr et al., 2012). Thus, extensive DNA damage to oocytes within primordial

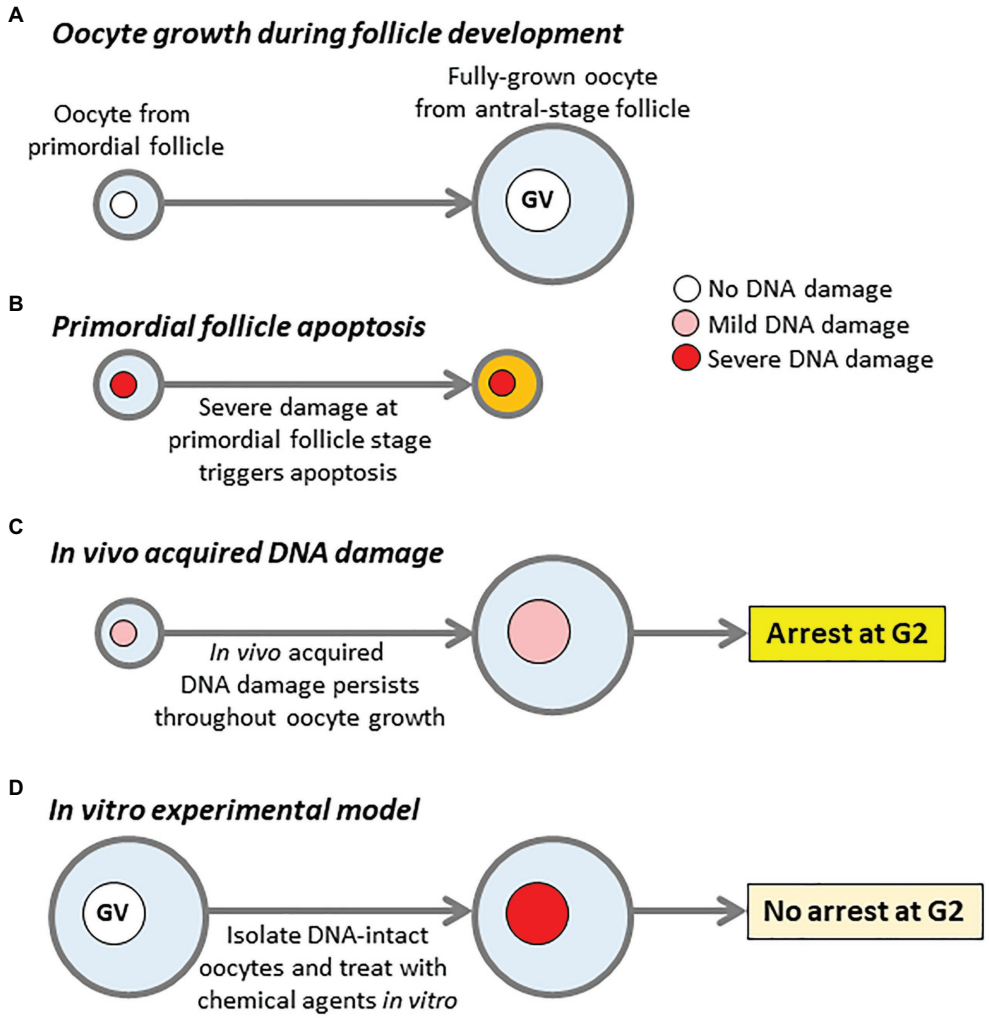

FIGURE 1 | DNA damage models and impact of DNA damage on oocytes. **(A)** Oocyte growth during follicle development. Oocytes undergo an extensive period of growth, while follicles develop from primordial to antral stages. Large antral-stage follicles contain fully-grown oocytes arrested at first meiotic prophase, identifiable by the presence of an intact germinal vesicle (GV). **(B)** Oocytes within primordial follicles, which sustain severe DNA damage trigger a TAp63-dependent pathway resulting in follicular apoptosis. **(C)** DNA damage sustained *in vivo* which does not trigger primordial follicle apoptosis persists throughout oocyte growth resulting in damaged fully-grown oocytes within antral-stage follicles. **(D)** The canonical model for studying the effects of DNA damage in fully-grown oocytes. Fully-grown oocytes with intact DNA are isolated from young female mice and subject to acute treatment with agents such as chemotherapy drugs to induce severe levels of damage. Note that the key differences between *in vivo* damage **(C)** and the *in vitro* experimental model **(D)** are, firstly, *in vivo* damage is much milder than *in vitro*-induced damage and, secondly, *in vivo* damage persists for weeks to months during oocyte growth vs. hours in the case of the *in vitro* model.

follicles triggers follicular apoptosis (**Figure 1B**) and severely threatens the ovarian reserve.

Severe depletion of the primordial follicle pool leading to menopause before 40 years of age – at least 10 years earlier than the average age of the menopause – is known as premature ovarian insufficiency (POI; Webber et al., 2016). A less severe form of accelerated decline in ovarian reserve is premature ovarian aging (POA), also known as occult POI (Gleicher et al., 2011). POI and POA can be brought on by gonadotoxic treatment such as chemotherapy but in most cases, the underlying cause is unknown.

Both primordial and fully-grown oocytes from reproductively aged females exhibit higher levels of DNA breaks than young oocytes (Titus et al., 2013). Notably, these physiological levels of damage seen with aging are substantially less severe than those induced by exogenous noxious agents like chemotherapy (Subramanian et al., 2020). Collectively, this suggests that physiological levels of primordial oocyte injury – as opposed to unusually severe drug- or radiation-induced damage – do not necessarily trigger TAp63-mediated apoptosis. Furthermore, this milder damage can persist throughout folliculogenesis and oocyte growth, culminating in damaged fully-grown oocytes (see **Figure 1C**). It is entirely plausible that primordial follicles with low levels of damage that escape apoptosis can persist to the antral stage since TAp63 expression wanes at more advanced

stages of follicular development (Suh et al., 2006). In keeping with this, high irradiation doses (0.45 Gy) fully activate TAp63 causing almost complete annihilation of the follicular pool, whereas most primordial follicles survive and TAp63 is only partially activated by milder degrees of injury induced by low doses of irradiation (0.1 Gy; Suh et al., 2006).

The combination of increased ROS and compromised DNA repair capacity associated with reduced expression of DNA repair genes predispose to DNA damage in aged oocytes (Titus et al., 2013; Winship et al., 2018). This has two major consequences, firstly, damage-induced attrition of the follicular pool due to TAp63-mediated primordial follicle depletion and, secondly, persistence of low levels of DNA damage throughout oocyte growth. Such damaged fully-grown oocytes could potentially mature into fertilizable eggs thereby posing risks to future offspring.

SETX IS CRITICAL FOR REPAIRING PHYSIOLOGICAL LEVELS OF AGING-INDUCED DNA DAMAGE

Since aging predisposes to DNA damage in oocytes, any additional compromise to DNA repair capacity would be expected to exacerbate damage and accelerate depletion of the follicular pool. This model based on DNA damage therefore provides at least one mechanistic basis for POI and POA (Turan and Oktay, 2020).

We recently studied female mice carrying either heterozygous ($SETX^{+/-}$) or homozygous ($SETX^{-/-}$) deletion of *SETX*. We found that at young ages, neither follicle-enclosed oocytes within ovaries nor isolated fully-grown oocytes from *SETX* mutants exhibited increased DSBs compared with wild-type mice (Subramanian et al., 2020). Moreover, young mutant females produced normal yields of fully-grown oocytes and these oocytes underwent maturation with normal spindle assembly and chromosome segregation indistinguishable from wild-type oocytes. Thus, at young ages, loss of Setx has no detrimental effects showing that Setx is dispensable in oocytes, at least at young ages. This contrasts with males in which, loss of Setx induces complete sterility due to failure to repair DSBs induced during reciprocal recombination (Becherel et al., 2013). Exactly why oocytes are able to complete repair of recombination-induced DSBs but spermatocytes cannot remains to be determined.

We then studied aging female mice bearing in mind that female mice typically exhibit overt signs of reproductive aging from around 12 months of age when both oocyte numbers and developmental competence decline markedly (Pan et al., 2008; Greaney et al., 2018; Iljas et al., 2020). Using the DSB marker, γH2AX, we found that by 8 months of age, there were low levels of damage in $SETX^{+/+}$ ovaries as expected (Subramanian et al., 2021). In contrast, follicle-enclosed oocytes within $SETX^{-/-}$ ovaries were already showing over 3-fold higher levels of damage. Moreover, 8-month-old $SETX^{-/-}$ follicles showed similarly elevated levels of TUNEL staining (Subramanian et al., 2021) altogether indicating that in the absence of Setx, oocytes within ovaries prematurely accumulated DNA damage that triggered apoptosis. Entirely in keeping with premature depletion of the ovarian reserve, 8-month-old $SETX^{-/-}$ ovaries contained less than half the numbers of all follicle stages seen in $SETX^{+/+}$ ovaries and produced less than half the numbers of fully-grown oocytes following hormonal priming (Subramanian et al., 2021). Collectively, therefore, these data show that in oocytes, Setx's role is restricted to aging and prevents early-onset accumulation of DNA damage that would otherwise induce premature depletion of the ovarian reserve.

At 4 months of age, isolated fully-grown $SETX^{+/+}$ and $SETX^{-/-}$ oocytes had low levels of DNA damage (Subramanian et al., 2020, 2021). This remained largely unchanged at 8 months of age for $SETX^{+/+}$ oocytes contrasting sharply with a 3–4-fold higher increase in $SETX^{-/-}$ oocytes consistent with the increased damage observed at this age in growing follicle-enclosed $SETX^{-/-}$ oocytes. Importantly, these data are the first to clearly demonstrate that physiological levels of damage brought about by natural aging – as opposed to extreme levels of damage induced experimentally by noxious agents – can, and does, persist throughout the growth stage of oocytes resulting in damaged fully-grown oocytes.

In summary, loss of Setx results in premature accumulation of DNA damage in oocytes during aging. Since damage is minimal in young $SETX^{-/-}$ oocytes, increased damage in older $SETX^{-/-}$ oocytes is not due to loss of Setx *per se* but the consequence of superimposed aging-related phenomenon. Given Setx's role in DNA repair, it is very likely that during aging, oocyte DNA acquires DSBs that are constantly being repaired by Setx-dependent mechanisms.

Due to the direct relationship between aging and oxidative stress, and Setx's importance in repairing oxidative stress-induced damage (Suraweera et al., 2007), we studied an *in vitro* model of increased oxidative stress (Subramanian et al., 2020). We found that long-term *in vitro* culture markedly increased ROS levels in oocytes (Subramanian et al., 2020). Importantly, young $SETX^{-/-}$ oocytes with inherently low levels of damage, acquired increased damage after ROS levels had increased by the end of prolonged *in vitro* culture whereas $SETX^{+/+}$ oocytes showed minimal damage. Significantly, preventing ROS increase by co-culture in the antioxidant, N-acetyl cysteine, prevented increased damage in young $SETX^{-/-}$ oocytes (Subramanian et al., 2020). This strongly supports that Setx is critical for repairing ROS-induced damage in oocytes. Furthermore, we showed that ROS levels are increased *in vivo* in $SETX^{-/-}$ oocytes after 8 months of natural aging (Subramanian et al., 2020).

Altogether, these data indicate that age-induced increases in oxidative stress constantly induce DNA damage that Setx-related mechanisms police and repair (**Figure 2**). We reason that by 12 months of age in mice, further increases in oxidative stress combined with compromised repair capacity ultimately tip the balance resulting in the higher basal levels of damage seen by this age in wild-type mouse oocytes (Titus et al., 2013).

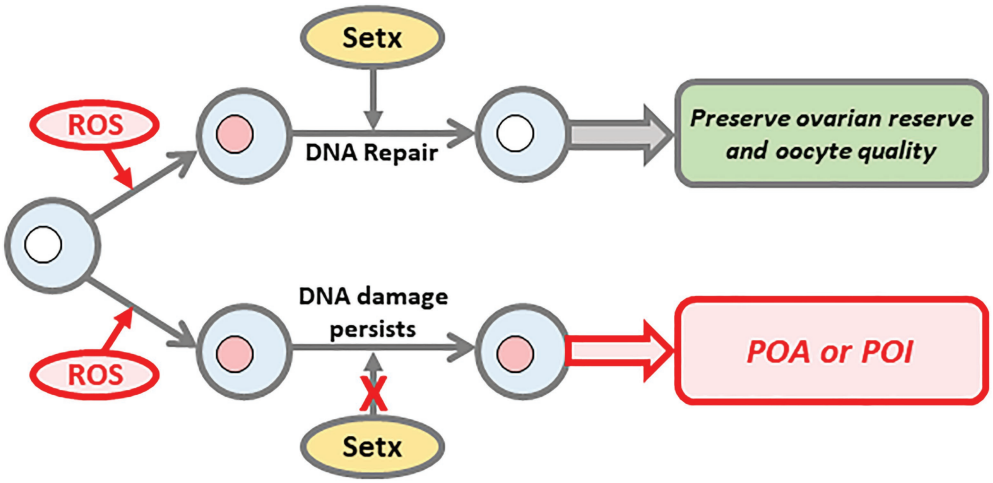

FIGURE 2 | Senataxin (Setx) promotes DNA repair to preserve ovarian reserve and oocyte quality. During their protracted arrest in the ovary, oocytes are increasingly exposed to aging phenomenon such as oxidative stress, which induces DNA damage. Setx is required to repair this damage thereby preserving both oocyte number and quality (upper pathway). In contrast, on a background of compromised Setx function, DNA damage persists ultimately resulting in premature depletion of the oocyte pool and decline in oocyte quality (lower pathway).

THE *SETX* MUTANT MODEL PROVIDES NEW INSIGHT INTO *IN VIVO* ACQUIRED DNA DAMAGE THAT PERSISTS THROUGHOUT OOCYTE GROWTH

The SETX Model for Studying Persistent Oocyte DNA Damage

The foregoing indicate that damage acquired *in vivo* can trigger primordial follicle apoptosis but that in some cases, follicles escape apoptosis leading to damage that persists throughout oocyte growth leading to damaged fully-grown oocytes (**Figure 1C**). While the molecular basis underpinning primordial follicle apoptosis has been extensively investigated *via* experiments involving exogenously induced injury, little was known about how fully-grown oocytes respond to physiological damage that had persisted throughout the oocyte's growth stage. The latter type of injury cannot readily be replicated by exogenous treatments since this typically produces severe levels of damage that trigger apoptosis (**Figure 1B**). Older *SETX* mutant females in which milder damage arises in oocytes, and persists throughout growth, therefore provides a very powerful model that not only enabled this to be definitively characterized as a distinct form of injury but also to investigate related molecular mechanisms for the first time. A major question was whether fully-grown oocytes that acquired damage *in vivo* could mount a cell-cycle response that would block meiotic maturation; this was a critically important question because if oocytes lacked this so-called DNA damage checkpoint, this could potentially result in the production of embryos with damaged DNA that would jeopardize pregnancy outcome. There is preliminary evidence in humans that oocyte DNA damage may well influence reproductive potential since women undergoing IVF treatment who produce oocytes with high levels of DSBs appear to have reduced pregnancy success rates (Astbury et al., 2020).

The Molecular Basis of Oocyte Prophase Arrest and Oocyte Maturation

Before delving further into cell-cycle responses of *in vivo*-damaged oocytes, it will first be important to outline some key regulatory pathways. Oocytes within follicles are arrested at the dictyate stage of prophase, which, from a cell-cycle point of view, is equivalent to a late G2-phase (Solc et al., 2010; Adhikari and Liu, 2014; Greaney et al., 2018). Dictyate-arrested oocytes are identifiable by the presence of an intact nucleus known as the germinal vesicle (GV; Solc et al., 2010; Greaney et al., 2018). Oocyte maturation is initiated by activation of the master cell-cycle regulator, cyclin-dependent kinase 1 (Cdk1; also known as maturation promoting-factor or MPF), a heterodimer comprised of a catalytic Cdk1 subunit and a cyclin B1 co-activator. First meiotic maturation (MI) begins with GV breakdown (GVBD) marking entry into M-phase of MI and concludes with an extremely asymmetric division marked by extrusion of a very small first polar body (PB) into which half of the oocyte's chromosomes are segregated (Greaney et al., 2018; Wei et al., 2018).

Maintenance of G2-arrest during the oocyte's protracted prophase arrest (lasting up to 4–5 decades in humans) therefore revolves around preventing Cdk1 activation. This occurs *via* inhibitory phosphorylation of Cdk1 by Wee1/Myt1 kinases (Wee1B in oocytes), which is counteracted by Cdc25 phosphatases (Han et al., 2005; Solc et al., 2010; Adhikari and Liu, 2014; Adhikari et al., 2016). Critically, in oocytes, Cdk1 inhibition also involves proteolysis of the Cdk1 activator, cyclin B1, orchestrated by the Cdh1-activated anaphase-promoting complex (APC-Cdh1; Holt et al., 2011; Homer, 2013). Hence, prophase

arrest is dependent upon Wee1B and APC-Cdh1 and is counteracted by Cdc25B.

Oocytes within follicles experience strong Cdk1 suppression due to Wee1B-mediated inhibition, which is further reinforced by inherently low levels of Cdk1 and cyclin B1 in incompletely grown oocytes (Kanatsu-Shinohara et al., 2000). Oocytes within antral-stage follicles have become fully grown and now contain adequate levels of cyclin B1 to support Cdk1 activation provided inhibitory Wee1B-mediated Cdk1 inhibition can be lifted. Release from Wee1B-induced inhibition is induced by the surge of luteinizing hormone (LH), which leads to suppression of Wee1B and activation of Cdc25B culminating in Cdk1 activation (Solc et al., 2010). The LH surge, therefore, triggers ovulation as well as oocyte maturation leading to the release of a mature metaphase II (MII)-arrested oocyte (or egg) capable of undergoing fertilization.

Identification of a Novel DNA Damage Response in Oocytes

In somatic cells, DNA damage at G2-phase triggers a checkpoint *via* the DNA damage response (DDR), which delays entry into M-phase thereby enabling DNA to be repaired (Harper and Elledge, 2007; Carroll and Marangos, 2013). The canonical DDR is a phosphorylation cascade involving the apical kinases, ATM, and ATR, as well as downstream checkpoint kinases, Chk1/Chk2 (Harper and Elledge, 2007; Carroll and Marangos, 2013). The latter result in inhibition of Cdc25 phosphatase thereby preventing Cdk1 activation. Thus, the canonical DDR is a phosphorylation-centerd response that blocks entry into M-phase by preventing activation of the Cdk1 activator, Cdc25B.

To determine whether damaged fully-grown oocytes mount a DDR, the standard approach has been to isolate fully-grown oocytes with intact DNA from young female mice and to subject these isolated GV-stage oocytes to exogenous treatments such as chemotherapy or radiation *in vitro* (**Figure 1D**; Marangos and Carroll, 2012; Collins et al., 2015; Marangos et al., 2015; Collins and Jones, 2016; Mayer et al., 2016). Surprisingly, and in contrast to somatic cells, oocytes carrying severe damage induced by such treatments failed to undergo a G2-arrest and readily progressed into M-phase (**Figure 1D**; Marangos and Carroll, 2012; Collins et al., 2015; Marangos et al., 2015; Collins and Jones, 2016; Mayer et al., 2016). From this it was inferred that oocytes may lack a checkpoint at the G2-M boundary (Marangos and Carroll, 2012). It appears that the inability to respond to sudden induction of injury is due to an inherently ineffectual ATM-mediated phosphorylation pathway (Marangos and Carroll, 2012). Intriguingly, although entry into M-phase was not impaired by DNA damage, oocytes subsequently arrested in M-phase due to activation of the spindle assembly checkpoint (SAC) thereby preventing completion of MI (Collins et al., 2015; Marangos et al., 2015; Collins and Jones, 2016). The SAC incorporates key players such as Mad2 and BubR1 (encoded by *BUB1B*) and prevents chromosome mis-segregation by delaying anaphase-onset in the presence of chromosomes that have not become properly attached to spindle microtubules (Musacchio and Salmon, 2007; Homer, 2011). Not surprisingly, poorer quality oocytes from aged women and mice characterized by high aneuploidy rates have reduced expression of *MAD2* and *BUB1B* (Steuerwald et al., 2001, 2007; Pan et al., 2008; Riris et al., 2014). Thus, acutely induced DNA injury does not trigger a DDR at the G2-M boundary in oocytes but activates the SAC during M-phase.

It is important at this stage to revisit salient characteristics of *in vivo* acquired DNA damage in fully-grown oocytes and how it differs from acute damage induced experimentally *in vitro*. Firstly, the extent of *in vivo* damage is very mild compared to that induced by drug or radiation treatment (**Figures 1C**,**D**); had it been severe, primordial follicle apoptosis would have been triggered thereby pre-empting the emergence of fully-grown oocytes (**Figure 1B**). Secondly, and highly significantly, *in vivo* damage persists for a prolonged period during the oocyte growth phase (**Figure 1C**) – lasting 2–3 weeks in mice and 2–3 months in humans (Gosden and Lee, 2010; Greaney et al., 2018) – whereas *in vitro* induced damage is acute and short-lived (**Figure 1D**). Since *in vivo* exposure to injurious agents (e.g., oxidative stress; environmental toxins) occurs over prolonged periods throughout the individual's lifetime, it is highly implausible for an oocyte to be completely free of damage throughout its entire growth phase within ovarian follicles *in vivo* and then to suddenly acquire very severe damage only at the very transient period when ovulation is about to occur. Previous experimental models involving short-term *in vitro* treatment of DNA-intact fully-grown oocytes (**Figure 1D**) do not therefore replicate the longer-term pattern of DNA damage occurring *in vivo*. In contrast, *SETX* mutant oocytes acquire modest levels of damage *in vivo* brought on by age-related increases in oxidative stress and not because of sudden exposure to artificial treatment (Subramanian et al., 2020). *SETX* mutant oocytes therefore provided a unique opportunity to study fully-grown oocytes that have acquired comparatively mild levels of DNA damage *in vivo*.

Using this model, we unexpectedly found that GVBD rates in $SETX^{-/-}$ oocytes from 8-month-old females, which possessed increased DNA damage, was severely reduced (Subramanian et al., 2020). This indicated that *in vivo* acquired DNA damage does induce an arrest at the G2-M boundary (**Figure 1C**). Because artificially induced short-lived injury in fully-grown oocytes does not impair the G2-M transition (**Figure 1D**; Marangos and Carroll, 2012; Collins et al., 2015; Marangos et al., 2015; Collins and Jones, 2016; Mayer et al., 2016), this suggested that a longer duration of injury may be critical for allowing this unique DDR to be mounted in oocytes. This was indeed the case since the same *in vitro* chemotherapy-induced damage, which did not impair meiotic maturation immediately after damage induction, did lead to a robust G2-arrest if a delay was imposed following chemical treatment (Subramanian et al., 2020).

Intriguingly, we found that this novel delayed-response DDR in oocytes does not suppress Cdk1 activation *via* canonical phosphorylation-related pathways but by increased APC-Cdh1-mediated proteolysis of cyclin B1 (Subramanian et al., 2020). Increased APC-Cdh1 activity in turn is the consequence of increased activity of the APC-Cdh1 activator,

Cdc14B, and reduced levels of the APC-Cdh1 inhibitor, Emi1 (Subramanian et al., 2020).

PREMATURE DECLINE IN FERTILITY AND ACUTE SENSITIVITY OF OOCYTES TO SETX COMPROMISE

The foregoing showed that in mice, compromised *SETX* function led to premature increases in oocyte DNA damage *in vivo* and to early-onset reduction in ovarian follicle numbers. We then asked how this might impact fertility during natural aging. For the first 6 months of a 12-month-long breeding trial, mean litter sizes and cumulative numbers of pups produced by $SETX^{-/-}$ and wild-type females were indistinguishable (Subramanian et al., 2021). From 7 months of age onwards, however, coinciding with the observed increase in *in vivo* DNA damage in $SETX^{-/-}$ oocytes, $SETX^{-/-}$ females produced significantly fewer pups than wild-types and this difference was sustained throughout the remainder of the trial to 13 months of age (Subramanian et al., 2021). This decline in fertility was due to reduced numbers of fully-grown oocytes as well as reduced oocyte quality manifested as failure of maturation. Thus, compromised Setx function predisposes to aging-induced DNA damage, which leads to a premature decline in female fertility due to reduced ovarian reserve and compromised oocyte quality.

We also studied heterozygous females carrying one mutated *SETX* gene ($SETX^{+/-}$ females) and surprisingly found that all reproductive parameters – oocyte DNA damage, follicle numbers, follicle atresia, numbers of fully-grown oocytes, oocyte maturation, and litter sizes – in 8-month-old $SETX^{+/-}$ females were equally impaired as in 8-month-old $SETX^{-/-}$ females (Subramanian et al., 2021). Further investigation identified that Setx expression from the remaining intact *SETX* gene in $SETX^{+/-}$ oocytes could only marginally compensate for the missing gene (Subramanian et al., 2021) indicating that both genes are simultaneously required to sustain adequate function in oocytes. Interestingly, Setx expression in somatic cells from $SETX^{+/-}$ females was significantly higher than in $SETX^{+/-}$ oocytes (Subramanian et al., 2021) pointing to an unusually high sensitivity of oocytes to Setx deregulation. The vulnerability of oocytes to Setx dysfunction suggests that females with heterozygous *SETX* mutations unlikely to produce an overt somatic phenotype could nevertheless be acutely vulnerable to POA making *SETX* one of the very few genes associated with silent POA.

SUMMARY AND CONCLUSION: IMPLICATIONS FOR UNDERSTANDING POA IN HUMANS

Although POA affects roughly 10 times more women than POI much less is known regarding POA's genetic causation. Gaining increased understanding about genetic causes of POA is particularly challenging since, unlike POI, which has overt manifestations and diagnostic criteria (Webber et al., 2016), POA often only comes to light if women seek fertility treatment. Our studies in mice show that *SETX* mutations produce a model of POA involving DNA damage secondary to compromised DNA repair capacity. Agents capable of inducing damage which accumulate with aging such as oxidative stress, pose a constant threat requiring robust Setx-dependent DNA repair mechanisms to maintain DNA integrity in oocytes (**Figure 2**). Any inherent threat to such repair capacity as with *SETX* mutations tips the balance toward damage at earlier ages with severe consequences for the ovarian reserve (**Figure 2**).

Importantly, new data now show that oocytes mount a DDR against *in vivo* damage and that this is a slow-evolving response (**Figure 1C**; Subramanian et al., 2020). In contrast, damage that is experimentally induced *in vitro* is an acute event, against which, oocytes are unable to mount a DDR (**Figure 1D**). However, the physiological relevance of the latter is questionable since it would require an *in vivo* scenario in which, only fully-grown oocytes within large antral follicles are exposed to a damage-inducing event during the transient peri-ovulatory period.

Oocytes appear to be especially dependent on Setx since loss of function from even one gene in mice has consequences equally as severe as loss of both genes. Since the best-known human disorder resulting from compromised Setx function, AOA2, involves homozygous mutations (Lavin et al., 2013), heterozygous Setx compromise may well be a cause of seemingly idiopathic POA. It is important to stress that we identified a link between *SETX* mutation and POA rather than the more severe end of the spectrum, POI. *SETX* mutant ovaries initially contained normal numbers of follicles and produced normal numbers of fully-grown oocytes in young adulthood but subsequently experienced accelerated follicular depletion (Subramanian et al., 2020, 2021). In contrast, mutation of *ATM*, which causes another ACRA, A-T, in humans (Savitsky et al., 1995), induces POI with almost all follicles becoming depleted within a few days after birth in mice (Di Giacomo et al., 2005).

Investigating the role of *SETX* in POA in humans may not be straightforward due to the pleiomorphic effects of *SETX* mutations. Indeed, different *SETX* mutations cause up to four distinct neurological disorders (Lavin et al., 2013). It is therefore likely that not all *SETX* mutations impact ovarian aging in the same way and that different mutations may have distinctly different effects. Indeed, there has been a case-report of an 18-year-old female with AOA2 caused by a rare non-coding mutation who had polycystic ovarian syndrome (PCOS), a condition characterized by *high* ovarian reserve (Fogel et al., 2009). We note as well that AOA2 is caused by a plethora of different mutations (Anheim et al., 2009; Nanetti et al., 2013) whereas in our mouse model, the *SETX* gene was disrupted by a single mutation – a deletion of exon 4 – which resulted in a complete absence of Setx protein in knockout animals (Becherel et al., 2013).

Premature ovarian aging remains an enigmatic condition with very few known genetic causes. Very recent studies in the mouse model suggest that *SETX* mutations can induce a POA phenotype. Based on the variability in neurological disease profile produced by different *SETX* mutations in humans, it may well be that only a fraction of mutations results in POA. Identifying which *SETX* mutations are linked with POA in women will be an important future undertaking.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: HH is a co-founder, shareholder, and advisor of Jumpstart Fertility Inc., which was founded to develop research into NAD^+-dependent pathways involved in female fertility.

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miR-29a/b$_1$ Regulates the Luteinizing Hormone Secretion and Affects Mouse Ovulation

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miR-29a/b$_1$ was reportedly involved in the regulation of the reproductive function in female mice, but the underlying molecular mechanisms are not clear. In this study, female mice lacking *miR-29a/b$_1$* showed a delay in vaginal opening, irregular estrous cycles, ovulation disorder and subfertility. The level of luteinizing hormone (LH) was significantly lower in plasma but higher in pituitary of mutant mice. However, egg development was normal in mutant mice and the ovulation disorder could be rescued by the superovulation treatment. These results suggested that the LH secretion was impaired in mutant mice. Further studies showed that deficiency of *miR-29a/b$_1$* in mice resulted in an abnormal expression of a number of proteins involved in vesicular transport and exocytosis in the pituitary, indicating the mutant mice had insufficient LH secretion. However, the detailed mechanism needs more research.

Keywords: *miR-29a/b$_1$*, knockout, LH, ovulation, reproduction

INTRODUCTION

The *miR-29* family consists of three related mature miRNAs, *miR-29a*, *miR-29b* and *miR-29c*, which are processed from two precursor sequences located at two distinct genomic clusters of *miR-29a/b$_1$* and *miR-29b$_2$/c*. Members of the miR-29 family are ubiquitously expressed, have considerable overall sequence homology with the same seed sequence. Although they have similar tissue expression patterns, *miR-29a* is the dominant member accounting for more than 50% of total *miR-29* expressed in all tissues (1). *miR-29* play important roles in regulating a number of physiological and pathological processes, including metabolism (1–3), inflammation (4, 5), fibrosis (6), cancer (7) and neurodegeneration (8).

As a potential clinical marker or new form of nucleic acid drug, much attention has been paid to *miR-29* research (9, 10). *miR-29* deficiency causes a wide range of physiological defects in mice. Premature cardiac fibrosis and atherosclerotic plaque remodeling is considered as a result of abnormal expression of *miR-29* target genes *Col4a* (11) and ECM (*Col1a* and *Col5a*) (12), and heart

Abbreviations: miRNA, microRNA; iTRAQ, isobaric tags for relative and absolute quantification; PCR, polymerase chain reaction; LH, luteinizing hormone; FSH, follicle-stimulating hormone; KO, knockout.

failure and metabolic disorders might be caused by up-regulating the target gene *PCG1α* (1). *miR-29a* responsible for repressing LPL in hepatocytes, contributes to physiological lipid distribution and protects hepatocytes from steatosis (13). Homozygous deletion of *miR-29a/b$_1$* in mice led to decreased self-renewal and increased apoptosis in hematopoietic stem cells (HSCs) through up-regulating *Dnmt3a* (14). In addition, early puberty in hypothalamic *miR-29* knockdown females is attributed to ectopic expression of *Tbx21*, a target gene of *miR-29* (15). Reproduction in *miR-29* brain-specifical knockdown mice was affected in a sex-dependent manner, with female mice exhibiting hyperfertility and males being subfertility (16); however, this result is inconsistent with the sterile phenotype reported in the *miR-29a/b$_1$* knockout mice (1). Therefore, the relationship between *miR-29a/b$_1$* and reproductive function is still not well understood.

In this work, we revealed that female *miR-29a/b$_1$* knockout mice exhibit severe fertility problems. We proposed that the lack of *miR-29a/b$_1$* in female mice may interfere with the secretion of luteinizing hormone in the pituitary, leading to ovulation failure and a subfertile phenotype.

MATERIALS AND METHODS

Generation of *miR-29a/b$_1$* Knockout Mice

A *miR-29a/b$_1$* knockout mouse line was established using CRISPR/Cas9 gene editing technology and was supplied by Shanghai Center for Model Organisms (SMOC) (17). *miR-29a/b$_1^{-/-}$* homozygous animals and their wild-type littermates were obtained by mating corresponding heterozygotes with each other. Genomic DNA was extracted from tail biopsies, using magnetic bead DNA isolation Kit (DE0596D, EmerTher, Shanghai). PCR was adopted for genotyping using 2 × Taq Plus Master Mix (P212-01, Vazyme) under the following conditions: denaturation at 98°C for 2 minutes, then 35 cycles of 98°C for 10 seconds, annealing at 63°C for 15 seconds, and extension at 68°C for 60 seconds. Primers used for genotyping are listed in **Table S1**.

Animals

All animals were housed in a specific pathogen-free environment (12 h light/12 h dark with lights on at 7.00 h at 21 ± 2°C) with food and water ad libitum. This study was performed in strict accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of Shanghai Model Organisms, and the IACUC permit number is 20090002.

Fertility Assessment

8-week-old *miR-29a/b$_1$* KO and wild-type virgin female or male mice were bred with wild-type male or female mice with known fertility at a proportion of ♀2: ♂1, and vaginal plug formation was examined every morning for 20 consecutive days. Pregnant female mice were separated and pups were recorded, while non-pregnant mice continued to mate. Female or male mice that did not conceive within 1 month of mating were defined as infertile.

Sexual Maturity and Vaginal Smear

Female *miR-29a/b$_1$* KO and wild-type mice from the age of 3-week-old were examined twice daily with respect to on vaginal opening as a marker of rodent sexual maturity. The date of vaginal opening in each mouse was recorded. Female *miR-29a/b$_1$* KO and wild-type (8-10 weeks old for each genotype) mice were caged individually for 3 weeks and at least two full estrous cycles were obtained in each mouse.

Vaginal smears were collected daily and the determination of estrous cycle was evaluated microscopically with the vaginal epithelium. The vaginal epithelium obtained from the vaginal opening by gently eluting 10 μl of physiological saline solution 2-4 times, then the vaginal epithelium transferred onto a microscopic slide and dried at room temperature and fixed with 100% methanol. The slides were stained with Wright's Giemsa (BASO) stain and examined with light microscopy. Proestrus cells are well-formed nucleated epithelial cells. Animals with 85% superficial epithelial cells were considered to be estrus. During metestrus, cornified squamous epithelial cells often in fragments, as well as leukocytes, may be observed. Otherwise, the predominant presence of leukocytes in the cytological smear was identified as diestrus.

Ovariectomy

Adult (8-10 weeks) *miR-29a/b$_1$* KO and control females in diestrus morning were injected subcutaneously with pentobarbital (effective dose 320 mg/kg). Mice were deeply anesthetized and placed on a heating pad. The back skin was shaved and cleaned. About 1.0 cm long incision was made through the muscle layer above the ovaries on each side of the midline. Through the incision, the ovaries were gently pulled outside the body and removed by cauterization below the oviduct. The skin incision was closed with sutures. The mice were left to recover on a heating pad. Adult sham-operated mice were in diestrus on the day of recording as determined by vaginal cytology. Sham-treated animals were processed in the same way, except for the intact ovaries retained. Mice were killed 7 days post-surgery, and their serum were measured for LH and FSH levels.

Hormone Measurement

For hormone measurement, orbital blood was collected in the morning (10.00 h-11.00 h) and evening (18.00 h-19.00h) (18) from freely-moving conscious animals during randomly estrous cycle stages, and were kept at room temperature for 30 minutes. Serum was obtained by centrifuging for 15 minutes at 3000 g at 4°C and was stored at -80°C until analysis. Serum levels of hormone and pituitary proteins LH level were analyzed by Shanghai WESTANG BIO-TECH cooperation using enzyme-linked immunosorbent assay (ELISA). The minimum detectable level of the LH assay was 0.1mIU/ml and the intra-and inter-assay coefficients of variation were 9.9% and 8.3%. The minimum detectable level of the FSH assay was 1mIU/ml and the intra-and inter-assay coefficients of variation were 9.8% and 8.6%, The minimum detectable level of the estrogen assay was 30 pg/ml and the intra-and inter-assay coefficients of variation were 9.3% and

8.5%, The minimum detectable level of the progesterone assay was 0.2ng/ml and the intra-and inter-assay coefficients of variation were 9.5% and 8.3%, The minimum detectable level of the testosterone assay was 0.1ng/ml and the intra-and inter-assay coefficients of variation were 9.4% and 8.2%, respectively.

GnRH Challenge

Animals received an intraperitoneally injection with 125ng/g (19) exogenous GnRH (L7134, Sigma-Aldrich, St Louis, MO, USA) or saline vehicle. Twenty minutes after GnRH or saline injection, orbital blood was collected, and the resultant serum samples were stored at −80°C for subsequent human-LH radioimmunoassay (RIA, performed by Beijing North Institute Biological Technology, Beijing, China) (20), with sensitivity and intra- and inter-assay coefficient of variation for LH of 0.5 mIU/ml, 15% and 20%, respectively.

Superovulation and Oocyte Collection

Superovulation: To induce superovulation, 8-week-old mice were intraperitoneally injected with 5 IU pregnant mare serum gonadotropin (PMSG, Sigma) at afternoon (15:00h-16:00 h), followed by 5 IU human chorionic gonadotropin (hCG, Sigma) 48 hour later to trigger oocyte maturation and ovulation. Female mice were mated with 10-week-old fertile wild-type males 16 h after injection and checked for vaginal plug formation the next morning.

Oocyte collection: Super ovulated or natural mated mice with a visible plug were sacrificed by cervical dislocation, the ovaries were removed and the ampulla was collected. Oocytes were harvested in M2 media and quantified by microscopy (Nikon SMZ800) following brief digestion in hyaluronidase (800IU/ml, Sigma) to strip cumulus and pipetting for 30-60 s. Oocytes were washed 5 times with PBS. The washed oocytes were transferred to M16 media and cultured overnight, and two-cell stage embryos were counted in the next morning.

LC-MS/MS Analysis

Total pituitary (P) protein from wild-type and *miR-29a/b*$_1$ KO females (8 weeks, n=3) were isolated and labelled with iTRAQ reagents 114, 115, 116, 117, 118, 119, 120 or 121, respectively, followed by Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) (Shanghai Wayen Biotechnologies Inc.). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (21) partner repository with the dataset identifier PXD017106.

Histological Analysis and Follicles Count

Wild-type and *miR-29a/b*$_1$ KO were euthanized and transcardially perfused with cold saline, followed by 4% paraformaldehyde 0.1 M phosphate buffer (PFA). Brain, pituitary and ovary were collected and fixed overnight at 4°C. Paraffin-embedded ovary samples were serially sectioned at 4 μm-thick sections. Brain coronal (20 μm) slices were cut with a Leica CM1950 following by dehydration in 30% sucrose saline solution.

Pituitary and ovary stained with hematoxylin and eosin using standard histological techniques (Servicebio). Stained sections were scanned using LEICA CTR6000 with a 10X, 20X and 40X objective. Ovarian follicles at different developmental stages were classified and quantified in serial sections according to the Pedersen and Peters method (22). To avoid double counting of follicles across sections, only follicles containing oocyte with a clearly visible nucleus were scored (23), and follicles were counted in every fifth serial section. Any follicle also appearing in the adjacent lookup section was not counted. The entire section was analyzed without subsampling. Each ovary was coded with no information about genotype group for blind counters and prevent bias. The mean count per section was calculated. All follicle types were summed together to determine the total number of follicles.

For immunohistochemistry, sections were subjected to antigen retrieval by incubation in 10 mM sodium citrate, pH 7.0, for 10 minutes at 95°C. The endogenous peroxidase activity of the sections was quenched with 3% H_2O_2 treatment (Sangon Biotech, Shanghai). Immunohistochemical staining was performed using mouse anti-Lutropin beta antibody (1:500, SANTA CRUZ, sc-373941) or rabbit anti-GnRHR antibody (24, 25) (1:100, Proteintech, 19950-1-AP) and HRP-conjugated donkey anti-mouse IgG (1:1000, ThermoFisher, A16017) or donkey anti-rabbit IgG (1:1000, ThermoFisher, A16035) for lutropin and GnRHR antibody.

For immunofluorescence, brain and pituitary sections permeabilized by incubation with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. After permeabilization, the sections were washed three times in PBST, and blocked with 5% normal donkey serum in PBS for 1h at room temperature, then were incubated with mouse anti-Lutropin beta antibody (1:1000, SANTA CRUZ, sc-373941), rabbit anti-GnRH1 (1:500, Immunostar, PA1-121) or rabbit anti-GnRHR antibody (26) (1:100, Proteintech, 19950-1-AP) overnight at 4°C, followed by staining with Alexa Fluor 647-conjugated donkey anti-mouse antibody (Invitrogen Molecular Probes) or Alexa Fluor 594-conjugated donkey anti-rabbit (Invitrogen Molecular Probes) antibody and DAPI dye to stain nuclei. The mouse liver and lung tissues were selected to negative control for GnRHR and Lutropin beta antibody respectively.

Stained sections were scanned using the 40X objective of a Zeiss Confocal microscope (LSM880). The area fractions of positive cells relative to entire area were determined using ImageJ (Fiji, NIH) software. Cell location was mapped to the atlas (27).

Real-Time Quantitative PCR

Total RNA was isolated using TRIzol (Tiangen Biotech, Beijing) according to the manufacturer's instructions and kept at -80°C subsequent for use. For microRNA measurement, 2 μg total RNA was transcribed into cDNA using the miRcute Plus miRNA First-Strand cDNA Synthesis Kit (Tiangen Biotech, Beijing). Expression level of mature *miR-29a*, *miR-29b* and *miR-29c* were measured using miRcute Plus miRNA qPCR Detection (Tiangen Biotech, Beijing). *U6* snRNA was used for normalization.

For mRNA measurement, total RNA (2 μg) from each sample was transcribed by using EasyScript First-Strand cDNA

Synthesis SuperMix (TransGen Biotech, Beijing), and mRNA levels of target genes were detected using TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing) according to the manufacturer's instructions. Murine *β-actin* was used as a reference to normalize target gene expression levels. Real-time PCR amplification was performed using the Realplex system (Applied Biosystems QuantStudio3, ThermoFisher Scientific). The sequences of the specific primers used are listed in Supplementary Material, **Table S1**. RNA levels were calculated using the $2^{-\Delta CT}$ method, where CT is the cycle threshold (28). Melting curve analysis for each primer set revealed only one peak for each product, and the sizes of PCR products were confirmed by comparing sizes with a commercial ladder after agarose gel electrophoresis. PCR products were further confirmed by sequencing.

Western Blot

Mice were euthanized and tissues were collected. Total tissue protein was extracted using RIPA buffer (ThermoFisher scientific) containing protease and phosphatase inhibitor cocktails (Selleck Chemicals). Protein concentration was quantified using the Enhanced BCA Protein Assay Kit (Beyotime). Protein (20 μg) from each sample was separated on 4%-20% SDS-PAGE (GenScript) and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were blocked with Western BLoT Blocking Buffer (Protein Free) (Takara) for 1 h at room temperature and then incubated with primary antibodies, Lutropin beta (1:1000, SANTA CRUZ, sc-373941) or anti-β-actin (1:1000, Santa Cruz, sc-47778) diluted in Western BLoT Immuno Booster PF (Takara) at 4°C overnight. After washing with TBST three times, membranes were incubated with fluorescent-conjugated secondary antibody for 1 h (1:10000, LI-COR Biosciences). Quantitative detection of protein expression was then performed using the Odyssey Infrared Imaging system (LI-COR Biosciences) and analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data analysis was performed using GraphPad Prism 7 (GraphPad software Inc.). Data are expressed as the mean ± SEM. Difference in mean values between two groups were analyzed using the Student's t-test (continuous variables) or Mann–Whitney test (discrete variables). For comparisons involving more than two groups, ANOVA (continuous variables) or Kruskal–Wallis (discrete variables) with *post hoc* testing was used, and survival profiles were constructed by Kaplan-Meyer survival analysis. Statistically significant differences are shown with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and **** $p < 0.0001$).

RESULTS

Genetic Ablation of *miR-29a/b$_1$* Leads to Female Sterility

A conventional *miR-29a/b$_1$* knockout mouse line (*miR-29a/b$_1$* KO) was previously established using CRISPR/Cas9 methods (17). The genotyping and the expression of *miR-29* in different genotypes of mice were detected by PCR and real-time PCR, respectively (**Figure S1**). To understand the role of *miR-29a/b$_1$* in fertility, the reproductive ability of *miR-29a/b$_1$* KO mice was evaluated. For data in **Table 1**, of the 25 females tested, 23 were sterile. The two pregnant *miR-29a/b$_1$* KO female mice gave birth to two offspring each and were not subsequently pregnant again. Among males, 66.7% *miR-29a/b$_1$* KO were still fertile. However, female *miR-29a/b$_1$* KO mice exhibited serious reproductive problems. Vaginal plugs were checked to study the mating behavior. *miR-29a/b$_1$* KO females had a significant lower mating frequency compared to wild-type females (**Table 1**), suggesting abnormal sexual maturity and estrous cycle. Sexual maturity indicated by vaginal opening occurred 5 days later in *miR-29a/b$_1$* KO female mice (postnatal day 28) compared to wild-type littermates (postnatal day 23) (**Figure 1A**). At the time of puberty onset, the mutant mice are significantly lighter than wild-type mice (**Figure 1B**). Meanwhile, abnormal estrous cycle with less time in estrus and metestrus and significantly more time in diestrus was observed in *miR-29a/b$_1$* KO female mice. (**Figures 1B, C** and **Figure S2**). RT-PCR analysis revealed that expression of *miR-29a* periodically changed in pituitary and ovarian tissues (**Figure S3A**), suggesting that *miR-29a/b$_1$* may play a role in the estrous period in mammals. Taken together, these data illustrate that loss of *miR-29a/b$_1$* induces growth retardation in mutant mice and subfertility in females.

miR-29a/b$_1$ Gene Knockout Leads to Decreased Plasma LH Level and Ovulation Disorder

Ovary and uteri weight in *miR-29a/b$_1$* KO females were significantly reduced compared to wild-type females (**Figures 2A, B**), whereas, in males, testis and seminal pouch in *miR-29a/b$_1$* KO mice and wild-type counterparts showed no difference (**Figure S4**). Fertilized eggs were collected from the

TABLE 1 | Fertility assessment. Body weight, number of plugs, offspring and pregnancy rate based on mating of wild-type male and female mice.

Fertility assessment	Females		Males	
Genotype	miR-29a/b$_1$ KO	Wild-type	miR-29a KO	Wild-type
Body weight (g)	16.35 ± 0.2228	20.72 ± 0.9777	20.02 ± 1.076	23.7 ± 0.6186
Number of plugs	1/25 (4%)	10/12 (83.3)	9/15 (60%)	5/6 (83.3%)
Mean litter size	2	7.6	5.5	7.6
Pregnancies rate (%)	8****	91.7	66.7**	83.3

$^{**}p < 0.01$, $^{****}p < 0.0001$.

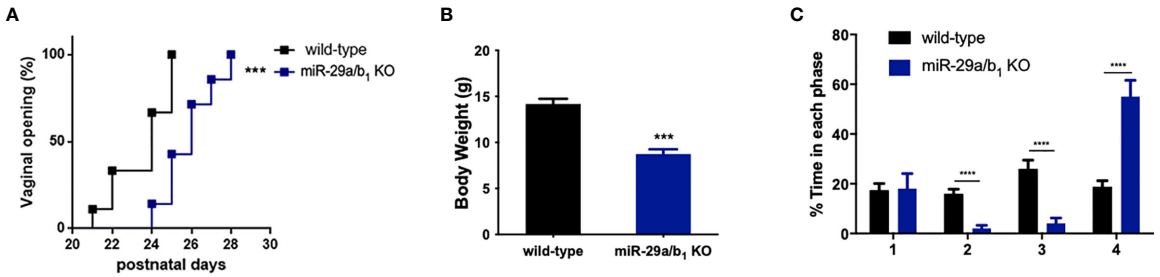


FIGURE 1 | Determination of pubertal onset and estrous cycle in *miR-29a/b*$_1$ KO females. **(A)** Pubertal onset was determined by vaginal opening in wild-type and *miR-29a/b*$_1$ KO mice (n=8). **(B)** Body weight of mice at the time of puberty onset (wild-type: 14.18 ± 0.5660, *miR-29a/b*$_1$ KO: 8.74 ± 0.5202, *p=0.0001*, n=5). **(C)** Estrous cycle quantitative measurements on wild-type and *miR-29a/b*$_1$ KO females (1: *p=0.9133*, 2-4: *p<0.0001*). ***$p < 0.001$, ****$p < 0.0001$.

oviducts of wild-type and *miR-29a/b*$_1$ KO females with vaginal plug after mating with wild-type males. In 20 females *miR-29a/b*$_1$ KO mice, only 2 oocytes were found and with no two-cell embryos the next day, while among five wild-type mice, 34 oocytes and 19 two-cell embryos were collected (**Figure 3A**). Histomorphometric analysis revealed that mutant ovaries contained normal primordial follicles, a similar number of secondary follicles with normal oocyte and a thick granulosa cell layer, indicating that the early follicles developed normally, but lacked corpora lutea formation (**Figures 3B–D**). These results suggest that subfertility of the mutant female mice may be caused by an ovulation disorder.

In females, hormonal control of the estrous cycle and ovulation is essential for the establishment of maturation and fertility in mammals (29). Thus, we examined hormone levels in the serum of wild-type and *miR-29a/b*$_1$ KO female mice. In the female *miR-29a/b*$_1$ KO mice, significant decreases in the serum LH and progesterone (P_4) (**Figure 4A**) were observed, while there was no apparent difference in serum content of follicle-stimulating hormone (FSH) or Testosterone (T) or Estradiol (E_2) compared to wild-type mice (**Figures S5A–C**). *Cyp19a*$_1$ and *Cyp17a*$_1$, encoding enzymes involved in estradiol and testosterone synthesis, were expressed at identical levels in ovaries from the two groups of mice, while the *Cyp11a*, which essential to the level of sex hormones, was significantly decreased in ovaries from mutant mice (**Figure S5D**). These results indicated that impaired corpora lutea formation in *miR-29a/b*$_1$ KO mice might be caused by a shortage of LH. This speculation was further confirmed by the superovulation experiment. Ovulation in the mutant mice was rescued by exogenous gonadotropin injection, indicating that responses to LH stimulation were not irreversibly lost in these mutant animals (**Figure 4B**). Ovaries from superovulated adult *miR-29a/b*$_1$ KO mice showed normal morphology, and the corpora lutea were formed (**Figure 4C**).

To determine whether the central regulated mechanisms mediating ovulation were altered in *miR-29a/b*$_1$ KO mice, females were subsequently treated with an intraperitoneally injection of 125ng/g GnRH or saline vehicle at 10.00 AM. Normal GnRH responsiveness was observed in *miR-29a/b*$_1$ KO pituitary, but serum LH level in *miR-29a/b*$_1$ KO females remained markedly below the levels observed in wild-type littermates (**Figure 4D**). Furthermore, The GnRHR-immunoreactivity in the pituitary of *miR-29a/b*$_1$ KO mice was increased compared to wild-type mice (**Figures 5A, B**), Again, to assess the impact of hyperstimulation with endogenous GnRH modulated by estrogen (30–33), female control and *miR-29a/b*$_1$ KO animals were castrated or underwent a sham surgery.

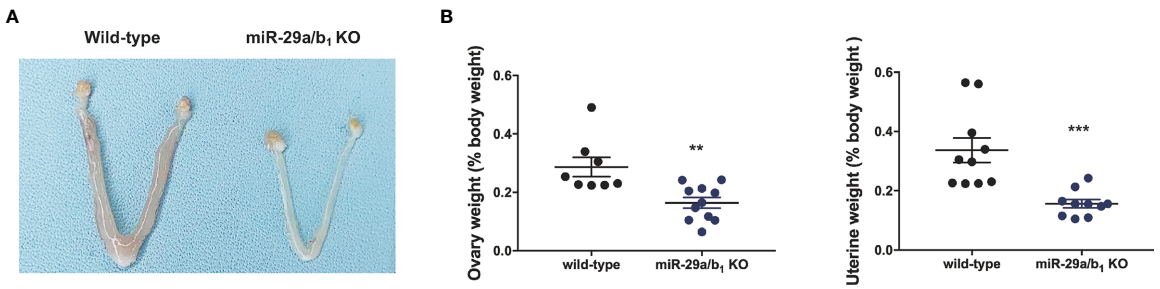


FIGURE 2 | Morphological study of reproductive system. **(A, B)** Macroscopic images, wet ovaries and uteri weight in females, normalized to body weight in the same animals (ovary: wild-type: 0.2868 ± 0.03286, n=8, *miR-29a/b*$_1$ KO: 0.164 ± 0.01834, n=11, *p=0.0028*; uteri: wild-type: 0.3369 ± 0.04175, *miR-29a/b*$_1$ KO: 0.1564 ± 0.01394, *p=0.007*, n=10). **$p < 0.01$, ***$p < 0.001$.

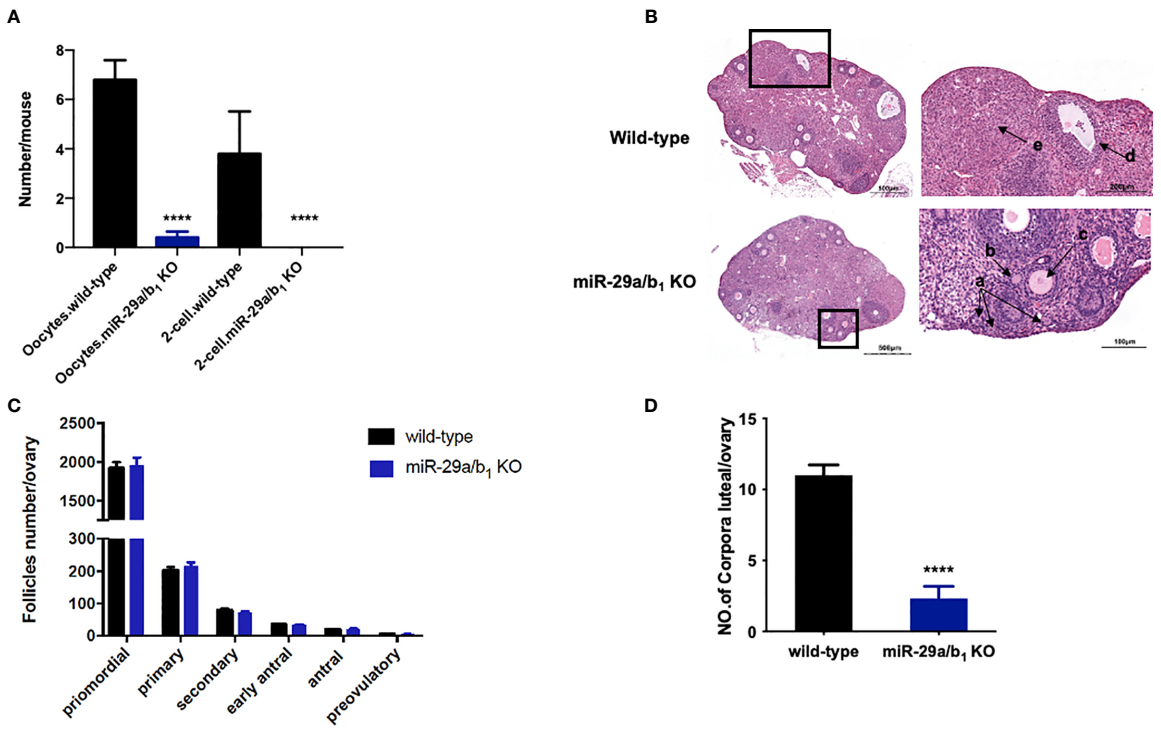


FIGURE 3 | Lacking of *miR-29a/b*$_1$ impairs ovulation in females. **(A)** Numbers of oocytes and 2-cell embryos in wild-type and *miR-29a/b*$_1$ KO mice during natural ovulation (n=5). **(B)** Histological sections of ovaries stained with haematoxylin and eosin (H&E) in wild-type and *miR-29a/b*$_1$ KO mice. Corpora lutea (CLs) and follicles at different stages are shown at higher magnification and denoted with arrows. a: Primordial follicles; b: Primary follicles; c: Secondary follicles; d: Antral follicles; e: Corpora lutea. **(C)** Numbers of follicles at different stages in ovaries from wild-type (n=10) and *miR-29a/b*$_1$ KO (n=12) mice. Primordial follicles: $p=0.8931$; Primary follicles: $p=0.9802$; Secondary follicles: $p=0.6842$; Early antral follicles: $p=0.5645$; Antral follicles: $p=0.8011$; Preovulatory: $p=0.5081$, respectively. **(D)** Lack of corpora lutea in the ovaries of *miR-29a/b*$_1$ KO females ($p<0.0001$). ****$p < 0.0001$.

Animals were euthanized after 7 days, and serum concentrations of LH and FSH were measured. Consistent with control females, castration resulted in an increase in both LH and FSH compared with sham-operated controls, however, the post-castration rise in LH secretion was blocked in *miR-29a/b*$_1$ KO females, while the FSH level was no significant differences in mutant mice serum from controls (**Figure 5C**). LH levels overall were markable lower in *miR-29a/b*$_1$ KO females relative to controls. There was no apparent difference in Kiss1and Gnrh1, which stimulating secretion of gonadotropin releasing hormone from the hypothalamus (34–37) and luteinizing hormone from the pituitary (35), respectively (**Figures 5D, E**). These results suggest that ovulation disorder in *miR-29a/b*$_1$ KO mice might be caused by dysregulation of related pituitary hormones, especially LH.

Dysregulated Pituitary LHβ Release in *miR-29a/b*$_1$ KO Mice

LH is synthesized in and secreted by the pituitary. A lack of *miR-29a/b*$_1$ was confirmed in mutant pituitary tissues (**Figure S3B**). The anterior pituitary undergoes rapid proliferation in neonatal mice, subsequently expanding the cells that produce factors required for growth and reproduction (38). Defective anterior pituitary development in animals contributes to many organism-level developmental defects (39). However, there was no difference in pituitary structure, size or position of the anterior pituitary between wild-type and *miR-29a/b*$_1$ KO mice (**Figure 6A**). No abnormalities were found upon pathological examination of mutant pituitary tissues (**Figure 6B**). Notably, transcript levels of the *Lhβ* gene in *miR-29a/b*$_1$ KO pituitary did not differ from control animals, but LH protein level and immunoreactivity were even higher in KO mice (**Figures 6C–F**).

To further elucidate the effects of *miR-29a/b*$_1$ gene knockout on pituitary function, iTRAQ analysis was performed to compare proteomic changes in the pituitary between mutant and wild-type mice. Total pituitary protein from three biological replicates of each genotype were subjected to LC-MS/MS analysis. The hierarchical clustering profile of differential proteins is shown in the heat map (**Figure 7A**). A total of 163 cellular proteins were statistically significant altered ($p<0.05$), including 75 upregulated proteins and 88 downregulated proteins (**Figure 7B**, **Table 2**). LHβ and FSHβ were significantly increased in the pituitary of *miR-29a/b*$_1$ KO mice according to b/y ion signal intensity (**Figure 7C**). Besides, TSHβ and Cga were also markedly upregulated as a result of the *miR-29a/b*$_1$ deficiency (**Figures 7C, G**). Through GO analysis, altered proteins

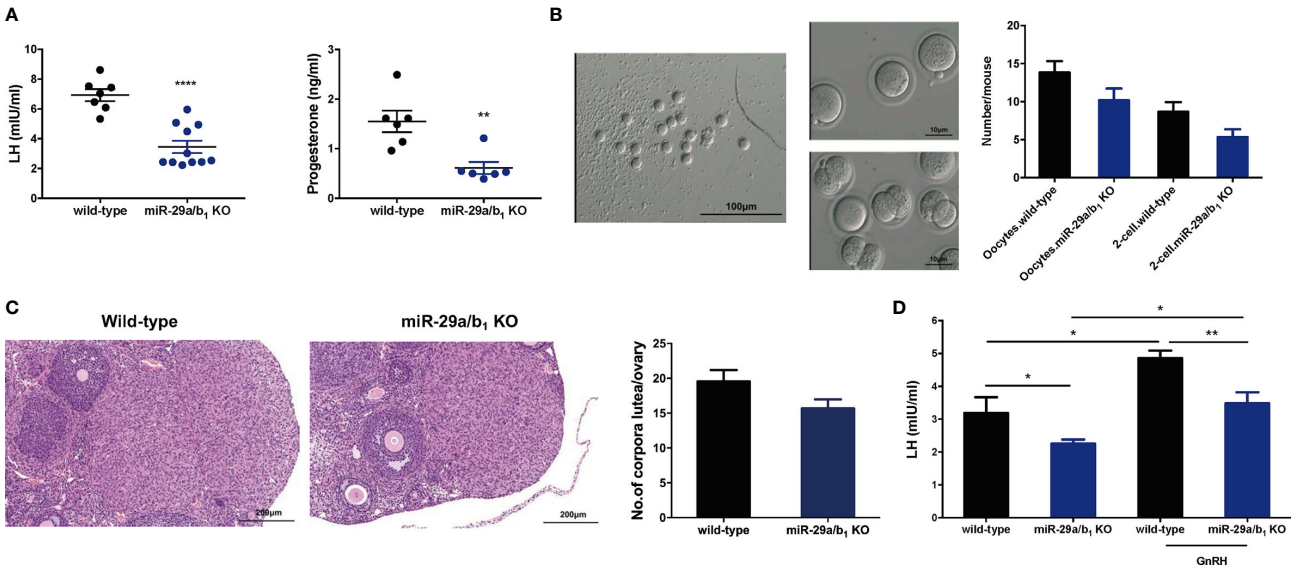


FIGURE 4 | Superovulation rescues the failure in corpora lutea formation in *miR-29a/b*$_1$ KO mice. **(A)** Serum LH (left) and progesterone (right) levels were significantly reduced in *miR-29a/b*$_1$ KO compared to wild-type mice (LH: wild-type: 6.927 ± 0.4062 mIU/ml, *miR-29a/b*$_1$ KO: 3.607 ± 0.5175 mIU/ml, *p*=0.0003, n=7; progesterone: wild-type: 8.166 ± 2.072 nmol/L, n=7, *miR-29a/b*$_1$ KO: 1.062 ± 0.1181 nmol/L, n=6, *p=0.0092*). **(B)** Numbers of oocytes and 2-cell embryos obtained in response to superovulation in *miR-29a/b*$_1$ KO and wild-type mice (Oocytes: wild-type: 13.83 ± 1.493, *miR-29a/b*$_1$ KO: 10.17 ± 1.558, *p=0.1201*; 2-cell embryos: wild-type: 8.667 ± 1.256, *miR-29a/b*$_1$ KO: 5.333 ± 1.022, *p=0.0666*, n=6). **(C)** Corpora lutea formation in ovaries of *miR-29a/b*$_1$ KO females after superovulation (wild-type: 19.56 ± 1.634, *miR-29a/b*$_1$ KO: 15.67 ± 1.302, *p=0.0811*, n=9). **(D)** GnRH challenge in *miR-29a/b*$_1$ KO and wild-type mice (wild-type: 3.19 ± 0.48 mIU/ml, *miR-29a/b*$_1$ KO: 2.255 ± 0.1287 mIU/ml, *p=0.0376*, n=5; GnRH: wild-type: 4.857 ± 0.2346 mIU/ml, *p=0.0138*, n=6, *miR-29a/b*$_1$ *KO*: 3.484 ± 0.3357 mIU/ml, *p=0.0145*, n=7). *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

identified in this study were found to be involved in a wide range of biological process, and most of the differential proteins were classified in the protein transport processes, which are essential for vesicle-mediated transport in the cytoplasm and exocytosis during plasma infusion (40–42) (**Figure 7D**).

The intersected gene between upregulated expression and miR-29a targets through miRDB (http://mirdb.org) were analyzed, 11 potential direct target transcripts of miR-29a were discovered (**Figure 7E**), and predicted target genes were expected to be upregulated in miRNA loss of-function models (**Figure 7G**). Among them, collagen family Col1a1, Col4a2 and Col5a1 are target genes of miR-29a-3p, and promote cancer cells invasion and migration (43–45). In addition, miR-29a can promote the neurite outgrowth by targeting extracellular matrix-related genes like Fibrillin 1 (Fbn1) and hyaluronan and proteoglycan link protein 1 (Hapln1) (46, 47), which dramatically increased in the pituitary of *miR-29a/b*$_1$ KO mice. Hdac4 (48), which is key epigenetic modified writer, may play important roles in the change of gene expression pattern in *miR-29a/b*$_1$ gene knockout mice, especially for down-regulated genes. For the 88 down-regulated proteins in the pituitary of *miR-29a/b*$_1$ KO mice, a considerable portion of them participate in vesicle-mediated transport and secretion (Ergic1, Fkbp2, Ssr3, Stat5a, Crhbp, **Figure 7F**). Notably, Ergic1, encodes a cycling membrane protein, and plays an important role in transport between endoplasmic reticulum and Golgi (49). Absence of trApγ (SSr3) impairs protein translocation into the endoplasmic reticulum and affects transport (50). Myosins were reported as core players in the final stages of regulated secretory pathways (51). Treatment of pituitary cells with the myosin light chain (Myl2/3) kinase inhibitor, wortmannin, attenuated GnRH-induced LH release (52). Further validated by quantitative PCR (qPCR) that the mRNA transcripts of these genes, which were consistent with LC-MS/MS (**Figure 7G**). These results indicated the deficiency of *miR-29a/b*$_1$ blocked proteins transportation, leading to impaired pituitary hormone secretion, especially LH released.

DISCUSSION

A lack of *miR-29a/b*$_1$ leads to female sterility in mice, which has been mentioned previously (1); however, the mechanisms underlying this result were not published or illustrated. In this work, we demonstrated that low serum LH level and ovulation disorder might be the direct cause of subfertility in female *miR-29a/b*$_1$ KO mice. This conclusion is further proved by the results that oocyte development is normal in the ovaries of mutant mice and normal eggs could be obtained through super-ovulated. Compared to wild-type mice, the pituitary gland in mutant mice stimulated with the same concentration of GnRH produced reduction LH secreted into the blood, indicating that

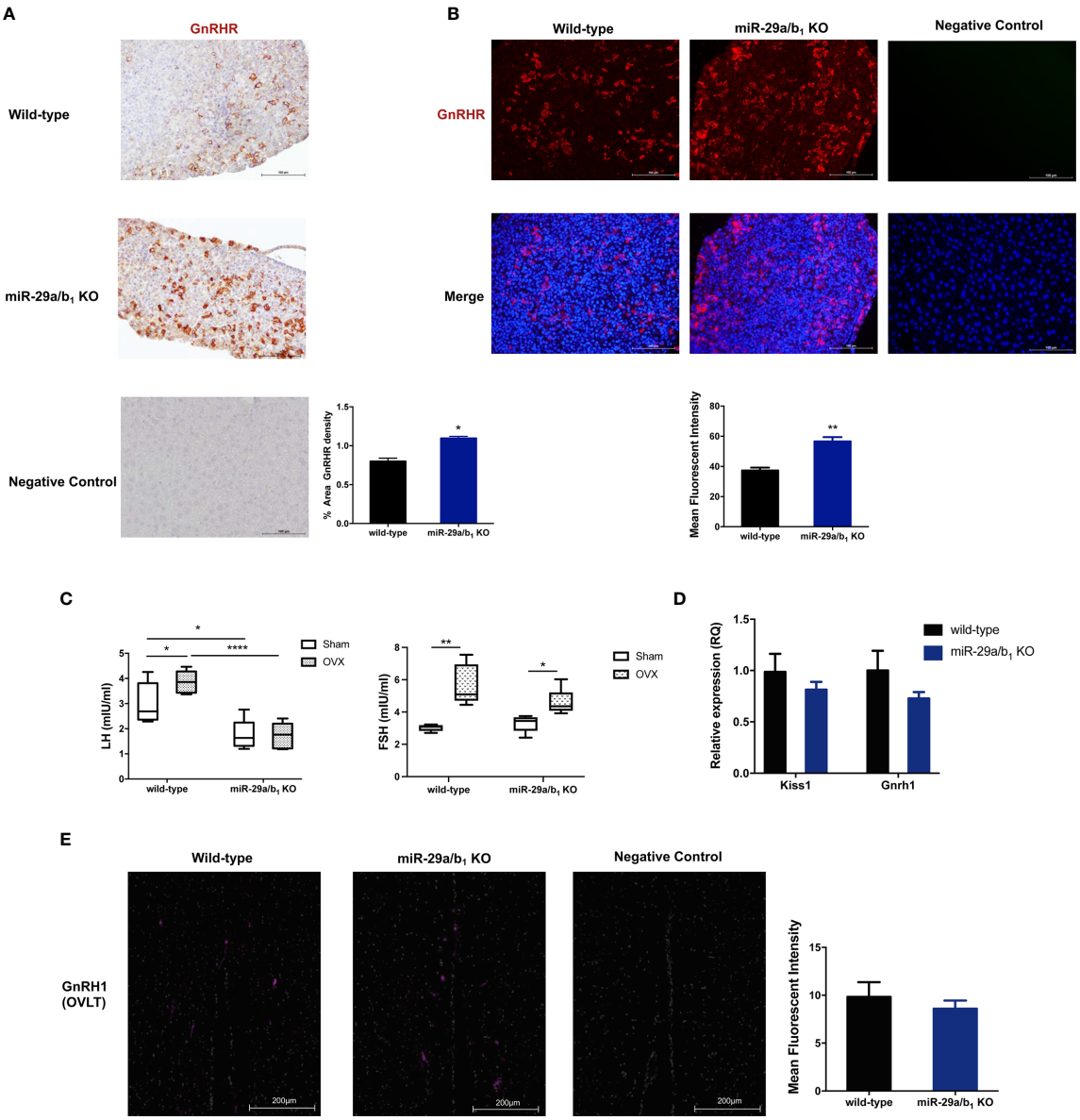


FIGURE 5 | Central mechanism in *miR-29a/b*$_1$ KO mice. **(A, B)** GnRHR immunoreactivity in pituitary of wild-type and *miR-29a/b*$_1$ KO females. The receptor was not detectable on the plasma membrane of control. (immunohistochemical: wild-type: 13.43 ± 0.7927, *miR-29a/b*$_1$ KO: 25.47 ± 0.534, *p=0.0249;* immunofluorescence: wild-type: 37.79 ± 1.858, *miR-29a/b*$_1$ KO: 56.64 ± 2.767, *p=0.0045*, n=3). **(C)** Serum LH and FSH levels in *miR-29a/b*$_1$ KO females and controls following ovariectomy (OVX) and sham-operated controls (Sham). (LH: wild-type: *p=0.0401*, *miR-29a/b*$_1$ KO: *p=0.9249*; FSH: wild-type: *p=0.0016*, *miR-29a/b*$_1$ KO: *p=0.0185*, n=6). **(D)** Expression of Kiss1 and Gnrh1 in hypothalamus (Gnrh1: wild-type: 1 ± 0.1912, *miR-29a/b*$_1$ KO: 0.7287 ± 0.06234, *p=0.1874*, Kiss1: wild-type: 1 ± 0.1305, *miR-29a/b*$_1$ KO: 0.8142 ± 0.0757, *p=0.8405*, n=15). **(E)** Normal distribution of GnRH neurons in *miR-29a/b*$_1$ KO mice compared to control littermates. OVLT, organum vasculosum of the lamina terminalis. Scale bars, 200μm. (wild-type: 9.827 ± 1.547, *miR-29a/b*$_1$ KO: 8.597 ± 0.8466, *p=0.5238*, n=3). $^*p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$.

miR-29a/b$_1$ KO females maintained normal pituitary responsiveness to GnRH, although expression of GnRHR was higher in *miR-29a/b*$_1$ KO females pituitaries which may represent compensation for plasma LH insufficiency (53). Meanwhile the expression of LH protein was higher in mutant pituitaries than that in wild types. This suggests that knockout of *miR-29a/b*$_1$ results in deficits in LH secretion from the pituitary but not in LH synthesis stimulated by GnRH (54, 55). Proteomic analysis of the pituitary showed that a large number of proteins related to cellular vesicle-mediated secretion and protein transport were significantly changed in *miR-29a/b*$_1$ KO mice. This effect seems to be omnidirectional, from the vesicle

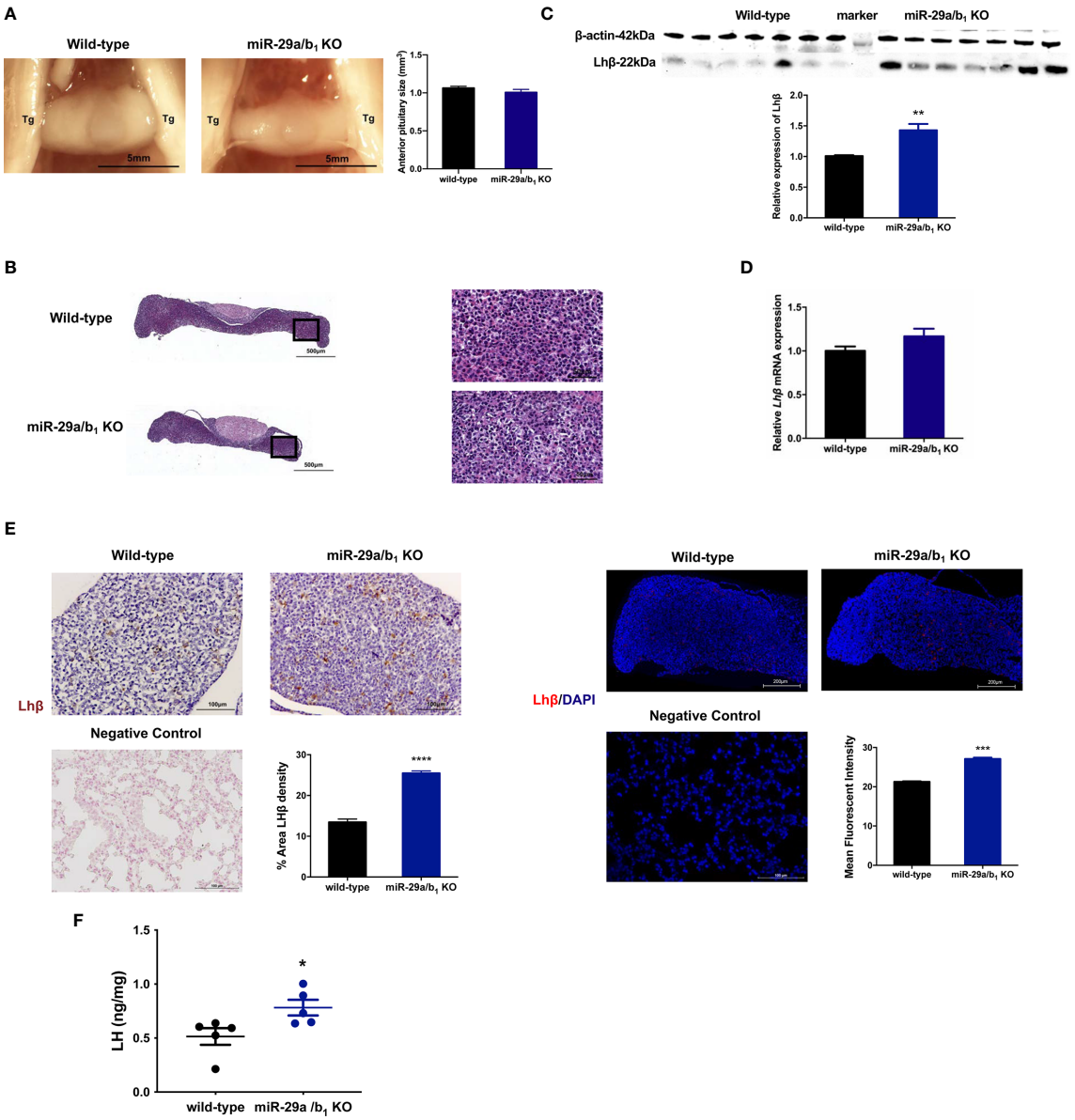
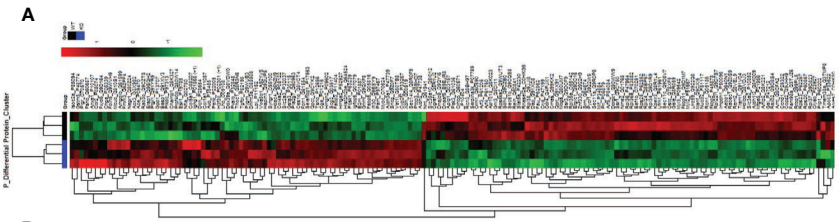


FIGURE 6 | Impairment of Lhβ protein export in the pituitary as a deficiency of *miR-29a/b*$_1$. **(A)** Pituitary from female wild-type mice (n=9) and *miR-29a/b*$_1$ KO mice (n=5) were photographed *in situ* during dissection. Trigeminal nerves that flank the pituitary are marked as Tg. Scale bar = 5 mm (2x magnification). Anterior pituitary size was statistically analyzed ($p=0.2411$, n=7). **(B)** The entire sagittal pituitary and higher magnification in the box from wild-type and *miR-29a/b*$_1$ KO females are shown. **(C, D)** LHβ protein ($p=0.0019$) and transcripts ($p=0.1278$) levels were determined in pituitary tissues from *miR-29a/b*$_1$ KO and wild-type mice (n=7). **(E)** Quantification of immunoreactivity LHβ in pituitary of *miR-29a/b*$_1$ KO or control mice (immunohistochemical: wild-type:13.43 ± 0.7927, n=5, *miR-29a/b*$_1$ KO: 25.47 ± 0.534, n=4, $p<0.0001$; immunofluorescence: wild-type: 21.27 ± 0.147, *miR-29a/b*$_1$ KO: 27.11 ± 0.3642, $p=0.0001$, n = 3). LHβ was not detectable on the plasma membrane of control. Scale bars: 200μm. Red indicates positive-LH cells, Cell nuclei (blue) were stained with haematoxylin or DAPI. **(F)** LH proteins relative contents in females. (Wild-type: 0.5147 ± 0.07769, *miR-29a/b*$_1$ KO: 0.7819 ± 0.07199, $p=0.0357$, n=5). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

transport between endoplasmic reticulum and Golgi apparatus, as well as the process of docking and priming of secretory vesicle on the cell membrane. As a result, many kinds of secretory proteins, including LHβ, were accumulated in pituitary cells. These secreted proteins accounted for 44% of the upregulated proteins in the pituitary of mutant mice.

It is worth noting that FSH required for follicle growth and development and maturation of the ovum (56–58) was less affected by the knockout of *miR-29a/b*$_1$. Different secretion modes between FSH and LH might be an important reason (59, 60). LH is secreted *via* a regulated pathway, while FSH release is primarily constitutive and controlled by synthesis. Increased FSH protein level in the

A



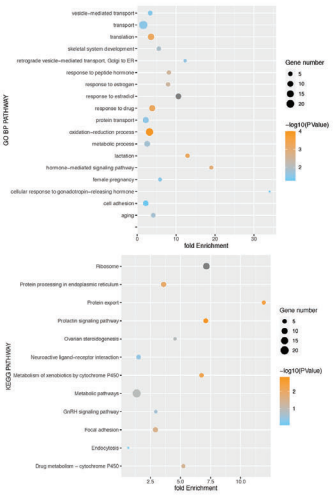
B

Differentially expressed proteins	Up-regulated	Down-regulated	Total
	75	88	163

C

Accession Number	Gene Symbol	Pvalue	P-KO_vs_P-WT Ratio	P-WT			P-KO		
O09108	Lhb	0.001	1.44	16.06	16.67	15.5	16.78	16.66	16.79
Q60687	Fshb	0.0039	1.72	19.22	18.85	18.96	20.55	19.72	19.55
P12656	Tshb	0.00035	1.22	18.48	18.41	18.31	18.74	18.66	18.67
P01216	Cga	0.00035	1.35	17.81	17.86	17.47	18.24	18.2	18.13

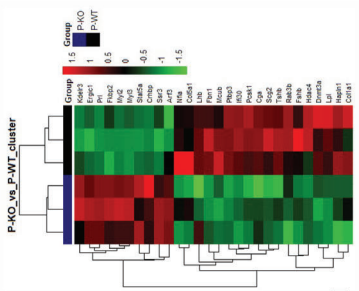
D



E

Gene Name	Representative transcript	Start	Predicted consequential pairing of target region (top) and miRNA (bottom)	End	P-value
Col1a1	ENSMUST00000001547.7	916	5' ...GUGGGAAGGAAUUUC---UGGUGCUA··· 3' AUUGGCUAAAGUCUACCACGAU 5' ...GUGGGAAGGCCACGUUGGUGCUU···	926	< 0.0001
Col4a2	ENSMUST00000033899.8	43	3' AUUGGCUAAAGUCUACCACGAU 5' ...AUAACUAUGAACAUUUGGUGCUG···	49	< 0.0001
Col5a1	ENSMUST00000028280.8	988	3' AUUGGCUAAAGUCUACCACGAU 5' ...AGCGGGGUCCCAAGCUGGUGCUA···	994	0.025
Dnmt3a	ENSMUST00000020991.9	825	3' AUUGGCUAAAGUCUACCACGAU 5' ...UGUGAGGGUUUGUAAUGGUGCUU···	831	< 0.0001
Fbn1	ENSMUST00000028633.7	656	3' AUUGGCUAAAGUCUACCACGAU 5' ...ACUUAGUAUAGGAAAUGGUGCUG···	662	< 0.0001
Hapln1	ENSMUST00000022108.7	2081	3' AUUGGCUAAAGUCUACCACGAU 5' ...AAACUUGAUUAAAACUGGUGCUU···	2087	< 0.0001
Hdac4	ENSMUST00000097644.3	395	3' AUUGGCUAAAGUCUACCACGAU 5' ...UGCCUAUUUUUCAAAUGGUGCUA···	401	0.016
Ifi30	ENSMUST00000034299.5	156	3' AUUGGCUAAAGUCUACCACGAU 5' ...UGCAGUAUAGAUUUG---UGGUGCUA···	163	0.00035
Lpl	ENSMUST00000157129	2264	3' AUUGGCUAAAGUCUACCACGAU 5' ...UGUCGUAAUUUCUCAUGGUGCUG···	2271	< 0.0001
Nfia	ENSMUST00000092532.7	268	3' AUUGGCUAAAGUCUACCACGAU 5' ...UUCUACUCAGAUUUU---UGGUGCUU···	274	0.037
Ptbp3	ENSMUST00000030076.6	1235	3' AUUGGCUAAAGUCUACCACGAU	1241	< 0.0001

F



G

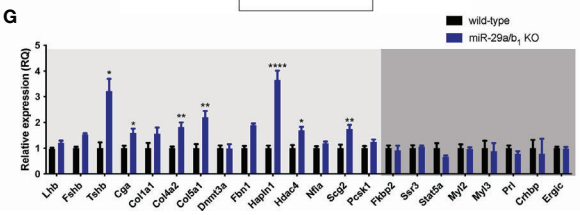


FIGURE 7 | Comparing protein expression profile in the pituitary of wild-type and *miR-29a/b*$_1$ KO mice. **(A, B)** Differential protein from pituitary of *miR-29a/b*$_1$ KO and wild-type mice (n=3 for each) detected by MS. **(C)** Pituitary hormone expression. **(D)** GO and KEGG analysis of the pituitary from *miR-29a/b*$_1$ KO compared to wild-type mice. **(E)** 11predicted miR-29a targets from up-regulated proteins. **(F)** Heat map of genes about vesicle-transport. **(G)** Quantification of up-regulation genes including coded pituitary hormone (light gray shaded area) and down-regulation vesicle-transport activators (dark gray shaded area) (Lhb: *p=0.2411, Fshb: p=0.0002, Tshb: p=0.0017, Cga: p=0.0117, Col1a1: p=0.1038, Col4a2: p=0.0012, Col5a1: p=0.0023, Dnmt3a: p=0.9237, Fbn1: p<0.0001, Hpln1: p<0.0001, Hdac4: p=0.0332, Nfia: p=0.1379, Scg2: p=0.0058, Pcsk1: p=0.0743, Fkbp2: p=0.6975, Ssr3: p=0.6844, Stat5a:0.1329, Myl2: p=0.7879, Myl3: p=0.7858, Prl: p=0.1706, Crhbp: p=0.7510, Ergic: p=0.7937*, n=6). *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

TABLE 2 | Differentially expressed proteins in pituitary involved in miR-29 regulation and protein transport (*p<0.05 and fold change≥1.2 or ≤ 0.83*).

Accession Number	Gene Symbol	Identified Proteins	Molecular Weight	P value	Ratio (KO vs wild-type)
Q60687	Fshb	Follitropin subunit beta OS=Mus musculus GN=Fshb PE=2 SV=1	15 kDa	0.0039	1.72
P32848	Pvalb	Parvalbumin alpha OS=Mus musculus GN=Pvalb PE=1 SV=3	12 kDa	0.0065	1.65
Q9Z0F7	Sncg	Gamma-synuclein OS=Mus musculus GN=Sncg PE=1 SV=1	13 kDa	< 0.0001	1.55
P01887	B2m	Beta-2-microglobulin OS=Mus musculus GN=B2m PE=1 SV=2	14 kDa	0.0039	1.54
Q80TB8	Vat1l	Synaptic vesicle membrane protein VAT-1 homolog-like OS=Mus musculus GN=Vat1l PE=1 SV=2	46 kDa	< 0.0001	1.48
Q03517	Scg2	Secretogranin-2 OS=Mus musculus GN=Scg2 PE=1 SV=1	71 kDa	< 0.0001	1.47
O09108	Lhb	Lutropin subunit beta OS=Mus musculus GN=Lhb PE=2 SV=2	15 kDa	0.001	1.44
Q9CYK2	Qpct	Glutaminyl-peptide cyclotransferase OS=Mus musculus GN=Qpct PE=1 SV=2	41 kDa	< 0.0001	1.44
Q9ESY9	Ifi30	Gamma-interferon-inducible lysosomal thiol reductase OS=Mus musculus GN=Ifi30 PE=1 SV=3	28 kDa	0.00035	1.41
Q60963	Pla2g7	Platelet-activating factor acetylhydrolase OS=Mus musculus GN=Pla2g7 PE=2 SV=2	49 kDa	< 0.0001	1.40
O70570	Pigr	Polymeric immunoglobulin receptor OS=Mus musculus GN=Pigr PE=1 SV=1	85 kDa	0.037	1.38
P33267	Cyp2f2	Cytochrome P450 2F2 OS=Mus musculus GN=Cyp2f2 PE=1 SV=1	56 kDa	< 0.0001	1.37
Q8R3N6	Thoc1	THO complex subunit 1 OS=Mus musculus GN=Thoc1 PE=1 SV=1	75 kDa	0.025	1.37
P01216	Cga	Glycoprotein hormones alpha chain OS=Mus musculus GN=Cga PE=2 SV=1	14 kDa	0.00035	1.35
P32037	Slc2a3	Solute carrier family 2, facilitated glucose transporter member 3 OS=Mus musculus GN=Slc2a3 PE=1 SV=1	53 kDa	< 0.0001	1.35
Q8VCT4	Ces1d	Carboxylesterase 1D OS=Mus musculus GN=Ces1d PE=1 SV=1	62 kDa	< 0.0001	1.34
P30115	Gsta3	Glutathione S-transferase A3 OS=Mus musculus GN=Gsta3 PE=1 SV=2	25 kDa	0.0039	1.34
P08122	**Col4a2**	**Collagen alpha-2(IV) chain OS=Mus musculus GN=Col4a2 PE=1 SV=4**	**167 kDa**	**< 0.0001**	**1.33**
P52927	**Hmga2**	**High mobility group protein HMGI-C OS=Mus musculus GN=Hmga2 PE=1 SV=1**	**12 kDa**	**0.00035**	**1.33**
O55100	Syngr1	Synaptogyrin-1 OS=Mus musculus GN=Syngr1 PE=1 SV=2	26 kDa	0.0039	1.33
Q64524	Hist2h2be	Histone H2B type 2-E OS=Mus musculus GN=Hist2h2be PE=1 SV=3	14 kDa	0.0039	1.32
Q9QXF8	Gnmt	Glycine N-methyltransferase OS=Mus musculus GN=Gnmt PE=1 SV=3	33 kDa	0.0039	1.32
Q8VDW0	Ddx39a	ATP-dependent RNA helicase DDX39A OS=Mus musculus GN=Ddx39a PE=1 SV=1	49 kDa	0.016	1.32
A9Z1V5	Vwa5b1	von Willebrand factor A domain-containing protein 5B1 OS=Mus musculus GN=Vwa5b1 PE=2 SV=1	134 kDa	0.0039	1.32
Q6NZM9	**Hdac4**	**Histone deacetylase 4 OS=Mus musculus GN=Hdac4 PE=1 SV=1**	**119 kDa**	**0.016**	**1.32**
G3X982	Aox3	Aldehyde oxidase 3 OS=Mus musculus GN=Aox3 PE=1 SV=1	147 kDa	0.0065	1.32
P47739	Aldh3a1	Aldehyde dehydrogenase, dimeric NADP-preferring OS=Mus musculus GN=Aldh3a1 PE=1 SV=2	50 kDa	< 0.0001	1.31
Q9D164	Fxyd6	FXYD domain-containing ion transport regulator 6 OS=Mus musculus GN=Fxyd6 PE=1 SV=2	10 kDa	0.0065	1.31
Q9EQH2	Erap1	Endoplasmic reticulum aminopeptidase 1 OS=Mus musculus GN=Erap1 PE=1 SV=2	107 kDa	< 0.0001	1.30
P01868 (+1)	Ighg1	Ig gamma-1 chain C region secreted form OS=Mus musculus GN=Ighg1 PE=1 SV=1	36 kDa	0.00035	1.30
Q9QUP5	Hapln1	Hyaluronan and proteoglycan link protein 1 OS=Mus musculus GN=Hapln1 PE=1 SV=1	40 kDa	< 0.0001	1.29
O88508	**Dnmt3a**	**DNA (cytosine-5)-methyltransferase 3A OS=Mus musculus GN=Dnmt3a PE=1 SV=2**	**102 kDa**	**< 0.0001**	**1.29**
Q07079	Igfbp5	Insulin-like growth factor-binding protein 5 OS=Mus musculus GN=Igfbp5 PE=1 SV=1	30 kDa	< 0.0001	1.28
P26339	Chga	Chromogranin-A OS=Mus musculus GN=Chga PE=1 SV=1	52 kDa	< 0.0001	1.27
P09602	Hmgn2	Non-histone chromosomal protein HMG-17 OS=Mus musculus GN=Hmgn2 PE=1 SV=2	9 kDa	< 0.0001	1.27
Q9CZT8	Rab3b	Ras-related protein Rab-3B OS=Mus musculus GN=Rab3b PE=1 SV=1	25 kDa	< 0.0001	1.27
P28654	Dcn	Decorin OS=Mus musculus GN=Dcn PE=1 SV=1	40 kDa	< 0.0001	1.27
P85094	Isoc2a	Isochorismatase domain-containing protein 2A OS=Mus musculus GN=Isoc2a PE=1 SV=1	22 kDa	0.0027	1.27
P22005	Penk	Proenkephalin-A OS=Mus musculus GN=Penk PE=1 SV=2	31 kDa	0.01	1.27

(Continued)

TABLE 2 | Continued

Accession Number	Gene Symbol	Identified Proteins	Molecular Weight	P value	Ratio (KO vs wild-type)
P02301 (+1)	H3f3c	Histone H3.3C OS=Mus musculus GN=H3f3c PE=3 SV=3	15 kDa	0.0039	1.27
Q91XV3	Basp1	Brain acid soluble protein 1 OS=Mus musculus GN=Basp1 PE=1 SV=3	22 kDa	< 0.0001	1.27
Q02780	**Nfia**	**Nuclear factor 1 A-type OS=Mus musculus GN=Nfia PE=1 SV=1**	**59 kDa**	**0.037**	**1.27**
Q8K327	Champ1	Chromosome alignment-maintaining phosphoprotein 1 OS=Mus musculus GN=Champ1 PE=1 SV=1	88 kDa	0.0017	1.27
Q6ZPF4	**Fmnl3**	**Formin-like protein 3 OS=Mus musculus GN=Fmnl3 PE=1 SV=2**	**117 kDa**	**0.037**	**1.27**
P82198	Tgfbi	Transforming growth factor-beta-induced protein ig-h3 OS=Mus musculus GN=Tgfbi PE=1 SV=1	75 kDa	< 0.0001	1.26
Q61599	Arhgdib	Rho GDP-dissociation inhibitor 2 OS=Mus musculus GN=Arhgdib PE=1 SV=3	23 kDa	0.00034	1.26
Q80W14	Prpf40b	Pre-mRNA-processing factor 40 homolog B OS=Mus musculus GN=Prpf40b PE=1 SV=2	99 kDa	0.0039	1.26
P97467	Pam	Peptidyl-glycine alpha-amidating monooxygenase OS=Mus musculus GN=Pam PE=1 SV=2	109 kDa	< 0.0001	1.25
P11152	**Lpl**	**Lipoprotein lipase OS=Mus musculus GN=Lpl PE=1 SV=3**	**53 kDa**	**< 0.0001**	**1.25**
Q00519	Xdh	Xanthine dehydrogenase/oxidase OS=Mus musculus GN=Xdh PE=1 SV=5	147 kDa	0.024	1.25
Q91YR9	Ptgr1	Prostaglandin reductase 1 OS=Mus musculus GN=Ptgr1 PE=1 SV=2	36 kDa	0.0013	1.25
P11087	**Col1a1**	**Collagen alpha-1(I) chain OS=Mus musculus GN=Col1a1 PE=1 SV=4**	**138 kDa**	**< 0.0001**	**1.24**
P10107	Anxa1	Annexin A1 OS=Mus musculus GN=Anxa1 PE=1 SV=2	39 kDa	< 0.0001	1.24
P13707	Gpd1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic OS=Mus musculus GN=Gpd1 PE=1 SV=3	38 kDa	< 0.0001	1.24
O88207	**Col5a1**	**Collagen alpha-1(V) chain OS=Mus musculus GN=Col5a1 PE=1 SV=2**	**184 kDa**	**0.025**	**1.24**
Q61554	**Fbn1**	**Fibrillin-1 OS=Mus musculus GN=Fbn1 PE=1 SV=2**	**312 kDa**	**< 0.0001**	**1.23**
Q05816	Fabp5	Fatty acid-binding protein, epidermal OS=Mus musculus GN=Fabp5 PE=1 SV=3	15 kDa	< 0.0001	1.23
P08074	Cbr2	Carbonyl reductase [NADPH] 2 OS=Mus musculus GN=Cbr2 PE=1 SV=1	26 kDa	0.0013	1.23
P97313	Prkdc	DNA-dependent protein kinase catalytic subunit OS=Mus musculus GN=Prkdc PE=1 SV=3	471 kDa	0.031	1.23
P19785	**Esr1**	**Estrogen receptor OS=Mus musculus GN=Esr1 PE=1 SV=1**	**67 kDa**	**0.0031**	**1.23**
P63239	Pcsk1	Neuroendocrine convertase 1 OS=Mus musculus GN=Pcsk1 PE=1 SV=1	84 kDa	< 0.0001	1.22
Q8BHD7	**Ptbp3**	**Polypyrimidine tract-binding protein 3 OS=Mus musculus GN=Ptbp3 PE=1 SV=1**	**57 kDa**	**< 0.0001**	**1.22**
Q9WUB3	Pygm	Glycogen phosphorylase, muscle form OS=Mus musculus GN=Pygm PE=1 SV=3	97 kDa	< 0.0001	1.22
P12656	Tshb	Thyrotropin subunit beta OS=Mus musculus GN=Tshb PE=2 SV=1	15 kDa	0.00035	1.22
Q9WVH9	Fbln5	Fibulin-5 OS=Mus musculus GN=Fbln5 PE=1 SV=1	50 kDa	0.016	1.22
P35455	Avp	Vasopressin-neurophysin 2-copeptin OS=Mus musculus GN=Avp PE=2 SV=1	18 kDa	< 0.0001	1.21
O70624	Myoc	Myocilin OS=Mus musculus GN=Myoc PE=1 SV=1	55 kDa	< 0.0001	1.21
P09470	Ace	Angiotensin-converting enzyme OS=Mus musculus GN=Ace PE=1 SV=3	151 kDa	0.0006	1.21
P11404	Fabp3	Fatty acid-binding protein, heart OS=Mus musculus GN=Fabp3 PE=1 SV=5	15 kDa	< 0.0001	1.21
Q80Z24	Negr1	Neuronal growth regulator 1 OS=Mus musculus GN=Negr1 PE=1 SV=1	38 kDa	0.01	1.21
P47738	Aldh2	Aldehyde dehydrogenase, mitochondrial OS=Mus musculus GN=Aldh2 PE=1 SV=1	57 kDa	< 0.0001	1.21
P17563	Selenbp1	Selenium-binding protein 1 OS=Mus musculus GN=Selenbp1 PE=1 SV=2	53 kDa	< 0.0001	1.21
P48774	Gstm5	Glutathione S-transferase Mu 5 OS=Mus musculus GN=Gstm5 PE=1 SV=1	27 kDa	< 0.0001	1.21
Q8R0F9	Sec14l4	SEC14-like protein 4 OS=Mus musculus GN=Sec14l4 PE=1 SV=1	46 kDa	< 0.0001	1.21
Q810S1	Mcub	Calcium uniporter regulatory subunit MCUb, mitochondrial OS=Mus musculus GN=Mcub PE=1 SV=1	40 kDa	0.00034	1.21
P81117	Nucb2	Nucleobindin-2 OS=Mus musculus GN=Nucb2 PE=1 SV=2	50 kDa	< 0.0001	0.83

(Continued)

TABLE 2 | Continued

Accession Number	Gene Symbol	Identified Proteins	Molecular Weight	P value	Ratio (KO vs wild-type)
P62852	Rps25	40S ribosomal protein S25 OS=Mus musculus GN=Rps25 PE=1 SV=1	14 kDa	< 0.0001	0.83
P84084	Arf5	ADP-ribosylation factor 5 OS=Mus musculus GN=Arf5 PE=1 SV=2	21 kDa	< 0.0001	0.83
P10852	Slc3a2	4F2 cell-surface antigen heavy chain OS=Mus musculus GN=Slc3a2 PE=1 SV=1	58 kDa	< 0.0001	0.83
Q9DC16	Ergic1	Endoplasmic reticulum-Golgi intermediate compartment protein 1 OS=Mus musculus GN=Ergic1 PE=1 SV=1	33 kDa	< 0.0001	0.83
Q9JJI8	Rpl38	60S ribosomal protein L38 OS=Mus musculus GN=Rpl38 PE=1 SV=3	8 kDa	< 0.0001	0.83
P50096	Impdh1	Inosine-5'-monophosphate dehydrogenase 1 OS=Mus musculus GN=Impdh1 PE=1 SV=2	55 kDa	< 0.0001	0.83
Q9QYI6	Dnajb9	DnaJ homolog subfamily B member 9 OS=Mus musculus GN=Dnajb9 PE=1 SV=2	26 kDa	0.0027	0.83
Q91V04	Tram1	Translocating chain-associated membrane protein 1 OS=Mus musculus GN=Tram1 PE=1 SV=3	43 kDa	< 0.0001	0.83
Q9JHH9	Copz2	Coatomer subunit zeta-2 OS=Mus musculus GN=Copz2 PE=1 SV=1	23 kDa	0.00093	0.83
P25322	Ccnd1	G1/S-specific cyclin-D1 OS=Mus musculus GN=Ccnd1 PE=1 SV=1	33 kDa	0.00049	0.83
Q922H9	Znf330	Zinc finger protein 330 OS=Mus musculus GN=Znf330 PE=1 SV=1	36 kDa	0.00035	0.83
Q80UM7	Mogs	Mannosyl-oligosaccharide glucosidase OS=Mus musculus GN=Mogs PE=1 SV=1	92 kDa	< 0.0001	0.82
Q99KK2	Cmas	N-acylneuraminate cytidylyltransferase OS=Mus musculus GN=Cmas PE=1 SV=2	48 kDa	< 0.0001	0.82
Q5I012	Slc38a10	Putative sodium-coupled neutral amino acid transporter 10 OS=Mus musculus GN=Slc38a10 PE=1 SV=2	117 kDa	< 0.0001	0.82
P62267	Rps23	40S ribosomal protein S23 OS=Mus musculus GN=Rps23 PE=1 SV=3	16 kDa	< 0.0001	0.82
P83882	Rpl36a	60S ribosomal protein L36a OS=Mus musculus GN=Rpl36a PE=1 SV=2	12 kDa	< 0.0001	0.82
P60867	Rps20	40S ribosomal protein S20 OS=Mus musculus GN=Rps20 PE=1 SV=1	13 kDa	< 0.0001	0.82
Q9D823	Rpl37	60S ribosomal protein L37 OS=Mus musculus GN=Rpl37 PE=3 SV=3	11 kDa	< 0.0001	0.82
Q3TJZ6	Fam98a	Protein FAM98A OS=Mus musculus GN=Fam98a PE=1 SV=1	55 kDa	0.00012	0.82
Q8K221	Arfip2	Arfaptin-2 OS=Mus musculus GN=Arfip2 PE=1 SV=2	38 kDa	0.00035	0.82
P62862	Fau	40S ribosomal protein S30 OS=Mus musculus GN=Fau PE=1 SV=1	7 kDa	0.00035	0.82
Q9Z0S9	Rabac1	Prenylated Rab acceptor protein 1 OS=Mus musculus GN=Rabac1 PE=1 SV=1	21 kDa	0.0039	0.82
B9EJR8	Dnaaf5	Dynein assembly factor 5, axonemal OS=Mus musculus GN=Dnaaf5 PE=1 SV=1	94 kDa	0.047	0.82
Q9CZB0	Sdhc	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial OS=Mus musculus GN=Sdhc PE=1 SV=1	18 kDa	0.016	0.82
Q8VDJ3	Hdlbp	Vigilin OS=Mus musculus GN=Hdlbp PE=1 SV=1	142 kDa	< 0.0001	0.82
Q8BP67	Rpl24	60S ribosomal protein L24 OS=Mus musculus GN=Rpl24 PE=1 SV=2	18 kDa	< 0.0001	0.82
Q9D1R9	Rpl34	60S ribosomal protein L34 OS=Mus musculus GN=Rpl34 PE=1 SV=2	13 kDa	< 0.0001	0.82
P45878	Fkbp2	Peptidyl-prolyl cis-trans isomerase FKBP2 OS=Mus musculus GN=Fkbp2 PE=1 SV=1	15 kDa	< 0.0001	0.82
P60202	Plp1	Myelin proteolipid protein OS=Mus musculus GN=Plp1 PE=1 SV=2	30 kDa	< 0.0001	0.82
P33622	Apoc3	Apolipoprotein C-III OS=Mus musculus GN=Apoc3 PE=1 SV=2	11 kDa	0.021	0.82
Q9DCF9	Ssr3	Translocon-associated protein subunit gamma OS=Mus musculus GN=Ssr3 PE=1 SV=1	21 kDa	< 0.0001	0.82
Q03157	Aplp1	Amyloid-like protein 1 OS=Mus musculus GN=Aplp1 PE=1 SV=1	73 kDa	0.019	0.81
Q8CI11	Gnl3	Guanine nucleotide-binding protein-like 3 OS=Mus musculus GN=Gnl3 PE=1 SV=2	61 kDa	< 0.0001	0.81
Q4PJX1	Odr4	Protein odr-4 homolog OS=Mus musculus GN=Odr4 PE=1 SV=2	50 kDa	0.00067	0.81
Q91XC8	Dap	Death-associated protein 1 OS=Mus musculus GN=Dap PE=1 SV=3	11 kDa	0.0065	0.81
Q01768	Nme2	Nucleoside diphosphate kinase B OS=Mus musculus GN=Nme2 PE=1 SV=1	17 kDa	< 0.0001	0.81
C0HK80	Arxes2	Adipocyte-related X-chromosome expressed sequence 2 OS=Mus musculus GN=Arxes2 PE=1 SV=1	20 kDa	0.00022	0.81
Q80WW9	Ddrgk1	DDRGK domain-containing protein 1 OS=Mus musculus GN=Ddrgk1 PE=1 SV=2	36 kDa	0.0032	0.81

(Continued)

TABLE 2 | Continued

Accession Number	Gene Symbol	Identified Proteins	Molecular Weight	P value	Ratio (KO vs wild-type)
P42230	Stat5a	Signal transducer and activator of transcription 5A OS=Mus musculus GN=Stat5a PE=1 SV=1	91 kDa	< 0.0001	0.81
Q3TMP8	Tmem38a	Trimeric intracellular cation channel type A OS=Mus musculus GN=Tmem38a PE=1 SV=2	33 kDa	0.031	0.81
Q922Q8	Lrrc59	Leucine-rich repeat-containing protein 59 OS=Mus musculus GN=Lrrc59 PE=1 SV=1	35 kDa	< 0.0001	0.80
O55142	Rpl35a	60S ribosomal protein L35a OS=Mus musculus GN=Rpl35a PE=1 SV=2	13 kDa	< 0.0001	0.80
P61961	Ufm1	Ubiquitin-fold modifier 1 OS=Mus musculus GN=Ufm1 PE=1 SV=1	9 kDa	0.00017	0.80
P47964	Rpl36	60S ribosomal protein L36 OS=Mus musculus GN=Rpl36 PE=3 SV=2	12 kDa	0.0039	0.80
Q99PL5	Rrbp1	Ribosome-binding protein 1 OS=Mus musculus GN=Rrbp1 PE=1 SV=2	173 kDa	< 0.0001	0.80
Q9R0P6	Sec11a	Signal peptidase complex catalytic subunit SEC11A OS=Mus musculus GN=Sec11a PE=1 SV=1	21 kDa	< 0.0001	0.80
Q9CY50	Ssr1	Translocon-associated protein subunit alpha OS=Mus musculus GN=Ssr1 PE=1 SV=1	32 kDa	0.0012	0.80
Q9D8S4	Rexo2	Oligoribonuclease, mitochondrial OS=Mus musculus GN=Rexo2 PE=1 SV=2	27 kDa	< 0.0001	0.80
Q8R1L4	Kdelr3	ER lumen protein-retaining receptor 3 OS=Mus musculus GN=Kdelr3 PE=1 SV=1	25 kDa	0.00035	0.80
P47199	Cryz	Quinone oxidoreductase OS=Mus musculus GN=Cryz PE=1 SV=1	35 kDa	< 0.0001	0.79
Q64674	Srm	Spermidine synthase OS=Mus musculus GN=Srm PE=1 SV=1	34 kDa	< 0.0001	0.79
Q8K009	Aldh1l2	Mitochondrial 10-formyltetrahydrofolate dehydrogenase OS=Mus musculus GN=Aldh1l2 PE=1 SV=2	102 kDa	< 0.0001	0.79
Q8R1U2	Cgref1	Cell growth regulator with EF hand domain protein 1 OS=Mus musculus GN=Cgref1 PE=1 SV=1	31 kDa	0.00015	0.79
Q8VEL9	Rem2	GTP-binding protein REM 2 OS=Mus musculus GN=Rem2 PE=1 SV=2	37 kDa	< 0.0001	0.79
P21956	Mfge8	Lactadherin OS=Mus musculus GN=Mfge8 PE=1 SV=3	51 kDa	< 0.0001	0.78
Q9D8V7	Sec11c	Signal peptidase complex catalytic subunit SEC11C OS=Mus musculus GN=Sec11c PE=1 SV=3	22 kDa	< 0.0001	0.78
Q9CXI5	Manf	Mesencephalic astrocyte-derived neurotrophic factor OS=Mus musculus GN=Manf PE=1 SV=1	20 kDa	< 0.0001	0.78
O70251	Eef1b	Elongation factor 1-beta OS=Mus musculus GN=Eef1b PE=1 SV=5	25 kDa	< 0.0001	0.77
Q78XF5	Ostc	Oligosaccharyltransferase complex subunit OSTC OS=Mus musculus GN=Ostc PE=1 SV=1	17 kDa	0.0039	0.77
Q9CQS8	Sec61b	Protein transport protein Sec61 subunit beta OS=Mus musculus GN=Sec61b PE=1 SV=3	10 kDa	< 0.0001	0.77
Q61036	Pak3	Serine/threonine-protein kinase PAK 3 OS=Mus musculus GN=Pak3 PE=1 SV=2	62 kDa	< 0.0001	0.77
Q91X91	Qprt	Nicotinate-nucleotide pyrophosphorylase [carboxylating] OS=Mus musculus GN=Qprt PE=1 SV=1	32 kDa	0.0031	0.77
Q61941	Nnt	NAD(P) transhydrogenase, mitochondrial OS=Mus musculus GN=Nnt PE=1 SV=2	114 kDa	< 0.0001	0.76
Q05186	Rcn1	Reticulocalbin-1 OS=Mus musculus GN=Rcn1 PE=1 SV=1	38 kDa	< 0.0001	0.76
C0HKG5	Rnaset2a	Ribonuclease T2-A OS=Mus musculus GN=Rnaset2a PE=1 SV=1	30 kDa	< 0.0001	0.76
Q8K023	Akr1c18	Aldo-keto reductase family 1 member C18 OS=Mus musculus GN=Akr1c18 PE=1 SV=2	37 kDa	0.0039	0.76
Q8R059	Gale	UDP-glucose 4-epimerase OS=Mus musculus GN=Gale PE=1 SV=1	38 kDa	< 0.0001	0.76
P61205	Arf3	ADP-ribosylation factor 3 OS=Mus musculus GN=Arf3 PE=2 SV=2	21 kDa	< 0.0001	0.75
P23927	Cryab	Alpha-crystallin B chain OS=Mus musculus GN=Cryab PE=1 SV=2	20 kDa	0.0039	0.75
Q8BH97	Rcn3	Reticulocalbin-3 OS=Mus musculus GN=Rcn3 PE=1 SV=1	38 kDa	< 0.0001	0.74
Q8CFA2	Amt	Aminomethyltransferase, mitochondrial OS=Mus musculus GN=Amt PE=1 SV=1	44 kDa	< 0.0001	0.74

(Continued)

TABLE 2 | Continued

Accession Number	Gene Symbol	Identified Proteins	Molecular Weight	P value	Ratio (KO vs wild-type)
Q922W5	Pycr1	Pyrroline-5-carboxylate reductase 1, mitochondrial OS=Mus musculus GN=Pycr1 PE=1 SV=1	32 kDa	< 0.0001	0.74
P34884	Mif	Macrophage migration inhibitory factor OS=Mus musculus GN=Mif PE=1 SV=2	13 kDa	< 0.0001	0.73
Q9D7S7	Rpl22l1	60S ribosomal protein L22-like 1 OS=Mus musculus GN=Rpl22l1 PE=1 SV=1	14 kDa	0.00049	0.73
Q9WUT3	Rps6ka2	Ribosomal protein S6 kinase alpha-2 OS=Mus musculus GN=Rps6ka2 PE=1 SV=1	83 kDa	< 0.0001	0.69
Q9D1M7	Fkbp11	Peptidyl-prolyl cis-trans isomerase FKBP11 OS=Mus musculus GN=Fkbp11 PE=1 SV=1	22 kDa	< 0.0001	0.69
P07759	Serpina3k	Serine protease inhibitor A3K OS=Mus musculus GN=Serpina3k PE=1 SV=2	47 kDa	< 0.0001	0.69
Q60841	Reln	Reelin OS=Mus musculus GN=Reln PE=1 SV=3	387 kDa	< 0.0001	0.66
P61750	Arf4	ADP-ribosylation factor 4 OS=Mus musculus GN=Arf4 PE=1 SV=2	20 kDa	0.00035	0.66
Q60590	Orm1	Alpha-1-acid glycoprotein 1 OS=Mus musculus GN=Orm1 PE=1 SV=1	24 kDa	0.00035	0.66
P47212	Gal	Galanin peptides OS=Mus musculus GN=Gal PE=2 SV=1	13 kDa	0.0039	0.65
P51667	Myl2	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform OS=Mus musculus GN=Myl2 PE=1 SV=3	19 kDa	< 0.0001	0.64
P06879	Prl	Prolactin OS=Mus musculus GN=Prl PE=2 SV=1	25 kDa	< 0.0001	0.61
Q640N1	Aebp1	Adipocyte enhancer-binding protein 1 OS=Mus musculus GN=Aebp1 PE=1 SV=1	128 kDa	< 0.0001	0.57
Q60571	Crhbp	Corticotropin-releasing factor-binding protein OS=Mus musculus GN=Crhbp PE=2 SV=1	36 kDa	0.00035	0.53
P09542	Myl3	Myosin light chain 3 OS=Mus musculus GN=Myl3 PE=1 SV=4	22 kDa	0.00035	0.46

mutant pituitary by 72% may compensate for the deficiency in the secretory mechanisms of the mutant mice (**Figure 7C**), which may also explain the fertility of male mutant mice. There is much agreement that FSH influences the mitotic activity of the spermatogonia and promote cellular differentiation during the pubertal phase (61). Testosterone regulated by LH also plays a role for spermatogenesis, however, completely T-independent spermatogenesis is possible if high-dose FSH treatment (62).

Of note, the use of intraventricular injection of *miR-29* inhibitor or overexpression of an antisense sequence targeting *miR-29* in the brain to knockdown expression of *miR-29* leads to earlier puberty onset or hyperfertility (63). These findings are not consistent with our results. It is possible that lack of *miR-29a/b*$_1$ function throughout development could result in compensatory effects which may lead to differences between our results and the results of the above literature. The underlying reasons for the different effects between knockout and knockdown need to be further studied. In addition, it should be noted that KO mice also showed growth retardation (64). We found that the weight of KO mice remained light, even though they had reached sexual maturity. So, the causal relation between two events cannot be confirmed now. We speculated that growth retardation and delayed maturity may come from the same reason, which happened in pituitary or upstream signal of KO mice.

In conclusion, LH secretion was impaired by *miR-29a/b*$_1$ knockout which caused ovulation deficiency in the mutant mice. Further studies revealed the effect of *miR-29a/b*$_1$ on hormone secretion function in the pituitary. Our work provides novel mechanistic insights into the relationship of *miR-29a/b*$_1$ and reproduction, opening the possibility of clinical approaches to reproductive studies based on the regulatory circuitry of *miR-29a/b*$_1$.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Shanghai Engineering Research Center for Model Organisms, SMOC. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YG, RSu, RSh and JF designed research. YG and JF analyzed data. YG, YW, HS, HZ, LC, QH, ZhiW, and YT performed research. YG, LX and JF wrote the paper. HY, MZ and ZhuW contributed to discussion and the proof reading of the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.636220/full#supplementary-material

Supplementary Table 1 | List of oligonucleotides used.

Supplementary Figure 1 | Genotyping of *miR-29a/b*$_1$ KO knockout mice. **(A)** The genotype of *miR-29a/b*$_1$ KO was identified by PCR amplification. There was 600bp deleted from the genomic DNA of *miR-29a/b*$_1$. +/+: wild-type, +/-: heterozygous, -/-: homozygous. **(B)** Mature *miR-29a* RNA was detected in different tissues of wild-type mice but not in those of homozygous knockout mice (n=3). The precursor of *miR-29a* **(C)** and *miR-29b*$_1$ **(D)** RNA level was measured by quantitative RT-PCR in different tissues. *Pre-miR-29a* or *pre-miR-29b*$_1$ levels were decreased in *miR-29a/b*$_1^{+/-}$ mice (n=10) and hardly detected in *miR-29a/b*$_1^{-/-}$ mice (n=10) compared to wild-type littermates (n=8).

Supplementary Figure 2 | Representative cell morphological changes in vaginal smears and estrous cycle pattern of four female mice in each wild-type and *miR-29a/b*$_1$ KO groups are shown.

Supplementary Figure 3 | *miR-29a* expression patterns. **(A)** *miR-29a* expression patterns in wild-type mice during the estrous cycle in pituitary and ovary tissues (n=10). 1, proestrus; 2, estrus; 3, metestrus; 4, diestrus. **(B)** Relative expression levels of *miR-29a*, *miR-29b* and *miR-29c* in hypothalamus, pituitary and gonad (HPG axis) of wild-type and *miR-29a/b*$_1$ KO mice (♀miR-29c: hypothalamus: *p=0.6648*, pituitary: *p=0.4896*, ovary: *p=0.8028*, ♂miR-29c: hypothalamus: *p=0.0727*, pituitary: *p=0.1225*, testis: *p=0.5042*, n=5).

Supplementary Figure 4 | Wet testis and seminal vesicle (SV) weight in males, normalized to body weight in the same animals (testis: wild-type: 0.7234 ± 0.02481, n=8, *miR-29a/b*$_1$ KO: 0.9563 ± 0.03834, n=9, *p=0.2107*; seminal: wild-type: 0.6085 ± 0.07193, n=8, *miR-29a/b*$_1$ KO: 0.5191 ± 0.03874, n=9, *p=0.2764*).

Supplementary Figure 5 | Levels of selected hormones in females. **(A–C)** Serum FSH (wild-type: 12.23 ± 1.415, n=8, *miR-29a/b*$_1$ KO: 13.62 ± 1.255, n=13, *p=0.4850*) **(A)** and Testosterone (wild-type: 2.774 ± 0.1147, n=10, *miR-29a/b*$_1$ KO: 2.711 ± 0.2052, n=9, *p=0.7867*). **(B)** and Estradiol (wild-type: 207.9 ± 13.89, n=17, *miR-29a/b*$_1$ KO: 179.3 ± 15.54, n=19, *p=0.1834*). **(C)** of wild-type and *miR-29a/b*$_1$ KO mice were determined. **(D)** Expression of hormone synthesis-related gene in the ovaries in wild-type (n=4) and *miR-29a/b*$_1$ KO (n=10) females (*cyp11a*: *p=0.0159*, *cyp17a*$_1$: *p=0.6094*, *cyp19a*$_1$: *p=0.9604*).

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BRIEF RESEARCH REPORT
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Identification of Novel Biallelic *TLE6* Variants in Female Infertility With Preimplantation Embryonic Lethality

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Preimplantation embryonic lethality is a rare cause of primary female infertility. It has been reported that variants in the transducin-like enhancer of split 6 (*TLE6*) gene can lead to preimplantation embryonic lethality. However, the incidence of *TLE6* variants in patients with preimplantation embryonic lethality is not fully understood. In this study, we identified four patients carrying novel biallelic *TLE6* variants in a cohort of 28 patients with preimplantation embryonic lethality by whole-exome sequencing and bioinformatics analysis, accounting for 14.29% (4/28) of the cohort. Immunofluorescence showed that the TLE6 levels in oocytes from patients were much lower than in normal control oocytes, suggesting that the variants result in the lower expression of the TLE6 protein in oocytes. In addition, a retrospective analysis showed that the four patients underwent a total of nine failures of *in vitro* fertilization and intracytoplasmic sperm injection attempts, and one of them became pregnant on the first attempt using donated oocytes. Our study extends the genetic spectrum of female infertility caused by variants in *TLE6* and further confirms previously reported findings that TLE6 plays an essential role in early embryonic development. In such case, oocyte donation may be the preferred treatment.

Keywords: preimplantation embryonic lethality, transducin-like enhancer of split 6 (TLE6), variant, whole-exome sequencing, oocyte donation

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INTRODUCTION

Infertility affects about 10–15% of couples worldwide and has become an increasingly common health problem (Tamrakar and Bastakoti, 2019). In recent years, assisted reproductive technology (ART) has become an important treatment for many women suffering from infertility. Recent evidence from ART and embryo research suggests that preimplantation embryonic lethality (PEL)

(OMIM:616814) may be a rare cause of primary female infertility (Yatsenko and Rajkovic, 2019). Women with PEL have normal ovarian follicle development and ovulation while suffering from recurrent failures of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) attempts due to fertilization failure and early embryonic arrest. It is challenging to identify genes that cause preimplantation embryonic lethality in humans.

Maternal effect genes (MEGs) are vital during embryonic cleavage stages. After fertilization, the zygotic genome is transcriptionally quiescent, and early embryo development relies on MEGs that encode many of the RNAs and proteins required for early divisions, chromatin remodeling, epigenetic reprogramming, and transcriptional activation cascades (Jukam et al., 2017). Subcortical maternal complex (SCMC), present in the oocytes and early embryos, contains multiple proteins encoded by MEGs and has been identified as being important for preimplantation mouse embryogenesis (Lu et al., 2017). The SCMC appears to be functionally conserved throughout mammalian species. It consists of at least eight proteins, including OOEP, NLRP5, TLE6, KHDC3L, PADI6, ZBED3, NLRP2, and NLRP7 (Zhu et al., 2015; Xu et al., 2016; Mahadevan et al., 2017; Monk et al., 2017; Gao et al., 2018). In mice, knockout of certain MEGs, namely, *Nlrp2*, *Mater*, *Padi6*, *Floped*, and *Tle6*, leads to infertility or subfertility owing to embryonic arrest (Tong et al., 2000; Li et al., 2008; Yurttas et al., 2008; Yu et al., 2014; Mahadevan et al., 2017). Recently, some MEGs have been identified in humans by way of whole-exome sequencing (WES) in a limited number of clinical cases. For example, *TLE6* (OMIM: 612399) variants have been shown to result in the earliest known PEL in human (Alazami et al., 2015). Furthermore, biallelic variants in *PATL2* (OMIM: 614661), *WEE2* (OMIM: 614084), *PADI6* (OMIM: 610363), *NLRP5* (OMIM: 609658), and *NLRP2* (OMIM: 609364) have been identified as the causes of a spectrum of PEL phenotypes, including oocyte maturation arrest, fertilization failure, and early embryonic arrest (Xu et al., 2016; Chen et al., 2017; Sang et al., 2018; Mu et al., 2019). However, variants in these genes can only explain a few cases, and the genetic basis of PEL is still largely unclear.

In the current study, we identified novel biallelic variants in *TLE6* in four patients (14.29%, 4/28) from three unrelated families in a small cohort of 28 women affected with PEL by WES, and these variants were confirmed by Sanger sequencing. In addition, immunofluorescence showed that the variants significantly reduced the amount of TLE6 protein in the oocytes from patients. These findings expand the variant spectrum of *TLE6*.

MATERIALS AND METHODS

Study Subjects

We recruited 28 women affected with PEL from the First Affiliated Hospital of Anhui Medical University, between January 2018 and November 2020. All of the 28 infertile women recruited in our study satisfied the following enrolled criteria for PEL: (1) women aged 20–40 years were diagnosed with primary infertility; (2) normal ovulatory status and the morphology of the oocytes without obvious abnormalities; (3) more than once failure of IVF/ICSI cycles caused by embryonic arrest (no high-quality blastocyst). We also excluded women suffering from other causes of infertility, containing chromosomal anomalies, male factors, endometriosis, and endocrinological causes. The clinical characteristics of all 28 infertile women are listed in **Supplementary Table 1**. Peripheral blood samples for DNA extraction were obtained from the affected individuals, their available family members, and control subjects. This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (number Quick-PJ2020-13-10). All of the subjects gave their informed consent to participate.

WES, Bioinformatic Analysis, and Sanger Sequencing

Genomic DNA was extracted from peripheral blood of the affected women using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Whole-exome capture was performed using SureSelect^{XT} Human All Exon Kit (Agilent Technologies, Santa Clara, CA, United States) following the manufacturer's instructions, and sequencing was carried out on the HiSeq X-TEN platform (Illumina, San Diego, CA, United States). The Burrows–Wheeler aligner was employed to map the original data to the human genome assembly GRCh37/hg19 (Li and Durbin, 2009). The Picard software was used to delete PCR duplicates and evaluate the quality of variants by obtaining valid reads, valid base, × 90– × 120 coverage ratio, and average coverage depth. We employed the Genome Analysis Toolkit to call and analyze the indels as well as single-nucleotide variants. We filtered out the single-nucleotide variants with read depths less than × 4 (McKenna et al., 2010). Detailed information about the analytical methods was described previously (Lv et al., 2020). Allele frequencies of the variants were searched using the Exome Aggregation Consortium (ExAC) database[1], the 1000 Genomes Project database[2], and the Genome Aggregation Database (gnomAD)[3]. The candidate variants and their parental origins were confirmed via Sanger sequencing.

Evaluation of Embryo Phenotypes

We used a light microscope (IX-71, Olympus, Japan) to observe morphologies of the embryos at different stages of development. Quality of each embryo was evaluated at several predefined timepoints during embryo development according to the conventional guidelines as described previously (Ding et al., 2020).

Immunofluorescence

Immature (germinal vesicle stage or metaphase I) and unfertilized (metaphase II, MII) oocytes were donated by the affected individuals and control subjects pursuing IVF/ICSI due to male infertility. These immature oocytes were matured *in vitro* following previously described methods (Zou et al., 2020).

[1]http://exac.broadinstitute.org/

[2]http://www.1000genomes.org/

[3]http://gnomad.broadinstitute.org/

Oocyte immunofluorescence staining was performed to assess TLE6 localization. Briefly, oocytes from all subjects (control as well as patients) were fixed with 4% paraformaldehyde for 30 min. The oocytes were then processed with a membrane permeabilizing solution [0.1% Triton X-100 in phosphate-buffered saline (PBS)] for 30 min, followed by blocking with 5% donkey serum for 1 h. Furthermore, the oocytes were incubated with a primary mouse anti-TLE6 antibody (1:50, sc-515065, Santa Cruz Biotechnology, Santa Cruz, CA, United States) overnight at 4°C. The oocytes were then incubated with Alexa Fluor 488 AffiniPure Donkey anti-mouse immunoglobulin G (IgG) (H + L) (1:100, 715-545-150, Jackson ImmunoResearch Laboratories, West Grove, PA, United States) and protected from light for 1 h at room temperature. Following this, the oocytes were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min to label DNA. Finally, the oocytes were visualized using an LSM800 confocal laser-scanning microscope (Zeiss, Jena, Germany).

RESULTS

Clinical Characteristics of Patients

The clinical information of the four patients carrying the biallelic *TLE6* variants are listed in **Table 1**, and their family pedigrees are shown in **Figure 1A**. Their menstrual cycles, karyotypes, transvaginal sonography, and sex hormone levels revealed no abnormalities. Furthermore, their husbands also showed normal semen parameters (sperm concentration, motility, and sperm morphology) as well as karyotypes (**Table 1**). These patients had been unable to get pregnant despite years of trying.

In family 1, subjects I-1 and I-2 raised four daughters. Three of four sisters (II-2, II-3, and II-4) in this family had infertility for several years. Two of the affected sisters (II-3 and II-4) underwent several IVF/ICSI attempts in the Reproductive Medicine Center of the First Affiliated Hospital of Anhui Medical University. The proband (II-3, 37 years old) had undergone two IVF/ICSI attempts. A total of 44 MII oocytes were retrieved in the two attempts. Only five oocytes were normally fertilized with two-pronucleus (PN) zygotes, while the others were abnormally fertilized with 0PN or degradation on day 1. After cultivation, a majority of her embryos were arrested at the early stages with heavy fragmentation, and only two poor quality blastocysts were available for transfer. Although the proband underwent one frozen–thawed embryo transfer cycle, she failed to obtain a successful pregnancy (**Supplementary Table 2**).

The other affected sister (II-4, 33 years old) in family 1 had undergone four IVF/ICSI attempts. A total of 55 MII oocytes were retrieved in the four attempts. A majority of oocytes were abnormally fertilized with 0PN; only 10 of them showed normal fertilization with 2PN zygotes on day 1. Most of her embryos were arrested at the early stages accompanied with heavy fragmentation. Only six embryos developed into poor quality blastocysts and were frozen. Patient II-4 underwent three frozen–thawed embryo transfer cycles, none of which was successful (**Supplementary Table 2**). Finally, she followed an oocyte donation ICSI cycle in which seven donated MII oocytes were obtained. The normal fertilization rate and blastocyst development rate in this cycle were 100.0 and 42.9%, respectively. Patient II-4 obtained three high-quality blastocysts at last and got pregnant on the first embryo transfer.

In family 2, the proband (II-1, 32 years old) had undergone an IVF attempt in which three MII oocytes were obtained. All oocytes had abnormal fertilization with 0PN or degradation on day 1 and were arrested during further blastocyst culture without blastocyst formation (**Supplementary Table 2**).

In family 3, the proband (II-1, 32 years old) had undergone two IVF/ICSI attempts, which also resulted in 17 retrieved MII oocytes. Most of the oocytes were abnormally fertilized with 0PN or degradation on day 1. Only one of the zygotes formed a poor quality blastocyst, while the others showed developmental arrest on day 3. The patient underwent a frozen–thawed embryo transfer cycle but failed to establish pregnancy (**Supplementary Table 2**).

Identification of Biallelic Variants in *TLE6*

We recruited 28 affected individuals with preimplantation embryonic lethality and identified four affected individuals (accounting for 14.29% of the cohort) from three unrelated families carrying biallelic variants *TLE6* (NM_001143986.1) by whole-exome sequencing and bioinformatics analyses. Two (II-3 and II-4 in family 1) of the four affected individuals were sisters from a non-consanguineous family in which three-quarters of the sisters were diagnosed with primary infertility, both of them carrying a homozygous *TLE6* frameshift variant c.1631_1632delCA (p.Pro544Argfs*5). In family 1, Sanger sequencing verified that the parents and one fertile elder sister were heterozygous carriers, while the other three sisters were homozygous for the variant, indicating a recessive inheritance pattern (**Figure 1A**). Another homozygous frameshift variant in *TLE6* (c.475_476delCT, p.Leu159Aspfs*14) was identified in the proband (II-1 in family 2) from a consanguineous family. Sanger sequencing confirmed that the proband's parents were both heterozygous carriers (**Figure 1A**). Moreover, we identified a compound heterozygous variant in *TLE6* (c.222G > C, p.Gln74His; c.798_799insG, p.Gln267Alafs*54) in the proband (II-1 in family 3) from another non-consanguineous family, and the two variants were also inherited from her heterozygous parental carriers, respectively (**Figure 1A**).

These *TLE6* variants were absent in the ExAC database, the 1000 Genomes Project database, and the gnomAD database, except that the *TLE6* frameshift variant c.475_476delCT was found at extremely low allele frequency (4.0×10^{-6}) in the general population in the gnomAD (**Table 1**). The three *TLE6* frameshift variants c.1631_1632delCA, c.475_476delCT, and c.798_799insG were all loss-of-function (LoF) variants that caused impaired function of the gene-encoded protein. Positions of the four *TLE6* variants and conservation of mutant residues in their expressed protein among different species are shown in **Figure 2**. Only the *TLE6* frameshift variant c.1631_1632delCA (p.Pro544Argfs*5) was located within the C-terminal WD40 repeat domain, while the other three variants were to the left of the WD40 repeat domain (**Figure 1B**). The positions of these four variants are highly conserved in primates (**Figure 1C**).

Phenotypic Spectrum of Patients With *TLE6* Variants

We used light microscopy to observe the development and morphology of the embryos from family 1 member II-4 for 5 consecutive days in her last ICSI attempt. Five of the embryos on day 3 were arrested, whereas the others had a high percentage of fragmentation, and all of them failed to form blastocysts (**Figure 2A**). An immunofluorescence analysis showed that an immunofluorescence signal for TLE6 was observed in the cytoplasm in the control oocytes, similar to previous reports of the localization of other SCMC component proteins in human oocytes, such as PADI6 and NLRP2 (Xu et al., 2016; Mu et al., 2019). However, the TLE6 signal was much weaker in oocytes from the affected individual (II-4 in family 1) compared with control oocytes (**Figure 2B**). These data suggest that the biallelic variants in *TLE6* result in lower expression of the TLE6 protein in oocytes.

DISCUSSION

Many infertile patients have experienced several failed attempts of IVF/ICSI. In some of them, ovulatory status is normal and the obtained oocytes look normal, but zygote formation and embryonic development are severely impaired; the phenotypes in these patients including failure of fertilization failure and early embryonic arrest are referred to preimplantation embryonic lethality. In the present study, using WES and bioinformatics analyses, we identified biallelic *TLE6* variants in 4 patients from a cohort of 28 infertile women with PEL, accounting for 14.29% of the cohort.

TLE6, also known as Groucho family member 6 (GRG6), is part of the SCMC that is necessary for mammalian embryonic development (Bajoghli, 2007; Bebbere et al., 2016). Research has shown that high levels of cyclic adenosine monophosphate (cAMP) in the oocyte maintain an increase in cAMP-dependent protein kinase (PKA) activity, which causes meiotic prophase I arrest. Following the luteinizing hormone surge during ovulation, PKA activity reduces due to a decrease in cAMP levels, leading to a resumption of meiosis, while PKA activity increases throughout the process of meiosis from the time of germinal vesicle breakdown (GVBD) until the MII arrest (Duncan et al., 2014). TLE6 is a substrate of PKA during mouse oocyte maturation. Inhibition of PKA activity can lead to delays in GVBD dynamics, abnormal spindle and chromatin structures, and a reduced ability of oocytes to undergo MII

TABLE 1 | Clinical laboratory evaluation for the patients carrying variants in *TLE6*.

		Patients			
		II-3, II-4 in family 1		II-1 in family 2	II-1 in family 3
Gene				***TLE6***	
cDNA variant[a]		c.1631_1632delCA		c.475_476delCT	c.798_799insG sG / c.222G > C
Protein alteration[b]		p.Pro544Argfs*5		p.Leu159Aspfs*14	p.Gln267Alafs*54 / p.Gln74His
Variant type		Frameshift		Frameshift	Frameshift / Missense
Zygosity		Homozygous		Homozygous	Heterozygous / Heterozygous
Exon		Exon 17		Exon 7	Exon 12 / Exon 5
Allele frequency	1KGP	NA		NA	NA / NA
	ExAC	NA		NA	NA / NA
	gnomAD	NA		4.0×10^{-6}	NA / NA
Karyotype					
Female		46, XX	46, XX	46, XX	46, XX
Male		46, XY	46, XY	46, XY	46, XY
Female sex hormone					
FSH (mIU/ml)		7.34	6.68	8.81	4.19
LH (mIU/ml)		5.14	3.45	4.64	2.45
E2 (pmol/L)		138.00	156.63	296.00	169.00
P (nmol/L)		4.30	0.76	5.62	1.59
T (nmol/L)		1.40	0.47	2.90	2.80
PRL (ng/ml)		13.39	17.49	13.32	13.83
Male semen parameters					
Sperm concentration (10^6/ml)		28.7	59.2	20.5	7.8
Progressive motility (%) (%)		27.8	47.7	29.8	23.9
Normal sperm morphology (%)		7	5	4	3

[a] *The GenBank accession numbers of TLE6 is NM_001143986.1.*

[b] *Full length protein has 572 amino acids. The "*" indicates the termination of protein translation and the number after "*" represents the number of amino acid position corresponding to the stop codon.*

1KGP, 1000 Genomes Project; ExAC, Exome Aggregation Consortium; gnomAD, Genome Aggregation Database; NA, not available; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; P, progesterone; T, testosterone; PRL: prolactin.

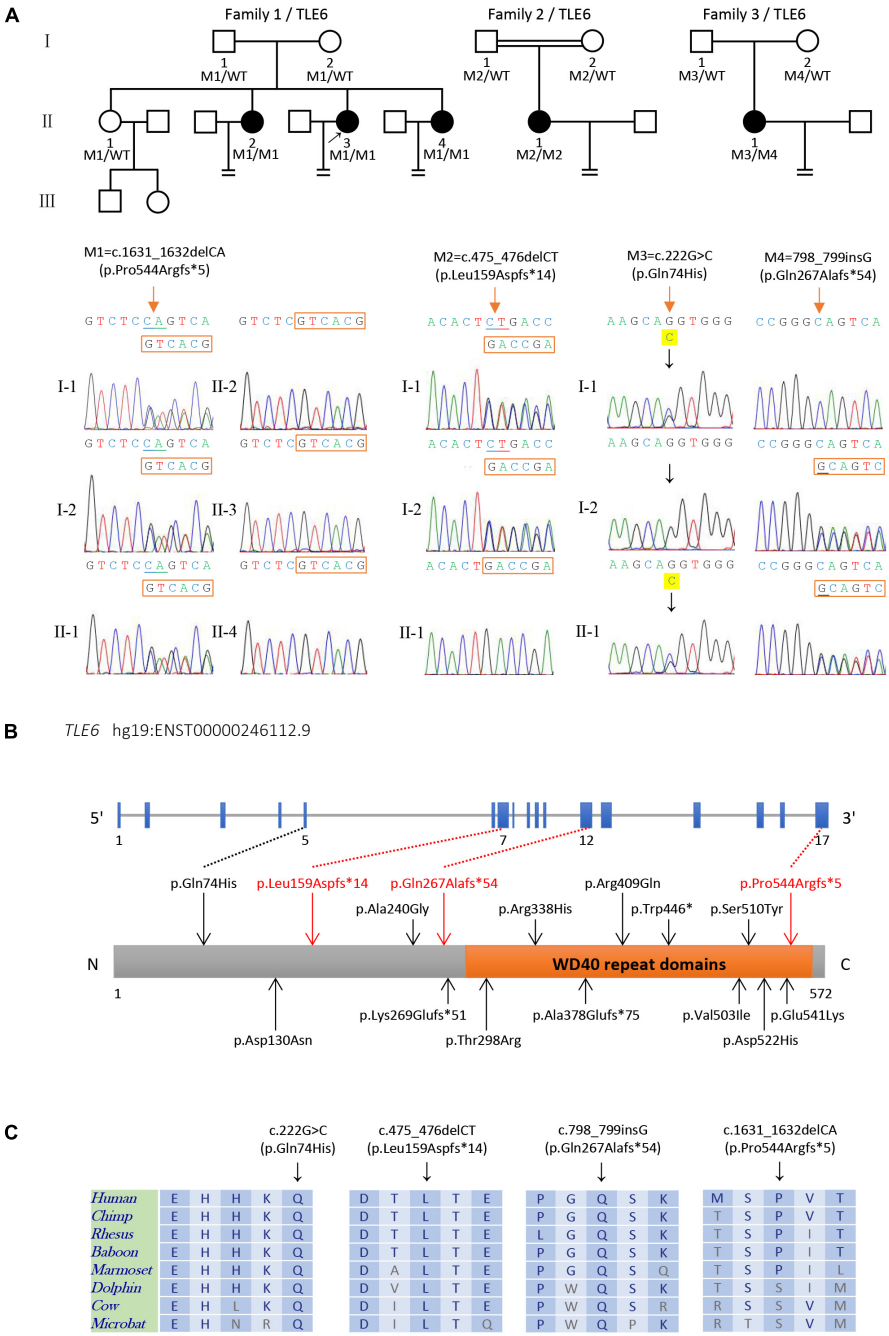

FIGURE 1 | Identification of biallelic *TLE6* variants in the patients and their family members. **(A)** Pedigrees structure of three families harboring variants in *TLE6*, circles represent female patients, squares represent male patients, and black circles denote patients with primary infertility. The double line represents consanguinity, equal signs indicate infertility, and the arrow indicates the proband. Sanger sequencing verification is shown below the pedigrees. Red arrows indicate the mutated positions. **(B)** Schematic representation of the *TLE6* gene and domain structure of its protein product. Black arrows indicate previously reported variants, while red arrows represent the novel variants identified in this study. Orange box represents a cluster of seven WD40 domain repeats. **(C)** Sequence alignment displays conservation of mutant residues in TLE6 among different species.

(Duncan et al., 2014). Therefore, it has been speculated that the PKA-dependent phosphorylation of TLE6 during GVBD may be relevant to oocyte maturation and subsequent embryonic development. In mouse models, knockout of the *Tle6* gene has no effect on folliculogenesis, oogenesis, and ovulation but instead results in embryonic development arrest at the two-cell stage, and some embryos showed significant fragmentation at embryonic days 2.5 and 3.5, eventually leading to female

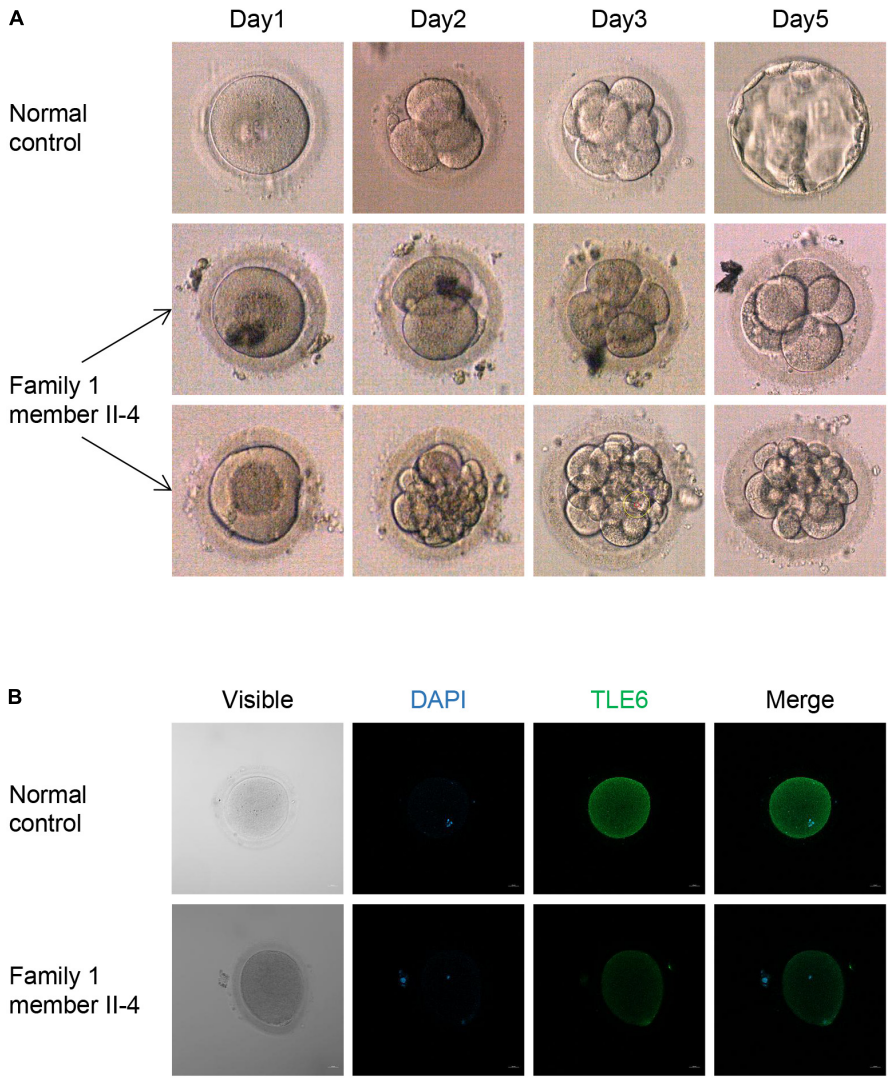

FIGURE 2 | Phenotypes of embryos and oocytes from control and patients. **(A)** Phenotype of control embryos from a subject underwent the ICSI attempt owing to male infertility and embryos from the patient II-4 in family 1. The light microscope was used to observe the morphologies of embryos on days 1, 2, 3, and 5 during cultivation. **(B)** The morphologies and immunofluorescence staining results of an oocyte from the patient II-4 in family 1 and a normal oocyte from a control individual. Oocytes were stained with DAPI (blue) in order to visualize the DNA and immunolabeled with antibodies against TLE6 (green). Scale bar, 20 μm.

mice infertility (Li et al., 2008; Yu et al., 2014). Further studies indicate that the SCMC might regulate spindle assembly by controlling formation of the F-actin cytoskeleton to ensure symmetric division of mouse zygotes, while the absence of TLE6 affects the formation of F-actin cytoskeleton due to destruction of the integrity and function of the SCMC and thus results in asymmetric cleavage as well as early embryonic arrest (Yu et al., 2014).

The currently reported mutational and phenotypic spectrum of *TLE6* is listed in **Supplementary Table 3**. Alazami et al. (2015) first identified a homozygous *TLE6* variant c.1529C > A (p.S510Y) in affected individuals from two Saudi families, which resulted in the earliest known human PEL phenotype, including fertilization failure and early cleavage failure. Their further research showed that the *TLE6* variant not only caused a significant reduction in the PKA-mediated TLE6 phosphorylation but also impaired its binding to other component proteins of the SCMC (Alazami et al., 2015). Wang et al. (2018) found the *TLE6* variant c.1133delC (p.A378Efs*75) being responsible for embryonic developmental arrest on day 3, similar to the phenotype of $Tle6^{Null}$ mice. Furthermore, another study also found that three patients carrying biallelic *TLE6* variants had fertilization failure and early embryonic arrest in several IVF/ICSI attempts. Two of them obtained a very low number of low-quality embryos but failed to establish pregnancy (Lin et al., 2020). Recently, using time-lapse imaging, Zheng et al. (2021) found that the *TLE6* missense variant c.1564G > C (p.Asp522His) is associated

with direct cleavage (zygotes directly cleaved into more than two blastomeres).

In our study, we mainly focused on patients with PEL and did not include cases of oocyte malformation, oocyte maturation arrest, and repeated implantation failure. Therefore, the incidence of *TEL6* variants in our study is higher than other studies (Lin et al., 2020; Maddirevula et al., 2020). Furthermore, phenotypes of infertile women harboring biallelic *TLE6* variants in this study are similar to the previously reported clinical cases (Alazami et al., 2015; Wang et al., 2018; Lin et al., 2020; Zheng et al., 2021). These four patients underwent a total of nine IVF/ICSI attempts in which the number and morphology of retrieved MII oocytes were not clearly abnormal. However, a majority of their oocytes were abnormally fertilized with 0PN or degradation on day 1, and the normal fertilization rate (the 2PN rate) was low (10.3 ± 7.6%). Patients II-4 and II-3 from family 1 had embryonic arrest with a high proportion of embryo fragmentation (36.1 ± 1.1%), while the others only represented embryonic arrest without heavy embryo fragmentation (**Supplementary Table 2**), indicating that patients harboring different *TLE6* variants showed a broad range of phenotypes, containing poor fertilization, embryonic arrest, and a high rate of embryo fragmentation. Patient II-4 from family 1 got pregnant successfully at the first attempt using donated oocytes. In contrast, subject II-1 in family 1, who had a heterozygous variant in *TLE6,* had normal fertility and two healthy children. Furthermore, in our cohort of control women pursuing IVF/ICSI due to male infertility, we also found that a subject harboring a heterozygous missense variant in TLE6 (c.1067T > C, p.L356P) followed a sperm donation ICSI cycle in which 11 MII oocytes were obtained. The normal fertilization rate and blastocyst development rate in this cycle were 100.0 and 18.2%, respectively. She obtained two high-quality blastocysts and achieved pregnancy on the first embryo transfer. These clinical cases combined with Sanger sequencing can help further understand the inheritance pattern of the *TLE6* mutant gene.

In addition, we used light microscopy for five consecutive days to observe the development and morphology of the embryos from one patient (II-4 in family 1) who carried the homozygous *TLE6* frameshift variant c.1631_1632delCA (p.Pro544Argfs*5) and found that three embryos had a high percentage of fragmentation during culture, whereas the other five embryos were arrested on the third day, showing similar phenotype in embryogenesis between infertile women carrying *TLE6* variants and $Tle6^{Null}$ female mice. Thus, combined with previous reports, we speculated that the *TLE6* missense/frameshift variants lead to embryo fragmentation by disrupting the F-actin and spindle dynamics. In addition, our study is the first to assess the expression levels of the TLE6 protein in the oocytes of affected individuals. Immunofluorescence staining showed that the biallelic variants in *TLE6* in the present study resulted in the lower expression of TLE6 in the oocytes of affected individuals. From this, we speculate that the lower expression of TLE6 protein might then affect the stability and function of the SCMC, eventually leading to preimplantation embryonic lethality.

There are a few limitations associated with the current study. First, the exact molecular mechanism of PEL could not be completely elucidated owing to the paucity of human oocytes and embryos. It will be worthwhile to study the molecular mechanism using knock-in mice for each variant in the future. Second, sample size of affected women was limited in the present study, so the incidence of *TLE6* variants in patients with PEL requires further research.

In conclusion, this study extends the spectrum of variants in *TLE6* and suggests that biallelic *TLE6* variants are likely responsible for preimplantation embryonic lethality. Oocyte donation may be the best ART available right now for patients harboring biallelic *TLE6* variants. Interestingly, very recently, nuclear transfer has been proposed to overcome embryo developmental arrest, as well as injection of wild-type *WEE2* cRNA into oocytes has been reported to overcome fertilization failure caused by *WEE2* variants (Sang et al., 2018; Christodoulaki et al., 2021). Further studies need to be performed in the future to explore whether these techniques could also be an option for patients with *TEL6* variants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MZ wrote the manuscript. CL and ML analyzed the data. BC, HZ, YL, and MZ conducted the experiments. YG, TW, QX, and YZ collected the sample and data. HW, XH, and YX designed and directed the study. ZZ, PZ, and ZW revised the manuscript. YC was responsible for the study supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Optimizing Fertility in Primary Ovarian Insufficiency: Case Report and Literature Review

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Primary ovarian insufficiency (POI) is a clinical spectrum of ovarian dysfunction. Overt POI presents with oligo/amenorrhea and hypergonadotropic hypogonadism before age 40 years. Overt POI involves chronic health problems to include increased morbidity and mortality related to estradiol deficiency and the associated osteoporosis and cardiovascular disease as well as psychological and psychiatric disorders related to the loss of reproductive hormones and infertility. Presently, with standard clinical testing, a mechanism for Overt POI can only be identified in about 10% of cases. Now discovery of new mechanisms permits an etiology to be identified in a research setting in 25–30% of overt cases. The most common genetic cause of Overt POI is premutation in *FMR1*. The associated infertility is life altering. Oocyte donation is effective, although many women prefer to conceive with their own ova. Surprisingly, the majority who have Overt POI still have detectable ovarian follicles (70%). The major mechanism of follicle dysfunction in Overt POI has been histologically defined by a prospective NIH study: inappropriate follicle luteinization due to the tonically elevated serum LH levels. A trial of physiologic hormone replacement therapy, clinically proven to suppress the elevated LH levels in these women, may improve follicle function and increase the chance of ovulation. Here, we report the case of a woman with Overt POI diagnosed at age 35 years. To attempt pregnancy, she elected a trial of intrauterine insemination (IUI) in conjunction with follicle monitoring and physiologic hormone replacement therapy. She conceived on the eighth cycle of treatment and delivered a healthy baby. Our report calls for a concerted effort to define the best methods by which to optimize fertility for women who have POI.

Keywords: primary ovarian insufficiency, pregnancy, luteinized follicles, hormone replacement therapy, intrauterine insemination

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INTRODUCTION

The diagnosis of primary ovarian insufficiency (POI) needs to be considered in the differential diagnosis for any woman of reproductive age who presents with infertility developing at <40 years of age. The disorder is a clinical spectrum of impaired ovarian function. Overt POI presents clinically as oligo/amenorrhea in association with menopausal level serum FSH values. Biochemical POI presents with infertility, regular menstrual cycles, and elevated cycle day 3 serum FSH levels.

Occult POI presents with infertility, regular menstrual cycles, normal cycle day 3 serum FSH values, and a poor response to exogenous gonadotropin stimulation (Nelson, 2009).

In this case report, we describe a woman who presented with oligo/amenorrhea at age 29 years. At age 35, she was given a diagnosis of Overt POI. She conceived and delivered a healthy child by employing follicle monitoring in conjunction with physiologic hormone replacement therapy (P-HRT) and intrauterine insemination. The P-HRT was employed to suppress elevated serum LH levels in order to avoid premature and inappropriate follicle luteinization (Nelson et al., 1994). As a rare disorder, POI puts many clinicians at a loss on how to manage Overt POI and a lack of appreciation for: (1) the pathophysiology of follicle dysfunction in Overt POI (Nelson et al., 1994), (2) the many benefits bestowed by providing P-HRT (Popat et al., 2014), and (3) the possibility of pregnancy in this condition (Fraison et al., 2019).

CASE REPORT

In January 2012, a previously healthy 29-year-old woman physician presented with a 7-month history of oligo-amenorrhea and a noticeable increase in fatigue and sleepiness despite adequate sleep hygiene. She had no vasomotor symptoms or night sweats. Laboratory evaluation revealed normal thyroid function and a markedly elevated serum FSH level of 37.6 mU/ml (normal follicular phase range 2.5–10.2). In February 2012, repeat laboratory examinations revealed serum FSH at 8.7 mU/ml, LH at 4.8 mU/ml (normal 2–15), estradiol at 132 pg/ml (normal 30–190), and testosterone at 11 ng/dl (normal range: 25–125). At age 31, she began taking the oral contraceptive. In August 2017, at age 35, she consulted a reproductive endocrinologist to discuss her desire to have a child. At that time, 2 weeks after stopping the oral contraceptive, her FSH was 19.4 mu/ml, AMH 0.2 ng/ml, and her estradiol was 52 pg/ml. She was given a diagnosis of Overt POI. Her POI was idiopathic. She had a normal 46,XX karyotype, negative genetic testing for the fragile X mental retardation 1 (*FMR1*) premutation (29/32 CCG repeats), negative 21 hydroxylase antibody testing (<1 U/ml), and small ovaries on ultrasound examination. A bone density measurement was normal. A saline sonogram revealed a small endometrial polyp, which was removed by hysteroscopy. Consultants suggested egg donation as her best option to have a child and advised IVF and oocyte preservation were not options for her.

Here is how the patient describes her care experience after seeing several consultants:

> "After my misdiagnosis and then difficulty accessing complete care for POI, I didn't know how to balance being a "good patient" and advocating for myself. I felt utterly dependent and distrusting at the same time. It made me sad that I couldn't find a physician to care for me the way I do for my own patients. I've been told that this should be humbling and help give me more empathy for my patients. I'm tired of being humbled. We – women with POI – deserve better. We deserve doctors who are informed about our condition, who know the limits of their knowledge, and who are not too proud to seek expertise."

The patient thus sought out the assistance of a health coach board certified by the National Board for Health and Wellness Coaching (National Board for Health and Wellness Coaching, n.d.). She wanted to learn how to better advocate with her clinician regarding her specific needs as a woman living with Overt POI. The health coach also helped her to "more confidently make decisions regarding her treatment plan" and "to better process and deal with the inherent stresses of fertility treatments." She elected to attempt conception with her own ova rather than using donor ova. She found a clinician willing to work with her special care needs.

In October 2018, while continuing the NIH P-HRT regimen to suppress LH levels, she conceived on the eighth cycle of intrauterine insemination (IUI) using donor sperm. On the NIH P-HRT regimen, her menstrual cycle day 3 serum LH level was in the normal range at LH 6.93 mIU/L. Her clinician monitored follicle development by serial transvaginal ultrasound, and the woman monitored urinary LH measurements. After the eighth cycle on this regimen, her home pregnancy test showed positive on day 13 post-IUI. This was confirmed by serial serum beta HCG measurements. The pregnancy was uncomplicated until at 35 weeks gestation when she developed gestational hypertension. There was concern also for intrauterine growth restriction. Due to ongoing concerns, her care team induced labor. The baby was born at 37 weeks and 4 days gestation. The neonatal course was complicated by being small for gestational age, with associated hypothermia, hypoglycemia, and hyperbilirubinemia. The baby was discharged home in good condition on the third day of life.

LIFE EXPECTANCY AND QUALITY OF LIFE

Importantly, convincing evidence has demonstrated women who have Overt POI in fact have a shorter life span. This is likely related to the long-term morbidity of chronic estradiol deficiency. A 2016 systematic review and meta-analysis addressed this issue. The report included an analysis of seven prospective studies producing nine articles. (Tao et al., 2016) the report showed women who have Overt POI as compared with controls have: (1) a higher risk of death from all causes and (2) a higher risk of death from ischemic heart disease. The study found no significant association with all-cancer mortality (Tao et al., 2016). If indeed the increased mortality is related to estradiol deficiency, one would expect a well-controlled prospective natural history study which provides physiologic HRT to improve their life expectancy. Importantly, an NIH prospective controlled study showed P-HRT restored the POI-related reduced bone density to normal by the end of 3 years (Popat et al., 2014). The NIH P-HRT regimen restored regular menses, relieved symptoms of estradiol deficiency, and was well-tolerated.

QUALITY OF CARE

Most women who have Overt POI are not satisfied with the quality of care provided by their clinician (Groff et al., 2005). Overall, 71% were displeased with how their clinician informed them of the diagnosis. Most (89%) experienced

moderate to severe emotional distress at the time. The degree of emotional distress was more severe in those women who were most dissatisfied with how they were told the diagnosis. The women reported several factors which helped then to cope with the diagnosis. These included getting thorough and accurate information about the disorder from the clinician, support of others, and spirituality. Most women find the diagnosis of Overt POI to be difficult to understand and emotionally traumatic. The findings suggest that clinicians can reduce the level of distress experienced by these women by better informing them about the diagnosis. Patients perceive a need to spend more time with clinicians and a need for more complete information about Overt POI (Groff et al., 2005).

EMOTIONAL HEALTH

Prospective controlled study has demonstrated women who have Overt POI as compared with controls have more symptoms of depression and anxiety, lower positive affect, and higher negative affect (Davis et al., 2010). In a controlled study, illness uncertainty was a significant independent risk factor associated with anxiety. Stigma was also a significant independent risk factor associated with depression. Goal reengagement was a significant and independent factor associated with positive affect. The findings suggest clinicians could improve the emotional well-being of their patients with Overt POI by: (a) informing them better about their condition, (b) educating to help them to feel less stigmatized by the disorder, and (c) assisting them in developing realistic goals with regard to family planning as well as other goals.

Social support is an important factor in quality of life. A prospective and controlled study demonstrated women who have Overt POI perceive less social support and greater stigma than control women (Orshan et al., 2009). Patients who perceived more social support in their life also had greater self-esteem. Marital status, whether or not the woman had children, or how long ago the diagnosis was established did not affect these findings. Overt POI is a life-altering diagnosis and strategies to improve social support and self-esteem are important considerations in reducing the emotional suffering that accompanies Overt POI.

Another important issue involves coping with the diagnosis of Overt POI. What is the evidence about the coping strategies women who have Overt POI? How do they address the associated emotional sequelae and behavioral health effects of the disorder? The NIH conducted a longitudinal study to address these questions (Driscoll et al., 2016). Importantly, analysis of the data identified a single stand-alone coping strategy which explained the outcome in affect at the end of 1 year. Avoidance is a coping strategy in which the patient refuses to acknowledge stress. Avoidance explained the association between the baseline vulnerability and the degree of distress measured at 12 months. Coping by avoidance has been linked to poorer psychosocial adaptation according to a systematic review published in 2019 (Livneh, 2019). The use of avoidance coping is largely ineffective when confronting stressful life events and health-related conditions. Furthermore, the reliance on avoidance strategies have been linked to poorer adaptation in a wide range of disorders (Livneh, 2019). Interventional studies targeted to reduce avoidance in women who have Overt POI are indicated.

One specialized area of particular concern is the emotional and behavioral health of teens who get a diagnosis of Overt POI. This diagnosis is both emotionally difficult and confusing not only for young girls but also their families. Parents require assistance to help their daughters understand the diagnosis. They need guidance to help the child maintain healthy growth and development. One published report provides a starting point on this path for parents and clinicians. A handout has been published entitled "Tips and Tools for Talking: Helping Your Daughter Understand Primary Ovarian Insufficiency" (Covington et al., 2011). Importantly, commonly the diagnosis of POI is delayed in teens. There is an assumption in teens that irregular menses are common, and therefore not in need of investigation. A family systems approach is an important component to the management plan for teens and their parents. Such an approach must incorporate mental health support. Ideally, a teen with Overt POI should have patient-centered and integrated comprehensive approach provided by a primary care clinician. The primary care clinician needs support from a multidisciplinary team (Gordon et al., 2015).

OVARIAN FUNCTION

Despite having oligo/amenorrhea and menopausal level serum FSH levels, 73% of women with Overt POI, nevertheless, have ovarian follicles remaining in the ovary (Nelson et al., 1994; Hubayter et al., 2010). Overt POI is ovarian function which is intermittent and unpredictable and may persist for many years. Graafian follicles grow in response to the high FSH levels, yet their function is impaired by the high serum LH levels. The high LH levels induce inappropriate luteinization and thus prevent normal follicle function and ovulation. Weekly blood sampling and sonography in 65 women with confirmed Overt POI revealed 50% demonstrated ovarian follicle function as defined by a serum estradiol >183 pmol/L (50 pg/ml) during 4 months of observation (Nelson et al., 1994). Importantly, 16% of these women achieved an ovulatory serum progesterone level as defined by >9.5 nmol/L (3.0 ng/ml).

In a 1994 histologic study of ovarian follicle function, the NIH POI research team biopsied antral follicles in six women who had Overt POI. They demonstrated luteinized Graafian follicles in every case (six of six, 95% confidence limit 60%). Inappropriate luteinization of Graafian follicles is thus the major pathophysiological mechanism of follicle dysfunction in patients who have Overt POI (**Figure 1**) (Nelson et al., 1994). By sonography, the NIH team found antral follicles in over 40% of women (27 of 65). When an antral follicle was present serum estradiol was significantly greater (Nelson et al., 1994). The follicles were dysfunctional in these women, however. Women with Overt POI had poor correlation between follicle diameter and serum estradiol as compared to controls (**Figure 2**).

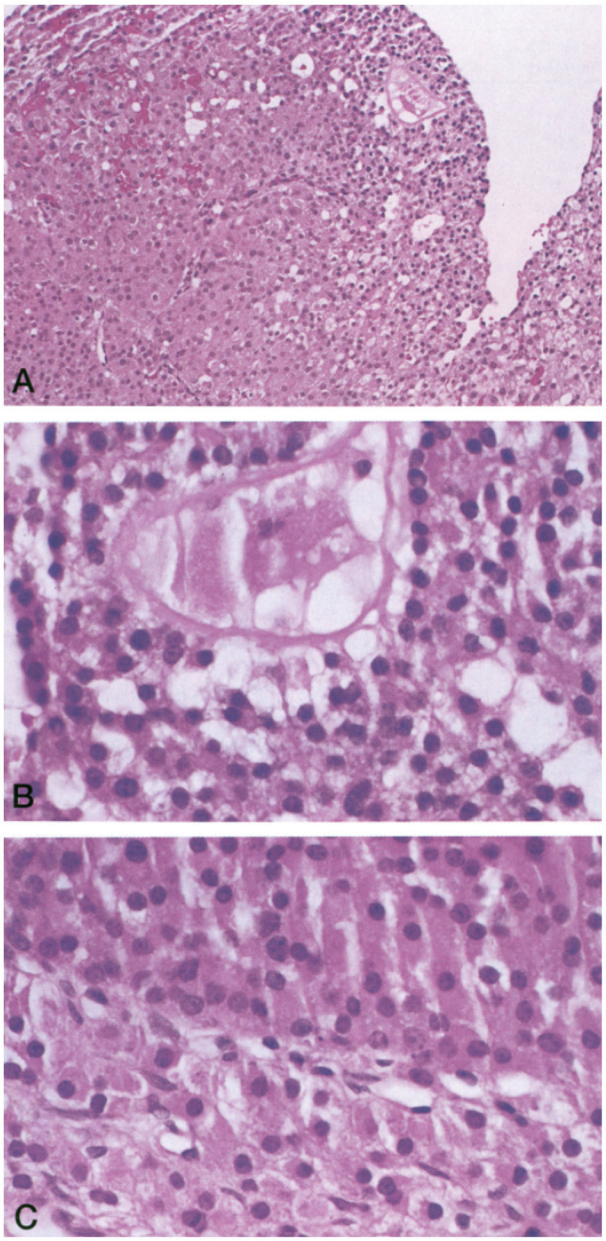

FIGURE 1 | (A) Luteinized follicle containing a structure suggestive of an ovum undergoing degeneration (hematoxylin and eosin stain; magnification, ×150). **(B)** Higher magnification of the putative ovum (hematoxylin and eosin stain; magnification, ×250). **(C)** Higher magnification of the same follicle showing luteinized cells characterized by their larger size, abundant eosinophilic cytoplasm, and prominent nucleus (hematoxylin and eosin stain; magnification, ×250).

In 2010, the NIH POI research team also developed an ovarian stimulation test (OST). The aim was to assess ovarian follicle function in response to FSH stimulation in women confirmed to have spontaneous 46,XX Overt POI (Hubayter et al., 2010). The team employed a case-control study with a nested prospective cohort. They administered the OST to 97 women who had Overt POI and had taken no HRT for the prior 2 weeks. The control group was 42 regularly menstruating women who were in the mid-follicular phase of the menstrual cycle. The OST consisted of a single injection of 300 IU hrFSH and monitoring of the change in serum estradiol levels 24 h later. Antral follicles >3 mm was detected in 73% (69/95) of women with Overt POI. Maximum follicle diameter correlated significantly with both serum estradiol and progesterone levels. Patients who had a follicle with a maximum follicle diameter ≥8 mm had significantly lower FSH and LH levels. They also had significantly higher serum estradiol and progesterone levels. In women with Overt POI, the serum estradiol levels did not significantly increase after FSH administration despite the presence of an antral follicle ≥8 antral and mm. In contrast with control women, their serum estradiol levels increased significantly in response to FSH (**Figure 3**) (Hubayter et al., 2010).

The case reported herein is consistent with the following hypothesis: by employing the NIH P-HRT regimen to suppress LH levels in women who have Overt POI one could prevent follicle luteinization, restore follicle function, permit ovulation, and increase the chance of pregnancy. The evidence that 70% of women with Overt POI have antral follicles on ultrasound examination provides convincing evidence against the hypothesis that downregulation of FSH receptors is the mechanism of follicle dysfunction in these women. Interestingly, now two different lines of evidence come together to confirm the pathogenesis of follicle dysfunction in Overt POI. The two lines of evidence are (1) evidence of significant progesterone secretion by antral follicles ≥8 mm in women with Overt POI and (2) published evidence demonstrating histologically that follicle luteinization is the major mechanism of abnormal follicle function in this condition (Nelson et al., 1994). Prospective controlled investigation designed to prevent follicle luteinization and improve ovulatory function and fertility in these women who have Overt POI is clearly indicated.

PHYSIOLOGIC HORMONE REPLACEMENT THERAPY

The estradiol deficiency of Overt POI is directly related to the associated health complications. Unfortunately, 52% of patients with Overt POI either never begin P-HRT at diagnosis, begin P-HRT many years later, and/or stop using P-HRT use before age 45 (Hipp et al., 2016). Unfortunately, findings regarding detrimental effects reported in the Women's Health Initiative (WHI) trial inappropriately causes many clinicians to refrain from prescribing hormone therapy for young women with Overt POI (Sullivan et al., 2016). The WHI was a study of older postmenopausal women, not young women who have Overt POI. It showed multiple increased health risks with hormone therapy. These included increased risk breast cancer, stroke, and cardiovascular disease (Rossouw et al., 2002). This is unfortunate because the clinical situation markedly differs. The clinical situation in young women with Overt POI is indeed pathological (an abnormal state of estradiol deficiency compared with women of the same age). Menopause is a normal state for older women.

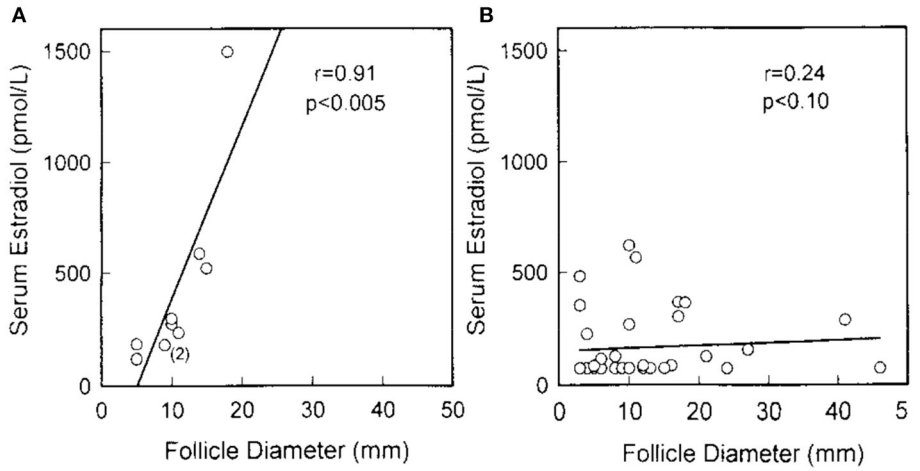

FIGURE 2 | Serum estradiol correlated with maximum follicle diameter in normal women but not in women with Overt POI. **(A)** Each point represents the findings in 1 of 10 normal women with regular menses examined during the follicular phase. Two congruent points are noted by (2). **(B)** Each point represents the findings in a patient with overt POI who had an ovarian follicle detected by sonogram (37 sonograms in 27 patients). There are 8 congruent points (Nelson et al., 1994).

In women with Overt POI, the term P-HRT accurately describes the clinical purpose. The prescribed hormones in this case replace the hormones that would normally be produced by young women (Sullivan et al., 2016). P-HRT is now considered standard of care for women with Overt POI (Committee Opinion No. 698: hormone therapy in primary ovarian insufficiency, 2017). It is generally recommended to continue P-HRT until age 50 (the average age when natural menopause occurs).

To test the hypothesis that P-HRT is an effective clinical approach, the NIH Intramural Research Program designed a prospective randomized controlled trial in young women who had Overt POI (Popat et al., 2014). The study lasted 3 years. The treatment employed physiologic estradiol replacement (transdermal estradiol 100 μg/day) and cyclic progestin (oral medroxyprogesterone 10 mg daily for 12 days/month). Importantly, this P-HRT resulted in lumbar spine and femoral neck BMD increasing significantly. In fact, by the end of the study, bone mineral density had increased to such a degree their BMD did not differ significantly from the control women who participated (**Figure 4**). Transdermal physiologic testosterone replacement provided no significant additional benefit to BMD (Popat et al., 2014). The NIH P-HRT regimen was well-tolerated by the study participants.

The ideal would be to mimic normal ovarian function as a hormone replacement strategy. An artificial ovary could theoretically be designed to employ a vaginal ring. This could deliver a steady parenteral release to mimic endogenous ovarian hormone production similar to a normal menstrual cycle. The transdermal patch and the vaginal ring that deliver 0.10 mg of estradiol per day are a fundamental and elegant step in this line of thinking. The design mimics the daily ovarian production rate of estradiol to put average serum estradiol levels of in the range of 100 pg/ml. This matches the average estradiol level across the menstrual cycle of control women who have normal ovarian function (Mishell et al., 1971). Transdermal and transvaginal routes of administration avoid the first pass effect of the liver (which occurs with orally delivered estrogen) by delivering hormones directly into the blood (Canonico et al., 2008). A systematic review concluded oral estrogen increases the risk of venous thromboembolism as compared with estradiol delivered transdermally (Mohammed et al., 2015). Importantly, in postmenopausal women, the relative risk of stroke is greater with oral estrogen compared with transdermal estradiol (Renoux et al., 2010).

The NIH study on P-HRT employed medroxyprogesterone acetate as the progestin. The NIH POI multidisciplinary team of clinicians and scientists had concerns regarding the use of oral micronized progesterone. They found inadequate evidence in the literature to demonstrate effectiveness in protecting the endometrium when used with full replacement doses of estradiol. Also of significant consideration, medroxyprogesterone acetate given orally is protected from metabolism by the liver by the addition of a simple medroxy group to the pure progesterone molecule. This agent has been in use for decades and has a wonderful safety record. In considering the possible use of oral micronized progesterone as part of the regimen, the NIH multidisciplinary team had significant concerns regarding the large number of metabolites resulting from the first pass effect on the liver (Sullivan et al., 2016). Some women who develop Overt POI in their younger years will be taking P-HRT for decades. The potential longer-term adverse health effects of these progesterone metabolites after exposure for decades are completely unknown. Another concern is the potential for adverse effects of these multiple progesterone metabolites on fertility and the developing embryo.

FERTILITY

Pregnancy may occur in women who have POI in the absence of any medical intervention (Soave et al., 2013). The authors of

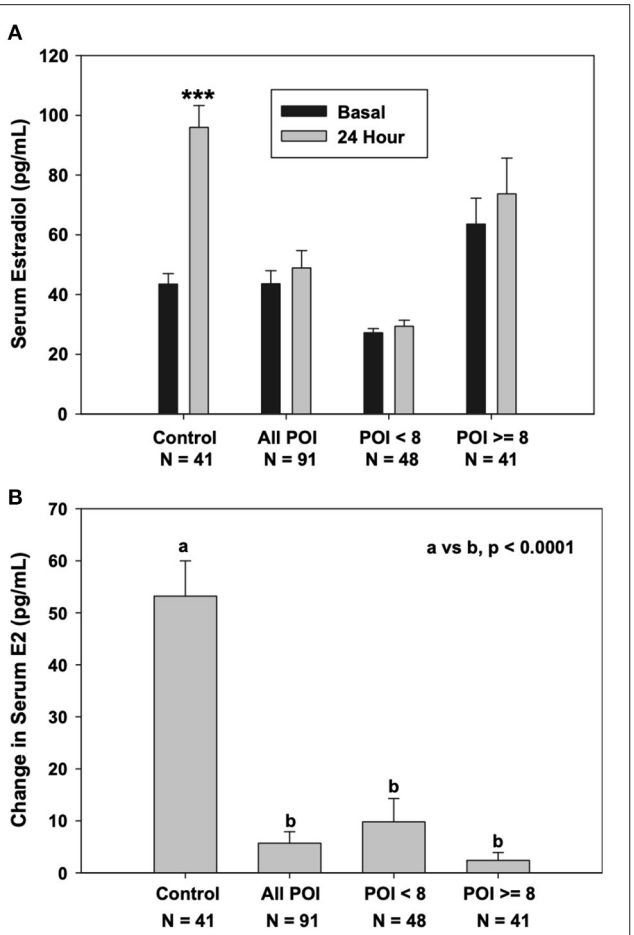

FIGURE 3 | Serum estradiol response to stimulation with 300 IU FSH. **(A)** Control women, women with Overt POI, and women with overt POI segregated by the absence or presence of an antral follicle 8 mm in diameter or greater (***$P < 0.0001$ vs. baseline). **(B)** Change in serum estradiol levels at 24 h.

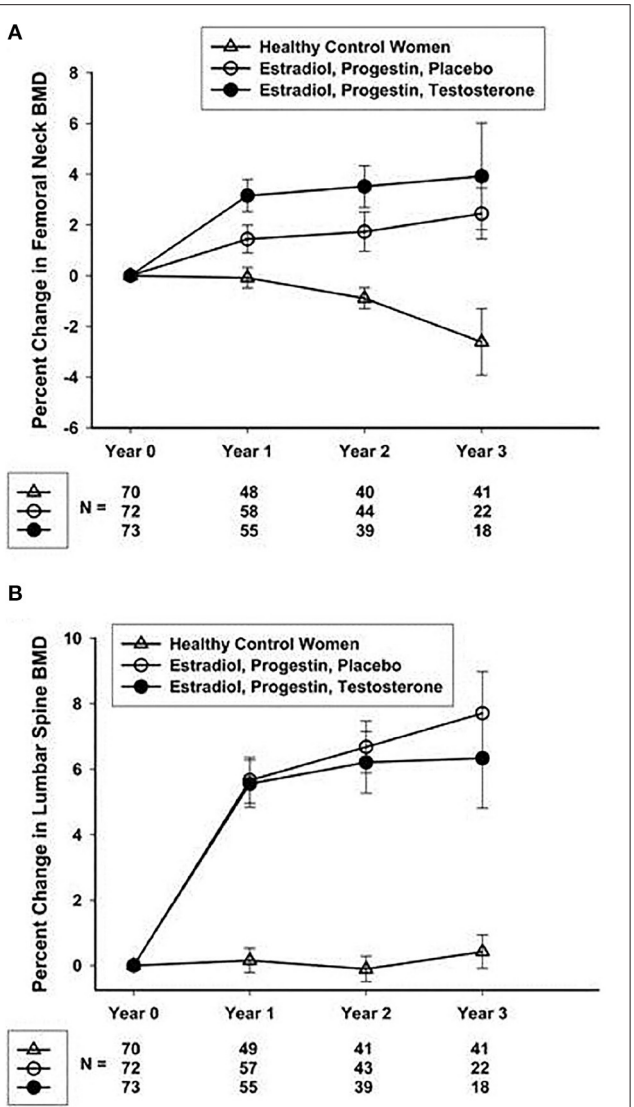

FIGURE 4 | (A) Mean (SEM) percentage change from screening in the femoral neck BMD. **(B)** Mean (SEM) percentage change from screening in the lumbar spine BMD (Popat et al., 2014).

a 2019 systematic review emphasized this important point, "To date no clinical test can determine the potential for conception in patients with POI." (Fraison et al., 2019). Also, the authors warn clinicians to be prudent and not to be hasty in claiming absolute sterility when counseling women who have the diagnosis of Overt POI. The authors suggest an approach which encourages patients to take their time deciding about ova donation rather than rushing the decision. Some women are uncomfortable with ova donation and desire a chance to have a pregnancy with their endogenous ova. As in the case reported herein, many women who live with Overt POI do indeed wish to have this chance to conceive with their own ova before moving forward to egg donation (Fraison et al., 2019).

This 2019 systematic review assessed fertility in women who have Overt POI. The authors searched electronic databases for the years 2000 to 2018 (Fraison et al., 2019). They included 15 studies and found pregnancy rates from 2.2 to 14.2%. The average age of patients who got pregnant was 30 years. The authors report this evidence supports a conclusion that oocyte quality in women who have Overt POI is likely normal.

As noted above, many young women find the diagnosis of Overt POI emotionally devastating. Many find it difficult to grieve their infertility and employ the regrettable course of avoidance coping (Driscoll et al., 2016). This makes these women extremely vulnerable to misguided approaches to restore fertility, some quite expensive, emotionally draining, and not based on credible evidence. Some women will request treatment with agents to induce super ovulation despite having been informed of the minimal chance of success (Fraison et al., 2019). It is difficult to justify using ovarian stimulation protocols to improve fertility in this population. As noted above, results of two NIH prospective studies to assess ovarian follicle function in these

women have demonstrated conclusively: follicles in women who have Overt POI are not capable of responding to FSH stimulation due to their luteinized state (Nelson et al., 1994; Hubayter et al., 2010).

The case presented herein raises an important question. How to proceed to optimize fertility in the face of Overt POI for those women and partners who do not pursue oocyte donation as a solution to their infertility? Many women and their partners find it very difficult to accept the idea of not passing their DNA on to their child. This is a bridge too far for some women and partners. Most donor oocyte programs offer pretreatment counseling to optimize the mental health of the child and family. Some will decide against using donor oocytes. In addition, ova donation is not always readily available. Many countries do not permit ova donation. Other countries do not permit payment to prospective donors, which severely limits availability of this approach. Importantly, questions still remain about the mental health of donor-conceived offspring. It is likely that many parents do no inform their donor conceived children about their origin (Bracewell-Milnes et al., 2018; Fraison et al., 2019).

As in any preconception situation, planning and preparing for pregnancy are important even if the chances of conceiving are limited. A visit to the care team is in order to: (1) assess any other medical conditions; (2) review medications; (3) review vaccinations; (4) review family history; (5) begin taking folic acid 400 μg/day; (6) stop smoking and exposure to any toxic substances; (7) establish a healthy weight and lifestyle; (8) get help with exposure to any violence; and (9) get mentally healthy (Stephenson et al., 2018).

> "A sharper focus on intervention before conception is needed to improve maternal and child health and reduce the growing burden of non-communicable disease. Alongside continued efforts to reduce smoking, alcohol and obesity in the population, we call for heightened awareness of preconception health, particularly regarding diet and nutrition. Importantly health professionals should be alerted to ways of identifying women who are planning a pregnancy" (Stephenson et al., 2018).

Published research has demonstrated the important role illness uncertainty plays as an independent contributor to anxiety in women who have Overt POI (Davis et al., 2010). Diabetes self-management education helps people with diabetes incorporate knowledge and navigate treatment decisions as well as establish new and healthier activities known to improve health outcomes (Powers et al., 2016; Chester et al., 2018). This case report demonstrates there is a similar need to develop certified POI Health Coach Educators (Jordan et al., 2015). Women who have Overt POI would benefit from a source of comprehensive guidance which is based on evidence.

Also, there is a need to maintain bone health while attempting conception. The best evidence supports the use of the NIH P-HRT regimen in this regard (Popat et al., 2014). As noted above, taking the NIH P-HRT regimen is known to permit pregnancy to occur (Nelson, 2009). Published expert opinion recommends women who desire to conceive take the following approach: (1) Keep a menstrual calendar while taking the NIH P-HRT regimen. (2) To assure sperm are always present in the event of an unpredictable ovulation have intercourse two or three times a week. (3) Take a pregnancy test if the menstrual period is late. (4) Notify the clinical team about the pregnancy and stop taking the P-HRT. (5) Allow time for conception to occur (up to 3 years in view of the reduced fecundity). (6) While attempting to conceive, make plans for the future in the event no birth takes place (Nelson, 2009).

Finally, as noted, there is no published evidence to support the use of any strategy to monitor follicle development or intervene with any treatment to increase pregnancy rates in women who have Overt POI (Fraison et al., 2019). However, there are specific situations in which monitoring and intervention may be indicated. For example, timing of ovulation becomes critical in the case of severe male factor parameters necessitating donor sperm. Similarly, for some couples having sexual intercourse 2 or 3 times a week is quite difficult. Infertility is known to be associated with a high incidence of sexual dysfunction in general (Riazi et al., 2020). Specifically, as compared with controls, women who have Overt POI, despite taking physiologic estradiol replacement score significantly lower on a validated sexual function scale (Kalantaridou et al., 2008). Presently, there are many options for women to employ in an attempt to time ovulation (Owen, 2013; Su et al., 2017). Cervical mucus monitoring deserves evaluation as a simple, free, readily available method which is supported by published evidence to improve pregnancy rates in the general population (Evans-Hoeker et al., 2013). None of these methods have been proven to be effective in women who have Overt POI. There is a need for prospective study to generate validated protocols for predicting ovulation in these women. Such a protocol might improve pregnancy rates by permitting timing of intercourse, donor insemination, intrauterine insemination, natural cycle *in vitro* fertilization (IVF), or intracytoplasmic sperm injection (ICSI) (Farquhar and Marjoribanks, 2018).

SOCIAL MEDIA

POI is a rare disorder. For those affected, finding reliable information on rare diseases is a major challenge. Limited data is available regarding the use of information sources for various rare diseases, or how the usefulness of the information is evaluated by patients and their families (Litzkendorf et al., 2020). One qualitative study reported patients with various rare diseases and their close relatives considered the internet as the most important and widespread information source. They considered searching online an expedient and practical manner for gathering information. Online information sometimes was insufficiently detailed to satisfy their needs.

Self-help associations and specialized clinicians help in filling the information gaps (Litzkendorf et al., 2020). Facebook is used commonly as a source of health-related information. However, social media involves the risk of getting misinformation and the risk of sharing of confidential health information. One online cross-sectional study surveyed patients with psoriasis to determine the relevance and suitability of Facebook as a source of disease-related information (Schuster et al., 2020). The study found Facebook helped them cope with psoriasis to a degree, but a majority (60%) reported finding unreliable information

regarding the disorder. The authors concluded Facebook is a relevant source of health information for patients who have psoriasis. However, the quality of information was deemed inadequate and is an area in need of improvement (Schuster et al., 2020).

There is a need to improve the quality of information about POI on Facebook. One specific closed group with over 1,500 members has a Facebook page working toward this goal. The group, *My Family Cares About Primary Ovarian Insufficiency and Early Menopause*, has been developed and maintained by the Conover Foundation (My Family Cares about Primary Ovarian Insufficiency and Early Menopause, n.d.). The page provides a health coach serving as a POI Clinical Navigator as well as an experienced physician/scientist to curate the page. The physician/scientist has 30 years of experience working with women who have dealt with the difficult life transitions which come with a diagnosis of POI/EM. The goal of the group is to maintain coherent discussion threads, provide peer-reviewed resources, and assure information shared is based on the best available medical evidence. On this Facebook page, women can read stories of international women expressing views regarding their experiences with POI/EM. They are able to share stories about getting the diagnosis, symptoms they have experienced, mention long-term health effects, describe treatments, report experiences with health services and health practitioners, and express their assessment regarding the impact POI/EM has had in their life and in their personal relationships. These are just a few aspects of the information women can find there. The page provides links to a comprehensive range of resources. When needed, the page offers a more personalized approach using videoconference to connect, educate, and coach clients with the help of a trained POI clinical navigator.

GENETIC CAUSES

Recent evidence has increased dramatically the number of candidate loci for POI (Huhtaniemi et al., 2018; Bestetti et al., 2019). Several new candidate genes account for mechanisms of DNA replication and repair. The systematic use of the Next-Generation Sequencing (NGS) approach with targeted exome sequencing has revealed frequent oligogenic involvement, mainly in cases with earlier onset of POI (Rossetti et al., 2017, in press; Cattoni et al., 2020).

Turner Syndrome X Chromosome Defects

Both X-linked and autosomal genes are involved in POI. Some are part of a syndrome and some lead to isolated POI (Rossetti et al., 2017). Both familial and sporadic forms of POI are associated with X chromosome abnormalities. Turner syndrome, Turner mosaic, triple X syndrome, partial X deletions, isochromosomes, and translocations between X chromosome and autosomes are well-known examples (Goswami et al., 2003; Toniolo, 2006). Either complete or partial loss of one X chromosome may result in Turner syndrome. The Turner syndrome phenotype includes POI, short stature, and various somatic abnormalities. Turner syndrome has an incidence of about 1:2,500 live female births (Rossetti et al., 2017).

Fragile X-Associated Primary Ovarian Insufficiency (*FMR1*)

Premutation in *FMR1* is an established genetic cause of Overt POI, termed fragile X-associated primary ovarian insufficiency (FXPOI) (Sherman, 2000). Impaired fertility is an important complication of FXPOI (Allen et al., 2007; Streuli et al., 2009). A premutation in FMRI is found in about 11% of women who have a family member with POI and in about 3.2% of women who have isolated POI (Sherman et al., 2007). A premutation is defined as 55–199 expanded CGG repeats located in the 5′ untranslated region (UTR) of the X-linked gene, *FMR1*. About 1/300 women carry a premutation (Hunter et al., 2014). Of women who have a premutation, overt FXPOI occurs in about 20% (Sherman, 2000; Sullivan et al., 2005; De Caro et al., 2008).

There are three risk factors associated with the development of FXPOI. Firstly, CGG repeat size has a non-linear relationship with the risk for FXPOI (Spath et al., 2011). The risk for FXPOI is about 10% for women with 55–79 repeats, 32% for women with 80–100 repeats, and 16% for women with more than 100 repeats (Allen et al., 2007). Earlier onset of the disorder is associated with women who have 80–100 repeats, in some cases even during adolescence (De Caro et al., 2008). Secondly, the risk of the disorder increases if there is a family history of early menopause (Spath et al., 2011). This indicates there are background genetic variants which contribute to the risk. Smokers have an earlier menopause, and there is a similarity in women who have a premutation (Allen et al., 2007).

For woman carrying a premutation, genetic counseling is essential. They are at risk of having a child with fragile X syndrome in addition to the risk of FXPOI and its clinical consequences. There is also risk for fragile X-associated tremor/ataxia syndrome (FXTAS), which is a neurodegenerative disorder developing at approximately age 60 (Hagerman et al., 2001; Hawkins and Matzuk, 2008; Harris et al., 2016). Women have a lifetime prevalence of FXTAS of 6–18% (Wheeler et al., 2014).

Basic science as well as clinical evidence suggest a novel mechanism for FXPOI. Little is known regarding the ovarian histopathology in women with FXPOI. One study with a small number of cases showed ovarian histology and follicle number similar to controls (Chang et al., 2011). Therefore, the mechanism of FXPOI appears to be one of follicle dysfunction rather than depletion. Similar findings have been reported in mouse models of FXPOI. Mice carrying an *FMR1* premutation were reported to have a normal complement of primordial follicles (Conca Dioguardi et al., 2016). Thus, FXPOI seems likely to be a disorder of impaired follicle function.

In one case report, a young woman who had FXPOI conceived without medical intervention while on the NIH P-HRT regimen (Fink et al., 2018). She terminated the pregnancy as prenatal genetic testing showed a full mutation in *FMR1* in the fetus. After that, she conceived two more pregnancies without medical intervention while on the same P-HRT regimen, one in 2013 and one in 2016. Both pregnancies and deliveries were unremarkable. Despite her history of having had two prior pregnancies after the diagnosis of FXPOI, a physician at an IVF clinic suggested she proceed to egg donation and quoted a current chance of

conception of <0.1%. This remarkable report about fertility in FXPOI while on the NIH P-HRT regimen suggests a need for more research into this approach to optimize fertility in women who have FXPOI, especially in view of the above evidence supporting follicle dysfunction rather than low number of follicles as the mechanism.

Autoimmune Polyendocrinopathy Syndrome Type I

Autoimmune polyendocrinopathy syndrome type I (*APS1*), made manifest by mutations in the autoimmune regulator gene (*AIRE*, MIM *607358), is a syndrome confirmed by the presence of two of these important clinical disorders: Addison's disease (AD), hypoparathyroidism, and chronic mucocutaneous candidiasis. Generally, AD usually presents as a child or young adult. The syndrome may be associated with primary hypogonadism, pernicious anemia, alopecia, malabsorption, or chronic active hepatitis (Rossetti et al., 2017).

Gonadotropin Receptors FSH and LH (*FSHR, LHCGR*)

FSH and LH receptors belong to the G-protein-coupled receptors (GPCRs) family. These receptors regulate reproductive hormone signaling by interacting with FSH and LH in both men and women. Loss-of-function mutations in these receptors result in resistance to gonadotropin stimulation and present with hypogonadotropic hypogonadism (Themmen and Huhtaniemi, 2000).

Blepharophimosis, Ptosis, Epicanthus Inversus Syndrome

Blepharophimosis, ptosis, epicanthus inversus syndrome (BPES) is an eyelid malformation characterized by BPES and telecanthus. The condition is autosomal dominant. BPES is considered type I when present in association with POI. Type II BPES is not associated with POI. Forkhead transcription factor L2 (*FOXL2*, MIM *605597) mutations are associated with BPES (Crisponi et al., 2001).

Galactosemia (*GALT*)

The normal ovary requires normal galactose metabolism. In galactosemia, galactose metabolism is abnormal due to a deficiency of the enzyme galactose-1-phosphatase uridyltransferase (GALT). The estimated incidence in Europe and North America is 1:30,000–1:50,000 (Rubio-Gozalbo et al., 2010). Organs with high GALT activity such as the ovary, liver, kidney, and heart are prone to the worst malfunction. Most women (80–90%) with *GALT* mutations which are homozygous, show a severe phenotype and exhibit POI (Rubio-Gozalbo et al., 2010).

Pseudo-Hypoparathyroidism Type 1a

Although pseudo-hypoparathyroidism type 1a (PHP1a, MIM #103580) is a renal resistance to parathyroid hormone (PTH), causing hypocalcemia and hyperphosphatemia, the disorder is in fact characterized by resistance to other hormones to include gonadotropins. Thus, delayed or incomplete gonadal function with associated amenorrhea, oligomenorrhea, or infertility is commonly part of the clinical disorder (Mantovani and Spada, 2006). Resistance to other hormones such as thyroid-stimulating hormone (TSH) or growth-hormone-releasing hormone (GHRH) may be present. The clinical disorder known as Albright hereditary osteodystrophy is part of the syndrome.

Progressive External Ophthalmoplegia (*POLG*)

The POLG gene encodes the enzyme that synthesizes mitochondrial DNA and corrects errors. Dysfunction in this gene results in a severe progressive multisystem disorder. The disorder includes parkinsonian symptoms as well as POI. These are not typical of mitochondrial disease (Luoma et al., 2004; Pagnamenta et al., 2006).

Ovarioleukodystrophies

By definition, ovarioleukodystrophies are genetic disorders in which POI is associated with neurological disorders characterized by abnormalities in the white matter of the central nervous system (Mathis et al., 2008).

Ataxia Telangiectasia

Mutation in the ataxia telangiectasia gene (ATM) is associated with primordial germ cell development defects. This gene encodes a cell-cycle checkpoint kinase. The gene contributes to the cellular response to DNA damage and processes DNA strand breaks. These occur with meiosis, immune system maturation, and telomere maintenance (Kastan and Bartek, 2004).

Demirhan Syndrome (*BMPR1B*)

Demirhan syndrome is a subtype of chondrodysplasia associated with amenorrhea, hypogonadism, and genital anomalies. A mutation in BMPR1B causes the disorder. This gene encodes bone morphogenetic protein receptor 1B (Demirhan et al., 2005).

Bloom Syndrome (*BLM*)

Bloom syndrome is an autosomal recessive disorder that is rare. Mutations in the gene coding for the DNA helicase *BLM* cause the disorder. The gene encodes the DNA helicase *BLM* (MIM #604610). Mutations lead to genomic instability. The disorder involves hypogonadism in both sexes, short stature, moderate immunodeficiency, increased cancer rate, and distinctive skin rashes on sun-exposed areas (Arora et al., 2014).

Werner Syndrome (*WRN*)

Recessive mutations in the *WRN* gene cause Werner syndrome, a form of adult progeria. WRN encodes a DNA helicase. This is a form of adult progeria. The disorder is characterized by atrophic gonads, scleroderma-like skin, cataracts, increased cancer rate, and premature arteriosclerosis (Rossi et al., 2010).

GAPO Syndrome (*ANTXR1*)

GAPO is a syndrome of premature aging associated with POI. It is characterized by distinctive facial features, optic atrophy, growth retardation, alopecia, and optic atrophy. Ovarian histology reveals extensive deposition of hyaline extracellular material and depletion of ovarian follicles (Benetti-Pinto et al.,

2016). *GAPO* is caused by recessive mutations in a gene involved in cell adhesion and migration, *ANTXR1*.

Bone Morphogenetic Protein 15

Bone morphogenetic protein 15 (BMP15) (MIM *300247) is a growth/differentiation factor specific to oocytes. The factor is involved in many cellular processes, including follicular development (Persani et al., 2011). The condition may present as either primary or secondary amenorrhea. Mutations in this gene account for the mechanism of POI in about 1.5–15% of cases (Persani et al., 2014).

MOVING FORWARD

POI is best managed by integrated personal care to address the spectrum of these women's clinical needs. Social media combined with a digital and centralized approach to health managed by an experienced team promises to improve this situation. Insights into molecular pathogenetic mechanisms are needed to permit prevention and early diagnosis. Most cases of spontaneous primary ovarian insufficiency are idiopathic. As a rare disease, POI diagnosis presents difficult problems in management. A strategy to improve care and facilitate research is needed. A comprehensive approach for managing POI is long overdue. A Clinical POI Navigator could be a valuable addition to improving the quality of care for these women and optimizing their overall health and fertility. A properly managed cloud-based system could connect women with POI, a POI navigator, primary care providers, and investigators who have the requisite knowledge and expertise (Martin et al., 2017).

In sum, there is a need for a new approach to POI (Cooper et al., 2011). There are major gaps in knowledge regarding this disorder's medical and psychosocial management, psychosocial effects, and natural history. An international disease registry and research consortium formed under an umbrella organization's guidance would provide a pathway to increase basic and clinical knowledge about the condition. Such a consortium and patient registry would also provide clinical samples and clinical data to define the disorder's specific mechanisms further. The effort would combine a patient registry and a community-based participatory research structure. The systematic application of a comprehensive NGS panel for genetic diagnoses may permit early diagnosis in young women who come from a family at higher risk of POI. This would facilitate fertility preservation for women identified as at high risk for POI.

SUMMARY AND CONCLUSIONS

Overt POI presents with oligo/amenorrhea and estradiol deficiency symptoms. Laboratory investigation reveals hypergonadotropic hypogonadism. The condition is a chronic disorder with increased morbidity and long-term mortality related to the reduced bone mineral density and increased risk of cardiovascular disease related to estradiol deficiency. The associated infertility is life altering. Here, we report the case of a young woman with Overt POI who had a successful pregnancy. She conceived on the eighth cycle of monitoring follicle development while taking the NIH P-HRT to reduce serum LH levels. There is a need for increased awareness regarding the fertility potential of women who have POI. The pathophysiology of follicle dysfunction has been defined as inappropriate luteinization of Graafian follicles. P-HRT has been demonstrated to lower LH levels in these women and would be expected to improve ovulation rates, as demonstrated in this case report.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because family consents to share data publicly was not allowed. Requests to access the datasets should be directed to Dr. Muhammad Imran Naseer, mimrannaseer@yahoo.com.

ETHICS STATEMENT

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KP helped develop the focus for the report and contributed content regarding managing POI. HS contributed the case report. LP contributed intellectual content regarding the pathophysiology of POI and the genetic mechanisms. LN conceived the purpose for the report and integrated the content to create the first draft. All authors approved the manuscript for submission.

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Vasopressinergic Activity of the Suprachiasmatic Nucleus and mRNA Expression of Clock Genes in the Hypothalamus-Pituitary-Gonadal Axis in Female Aging

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The important involvement of the suprachiasmatic nucleus (SCN) and the activity of vasopressinergic neurons in maintaining the rhythmicity of the female reproductive system depends on the mRNA transcription-translation feedback loops. Therefore, circadian clock function, like most physiological processes, is involved in the events that determine reproductive aging. This study describes the change of mRNA expression of clock genes, *Per2*, *Bmal1*, and *Rev-erbα*, in the hypothalamus-pituitary-gonadal axis (HPG) of female rats with regular cycle (RC) and irregular cycle (IC), and the vasopressinergic neurons activity in the SCN and kisspeptin neurons in the arcuate nucleus (ARC) of these animals. Results for gonadotropins and the cFos/AVP-ir neurons in the SCN of IC were higher, but kisspeptin-ir was minor. Change in the temporal synchrony of the clock system in the HPG axis, during the period prior to the cessation of ovulatory cycles, was identified. The analysis of mRNA for *Per2*, *Bmal1*, and *Rev-erbα* in the reproductive axis of adult female rodents shows that the regularity of the estrous cycle is guaranteed by alternation in the amount of expression of *Bmal1* and *Per2*, and *Rev-erbα* and *Bmal*1 between light and dark phases, which ceases to occur and contributes to determining reproductive senescence. These results showed that the desynchronization between the central and peripheral circadian clocks contributes to the irregularity of reproductive events. We suggest that the feedback loops of clock genes on the HPG axis modulate the spontaneous transition from regular to irregular cycle and to acyclicity in female rodents.

Keywords: suprachiasmatic nucleus, clock genes, aging, hypothalamus-pituitary-gonadal axis, kisspeptin, vasopressin

INTRODUCTION

The physiological rhythms of development, growth, maturity, and aging are regulated in the suprachiasmatic nucleus (SCN) of the mammalian brain. This nucleus exhibits a marked circadian rhythm in neuronal activity (1, 2) and neurotransmitter synthesis and release (3, 4). In turn, the rhythms are under the regulation of transcription factors generally called clock proteins (5), which are expressed in practically all cell bodies and are the molecular basis of circadian clocks (6, 7). Additionally, the rhythms depend on the activity of vasopressinergic neurons present in the SCN dorsomedial portion, from where they project to important areas in relation to reproduction, such as preoptic area (POA), anteroventral periventricular nucleus (AVPv), dorsomedial hypothalamus (DMH), and arcuate nucleus (ARC) (8). At the cellular levels, inhibitory and stimulatory transcription-translation feedback loops regulate clock proteins. Hormones and signals resulting from the activation of neural networks, such as Kisspeptin neurons, are related to fertility and regularity of reproductive cycles and influence these feedback loops (9, 10). Changes in the pattern of gonadotropin secretion provide powerful information about physiological control mechanisms, such as the underlying characteristics of pulsatile GnRH secretion (11). Furthermore, GnRH neurons reside mainly in POA and ARC, where neurons are sensitive to kisspeptin, steroids, and circadian regulation of AVP (12–14). Over the last decade, a significant body of evidence has accumulated on the interconnections between circadian clocks and aging (15). Among the theories of aging, the neuroendocrine proposes that programmed functional changes in neurons and associated hormones are central to the aging process (16). Studies in physiological aging animal models have demonstrated that there is higher neuronal activity in brain regions known to be involved with reproduction during the transition to reproductive aging (17, 18). The involvement of clock genes in the hypothalamus-pituitary-gonadal axis (HPG) of female rats in the spontaneous transition from regular to irregular cycle and to acyclicity still requires further investigation. It is still unclear to what extent the different physiological phases in life result from effects of the central pacemaker (SCN), peripheral oscillators, both, or on the mechanisms that provide synchronization between the contributing oscillators. In order to characterize the effects of circadian clocks on the aging of the HPG axis, this study evaluated whether the expression of the clock genes on the HPG axis and the vasopressinergic activity of SCN contribute to female reproductive senescence.

MATERIALS AND METHODS

Characterization of the Animal Model

The local Ethics Committee of the Universidade Estadual Paulista approved this protocol for Research Involving Animals (Process n. 2014-00269). The animals were treated according to the laboratory principles of animal care (19). They were housed with *ad libitum* access to food and water. Four-month-old female Wistar rats (adult-regular cycle in diestrus phase), referred to as the regular cycle group (RC), and 18-month IC group (old-irregular cycle in persistent diestrus phase), referred to as the irregular persistent diestrus cycle group (IC), were obtained from the animal facilities of Universidade Estadual Paulista. The analysis of the increase in the duration of the estrous cycle phases, the decrease in phase variability, and the high frequency of days with leukocyte vaginal cytology characterized the irregularity of the estrous cycle in 18-month-old Wistar rats. The animals in the IC group had persistent diestrus lasting 10–12 days longer, with recurrence in three or four cycles. The adult females showed a regular cycle, and the experimental protocols were performed in the diestrus phase. Only adult multiparous rats with regular estrous cycles and senescent rats with irregular estrous cycles and in persistent diestrus participated in this study, presented in at least three consecutive cycles. The experimental procedures during the dark phase were performed using a light emission diode (LED) emission with emission wavelength of 720 nm (red light) and intensity less than 1 lux (20, 21), controlled by a luximeter (Minipa® digital lux meter MLM-1011).

Collection of Material and Hormone Assays

Rats from RC and IC groups housed under 12/12 h light/dark cycle, lights on at 7:00 h (n=5–10 animals/time/group) were killed by decapitation at 6 h intervals. Blood samples were obtained from the trunk, and brains were rapidly removed and frozen at −70°C.

Blood samples collected in heparinized tubes were centrifuged, and the plasma was stored frozen (−20°C) for hormone assays. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) plasma concentrations were determined using double antibody radioimmunoassay (RIE) with reagents from the National Hormone and Peptide Program (Harbor-UCLA, Torrance, CA, USA). The specific antibodies (anti-rat) used were LH-S10 and FSH-S11 diluted in phosphate buffer with rabbit serum. Standard reference preparations, LH-RP3 and FSH-RP3 ng/ml were diluted in 0.1% phosphate buffer (0.01 M, pH=7.5). After the hormones were iodinated and purified (Celso Rodrigues Franci Laboratory, Medical School of Ribeirão Preto-USP, Brazil), the non-specific antibody was also produced in sheep for precipitation of the reaction in the tests. All samples were dosed in duplicate in the same assay to avoid inter-assay variation. The minimum detectable dose was 0.04 ng/ml for LH and 0.09 ng/ml for FSH. Intra-assay coefficients of variation were 1.8 and 3% for LH and FSH, respectively. The plasma estradiol (E_2) and progesterone (P_4) concentrations were measured by Competetive-ELISA using commercial kits (Cayman Chemical Company, MI, USA, for steroids and LSBio, Seattle, USA, for melatonin). E_2 detection range: 6.6–4.000 pg/ml and sensitivity: 20 pg/ml. P_4 detection range: 7.8–1.000 pg/ml and sensitivity: 10 pg/ml. All the results were expressed in ng/ml.

Fixation of Brain Tissue and Double-Immunolabeling Analysis

AVP/cFos

RC and IC rats were deeply anesthetized with ketamine (ketamine hydrochloride, Syntec® 80 mg/Kg pc/ip) and xylazine (xylazine-hydrochloride, Syntec® 40 mg/Kg pc/ip) and transcardially perfused with phosphate-buffered saline (PBS), followed by ice-cold 4% paraformaldehyde at 08:00, 14:00, 20:00, or 02:00 on the day of diestrus. Serial coronal brain sections of 30 μm were then cut in four series that represented the anteroposterior length of the SCN. The double-immunolabeling of cFos and argine vasopressin (AVP) was performed on free-floating sections. Sections were first processed for Fos immunoreactivity (anti-Fos antibody, 1:10.000; anti-cFos AB5, PC38, Calbiochem®) followed by rabbit anti-AVP (1:20.000, anti-AVP, Bachem T4563). The double labeling were performed using the immunoperoxidase method as described previously (22, 23). Briefly, for cFos, the following were used: anti-rabbit IgG as secondary antibody (1:200; BA 1000, Vector Laboratories), avidin-biotin kit (1:100; Vector Laboratories), cobalt chloride solution 1% (12.5 μl/ml), nickel sulfate solution 1% (1 μl/ml), 0.075 mg/ml 3,3′-diaminobenzidine-HCl (DAB; Sigma-Aldrich, St. Louis, MO, USA), and 0.015% H_2O_2 in 0.1 M PB buffer. AVP exposure was performed using DAB (0.075 mg/ml) and H_2O_2 (0.3 μl/ml of 30% stock solution in 0.1 M PBS). Next, the sections were mounted on slides (Fisherbrand Superfrost Plus; Fisher Scientific), treated with subbing solution (0.1% gelatin and 0.01% chromium potassium sulfate), and cover slipped with Entellan (Merck®).

Immuno-double-stained cells were quantified with the aid of a computerized system that included a Leica microscope (Leica DM 4000B LED) equipped with a Leica digital camera (Leica DFC 450) attached to a contrast enhancement device. Representative sections from three to five animals of each experimental group from similar anatomic levels were analyzed (Bregma −0.60 mm and −0.72 mm were considered) (24). The counting of the double-labeled neurons was performed bilaterally, and neurons with high- and medium-intensity nuclear labeling were considered in this analysis.

Kisspeptin/Fos-Related Antigen (FRA)

Sections were incubated with the anti-FRA rabbit antibody (K-25; Santa Cruz Biotechnology, Santa Cruz, CA, USA), at 1:2,000 for 40 h, biotinylated anti-rabbit goat IgG (Vector Laboratories, Burlingame, CA, USA) at 1:600 for 90 min, and avidin-biotin complex solution at 1:100 for 1 h (Elite ABC kit, Vector Laboratories). A solution of nickel sulphate (25 mg/ml), 3,3'-diaminobenzidine-HCl (DAB, 0.2 mg/ml), and 0.03% H_2O_2 (Ni-DAB) was used as the chromogen. Then, sections were then incubated with anti-mouse Kp-10 antibody raised in rabbits (A.C. 564), at 1:10,000 for 40 h, biotinylated anti-rabbit goat IgG (Vector Laboratories) at 1:600 for 90 min, and Elite ABC kit at 1:100 for 1 h. DAB solution was used as chromogen. Brain sections were blindly analyzed for experimental groups under a light microscope with an image analysis system (Motic). The number of FRA-, kisspeptin-, and FRA/kisspeptin-immunoreactive (ir) neurons was quantified bilaterally in the ARC (−1.88 to −4.2 from Bregma), according to Paxinos and Watson (24).

Profile Clock Genes mRNA Expression in HPG Axis

The mRNA quantitative expression for vasopressin (*AVP*), *Period 2* (*Per2*), *Bmal1*, and *Rev-Erbα* was performed in microdissections of the SCN, preoptic area (POA), and medio-basal-hypothalamus (MBH) obtained using the *punch* technique according to Palkovits (25). Coronal brain sections containing the POA, SCN, and MBH according to the Paxinos and Watson (24) atlas were obtained in a cryostat (Leica® 3050S) at −20°C (25). For the POA, a 1,500 μm section was obtained starting at approximately +0.48 mm from the bregma. A single slice of 600 μm, immediately after the POA, was made to the SCN from −0.48 mm posterior to the bregma and two subsequent 1,000 μm slices from −1.72 mm posterior to the bregma for the MBH. The POA and SCN were dissected with 1.5 and 1.0 mm diameter needles, respectively. The SCN and MBH were dissected medially to the third ventricle. The MBH was dissected bilaterally using a 1-mm "square puncher". All isolated regions were stored in RNA later® (Sigma-Aldrich) solution at −70°C until RNA extraction. After pituitary removal, the adenohypophysis was isolated, stored in RNAse-free Eppendorf tubes, and frozen in liquid nitrogen. Ovaries were isolated from with the adipose tissue and subsequently frozen in liquid nitrogen. The structures were kept at −70°C until RNA extraction.

RNA Extraction, cDNA Synthesis, and qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen®) according to the manufacturer's protocol. The RNA concentrations were determined using a Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Scientific). A concentration of 500 ng/μl of RNA was used for cDNA synthesis using the high-capacity complementary DNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR for *Per2*, *Bmal1*, *Rev-erbα*, and *AVP* were performed using a Step One Plus real-time PCR system purchased from Applied Biosystems. The qPCR reactions were performed using two different protocols: TaqMan® for the *Bmal1* gene and SYBR® GreenER™ for *Per2*, *Rev-erbα*, and *AVP*. The access number of each gene, respective primer sequences, and concentrations are shown in **Table 1**. Each experimental cDNA was run in triplicate (2 μl of cDNA per reaction) in 96-well plates. Differences above 0.2 between replicates were automatically excluded. The assays were performed under the following conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, 15 s at 95°C, and 1 min at 60°C and 15 s at 95°C, with a gradual increase of 0.3°C. Water (instead of cDNA) was used as a negative control. The housekeeping gene for normalizing *Per2*, *Rev-erbα*, and *AVP* expression was ribosomal 26S RNA, and for *Bmal1* expression was β-actin (**Table 1**). The determination of the gene transcript levels in each sample was obtained using the ΔΔCT method. *Per2*, *Rev-erbα*, and *AVP* relative mRNA level in

TABLE 1 | Sequences and concentrations of primers, and gene access numbers.

Template access numbers	Primers and probes	Final concentration
*m*Per2 (NM_031678.1)	Forward: 5'-GGTCGAGCAAAGGACCGAC-3' Reverse: 5'-GCTGCTCATGTCCACGTCTT-3'	10 μM
*m*AVP (NM_016992.2)	Forward: 5'-TGCCTGCTACTTCCAGAACTGC-3' Reverse: 5'-AGGGGAGACACTGTCTCAGCTC-3'	10 μM
*m*Rev-erbα (NM_001113422.1)	Forward: 5'-ACAGCTGACACCACCCAGATC-3' Reverse: 5'-CATGGGCATAGGTGAAGATTTCT-3'	10 μM
26S RNA	Forward: 5'-CGATTCCTGACAACCTTGCTA-3' Reverse: 5'-CGTGCTTCCCAAGCTCTATGT-3'	10 μM
*m*Bmal1 (NM_024362.2)	(Rn00577590_m1)	*
*m*ACTB (NM_031144.2)	(Rn00667869_m1)	*

**Inventoried Assay.*

the unknown sample was calculated using the $2^{-\Delta\Delta Ct}$ method (26). The *Bmal1* relative mRNA level was determined using the relative standard curve method (27) with β-actin as an internal reference. The calibrator group used in both quantifications was CD group/8 h. All results were expressed in arbitrary units of gene expression.

Statistical Analysis

The Shapiro-Wilk normality test was used to verify the normal distribution of data. Statistical differences in qPCR, hormonal levels, and immunohistochemistry assays were determined using two-way ANOVA performed on Graph Pad Prism® software (CA, USA), version 7.0 for Windows, followed by the Tukey post-test for multiple comparisons. The Mann-Whitney test was used to determine differences in body mass. Values are presented as the means ± standard error of mean (S.E.M). $P < 0.05$ was considered statistically significant for all comparisons.

RESULTS

Gonadotropins and Steroids Plasma Concentrations

To understand the temporal profile of gonadotropin and steroid release in persistent diestrus, analyses of the plasma concentrations of these hormones were made. Statistical analysis of plasma concentrations of FSH and LH indicated an interaction of age and time of day ($axb_{\mathbf{FSH}}<0.0001$, $F_{(3,31)} = 14.13$; and $axb_{\mathbf{LH}}<0.0001$, $F_{(3,33)} = 9.245$). Temporal analysis revealed no changes in the FSH plasma levels of the RC group (**Figure 1A**). However, the IC group showed higher FSH levels at the light phase compared to dark phase, at 08:00 and 14:00 ($p_{20h}<0.0001$; $p_{02h}<0.05$), as well as those at 02:00 from those at 20:00 ($p_{02h}<0.05$). Intergroup analysis revealed higher plasma FSH concentration in rats in the IC group at 08:00, 14:00, and 02:00 ($p<0.0001$). The results for LH (**Figure 1B**) in the RC group showed no significant changes in plasma concentrations. For the IC animals, there was a higher plasma concentration of LH at the beginning of the dark phase relative to the light period ($p<0.01$). RC and IC animals were significantly different at 20:00 ($p<0.01$). E_2 plasma concentration (**Figure 1C**) did not show interaction between age and time of day ($axb_{\mathbf{E2}} = 0.5370$, $F_{(3,38)} = 0.7361$). IC group showed a considerable increase in the E_2 plasma concentration until the beginning of the dark phase ($p_{20h} <0.05$), while in the RC group, basal estradiol levels were observed on diestrus day. In regard to P4 plasma concentrations, there was an interaction between age and time of day ($axb_{P4}<0.05$, $F_{(3,40)} = 4.023$) (**Figure 1D**). IC rats showed a higher plasma P_4 concentration, except the 14 h (**Figure 2D**), than RC rats in the diestrus, which showed the lowest levels of P_4. Statistical differences were observed in the IC group at 14:00 compared to 08:00 ($p <0.01$), 20:00, and 02:00 ($p <0.001$). In intergroup comparisons, the differences were found at 08:00 ($p <0.05$), 20:00, and 02:00 ($p <0.001$), and there was an interaction between age and time of day ($axb_{\mathbf{P4}} <0.05$, $F_{(3,40)} = 4.023$).

These results show that during the spontaneous transition from regular to irregular cycle and to acyclicity, there is greater secretion of FSH and P_4 and a slight increase in the profile of LH and E_2 secretion in relation to RC on the day of diestrus.

Circadian Activity of the AVP Neurons in the SCN Nucleus

This experiment examined the light/dark synchronization of the dorsomedial SCN in persistent diestrus during reproductive aging. Our results showed higher *AVP* gene expression (**Figure 2A**) and AVP neuronal activity (**Figure 2B**) in IC group rats during dark period. RC animals showed higher relative mRNA expression at 02:00 than at 08:00 ($p<0.05$), while in IC group the increase occurred at 20:00 and 02:00, intra- and intergroup (**Figure 2A**). In addition, the IC animals showed greater vasopressinergic activity at 20:00 than at 08:00 ($p<0.05$) and at 02:00 ($p<0.001$), as well as at 14:00 relative to at 02:00 ($p<0.05$) and the intergroup at 20:00 ($p<0.01$) (**Figure 2B**). The statistical analysis detected that there was an interaction between age and time of day ($axb<0.05$, $F_{(3,31)} = 3.172$). In photomicrographs (**Figures 2C–F**), it is possible to check a greater amount of cFos-labeled double vasopressinergic neurons at 20:00 on the day of diestrus in the dorsomedial portion of the SCN of IC rats (**Figures 2E, F**) and a smaller amount in RC rats (**Figures 2C, D**). The results show an increase in SCN dorsomedial neuronal activity, in the dark period, during persistent diestrus.

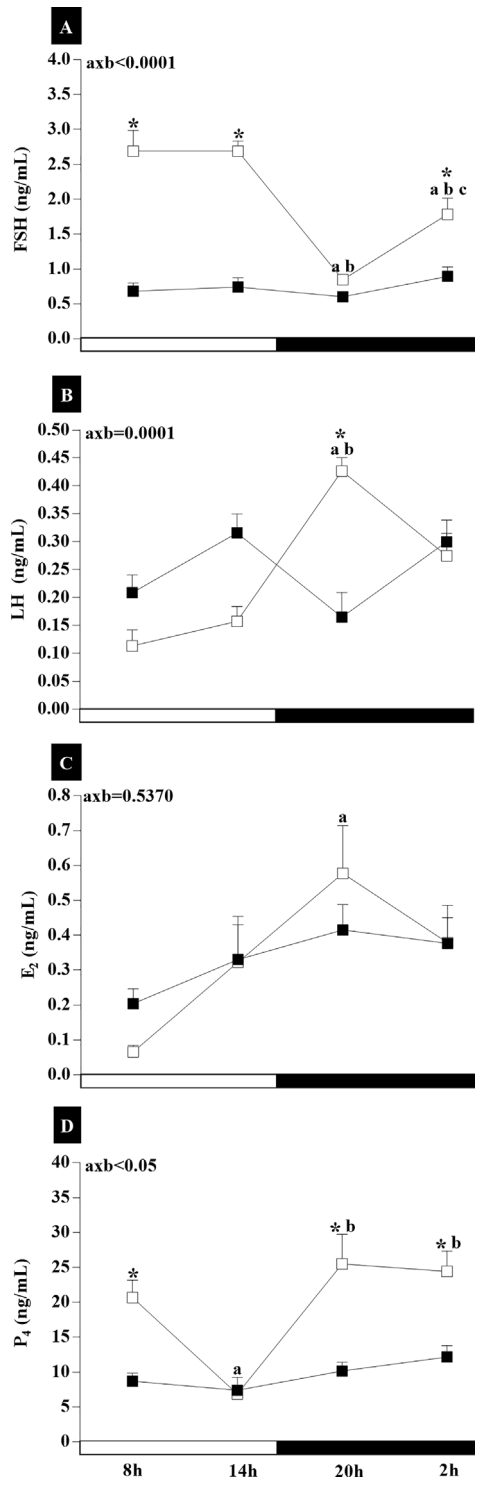

FIGURE 1 | The plasma levels of gonadotropins **(A, B)** and steroids **(C, D)** of adult rats with a regular estrous cycle (RC) on diestrus day (■) and IC group rats with an irregular cycle (IC) in persistent diestrus (□) at 8:00, 14:00, 20:00, and 02:00. The data are expressed as the mean ± SEM. *$p < 0.05$ *vs.* RC at the same time; [a]vs. 08:00, [b]vs.14:00, and [c]vs. 20:00; axb = interaction of age and time.

Circadian Activity of the Kisspeptin Neuron in the Arcuate Nucleus

To evaluate whether there is loss of rhythm in the activity of kiss neurons in the ARC nucleus during the circadian cycle of IC animals, analyses of kisspeptin-ir neurons were conducted. Two-way ANOVA showed that there was no interaction between the two variables, groups (RC and IC), and time (8, 14, 20, and 2h) on the number of FRA-ir neurons in the ARC (axb=0.4572, $F_{3.53}$ = 0.8805). There was no statistical difference in the number of FRA-ir neurons in the ARC of both groups (**Figure 3A**). Analysis of kisspeptin-ir neurons (**Figure 3C**) showed that there was no interaction between age (RC and IC) and time of day (axb = 0.1961, $F_{(3.53)}$=1.618). The number of kisspeptin-ir neurons in the ARC was lower ($p<0.05$) in IC than in RC group at 08:00, 20:00, and 02:00. In the RC group, differences were observed at 20:00 ($p<0.05$) and 02:00 (<0.0001) in relation to at 14:00. There was no interaction between groups (RC and IC) on the number of FRA/Kiss-ir neurons (axb = 0.3975, $F_{(3,51)}$=1.007), and statistical differences were also not observed in both groups (**Figure 3B**). There was no interaction between groups (RC and IC) and time (8:00, 14:00, 20:00, and 02:00h) on the % of FRA/Kisspeptin-ir neurons in the ARC (axb = 0.2826, $F_{3.51}$ = 1.306) (**Figure 3D**). The results suggest that the circadian signaling of kiss neurons from IC animals is attenuated, contributing to the reduction in the interaction of kiss and GnRH neurons.

Relative Expression of mRNA Clock Genes in HPG Axis

In this analysis, we aimed to verify if there is an alteration in the expression of clock genes in the HPG axis of senescent rats during the spontaneous transition from regular to irregular cycle and to acyclicity, in relation to cyclic female rats. The results demonstrate temporal changes in circadian rhythm amplitude in 18-month-old animals whose cycles are irregular and remain in persistent diestrus. The experiments demonstrated the synchronization of clock gene transcription with gene activity during the SCN light/dark cycle (**Figure 4**). Statistical analyses for *Bmal1*and *Rev-erbα* (**Figures 4B, C**) in the SCN showed an interaction between age and time of day (axb_{Bmal1}<0.001, $F_{(3,105)}$ = 5.913; $axb_{Rev\text{-}erb\alpha}$<0.0001, $F_{(3,112)}$ = 15.16; and axb_{AVP}<0.0001, $F_{(3,\ 105)}$ = 21.16). There was greater expression of *Per2* at 20:00 (**Figure 4A**) in the SCN of RC groups. The IC group did not present any statistical difference between the dark and light phase schedules. The intergroup comparison also showed no statistical difference (**Figure 4A**). Higher *Bmal1* mRNA were observed in the light phase of RC group compared to the dark phase (p_{14x20h}<0.01; p_{8x20h}<0.0001; p_{8x02h}<0.01) (**Figure 4B**). However, in the IC group, there was no temporal changes in the *Bmal1* mRNA (**Figure 4B**). The expression of *Rev-erbα* mRNA in the SCN of RC group was higher at 14:00 (p<0.0001), decreasing at 20:00 and 02:00 (**Figure 4C**). On the other hand, IC rats showed increases in *Rev-erbα* mRNA expression at 20 h compared at 08:00 ($p<0.05$) in the SCN (**Figure 4C**). Decreased *Rev-erbα* mRNA occurred at 14:00 h ($p<0.01$), while it increased at 20:00 h ($p<0.01$) in the animals of IC group compared to RC group (**Figure 4C**).

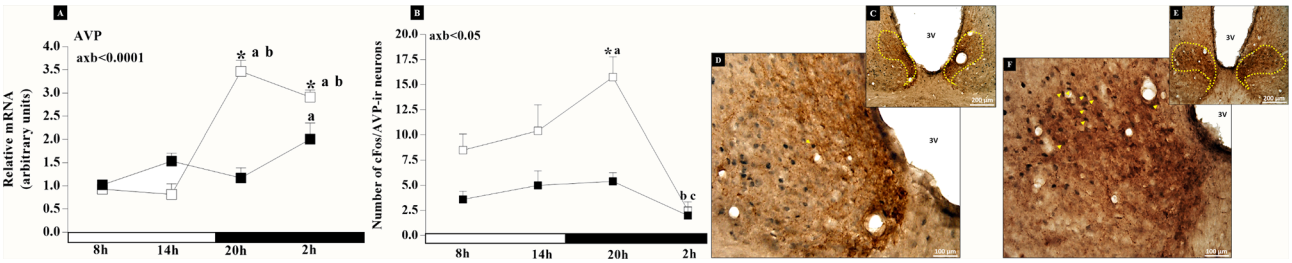

FIGURE 2 | Graphical representation of higher *AVP* gene expression **(A)** and AVP neuronal activity **(B)** observed in rats with irregular cycle (IC group) in persistent diestrus (□) compared to adult rats with regular estrous cycle (RC) on diestrus day (■). The mean ± SEM number of cFos/AVP-ir neurons are shown on the graph. *$p < 0.05$ *vs.* RC at the same time interval; [a]vs. 08:00, [b]vs.14:00, and [c]vs. 20:00; axb = interaction of age and time. Photomicrographs of vasopressinergic neurons in the dorsomedial portion of the SCN doubly labeled with cFos (arrows) at 20:00 on diestrus day. Animal RC increased by 200× **(C)** and 400× **(D)**; animal IC increased by 200× **(E)** and 400× **(F)**.

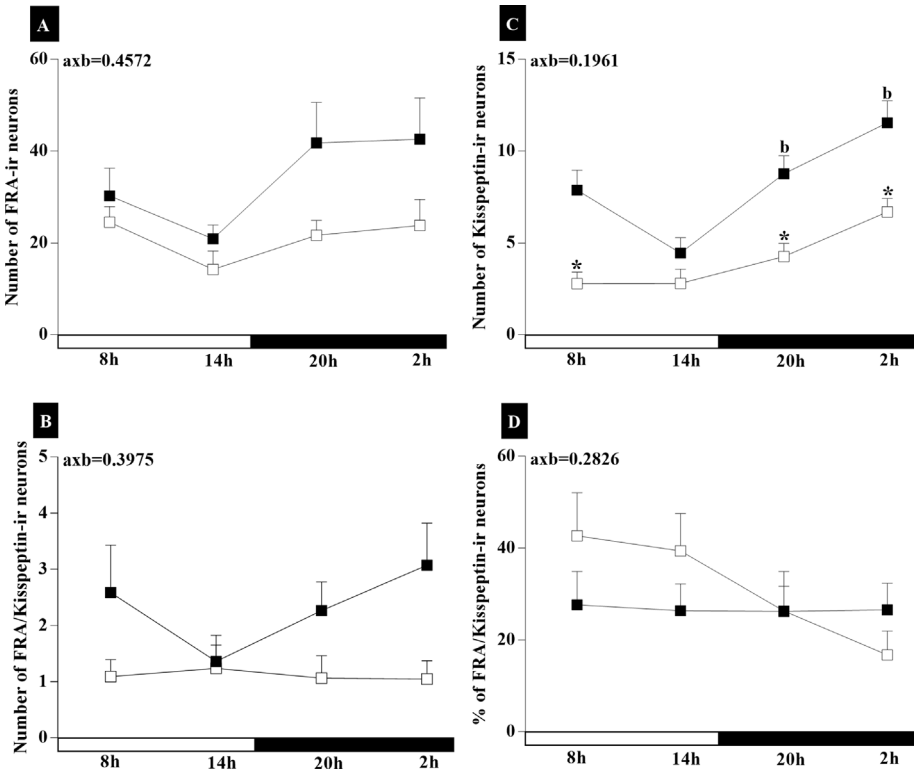

FIGURE 3 | Graphical representation of higher Kisspeptin neuronal activity observed in IC group rats with irregular cycle (IC) in persistent diestrus (□) compared to adult rats with regular estrous cycle (RC) on diestrus day (■). The mean ± SEM number of FRA-ir neurons **(A)**, Kiss-ir neurons **(B)**, FRA/Kiss-ir neurons **(C)**, and % FRA/Kiss-ir neurons **(D)** are shown on the graph. *$p < 0.05$ *vs.* RC at the same time interval; [b]vs.14:00; axb = interaction of age and time.

The POA analysis of clock genes mRNA indicated an interaction of age and time of day for *Per2* and *Rev-erbα* ($axb_{Per2}<0.0001$, $F_{(3,108)} = 8{,}098$; and $axb_{Rev\text{-}erb\alpha}<0.05$, $F_{(3,130)} = 5{,}105$) but not for *Bmal1* ($axb_{Bmal1} = 0.0834$, $F_{(3,83)} = 2{,}299$) (**Figure 5A**). In IC group, the greatest *Per2* mRNA was found at 20:00 ($p<0.0001$). Whereas, in the RC group, *Per2* expression was higher at 20:00 h and 02:00 h compared to 08:00 ($p_{20h}<0.0001$ and $p_{02h}<0.05$) (**Figure 5A**). In the RC group, the *Bmal1* expression was higher at 02:00 h compared to that at 08:00 ($p<0.05$) and at 20:00 h ($p<0.01$) (**Figure 5B**). While, in the IC group, lower *Bmal1* expression was found at 20:00 h compared to 14:00 h ($p<0.05$). In the IC group, *Per2* expression increased at 20:00, while *Rev-erbα* decreased at 14:00 h. *Rev-erbα* in the RC group had higher expression at 14:00 ($p_{14hx08h}<0.01$;

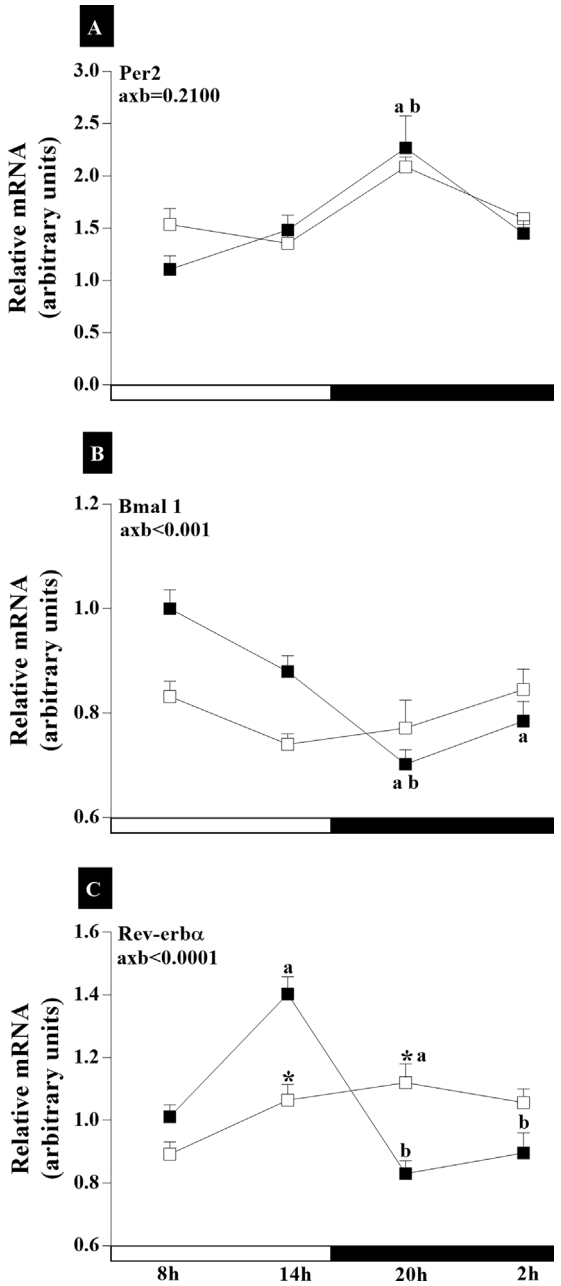

FIGURE 4 | Daily oscillations in relative mRNA expression for *Per2*, *Bmal1*, and *Rev-erbα* in the suprachiasmatic nucleus. Light and dark phases are represented along the X-axis by their respective schedules. Beginning of the light phase at 07:00, when the lights are turned on. The dark phase started at 19:00. All samples were collected at six-hour intervals after the first hour (i.e., 08:00), for 24 h. Light/dark cycle = 12/12 h. **(A)** Relative temporal expression of mRNA for *Per2*, **(B)** *Bmal1*, and **(C)** *Rev-erbα* in animals with a regular cycle on diestrus day and irregular cycle in persistent diestrus. * indicates intergroup variation at the same time: (■) Regular estrous cycle on diestrus day × (□) irregular cycle in persistent diestrus. Letters indicate temporal variation within the group. [a]vs. 8:00; [b]vs. 14:00, and [c]vs. 20:00. axb = age and time of day interaction. n = 3–7. Replicates with a variation above 0.2 were automatically excluded from the groups. ΔΔCt for *Per2* and *Rev-erbα*; relative standard curve for *Bmal1*.

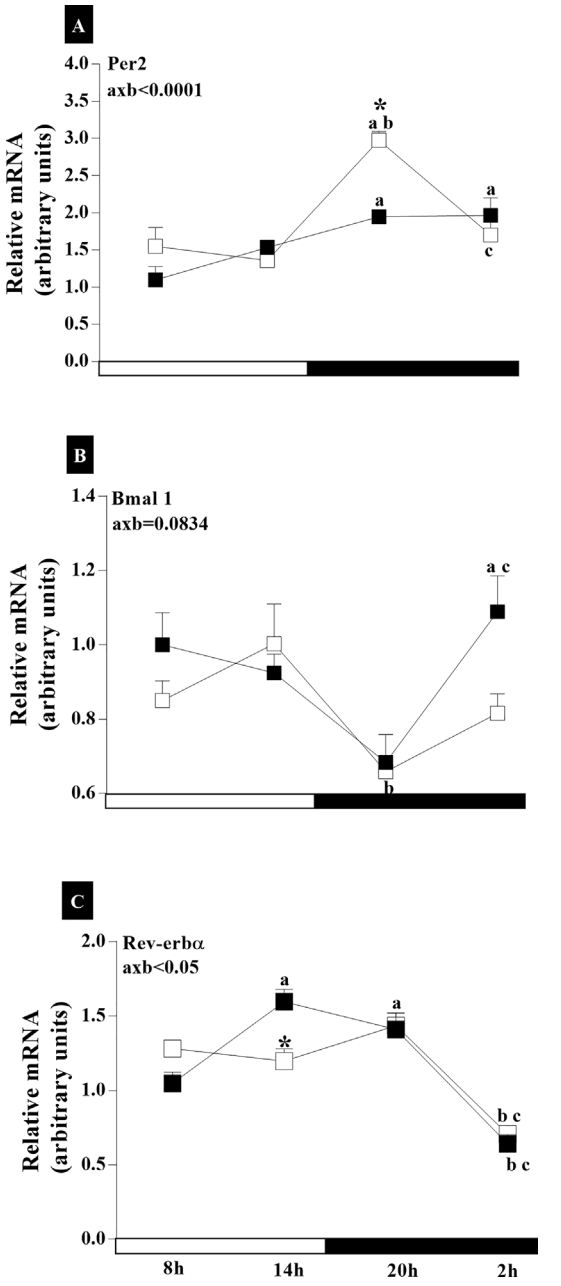

FIGURE 5 | Daily oscillations in relative mRNA expression for *Per2*, *Bmal1*, and *Rev-erbα* in the preoptic area. Light and dark phases are represented along the X-axis by their respective schedules. Beginning of the light phase at 07:00, when the lights are turned on. The dark phase started at 19:00. All samples were collected at six-hour intervals after the first hour (i.e., 08:00), for 24 h. Light/dark cycle = 12/12 h. **(A)** Relative temporal expression of mRNA for *Per2*, **(B)** *Bmal1*, and **(C)** *Rev-erbα* in animals with a regular cycle on diestrus day and irregular cycle in persistent diestrus. * indicates intergroup variation at the same time: (■) Regular estrous cycle on diestrus day × (□) Irregular cycle in persistent diestrus. Letters indicate temporal variation within the group. [a]vs. 8:00; [b]vs. 14:00; and [c]vs. 20:00. axb = age and time of day interaction. n = 3–7; Replicates with a variation above 0.2 were automatically excluded from the groups. ΔΔCt for *Per2* and *Rev-erbα*; relative standard curve for *Bmal1*.

$p_{14hx02h}$<0.001) and 20:00 ($p_{20hx08h}$<0.05; $p_{20hx02h}$<0.001) than at 08:00 and 02:00 (**Figure 5C**). On the other hand, the animals with IC expressed a lower amount of *Rev-erbα* mRNA, with similar amounts expressed in the light phase and dark phase, with lower amounts at 02:00 (p_{08x02h}<0.0001; p_{14x02h}<0.001; p_{20x02h}<0.0001), but did not differ from RC rats at this time.

In the MBH, the RC group exhibited slightly higher *Per2* expression at 02:00 h compared to 14:00 h (p<0.01) (**Figure 6A**). In the IC group, there was a decrease in *Per2* mRNA at 8:00 (p<0.0001), 14:00 h (p<0.0001), and 20:00 h (p<0.0001) (**Figure 6A**). *Bmal1* gene expression was higher at 14:00 (p<0.001) and 02:00 (p<0.01) than at 20:00 in the RC group (**Figure 6B**). *Rev-erbα* exhibited higher expression in RC rats at 08:00 and 14:00 than at 20:00 and 02:00 (p<0.05). The IC group showed no time-of-day differences. However, in the group of animals with IC, we found a lower expression of *Rev-erbα* at 08:00 (p <0.01) and 14:00 (p <0.0001) compared to animals with RC (**Figure 6C**). An interaction of age and time of day was observed for analyses of *Per2* (axb<0.0001, $F_{(3,90)}$ = 27) and *Rev-erbα* (axb<0.0001, $F_{(3,106)}$ = 18.04) (**Figure 6**).

In the adenohypophysis, there were significant interactions of age and time-of-day factors on the expression of *Per2* mRNA (axb_{Per2}<0.0001, $F_{(3,76)}$ = 10.93) (**Figure 7A**). The RC group showed a peak in *Per2* gene expression at 20:00 h ($p_{08hx20h}$<0.0001; $p_{14hx20h}$< 0.0001), while minimal expression was seen at 08:00 h (**Figure 7A**). In the IC group, *Per2* expression was higher at 20:00 h compared to that at 8:00 h (p<0.05) (**Figure 7A**). However, the expression of *Per2* at 20:00 h (p<0.0001) was lower in senescent females compared to adult female rats (**Figure 7A**). In both RC and IC groups, *Bmal1* mRNA was highest at 8:00 h compared to the times analyzed in this study (**Figure 7B**). For this gene, no interaction of age and time-of-day factors was observed (axb = 0.3964, $F_{(3,50)}$ = 1.009). *Rev-erbα* gene analysis demonstrated a significant interaction of age and time of day (axb<0.001, $F_{(3,90)}$ = 130.3) (**Figure 7C**). The RC group (**Figure 7C**) showed higher *Rev-erbα* expression at 20:00 than at 08:00 and 02:00 (p<0.001). The IC group showed higher *Rev-erbα* expression at 20:00 than at 08:00, 14:00, and 02:00 (p<0.0001). Increase in *Rev-erbα* expression occurred at both times of the dark phase in the IC group (**Figure 7C**).

In the ovaries, statistical analyses reveled interaction of age and time of day on *Per2* expression (axb<0.0001, $F_{(3,71)}$ = 38.62), on *Bmal1* expression (axb<0.0001, $F_{(3,58)}$ = 9.448), and on *Rev-erbα* expression (axb<0.0001, $F_{(3,90)}$ = 26.28) (**Figure 8**). The highest level of *Per2* expression in the RC group was at 20:00 (p<0.0001). The IC group showed lower expression at 08:00 h (p<0.0001) and reduced expression at 20:00 h (p<0.0001) (**Figure 8A**). The animals in the IC group showed higher expression of *Per2* at 08:00 h (p <0.05), 14:00 h, and 02:00 h (p <0.0001) than the animals in the RC group (**Figure 8A**). Similar temporal differences in the ovarian *Bmal1* mRNA were found between the RC and IC groups (**Figure 8B**). *Bmal1* expression was greater at 08:00 ($p_{RC14\ and\ 20h}$<0.001 and $p_{IC14\ and\ 20h}$<0.0001) and lower at 14:00 and 20:00 than at 02:00 ($p_{RC8\ and\ 20h}$<0.0001, p_{RC14h}<0.05, and $p_{IC14\ and\ 20h}$<0.0001) (**Figure 8B**). There was increased *Bmal1* expression at 02:00 h

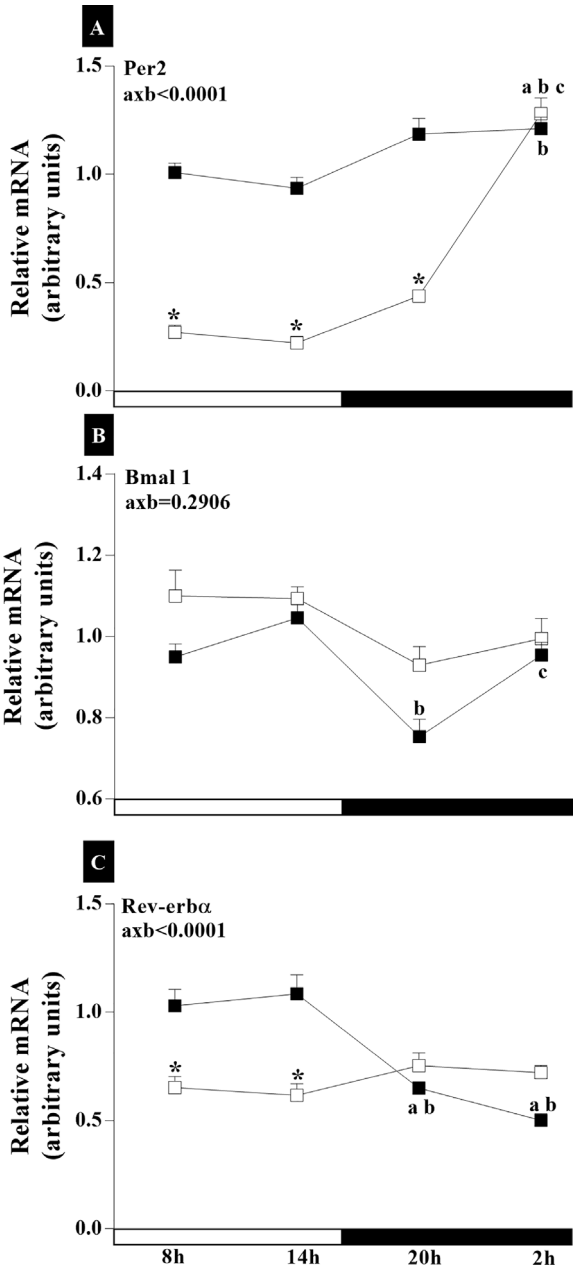

FIGURE 6 | Daily oscillations in relative mRNA expression for *Per2*, *Bmal1*, and *Rev-erbα* in the medio-basal hypothalamus. Light and dark phases are represented along the X-axis by their respective schedules. Beginning of the light phase at 07:00, when the lights are turned on. The dark phase started at 19:00. All samples were collected at six-hour intervals after the first hour (i.e., 08:00), for 24 h. Light/dark cycle = 12/12 h. **(A)** Relative temporal expression of mRNA for *Per2*, **(B)** *Bmal1*, and **(C)** *Rev-erbα* in animals with a regular cycle on diestrus day and irregular cycle in persistent diestrus. * indicates intergroup variation at the same time: (■) Regular estrous cycle on diestrus day × (□) Irregular cycle in persistent diestrus. Letters indicate temporal variation within the group. [a]vs. 08:00; [b]vs. 14:00; and [c]vs. 20:00. axb = age and time of day interaction. n = 3–7. Replicates with a variation above 0.2 were automatically excluded from the groups. ΔΔCt for *Per2* and *Rev-erbα*; relative standard curve for *Bmal1*.

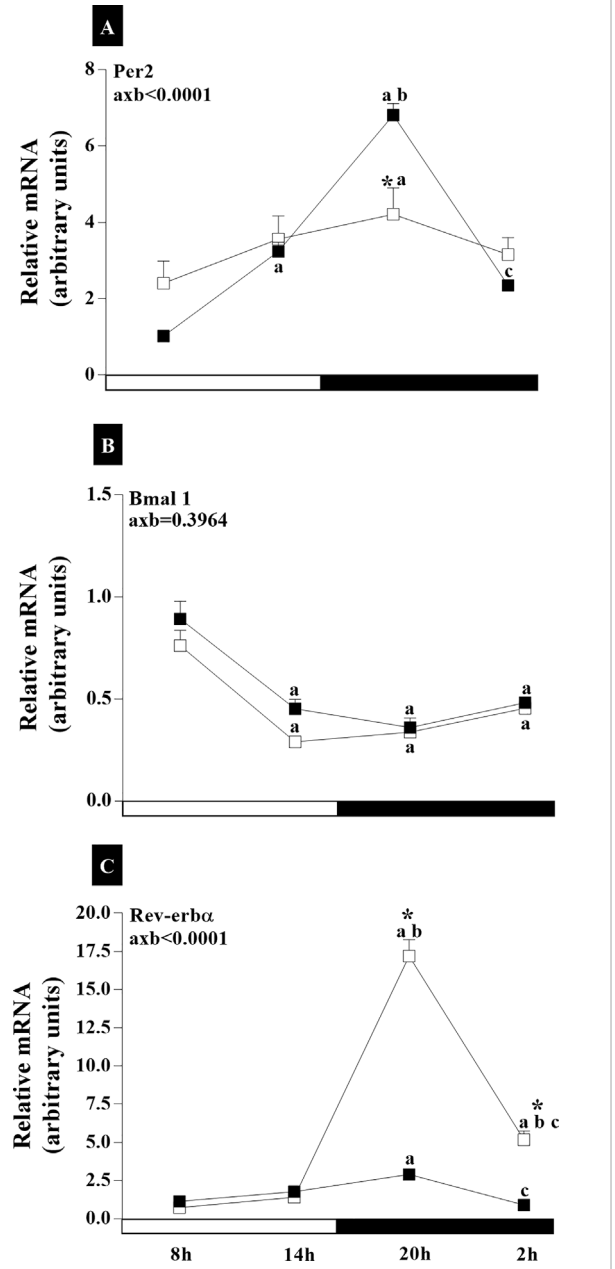

FIGURE 7 | Daily oscillations in relative mRNA expression for *Per2*, *Bmal1*, and *Rev-erbα* in the adenohypophysis. Light and dark phases are represented along the X-axis by their respective schedules. Beginning of the light phase at 07:00, when the lights are turned on. The dark phase started at 19:00. All samples were collected at six-hour intervals after the first hour (i.e., 08:00), for 24 h. Light/dark cycle = 12/12 h. **(A)** Relative temporal expression of mRNA for *Per2*, **(B)** *Bmal1*, and **(C)** *Rev-erbα* in animals with a regular cycle on diestrus day and irregular cycle in persistent diestrus. * indicates intergroup variation at the same time: (■) Regular estrous cycle on diestrus day × (□) Irregular cycle in persistent diestrus. Letters indicate temporal variation within the group. [a]vs. 08:00; [b]vs. 14:00; and [c]vs. 20:00. axb = age and time of day interaction. n = 3–7. Replicates with a variation above 0.2 were automatically excluded from the groups. ΔΔCt for *Per2* and *Rev-erbα*; relative standard curve for *Bmal1*.

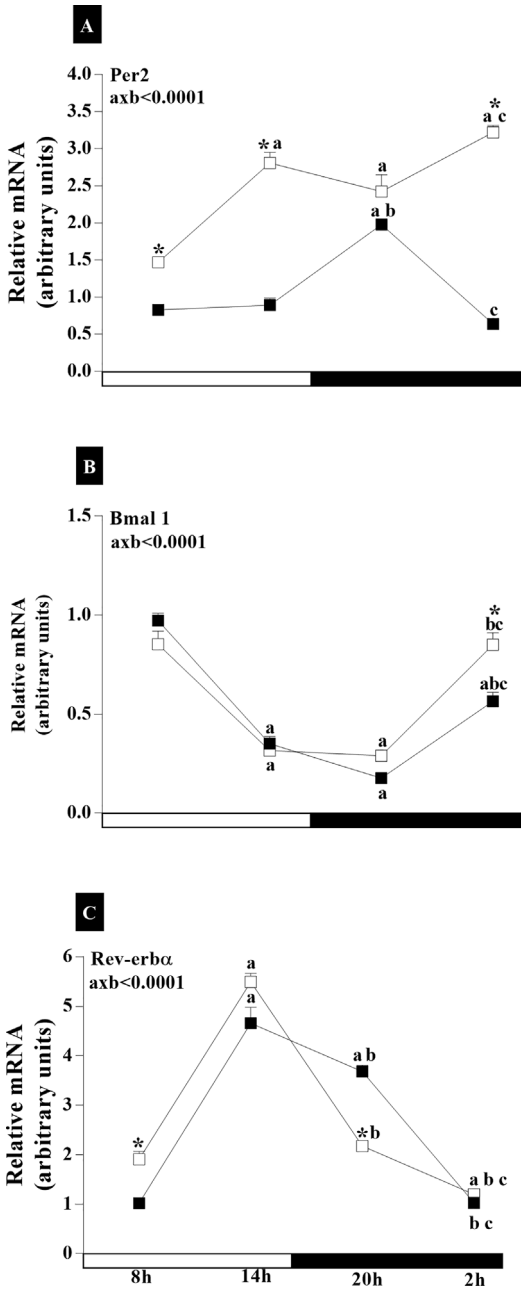

FIGURE 8 | Daily oscillations in relative mRNA expression for *Per2*, *Bmal1*, and *Rev-erbα* in the ovary. Light and dark phases are represented along the X-axis by their respective schedules. Beginning of the light phase at 07:00, when the lights are turned on. The dark phase started at 19:00. All samples were collected at six-hour intervals after the first hour (i.e., 08:00) for 24 h. Light/dark cycle = 12/12 h. **(A)** Relative temporal expression of mRNA for *Per2*, **(B)** *Bmal1*, and **(C)** *Rev-erbα* in animals with a regular cycle on diestrus day and irregular cycle in persistent diestrus. * indicates intergroup variation at the same time: (■) Regular estrous cycle on diestrus day × (□) Irregular cycle in persistent diestrus. Letters indicate temporal variation within the group. [a]vs. 08:00; [b]vs. 14:00; [c]vs. 20:00. axb = age and time of day interaction. n = 3–7. Replicates with a variation above 0.2 were automatically excluded from the groups. ΔΔCt for *Per2* and *Rev-erbα*; relative standard curve for *Bmal1*.

($p<0.0001$) in IC group. There was a significant interaction of age and time of day on *Bmal1* expression (axb<0.0001). The RC and IC groups presented similar gene expression profiles for *Rev-erbα* (**Figure 8C**). There was higher *Rev-erbα* expression at the end of the light phase and the beginning of the dark phase (**Figure 8C**). Increased *Rev-erbα* mRNA at 08:00 h ($p<0.01$), while it decreased at 20:00 h ($p<0.01$), occurred in the animals of IC group (**Figure 8C**).

The results showed irregularity in the expression of *Bmal1* and *Per2*, of *Rev-erbα* and *Bmal1* in the HPG axis, between the light and dark phases, in the period of spontaneous transition from regular to irregular cycle and to acyclicity.

DISCUSSION

In this study, we observed that clock genes expression rates varied between light and dark phases on the HPG axis, as well as SCN neuronal AVP activity in the persistent diestrus of senescent females compared to the diestrus of adult females. Remarkably, these results are important because the synchronization between neural, endocrine, and neuroendocrine signals, coordinated by the SCN and HPG axis, is important for the occurrence and maintenance of reproduction in mammals (28, 29). Also noteworthy is the circadian rhythm for the synthesis activity of vasopressin-releasing neurons in the SCN and its participation in the communication of suprachiasmatic cells. However, the capacity of circadian rhythm regulator genes seems to be diminished during the senescence period. In this sense, studies in the late 1990s showed the importance of the SCN in regulating the circadian system in aged organisms (30–32). We detected higher nocturnal gene expression of *AVP* and neuronal activity in the SCN, mainly in females with irregular estrous cycle and reproductive senescence, when compared to adult female rats in the diestrus phase of the regular cycle. However, there was found a smaller number of kisspeptinergic neurons in the ARC of IC rats. We highlight here the studies carried out by Padilla et al. (33), showing that only female rodents have kisspeptin neurons in the SCN, some of which corresponded to AVP neurons. These authors also showed that the silenced kisspeptin ARC neurons in female mice resulted in the loss of the normal estrous cycle, remaining in diestrus (33, 34). Thus, circadian information from the vasopressinergic and kisspeptinergic neural pathways are essential for the activity of GnRH cells (35–37). Study carried out by our group (38) showed a smaller and constant GnRH content in 18-month-old IC group, similar to the immunostaining data of Kiss neurons in the ARC nucleus. Therefore, our data suggest that communication with GnRH cells *via* kisspeptin in ARC may be compromised and lead to a period of persistent diestrus in IC rats. Furthermore, the SCN analysis showed different expression of *Per2*, *Bmal1*, and *Rev-erbα* in the diestrus of RC rats compared to IC rats with persistent diestrus, especially at 20 h, reinforcing the idea of a weakening of the SCN output system (39). These results together reflect the loss of circadian oscillation, which in RC animals is marked by a reduction in the activity of kisspeptin neurons at the end of the light phase with a consequent increase in the dark phase.

An interesting finding of this study was the higher expression of *Per2* at 20:00 in the POA of animals in the IC group, similar to that observed in the SCN and coinciding with the greater expression and activity of AVP neurons in the SCN. In addition, aged animals show phase misalignment and lack of rhythmic profile in plasma LH secretion, whose concentration was slightly higher in the dark phase, similar to estradiol concentrations. A subpopulation of the GnRH neurons in the MBH expresses endogenous clock functions, and gene expression is necessary for continuous and typical GnRH secretion (40–42). We verified that the relative expression of *Per2* did not change in the MHB of the RC rats in diestrus. However, in IC rats in persistent diestrus, the expression of *Per2* was lower at 8, 14, and 20 h. These data allow us to suggest linearity of gene expression in the MBH related to the linearity of the GnRH content observed in rodent females from the IC group, as verified in a previous study of our group (38). We also found differences in the expression of *Bmal1* and *Rev-erbα* in SCN and MBH in senescent rats. The absence of circadian rhythmicity of these genes leads us to speculate about the physiological activity of the molecular clock as a determining factor for the absence of ovulatory peaks during female aging. In addition to our data, studies have shown that rodent knockouts of the central *Bmal1* gene are infertile and the mutation/deletion of the gene in the periphery contributes to infertility by compromising the production of steroid hormones, gametogenesis, and abnormalities of delivery and implantation in female (43, 44).

Although pituitary cells are under the control of hypothalamic factors, the adenohypophysis cells have a molecular clock capable of measuring time autonomously (45). The molecular clock control in the different cell types of the adenohypophysis provides circadian oscillation in pituitary gonadotrophs, and the daytime rhythm of cell proliferation is synchronized with the estrous cycle in adult rats (29, 46, 47). Our study demonstrates that these cells are the first extra-SCN elements of the HPG axis to signal changes and contribute to reproductive aging. In IC rats, there was no alteration in the expression of *Per2* and *Bmal1*, although we verified a strong inhibition of *Rev-erbα* at the same time (20 h) as there is an increase in LH, E_2 and P_4, and lowest in FSH. Interestingly, IC rats exhibit the same response profile for *AVP* mRNA in the SCN, *Rev-erbα* in the adenohypophysis, and plasma LH concentration, suggesting that the non-oscillation in the expression of *Bmal1* and *Rev-erbα* in the SCN at the times analyzed contribute to the increase in the expression of AVP in SCN and plasma LH. In addition, molecular clock components have been detected in the pituitary gland of the rat with autonomous activity and rhythmic LH release, independent of hypothalamic control (48). Perhaps higher plasma E_2 and P_4 concentrations in IC animals modulate the axis, as estrogen itself alters circadian rhythms, increasing their amplitude (49, 50).

Reproductive senescence in laboratory rodents is characterized by follicular atresia, irregular cycles, and steroid hormone

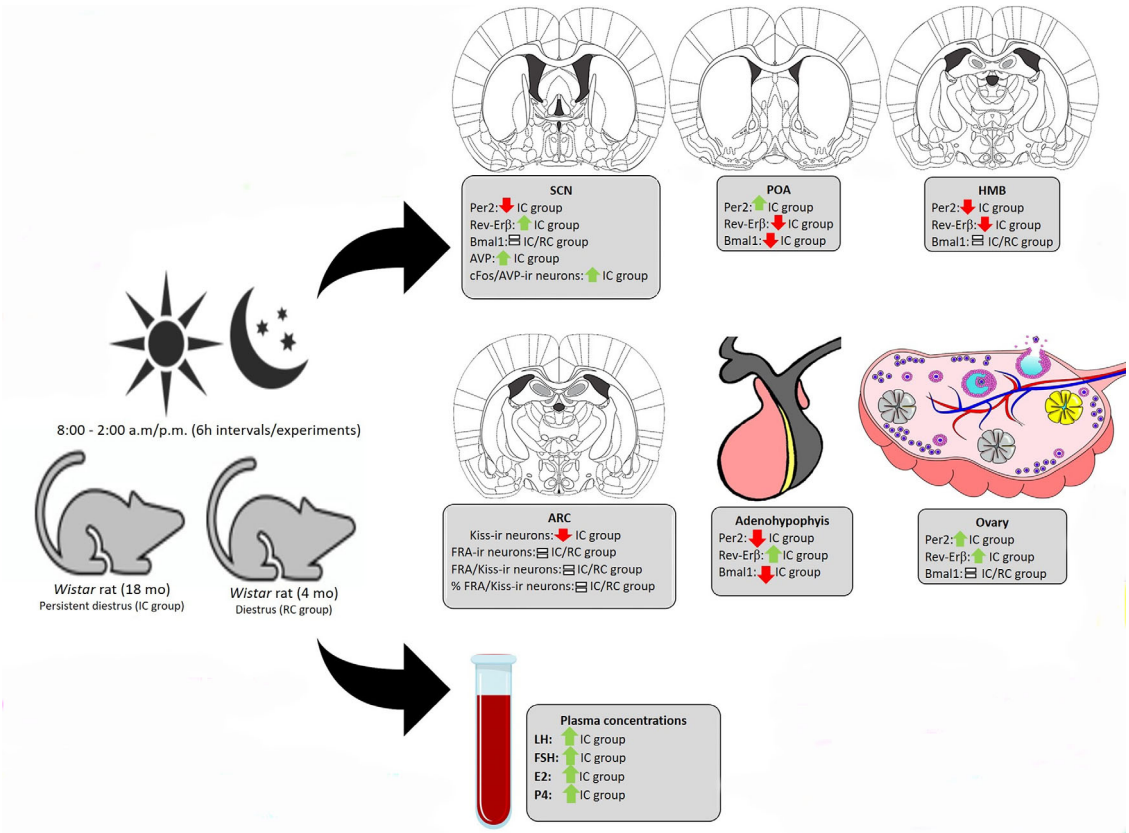

FIGURE 9 | Results summary. Expression profile of clock genes on the HPG axis and activity of SCN AVP neurons and ARC Kiss neurons of rats in periestropause.

fluctuations (51). Remarkably, several studies have shown that there are temporal variations in molecular clock components in ovarian rodent tissues, whose function is related to the timing of gene expression in mature granulosa cells, including genes related to steroidogenesis, gonadotropin responsiveness, and ovulation (52–55). Results of the analyses carried out in the diestrus period of RC rats showed the synchrony of the expression of *Per2* and *Bmal1* in the ovary and SCN, reinforcing the important synchronization in the expression of clock genes and the integration in the HPG axis modulating the reproductive system. However, this study shows the desynchronization of the expression of these genes in the ovary and that this desynchronization occurs in a subtle way in relation to the SCN and POA. In these animals, transcription of ovarian genes seems to occur autonomously, with the pattern of response of the *Per2* transcript contrary to that of RC animals, suggesting an ovarian temporal window in response to the central stimulus, contributing to persistence in the diestrus phase.

Together, these data support our hypothesis that the feedback loops of clock genes on the HPG axis contribute to modulating the spontaneous transition from regular to irregular cycle and to acyclicity in female rodents (**Figure 9**). Additionally, it was evidenced that the desynchronization between the central and peripheral circadian clocks contribute to the irregularity of reproductive events.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at: http://hdl.handle.net/11449/152720 and https://repositorio.unesp.br/bitstream/handle/11449/152720/nicola_ml_dr_araca_int.pdf?sequence=3&isAllowed=y.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Universidade Estadual Paulista (Process n. 2014-00269).

AUTHOR CONTRIBUTIONS

AN: care of experimental animals, data collection, data analysis, data tabulation, discussion of results, and paper

editing. LF: data collection. MM: gene expression data collection and analysis of results. TV-F: standardization of the cFos/AVP immunohistochemistry analysis in SCN nucleus. CL: standardization of the FRA/Kiss immunohistochemistry and data analysis in Arcuate nucleus. AM: FRA/Kiss data analysis in Arcuate nucleus. JA-R: standardization of gene expression and cFos/AVP immunohistochemistry. MP: data analysis, discussion of results, and paper editing. RD: guidance and monitoring of all experimental steps, data analysis, data tabulation, discussion of results, and paper editing. All authors contributed to the article and approved the submitted version.

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Shared Genetics Between Age at Menopause, Early Menopause, POI and Other Traits

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Reproductive ageing leading to menopause is characterized by depletion of follicles and its regulating mechanisms are only partly understood. Early age at menopause and premature ovarian insufficiency (POI) are associated with several other traits such as cardiovascular disease, dyslipidemia, osteoporosis and diabetes. In large cohorts of Northern European women hundreds of Single Nucleotide Polymorphisms (SNPs) have been identified to be associated with age at menopause. These SNPs are located in genes enriched for immune and mitochondrial function as well as DNA repair and maintenance processes. Genetic predisposition to earlier menopause might also increase the risk of other associated traits. Increased risk for cardiovascular disease in women has been associated with age at menopause lowering SNPs. Pleiotropy between early age at menopause and increased mortality from coronary artery disease has been observed, implicating that genetic variants affecting age at menopause also affect the risk for coronary deaths. This review will discuss the shared genetics of age at menopause with other traits. Mendelian Randomization studies implicate causal genetic association between age at menopause and age at menarche, breast cancer, ovarian cancer, BMD and type 2 diabetes. Although the shared biological pathways remain to be determined, mechanisms that regulate duration of estrogen exposure remain an important focus.

Keywords: menopause, premature ovarian insufficiency, primary ovarian insufficiency, postmenopause, genetics, genome-wide association study, genetic pleiotropy, mendelian randomization analysis

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INTRODUCTION

Menopause is defined as the cessation of reproductive function and age at menopause is determined retrospectively as the absence of menses for 12 months (te Velde and Pearson, 2002). The mean age of natural menopause (ANM) in women of European descent is 51 years, showing a normal distribution ranging from 40 to 60 years (te Velde and Pearson, 2002). An early menopause (EM) before the age of 45 years occurs in ~5% of women in the general population. Menopause before the age of 40 years is present in 1% of women and is considered a pathological condition referred to as premature ovarian insufficiency (POI) (Coulam et al., 1986; Webber et al., 2016). Importantly, already prior to menopause fecundity begins to decline (te Velde and Pearson, 2002). The latter has gained more interest given that women in the Western world decide to have their first child later in life, and thereby increasing the risk of age-related involuntary infertility (Broekmans et al., 2009).

Menopause results from exhaustion of the follicle pool. After the formation of the primordial follicle pool *in utero*, the ovary contains on average one million follicles at birth. Primordial follicles

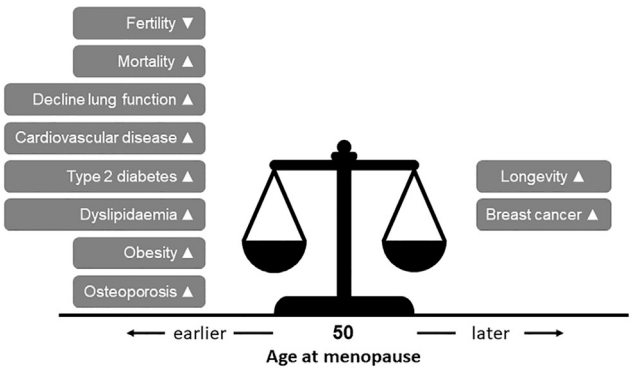

FIGURE 1 | The impact of age at menopause on women's health. Data of numerous conventional epidemiological studies indicate that the timing of age of menopause increases (▲) or decreases (▼) the risk for various diseases.

are continuously activated and recruited into the growing follicle pool of which, after puberty, only one is destined to reach the preovulatory stage. When the pool of primordial follicles is exhausted to 100–1,000 follicles, this number is too low to support reproductive cycles. In addition to the decline in follicle number, oocyte quality declines with increasing age (Hall, 2015). This reproductive aging also leads to changes in the hypothalamic-pituitary axis. The reduction in number of growing follicles results in decreased levels of inhibin B, estradiol and progesterone, and thereby loss of the negative feedback on GnRH pulse frequency and secretion and subsequently in significantly increased gonadotropin levels. In addition to ovarian aging, hypothalamic and pituitary aging have also been proposed to contribute to reproductive aging (Hall, 2015).

POI is often considered the result of premature ovarian aging, as a consequence of a reduced primordial follicle pool at birth, accelerated depletion of the follicle pool, or increased atresia of follicles. However, POI can also develop despite follicles being present due to insensitivity to gonadotropins. Therefore, POI can present as primary amenorrhea or as secondary amenorrhea, with the latter more closely resembling the process of natural menopause (Visser et al., 2012).

EM and POI not only lead to infertility but also increase the risk for osteoporosis, type 2 diabetes and cardiovascular disease, among others (Forman et al., 2013), as shown by numerous epidemiological studies (**Figure 1**). The lower estrogen levels are considered an important contributing factor to this increased risk. In contrast, an earlier age at menopause reduces the risk of breast cancer (**Figure 1**), possibly due to a reduced period of estrogen exposure (Forman et al., 2013).

GENETICS OF MENOPAUSE, EARLY MENOPAUSE AND POI

Age of natural menopause is determined by genetic and environmental factors. Particularly smoking has been demonstrated to be an important factor negatively impacting ANM (Forman et al., 2013). However, the genetic component in ANM is relatively strong, as twin studies have estimated that the heritability of ANM varies between 44 and 85% (te Velde and Pearson, 2002). Nevertheless, previous linkage studies have only been moderately successful in identifying genes influencing ANM, which can be explained by the fact that this technique is more suited to detect variants with large effect sizes. Candidate gene association studies have been more successful, although conflicting results have been reported and replication in independent cohorts is mostly lacking. For candidate gene studies, an a priori assumption is made that the selected gene is involved in the trait of analysis. Thus for ANM, genes involved in sex steroid biosynthesis and signaling, gonadotropin biosynthesis and signaling, and folliculogenesis have been studied mostly. Indeed, SNPs in FSHB and ESR1 showed consistent associations with ANM [reviewed in (Huhtaniemi et al., 2018; Jiao et al., 2018)]. A major limitation of candidate gene association studies is that it does not identify novel genes or pathways. This limitation is overcome with genome wide association studies (GWASs), which have an hypothesis-free approach (Hirschhorn and Daly, 2005). Indeed, GWAS of ANM have identified previously unpredicted genes and pathways, such as genes implicated in DNA damage response (DDR), immune function and mitochondrial biogenesis (Stolk et al., 2012; Day et al., 2015). Our GWAS meta-analysis of age at menopause, in nearly 70,000 women with European ancestry, identified 54 independent signals located in 44 genomic regions, with minor allele frequency (MAF) ranging between 7 and 49% (Day et al., 2015). Effect sizes ranged from 0.07 to 0.41 years per allele, with one SNP having an effect of 0.88 years. There was no clear pattern whether less frequent variants had a larger effect size, and the ANM lowering effects were observed for both minor and major alleles (Day et al., 2015). These 54 genome-wide significant SNPs explain 6% of the variance in ANM. Pathway analysis again highlighted a role for DDR pathways in age of menopause as nearly two-third of the ANM SNPs are involved in these pathways. Furthermore, five of the identified loci contain genes involved hypothalamic-pituitary function, including *FSHB*, suggesting also a neuro-endocrine component of ovarian aging (Day et al., 2015). In our latest GWAS in over 200,000 women of European ancestry, the number of ANM-genetic loci increased to 290 and strengthened the involvement of DDR pathways in the regulation of age of menopause (Ruth et al., 2021). These GWAS-ANM loci identified in women of European descent were also identified in cohorts of diverse ethnicities suggesting shared genetics in reproductive aging among ethnic groups (Chen et al., 2014; Fernández-Rhodes et al., 2018; Horikoshi et al., 2018; Zhang et al., 2021). However, assessment of the 290 ANM loci in a cohort of approximately 78,000 women of East Asian ancestry also highlighted the presence of heterogeneity in effect sizes and allele frequencies (Ruth et al., 2021).

To date four GWASs (Kang et al., 2008; Knauff et al., 2009; Pyun et al., 2012; Qin et al., 2012; Park et al., 2020) and two genome-wide linkage analyses (Oldenburg et al., 2008; Caburet et al., 2012) have been performed to identify POI-associated loci. However, cohorts of POI are relatively small and a GWAS may therefore lack sufficient statistical power. Indeed, only one

genome-wide significant locus was identified in these studies (APBA3) (Park et al., 2020), and a few suggestive significant associations were observed (ADAMTS19; LAMC1; a region at 8q22.3) (Knauff et al., 2009; Pyun et al., 2012; Qin et al., 2012). Untangling the genetics of POI is further complicated by the fact that POI has syndromic and non-syndromic presentations, making POI a heterogeneous disease. However, recent technological advancement in Next Generation Sequencing (NGS), in particular Whole Exome Sequencing (WES), has led to the identification of novel causative genes in POI. Combined with previous candidate approaches, mutations in >60 genes have currently been identified (Huhtaniemi et al., 2018; Jiao et al., 2018). Interestingly, the increasing list of genes identified by WES shows enrichment of genes involved in DDR, homologous recombination, and meiosis (reviewed in (Huhtaniemi et al., 2018; Jiao et al., 2018)). This suggests that similar biological pathways may underlie POI and menopause. This hypothesis is supported by the finding that GWAS-ANM loci are enriched in genes linked to monogenic POI (Day et al., 2015). Other studies also reported shared genetics between EM and POI with ANM (He et al., 2010; Murray et al., 2011; Perry et al., 2013). Furthermore, in our study the combined effect of ANM lowering SNPs was estimated to explain 30% of the variance in EM (Perry et al., 2013). Thus, ANM, EM and POI may have an overlapping polygenic etiology, with women with POI carrying more ANM lowering variants and representing the extreme of the trait (Day et al., 2015; Ruth et al., 2021). The enrichment of DDR genes in ANM, EM, and POI furthermore suggests that reproductive aging may be part of systemic aging, as accumulation of DNA damage has been shown to be a major driver of aging (López-Otín et al., 2013). Thus, factors underlying genetic predisposition to earlier menopause might also be involved with other traits.

SHARED GENETICS BETWEEN AGE AT MENOPAUSE AND OTHER TRAITS

Improved knowledge of a shared genetic background between complex traits and diseases can highlight specific biological mechanisms underlying those traits and can identify causal relationships (Bulik-Sullivan et al., 2015a). Over the last 2 decades an enormous effort has been made to further identify common genetic variants in relation to complex traits, such as ANM. Large studies have been conducted analyzing hundreds of thousands of individuals which has led to the successful identification of tens of thousands of genetic variants associated with complex traits (Visscher et al., 2017). As a consequence of the identification of these genetic variants, studies are more and more focusing on further elucidating the complex interplay between all these associated genetic variants with different traits.

Several methods are available to further explore whether different traits have a shared underlying genetic mechanism. One of these, is to estimate the genetic correlation between traits, also interpreted as "shared heritability" (i.e., the correlation between the underlying genetic variance of the traits). Such genetic correlation is estimated from summary statistics using different methods. One of those, is based on regressing the association test statistics against a linkage disequilibrium (LD) score or LD-score regression (Bulik-Sullivan et al., 2015b). LD Score regression assumes that the GWAS effect size estimate for a given SNP reflects the effects of all SNPs in LD with that SNP (Bulik-Sullivan et al., 2015a). While the method was initially developed to differentiate "polygenicity" from "stratification," the use of the regression can be extended to the estimation of heritability and genetic correlation (when evaluating multiple traits).

With the advent of numerous well-powered GWAS, genetic risk score (GRS) or polygenic risk scores (PRS) have leveraged their use as instrumental variables (IV) in the context of Mendelian randomization (MR). MR is an approach allowing to scrutinize evidence for causal relationships among associations between two traits in an observational setting. **Figure 2** illustrates schematically the use of significantly associated SNPs as instrumental variables (IV), in the context of an MR setting and its underlying assumptions (discussed below) (Pasaniuc and Price, 2017; Davey Smith and Hemani, 2014; Davey Smith and Ebrahim, 2003). Mendel stated that inheritance of one trait is independent of the inheritance of other traits, in other words, there is a natural randomization of known and unknown confounders. MR is an effective method when instruments are robust, i.e., hold robust associations between SNPs and traits/diseases arising from well-powered settings (Bulik-Sullivan et al., 2015a). MR can be a reliable test to infer evidence for causal associations provided that several IV assumptions are met. These assumptions are shown in *italic* in **Figure 2**: 1) IVs should be strongly associated with the trait, 2) IVs share no common cause with the outcome, and 3) IVs affect the outcome solely through the exposure (absence of horizontal pleiotropy). Horizontal pleiotropy occurs when a certain SNP influences two traits independently, which constitutes a critical violation of the MR assumption and as such a threat for the validity of MR studies. The MR-Egger method has been proposed as an adequate method to evaluate whether there is evidence of bias-generating pleiotropy invalidating the genetic instrumental variables. Thus, it is important that sensitivity analyses and adequate methodology is used to verify compliance of the MR assumptions (Bulik-Sullivan et al., 2015a).

Below we will review examples of studies applying these methods to infer causal relationships between ANM and other health-related traits. The results of the MR studies are summarized in **Table 1**, in which we also indicated whether sensitivity tests were applied and reported.

Metabolic Traits

Obesity and Waist Hip Circumference

Menopause is accompanied by an elevated prevalence of obesity (Davis et al., 2012). Menopause does not necessarily lead to weight gain, but it does cause an increase in total body fat and a redistribution of body fat, resulting in increased visceral adiposity (Davis et al., 2012; Tchernof and Després, 2013; Davis et al., 2015). Development of obesity after menopause seems the result of several factors, namely reduced physical

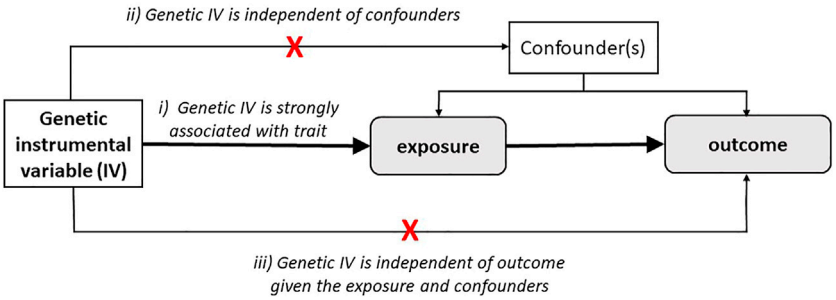

FIGURE 2 | Schematic representation of Mendelian Randomization strategy. Mendelian randomization estimates the causal relationship of the trait (exposure) on other traits (outcome) through genetic variants as instrument variables (IVs). MR studies rely on three main assumptions: i) IVs are strongly associated with the exposure, ii) IVs are not associated with confounders, and iii) IVs affect the outcome solely through the exposure.

activity and reduced energy expenditure, whether or not accompanied by depression, in combination with muscle atrophy and a lower basal metabolic rate (Davis et al., 2012). Whether obesity influences the timing of menopause is less well established. A large pooled analysis of prospective studies found that women with underweight (BMI <18.5 kg/m^2) had a higher risk of EM (RR 2.15, 95% CI 1.50–3.06), while women with overweight (BMI 25–29.9 kg/m^2) or obesity (BMI ≥30 kg/m^2) more often had a later menopause (RR 1.52, 1.31–1.77 and RR 1.54, 1.18–2.01, respectively) compared to women with a normal BMI (Zhu et al., 2018). This suggest some sort of protective effect of obesity for EM. However, moderate heterogeneity between studies, self-reported BMI, and potential confounders including smoking, could influence these results. In contrast, genetic studies using cross-trait LD score regression have shown a significant negative genetic correlation between ANM and obesity and additional related anthropometric traits such as BMI and waist circumference in women only. ANM lowering SNPs were associated with higher BMI levels (Day et al., 2015; Sarnowski et al., 2018). Furthermore, a GRS for adult BMI increasing variants with BMI profiles from early to late adulthood showed that this association was strongest in women with EM (Song et al., 2018). However, (bidirectional) MR analysis did not identify evidence for a causal relationship between ANM and BMI (Day et al., 2015; Ruth et al., 2021; Sarnowski et al., 2018), while a study of Ding et al. suggests that there is genetic support for a causal association between higher BMI and earlier ANM (Ding et al., 2020). These contrasting results are likely due to differences in SNP selection and differences in sample size. Whether appropriate sensitivity analyses were performed, was not reported for all studies (**Table 1**). These data support the existence of shared biological mechanisms between ANM and BMI, however ANM lowering variants may not be causal for higher BMI. Importantly, the observed association between ANM and BMI by the MR study of Ding et al. (2020) shows an opposite direction than observed in the epidemiological study by Zhu et al. (2018). Assessment of BMI before or after menopause may account for this difference. This raises an interesting question whether BMI could be both a cause and consequence of ANM, which should be taken into account in the study design.

Lipid Regulation

Menopause transition results in lipid profile changes, with a 10–15% higher LDL-cholesterol and triglyceride levels and slightly lower HDL cholesterol levels (Choi et al., 2015). In agreement, POI is associated with similar changes in lipid profile (Knauff et al., 2008; Gulhan et al., 2012). Hence, a shared genetic background for reproductive ageing and lipid regulation has been proposed. Indeed, we observed overlapping signals between ANM SNPs and proxies ($r^2 > 0.5$) and SNPs influencing lipid levels, including genetic variants in STARD3, and the cross-trait LD score regression was nominally significant for HDL levels (Teslovich et al., 2010; Day et al., 2015). Although the same gene region is associated with ANM and lipid levels, it remains to be determined whether the same variant drives this association. Colocalization methods (Burgess et al., 2018) may need to be applied to confirm whether there is indeed shared genetic etiology between ANM and lipid levels. Whether loci for lipid levels are associated with ANM has not been addressed yet. However, when inferring causality, potential pleiotropy between ANM and lipid levels, as observed in our initial study, should be taken into account. This is underlined by the involvement of steroidogenic acute regulatory protein (STAR) genes in ANM (STAR and STARD3) (Day et al., 2015; Perry et al., 2015). The proteins encoded by these genes function as cholesterol-binding proteins and play a role in translocation of cholesterol to the inner mitochondrial membrane. Also, by facilitating the conversion of cholesterol into pregnenolone, they are key players in the acute regulation of steroid hormone synthesis (Reitz et al., 2008). Our recent MR analysis, however, did not support causality between ANM and lipid levels (Ruth et al., 2021).

Cardiovascular Disease

It had been well established that menopause transition is related to an increase in cardiovascular risk and a reduced quality of life, both requiring preventive care (Maas et al., 2021). The reduction in estrogen levels are considered to play a critical role in this as estrogens regulate vascular reactivity, blood pressure (BP), endothelial function and cardiac remodeling (Turgeon et al., 2006; Miller and Duckles,

TABLE 1 | Overview of mendelian randomization studies for age at natural menopause.

Cohorts	Genetic variants with associated exposure	Outcome	MR method	Causal effect estimate	*p*	Ref	Sensitivity tests
Summary data from GIANT consortium	54 SNPs - ANM	BMI	Bidirectional-MR: weighted genetic risk score	Not reported	0.668	Day et al. (2015)	Not reported
Summary data from Reprogen consortium	32 SNPs - BMI	ANM	Bidirectional-MR: weighted genetic risk score	Not reported	0.683		
	54 SNPs - ANM	Age at menarche	Bidirectional-MR: weighted genetic risk score	Not reported	0.571		
	123 SNPs - age at menarche	ANM	Bidirectional-MR: weighted genetic risk score	Not reported	0.0005		
	56 SNPs - ANM	Breast cancer risk	Unconditional logistic regression	OR = 1.064; 95%CI 1.050–1.081	2.78×10^{-14}		
Summary data from Reprogen consortium	210 SNPs - ANM	BMI	Two-sample MR: IVW method	β = −0.003; 95%CI -0.008–0.003	3.5×10^{-1}	Ruth et al. (2021)	Yes
	227 SNPs - ANM	CAD	Two-sample MR: IVW method	OR = 1.002; 95%CI 0.992–1.013	6.5×10^{-1}		
	229 SNPs - ANM	Breast cancer risk	Two-sample MR: IVW method	OR = 1.035; 95%CI 1.027–1.042	3.7×10^{-17}		
	256 SNPs - ANM ER negative set	Breast cancer risk	Two-sample MR: IVW method	OR = 1.015; 95%CI 1.002–1.029	2.1×10^{-2}		
	227 SNPs - ANM ER positive set	Breast cancer risk	Two-sample MR: IVW method	OR = 1.041; 95%CI 1.032–1.05	2.7×10^{-16}		
	223 SNPs - ANM	Ovarian cancer risk	Two-sample MR: IVW method	OR = 1.028; 95%CI 1.013–1.043)	2.9×10^{-4}		
	194 SNPs - ANM	Type 2 diabetes	Two-sample MR: IVW method	OR = 0.981; 95%CI 0.97–0.992	1.1×10^{-3}		
	117 SNPs - ANM	Fasting glucose	Two-sample MR: IVW method	β = 0; 95%CI -0.006–0.005	8.9×10^{-1}		
	127 SNPs - ANM	Fasting insulin	Two-sample MR: IVW method	β = −0.004; 95%CI -0.01–0.002	1.6×10^{-1}		
	212 SNPs - ANM	Fracture risk	Two-sample MR: IVW method	OR = 0.983; 95%CI 0.974–0.992	4.7×10^{-4}		
	243 SNPs - ANM	BMD (45–60 years)	Two-sample MR: IVW method	OR = 0.033; 95%CI 0.021–0.045	6.9×10^{-8}		
Summary data from Reprogen consortium	298 SNPs - age at menarche	ANM	Two-sample MR: IVW method	β = 0.13; 95%CI 0.086–0.174	2.0×10^{-8}		
Summary data from UK Biobank	110 SNPs - BMI	ANM	Two-sample MR: IVW method	β = −0.05; se = 0.01	0.027	Ding et al. (2020)	Yes
	111 SNPs - age at menarche	ANM	Two-sample MR: IVW method	β = 0.34; se = 0.16	0.035		
Summary data from Reprogen consortium	61 SNPs - age at menarche	ANM	Two-sample MR: IVW method	β = 0.23; se = 0.07	0.001		
Summary data from Social Science Genetic Association Consortium	16 SNPs - education	ANM	Two-sample MR: IVW method	β = 1.19; se = 0.41	0.004		
Summary data from UK Biobank	2 SNPs - smoking	ANM	Two-sample MR: IVW method	β = 0.26; se = 1.46	>0.05		
3 cohorts part of CHARGE consortium	54 SNPs - ANM	CAD	Not clearly reported	Not reported	0.52	Sarnowski et al. (2018)	Not reported

(Continued on following page)

TABLE 1 | (*Continued*) Overview of mendelian randomization studies for age at natural menopause.

Cohorts	Genetic variants with associated exposure	Outcome	MR method	Causal effect estimate	*p*	Ref	Sensitivity tests
Summary data from Reprogen consortium	54 SNPs - ANM	Rheumatoid arthritis	Two-sample MR: IVW method	OR = 1.05; 95%CI 0.98–1.11	0.15	Zhu et al. (2021)	Yes
WHI data	2 SNPs - ANM (rs11668344; rs16991615	Accelaration epigenetic aging	IVW method	β = 0.506 (rs11668344) β = 0.151 (rs16991615)	0.031 0.763	Levine et al. (2016)	Not reported
Summary data from Reprogen consortium	34 SNPs - ANM	Breast cancer survival	Two-sample MR: IVW method	HR = 1.01; 95%CI 0.98–1.05	0.49	Escala-Garcia et al. (2020)	Yes
	Premenopausal set	Breast cancer survival	Two-sample MR: IVW method	HR = 1.03; 95%CI 0.698–1.08	0.21		
	Postmenopausal set	Breast cancer survival	Multivariable model	HR = 1.01; 95%CI 0.93–1.08	0.9		
Summary data from Reprogen consortium; 3 consortia: GECCO. CCFR. CORECT (12,944 female cases. 10,741 female controls)	51 SNPs - ANM as GRS (risk increasing alleles)	Colorectal cancer	Two-sample MR: IVW method	OR = 0.99; 95%CI 0.83–1.17	—	Neumeyer et al. (2018)	Yes
Summary data from Reprogen consortium	54 SNPs - ANM	Fracture risk	Two-sample MR: IVW method	OR = 1.10; 95%CI 1.00–1.21	0.05	Trajanoska et al. (2018)	Yes
		Femoral Neck BMD	Two-sample MR: IVW method	EE = –0.063; 95%CI -0.080–0.047	0.038		
		Lumbar Spine BMD	Two-sample MR: IVW method	EE = –0.018; 95%CI -0.033–0.004	0.01		
UK Biobank	63 SNPs - ANM (39 strong IV for EM; 40 strong IV for late ANM)	Lung function FEV1/ FVC(%) (EM)	Two-sample MR: IVW method	β = 0.29; 95%CI 0.22–0.36	1.48×10^{-16}	van der Plaat et al. (2019)	Yes
		Lung function FEV1/FVC < LLN (EM)	Two-sample MR: IVW method	OR = 0.85; 95%CI 0.82–0.89	5.88×10^{-14}		
		Lung function FEV1/FVC(%) (late ANM)	Two-sample MR: IVW method	β = –0.18; 95%CI -0.26–0.1	1.09×10^{-5}		
		Lung function FEV1/FVC < LLN (late ANM)	Two-sample MR: IVW method	OR = 1.06; 95%CI 1.01–1.11	0.018		
Summary data from Reprogen consortium	35 SNPs -ANM	Invasive epithelial ovarian cancer	Two-sample MR: IVW method	OR = 1.03; 95%CI 1.00–1.06	0.07	Yarmolinsky et al. (2019)	Yes
		Ovarian cancer histotypes: endometriod	Two-sample MR: IVW method	OR = 1.19; 95%CI 1.05–1.36	0.008		

2008; Menazza and Murphy, 2016; Maas et al., 2021). Women with POI have a shorter life expectancy than women with a late menopause due to cardiovascular disease and osteoporosis (Ossewaarde et al., 2005; Atsma et al., 2006; Muka et al., 2016). Indeed, a meta-analysis showed an increased risk of CVD not only for women with EM and POI, but for all women with an ANM before the age of 50 (Zhu et al., 2019). Each earlier year of menopause was associated with a 3% increased risk of cardiovascular disease (Zhu et al., 2019).

Genetic data of more than 22,000 men and women derived from three population-based cohorts were analyzed and related to development of their first cardiovascular event (Sarnowski et al., 2018). The authors composed a GRS consisting of genetic variants associated with EM and POI in large GWASs (Day et al., 2015). Sex-stratified analyses showed that this GRS increased the risk of coronary heart disease deaths in women while no effect was observed in men (Sarnowski et al., 2018). In women, a 1-unit decrease in genetically predicted ANM increased the hazard of coronary heart disease death by 12% and increased the risk of

the combined endpoint (including myocardial infarction, stroke, congestive heart failure, or death from coronary heart disease) by 10% (Sarnowski et al., 2018). Even after adjusting for menopause timing, the GRS remained associated with time to the first cardiovascular event. This suggests genetic pleiotropy between ANM and coronary deaths. In other words, some SNPs affect ANM as well as coronary deaths (Sarnowski et al., 2018). However, similar to BMI, causal inference by MR analysis could not be detected (Sarnowski et al., 2018; Ruth et al., 2021).

Diabetes

EM and POI are an independent riskfactor for type 2 diabetes (Anagnostis et al., 2019). Also, patients with type 1 diabetes have menopause at an earlier age than controls (Dorman et al., 2001; Kjaer et al., 1992). However, two large cohort studies found contrary results and were unable to link type 1 diabetes to EM (Kim et al., 2016; Yarde et al., 2015). It is likely that in the more recent type 1 diabetes cohort studies better glycemic control was achieved than in the previous cohorts, explaining the relatively low occurrence of substantial microvascular disease (Thong et al., 2020). Follicle recruitment and growth could be negatively affected by chronic hyperglycaemia, thereby leading to an impaired ovarian function in type 1 diabetes. Advanced glycation end-products, which are enhanced upon hyperglycaemia, have been suggested to contribute to ovarian ageing (Thong et al., 2020; Codner et al., 2012). In our latest GWAS study we did observe genetic pleiotropy between the GWAS-ANM SNPs and proxies ($r^2 > 0.5$) and GWAS-identified loci for fasting glucose (Day et al., 2015). Amongst the identified overlapping GWAS loci was the *GCKR* gene, suggesting that an altered glucokinase regulation and glucose sensing might be a shared genetic etiology for reproductive ageing, type 2 diabetes, cardiovascular disease and dyslipidaemia (Dupuis et al., 2010; Day et al., 2015; Perry et al., 2015). However, as discussed for lipid regulation, it remains to be determined whether the same variant drives this association. Particularly, since cross-trait LD score regression did not reveal a genetic correlation (Day et al., 2015), suggesting that the observed associations with ANM and fasting glucose are independent. Likewise, causal inference between ANM and fasting glucose or fasting insulin was not identified in our latest GWAS study, while a significant causal association with type 2 diabetes was observed (Ruth et al., 2021).

Breast Cancer and Ovarian Cancer

There is very convincing epidemiological evidence of an inverse relationship between ANM and breast cancer risk. The development of breast cancer is associated with later ANM, while breast cancer incidence decreases substantially in women with EM (Monninkhof et al., 1999; Collaborative Group on Ho, 2012). In line with this epidemiological association, our genetic analysis showed that carrying more ANM increasing SNPs increased the risk for breast cancer (Day et al., 2015). Furthermore, Mendelian randomization analyses supports the existence of a causal relationship between a later age at menopause and breast cancer risk. For each 1-year increase in ANM, a 5–6% increase in breast cancer risk was observed (Day et al., 2015; Ruth et al., 2021). Given that DDR genes are enriched in the association with ANM, this causal relationship between ANM and breast cancer may seem counterintuitive as entering menopause at a later age may reflect having more efficient DNA repair mechanisms (Laven et al., 2016). We therefore hypothesized that the increased susceptibility to breast cancer is not directly due to DNA repair mechanisms but rather results from longer exposure to sex steroids, established by a more efficient DDR pathway (Day et al., 2015; Laven et al., 2016). This is consistent with our observation that significantly larger effect estimates were found for ANM GRS in estrogen receptor (ER)-positive versus ER-negative breast cancer cases (Day et al., 2015). Furthermore, MR analysis stratified for ER-positive and ER-negative breast, only resulted in a significant causal inference for ER-positive cases (Ruth et al., 2021). Thus the causal genetic association between ANM and breast cancer risk appears complex, involving at least interactions between DDR pathways and estrogen signaling.

In addition to breast cancer risk, also breast cancer survival in relation to ANM has been studied. Earlier ANM has been associated with poorer breast cancer survival, where ANM did not (Orgéas et al., 2008). MR approach testing for a genetic association between breast cancer survival and ANM found no evidence for a causal relation (**Table 1**) (Escala-Garcia et al., 2020).

A later age at menopause, and thereby a longer period of exposure to sex steroids, has also been suggested to be a risk factor for ovarian cancer (Franceschi et al., 1991), although the number of epidemiological studies analyzing the association between ANM and ovarian cancer are scarce and show inconsistent results (Schildkraut et al., 2001). However, the identification of DDR genes in determining ANM, including BRCA1 and BRCA2 which are associated with both breast and ovarian cancer (Antoniou et al., 2003), warrants further analysis. MR analysis based on 35 ANM SNPs showed a trend in causal inference with epithelial ovarian cancer risk (Yarmolinsky et al., 2019). Furthermore, stratification based on ovarian cancer subtypes identified a significant causal association between ANM and endometriod ovarian cancer (**Table 1**) (Yarmolinsky et al., 2019). Increasing the number of ANM SNPs as instrument variables, as shown in our recent MR analysis, resulted in a significant causal association between ANM and ovarian cancer, with a later age of menopause increasing the risk of ovarian cancer (**Table 1**) (Ruth et al., 2021). These findings suggests that a potential genetic overlap exists between ANM and ovarian cancer risk. As for breast cancer risk, an interaction between estrogen signaling and DDR pathways may be the underlying biological mechanism.

Osteoporosis

Estrogen deficiency results in increased bone remodeling leading to osteoporosis. The underlying biological mechanisms are increased bone resorption due to increased osteoclast activity and reduced bone formation by osteoblasts, and as a result, bone resorption exceeds bone formation. The net loss of bone due to remodeling upon estrogen deficiency is estimated to be around 2–3% per year

after menopause (Webber et al., 2016). Development of osteoporosis is an important concern for women with EM and POI. Epidemiological studies found a prevalence of 8–27% of osteoporosis in women with POI (Francucci et al., 2008; Sullivan et al., 2017; Xu et al., 2020). In our GWAS study, we did not report on shared genetics between ANM and osteoporosis or related traits (Day et al., 2015). However, in a cohort of Han Chinese postmenopausal women, out of 22 osteoporosis SNPs, selected from a bone mineral density (BMD) GWAS, it was observed that a SNP in MHC was significantly associated with ANM after correction for multiple testing (Zhao et al., 2011). However, the MHC region is associated with a multitude of diseases (de Bakker and Raychaudhuri, 2012), and combined with the candidate gene-like approach, it thus remains to be determined whether the association of MHC SNP with ANM persists in an unbiased analysis. Indeed, while cross-trait LD Score regression did showed a negative genetic correlation between ANM and fracture risk, upon correction for multiple testing this failed to reach significance (Trajanoska et al., 2018). This study by Trajanoska et al. (2018) also revealed that based on MR there is no evidence for a causal effect of ANM SNPs on fracture risk, while only a marginal effect was observed for BMD. However, increasing the number of ANM SNPs as instrument variables did indicate a causal relationship between ANM increasing SNPs and fracture risk and BMD (Ruth et al., 2021). Thus, a genetic link between earlier ANM and osteoporosis or BMD may be present. Interestingly, shared genetic variants between bone mineral density (BMD) and other reproductive traits including age at menarche and puberty timing, were found (Christou et al., 2020). It has been shown though that shared genetic factors by BMD and age at menarche were only identified in premenopausal women but not in postmenopausal women (Zhang et al., 2009). This GWAS result suggests that ANM may be a confounding factor in this shared genetic background possibly by affecting the duration of estrogen exposure. However, it should be noted that this study was performed in a relatively small cohort of <3,000 pre- and postmenopausal women. Replication in well-powered sample sizes is therefore needed to unravel the relationship between ANM, age at menarche, and bone-related phenotypes.

Age at Menarche and Puberty Timing

Several epidemiological studies have linked onset of menarche as well as puberty timing to ANM. Nine studies in the UK, Scandinavia, Australia and Japan including over 50,000 women observed that having menarche before the age of 11 years increased the risk of EM and POI compared to having menarche at 13 years (Mishra et al., 2017). The association of early menarche and EM was also observed in American and Chinese women (Wang et al., 2018; Whitcomb et al., 2018).

In line with the above, while initial GWAS observed that ANM loci were different from those for age at menarche (He et al., 2009), subsequent GWAS analysis show that they partially overlap with genes implicated in disorders of puberty (Perry et al., 2014; Day et al., 2015; Perry et al., 2015). These SNPs are located in or near genes involved in the regulation of the hypothalamic–pituitary–gonadal axis (such as CHD7, FGFR1, SOX10, KISS1 and TAC3) (Day et al., 2015), suggesting that these reproductive milestones are at least partly regulated by shared biological mechanisms (Perry et al., 2015). Indeed, our cross-trait LD Score regression identified a genetic correlation between these two traits (Day et al., 2015), and a causal relationship was implied in recent MR analyses showing that a later age of menarche is associated with a later ANM (Ding et al., 2020; Ruth et al., 2021). Knowledge of this shared genetics of ANM and age at menarche may proof to be important when analyzing traits impacted by the duration of estrogen exposure, which applies to many of the postmenopausal diseases.

Other Traits

In this paragraph we describe other traits for which the association with ANM has been studied in observational epidemiological studies and for which the causal relationship was further explored using the MR approach or GRS strategy.

The relationship between SNPs associated with ANM and polycystic ovary syndrome (PCOS), as well as reproductive markers such as gonadotrophin levels and ovarian volume, has been studied using a GRS strategy. One small longitudinal study suggested that PCOS is associated with entering menopause at a later age (Mulders et al., 2004). Throughout their entire life, women with PCOS encounter a larger number of follicles than women without PCOS at similar age (Mulders et al., 2004). This, together with a potential later ANM, raises the hypothesis that SNPs associated with ANM might also be associated with risk for PCOS. Indeed, analysis in a discovery and validation cohort showed that ANM increasing genetic variants were associated with risk for PCOS (Saxena et al., 2015). Furthermore, ANM lowering SNPs yield an opposing effect on ANM in women with PCOS, when corrected for BMI. This opposing effect could be partly explained by the association of ANM SNPs with higher luteinizing hormone (LH) levels (Saxena et al., 2015). It should be noted that in this study the GRS was calculated from a limited number of ANM variants, based on initial GWAS studies. It would be interesting to repeat this study using the SNPs from the latest GWAS study on ANM.

Observational studies suggest that lower forced vital capacity (FVC) and a higher risk of spirometric restriction is associated with an earlier age at menopause. A Mendelian randomization study suggests that ANM lowering SNPs were associated with a 15% lower risk of spirometric restriction (van der Plaat et al., 2019). Entering menopause at an earlier age seems to have a positive effect on airflow obstruction (van der Plaat et al., 2019). Further studies are needed to investigate the underlying mechanisms and the role of female sex hormones in lung function.

Development of colorectal cancer seems inversely associated with the use of menopausal hormone replacement therapy (Lin et al., 2012). This suggests a role of prolonged estrogen exposure in colorectal cancer development. However, a relationship between endogenous estrogen exposure and colorectal cancer is inconclusive. A large Mendelian randomization study did not find a causal relationship between GRS for ANM and colorectal cancer risk (Neumeyer et al., 2018).

Several large epidemiologic studies have observed an association with earlier ANM and increased risk of

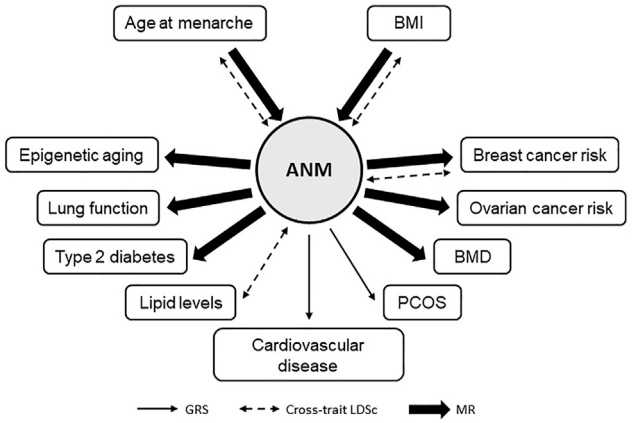

FIGURE 3 | Mendelian randomization studies for age at natural menopause. Schematic summary of MR studies for age at natural menopause (ANM) with other health-related traits using ANM SNPs as instrument variables. Only traits with a significant result are depicted.

rheumatoid arthritis (Karlson et al., 2004; Bengtsson et al., 2017). Methodologically solid MR analysis by Zhu et al. did not support a causal relationship between ANM and the development of rheumatoid arthritis (Zhu et al., 2021).

A relationship between ANM and biological aging rate has been proposed. Data from four large observational studies found an association between ANM and methylation in blood as a marker of epigenetic aging (Levine et al., 2016). The authors also conducted a well-designed MR approach in which they selected the two most significant GWAS ANM SNPs as IVs. One of these SNPs also showed a significant association with epigenetic age acceleration (Levine et al., 2016). However, whether evidence for causal inference would be obtained using the complete GWAS summary statistics was unfortunately not addressed. MR analysis using our recent identified ANM SNPs did not infer causality between ANM and longevity (Ruth et al., 2021). Given that the DDR pathway has been implicated in longevity (Debrabant et al., 2014; Vermeij et al., 2014) this raises the question whether different DNA repair mechanisms are involved in reproductive aging and longevity.

CONCLUSION

Conventional observation studies have identified several associations between POI, early menopause as well as menopause and other traits. However, confounding, reverse causation and other potential bias complicate translation of these associations into causal inference. Large GWAS studies have been performed in which hundreds of SNPs are found to be associated with the abovementioned traits. Advanced genetic approaches like MR are a promising way of identifying causality provided that all the required assumptions are met. It is therefore important that the statistical methods for IV analysis and several sensitivity tests are clearly reported.

The studies discussed in this review (significant findings are summarized in **Figure 3**) suggest that shared genetics may exist between ANM and cardiovascular disease and dyslipidemia, although evidence for causal associations were not observed. For the well-known epidemiological association between ANM and osteoporosis, evidence for a causal genetic relationship was identified when the number of ANM SNPs as instrument variables was largely increased. The same was observed for type 2 diabetes. Based on studies published to date, genetic associations between ANM and age at menarche, breast cancer, and ovarian cancer risk appear causal and robust. Biological mechanisms that regulate duration of estrogen exposure therefore remain an important focus. The negative correlation between ANM and BMI, potentially causal, suggests that BMI is an important confounding factor that could impact the validity of MR studies. The majority of the genetic analyses for ANM discussed in this review have analyzed cross-trait associations in women only. Only two studies, analyzing the association of ANM with BMI and cardiovascular disease, used a sex-stratified approach, and observed associations in women only. While more studies are needed and causality remains to be determined, this may suggest that the biological pathways underlying menopause-related traits are women-specific.

It should be noted that the reported causal associations have been mostly identified in cohorts of European descent. However, the timing of menopause differs by geographical region, race/ethnicity and socioeconomic status (El Khoudary, 2020). Indeed, although shared genetics in ANM among different ethnic groups were reported, differences in allele frequencies and effect sizes were also observed. Furthermore, the risk for post-menopausal traits, such as breast cancer, CVD, and osteoporosis, differs between ethnicities (Cauley, 2011; Danforth Jr, 2013; Bays et al., 2021). Hence, it is suggested that valid causal estimates are obtained when cohorts have a similar racial/ethnic background (Burgess et al., 2015). Although receiving increased attention, more studies are needed to identify transethnic causal associations between ANM and other traits.

These studies also highlight that a critical analysis of the genetic variants and methods used is important. We advocate for multidisciplinary research teams, consisting of epidemiologists, geneticists, clinicians and basic scientists, to allow a proper design and interpretation of the results. Although in its infancy, it is expected that these studies will further elucidate the shared genetics of menopause, early menopause, and POI with other traits and thereby provide new insights in shared biological pathways.

AUTHOR CONTRIBUTIONS

YL and JV both reviewed the literature, wrote the manuscript, and approved the submitted version.

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Targeted Next-Generation Sequencing Indicates a Frequent Oligogenic Involvement in Primary Ovarian Insufficiency Onset

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Primary ovarian insufficiency (POI) is one of the major causes of female infertility associated with the premature loss of ovarian function in about 3.7% of women before the age of 40. This disorder is highly heterogeneous and can manifest with a wide range of clinical phenotypes, ranging from ovarian dysgenesis and primary amenorrhea to post-pubertal secondary amenorrhea, with elevated serum gonadotropins and hypoestrogenism. The ovarian defect still remains idiopathic in some cases; however, a strong genetic component has been demonstrated by the next-generation sequencing (NGS) approach of familiar and sporadic POI cases. As recent evidence suggested an oligogenic architecture for POI, we developed a target NGS panel with 295 genes including known candidates and novel genetic determinants potentially involved in POI pathogenesis. Sixty-four patients with early onset POI (range: 10–25 years) of our cohort have been screened with 90% of target coverage at 50×. Here, we report 48 analyzed patients with at least one genetic variant (75%) in the selected candidate genes. In particular, we found the following: 11/64 patients (17%) with two variants, 9/64 (14%) with three variants, 9/64 (14%) with four variants, 3/64 (5%) with five variants, and 2/64 (3%) with six variants. The most severe phenotypes were associated with either the major number of variations or a worse prediction in pathogenicity of variants. Bioinformatic gene ontology analysis identified the following major pathways likely affected by gene variants: 1) cell cycle, meiosis, and DNA repair; 2) extracellular matrix remodeling; 3) reproduction; 4) cell metabolism; 5) cell proliferation; 6) calcium homeostasis; 7) NOTCH signaling; 8) signal transduction; 9) WNT signaling; 10) cell death; and 11) ubiquitin modifications.

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Consistently, the identified pathways have been described in other studies dissecting the mechanisms of folliculogenesis in animal models of altered fertility. In conclusion, our results contribute to define POI as an oligogenic disease and suggest novel candidates to be investigated in patients with POI.

Keywords: primary ovarian insufficiency, primary amenorrhea, secondary amenorrhea, oligogenic disease, next-generation sequencing

1 INTRODUCTION

Female factors account for one-third of all causes of infertility. Besides tubal disease and endometrial pathology, the dysregulation of any essential step involved in the ovulation of a competent oocyte may cause primary ovarian insufficiency (POI), a clinical syndrome defined by the premature loss of ovarian function. A recent meta-analysis of 31 epidemiological studies on the prevalence of POI in different countries between 1987 and 2018 reports an overall occurrence up to 3.7% in women younger than 40 years (1). At present, this disease is currently diagnosed when fertility is irreversibly affected. POI can manifest with a wide variety of clinical phenotypes, ranging from ovarian dysgenesis (OD) and primary amenorrhea (PA) to post-pubertal secondary amenorrhea (SA) for more than 4 months with raised gonadotrophins and low estradiol. The consequent long-standing estrogen deficiency exposes these women to an increased risk of complications such as cardiovascular diseases, reduced bone mineral density, and cognitive impairment (2). This disorder is highly heterogeneous in its etiology and several causes have been reported, mainly genetic, associated with chromosomal abnormalities (especially including X chromosome, such as in Turner syndrome), but also autoimmune, infectious, or iatrogenic. However, most causes of POI are still unknown, and the identification of novel causative genes is challenging. More recently, the advent of next-generation sequencing (NGS) technique and, especially, the whole exome screening (WES) of large POI families expanded the list of candidate genes to be screened in patients and consequently empowered the possibilities of a genetic diagnosis in idiopathic cases (3). Some WES studies demonstrated that pathogenic variants in meiotic chromosome pairing and synaptonemal complex (4–6) or alterations of other proteins of DNA recombination and repair (7, 8) could be responsible for POI onset by usually impairing meiotic progression and triggering oocyte death, as further evidenced by murine models (9). Other studies identified variants in the folliculogenesis players of all stages of ovarian follicle maturation, which involves the precise interaction of hundreds of genes: from the primordial follicle stock establishment of ovarian reserve (10) to the primordial to primary follicle activation (11, 12), throughout the follicular development in the gonadotropin-independent (13, 14) and gonadotropin-dependent stages (15, 16). Furthermore, alterations in genes contributing to extracellular matrix (ECM) remodeling by proteolytic activity on specific substrates within the ovarian context have been linked to the ECM turnover of abnormal somatic cells, thus leading to defects in follicular development (17). Since biological processes related to metabolism and immune system activation resulted to be enhanced in gene expression dynamics along with ovary development, including pathways associated with cell cycle, proliferation, apoptosis, ovulation, angiogenesis, and steroidogenesis (18), the disturbance of any of these pathways has been associated with reproductive diseases like POI (19–22). Moreover, the remarkable point that emerged from recent NGS studies is the occurrence of oligogenic defects (6, 23). From this perspective, various interacting genes might affect several mechanisms and pathways, and the synergistic and/or cumulative effect of several variants may contribute to POI phenotype. The NGS results in 64 patients with PA or early onset SA based on our panel of 295 POI candidate genes presented here aims to contribute to expand the list of potentially causative candidate genes and to support a genetically heterogeneous architecture of POI, resulting from defects in multiple complementary pathways.

2 MATERIALS AND METHODS

2.1 Next-Generation Sequencing Panel Construction and Analysis

Genomic DNA (gDNA) was extracted from peripheral blood of enrolled patients. The Ampliseq Custom DNA panel (Illumina) was designed *ad hoc* to include the coding exons and flanking splice sites of 295 genes. The NGS panel, called *OVO-Array* (**Table S1**), has been obtained by joining data from: a) literature search of genes known to be previously associated with female infertility, with a role in ovary and in menopause onset (n = 159) (23); b) transcriptomic analysis of human granulosa cells treated with the oocyte-derived growth factor BMP15 (n of genes: 19; focus of another study with manuscript under review); and c) WES on 10 Italian women selected with the most severe phenotypes of PA and OD, six of them with familial inheritance (n = 117). The latter were identified among a total of 18,570 rare variants (missense, 86%; nonsense, 3.5%; indels, 5.6%). Briefly, pathway analysis (by Reactome v.74) of 1,916 genetic identifiers, found mutated at least once in WES, revealed an enrichment in the following biological processes with a key role in ovarian functionality (e.g., chromatin organization, cell cycle and meiosis, extracellular matrix organization, and cell–cell communication). Within these pathways, we further selected 117 genes potentially correlated with POI onset. The total coverage of the target genes by the designed amplicons was 100%. Library was

prepared using enzymatic DNA fragmentation, with 50 ng of total gDNA and quantified with Quant-iT PicoGreen (Thermo Fisher Scientific). Nextera Rapid Capture Enrichment protocol (Illumina) was followed to tagment gDNA, amplify tagmented gDNA, hybridize probes, capture hybridized probes and for library capture and amplification. The library was then loaded onto the reagent cartridge (Illumina) and sequencing was performed on a NextSeq 500 (Illumina). Reads were then examined to identify single nucleotide alterations or small insertions/deletions, and a first *in silico* analysis (including base calling and demultiplexing) has been performed using MiSeq provided software (Real Time Analysis RTA v.1.18.54 and Casava v.1.8.2, Illumina). FastQ files for each sample, containing mate paired-end reads after demultiplexing and adapter removal, have been used as input for MiSeq pipeline. Briefly, FastQ files have been processed with MiSeq Reporter v2.0.26 using the Custom Amplicon workflow. This analytical method required FastQ files, a "Manifest file" containing information about the sequences of primer pairs, the expected sequence of the amplicons, and the coordinates of the reference genome (*Homo sapiens*, hg19, build 37.2) as input. Each read pair has been aligned using the MEM algorithm of the BWA software. Local Indel realignment and base recalibration step were performed using the software GATK. The realigned and recalibrated BAM file was used as input to GATK Unified Genotyper thus generating a VCFv4.2 file for each sample. Quality control of sequencing data was performed directly on FastQ files using the FastQC software. Reads were also filtered based on quality mapping and removed if their quality mapping was <20. Genetic variants showing a PHredScore lower than 20 were also filtered out.

2.2 POI Patients

We collected a cohort of patients to be analyzed through the *OVO-Array* panel, following IRCCS Istituto Auxologico Italiano Ethics Committee approval (BIOEFFECT, code 05M101_2014), composed of a total of 64 women with either primary (PA, n = 21) or early onset secondary amenorrhea (SA, n = 43), with onset before 25 years, all characterized by FSH values >40 IU/L and low estradiol. We excluded karyotype abnormalities, FMR1 premutations, and ovarian autoimmunity in all of them. Moreover, we selected additional 43 patients (PA, n = 18 and SA, n = 25) with the same inclusion criteria and early onset POI before 25 years of age. These patients have been analyzed between the years 2013 and 2020 for variants on a selected subset of only nine causative genes for POI, mainly involved in folliculogenesis and meiosis (*BMP15*, *FIGLA*, *FOXL2*, *FSHR*, *GDF9*, *NOBOX*, *NR5A1*, *SYCE1*, *STAG3*) for diagnostic purposes at IRCCS Istituto Auxologico Italiano. The results obtained with the different screening approaches (*OVO-Array* vs. diagnostic routine) have been then compared. Informed consent was obtained from all patients prior to blood sample collection and molecular studies.

2.3 *In Silico* Analysis of NGS Results and Variant Interpretation

The genetic variants resulting from the *OVO-Array* experiment and those derived from the diagnostic routine were annotated using Annovar software (24) and then classified as *rare* if resulting unknown or with a minor frequency allele (MAF) <0.01 in 1000 genomes, dbSNP, or EXAC databases. By checking for pathogenicity prediction the VarSome database until July 2021 (25), only those rare variants classified as likely pathogenic (LP), pathogenic (P), or variant of unknown significance (VUS), according to the American College of Medical Genetics (ACMG) classification guidelines (26), were considered for further analysis and confirmed by using Sanger sequencing. Gene ontology analysis of all the significatively altered genes was performed against DAVID Bioinformatics v6.8 (27) and Reactome Pathway Browser version 74 (28) on October 26, 2020. Both databases cross-reference other resources (e.g., NCBI, Ensembl, UniProt, KEGG, ChEBI, PubMed, and GO).

3 RESULTS

3.1 NGS Identified Novel Variants in Putative Candidates for POI

We recruited 64 patients presenting PA or SA with early onset (from 10 to 25 years) and performed NGS analysis on 295 selected genes, including known candidates and novel potentially causative genes. The mean coverage depth of the target regions was greater than 90% at 50× for all patients. Variants with MAF >0.01 were filtered out. We considered the identified variants relevant only if they were *rare* (MAF <0.01) or never described; predicted as P, LP, or VUS; and already known to be associated with POI. We also considered nine variants predicted as likely benign, found in recurring genes with a higher frequency in our patients than in the general female population or previously associated to POI and functionally characterized.

We report a total of 114 rare variants in 78 different genes (**Figure 1A**, **Table S2**) in 64 patients. In 75% of patients analyzed by means of *OVO-Array*, we could find at least one alteration (48/64) possibly related to POI. Noteworthy, 2 or more variants have been identified in 34 of them; in particular, we found 11 patients (11/64, 17%) with two variants, 9 patients (9/64, 14%) with three variants, 9 patients (9/64, 14%) with four variants, 3 patients (3/64, 5%) with five variants, and 2 patients (2/64, 3%) with six variants (**Figure 2A**, **Table S3**). No significative alterations in the selected 295 genes were found in only 16 patients of the cohort.

Moreover, among the 43 patients screened for diagnostic purposes, we could identify at least one genetic variant in known POI genes in 11 of them, thus providing a genetic diagnosis in about 25% of POI patients through NGS. Only one patient harbored two different alterations and another one harbored three variants (**Figures 1B**, **2B**, **Tables S4**, **S5**). All the identified variants were confirmed by Sanger sequencing.

3.1.1 Correlation Among the Number of Variants per Patient, Predicted Pathogenicity, and Phenotype

We summarized the prioritized variants harbored by each patient analyzed by *OVO-Array* NGS panel in **Supplementary**

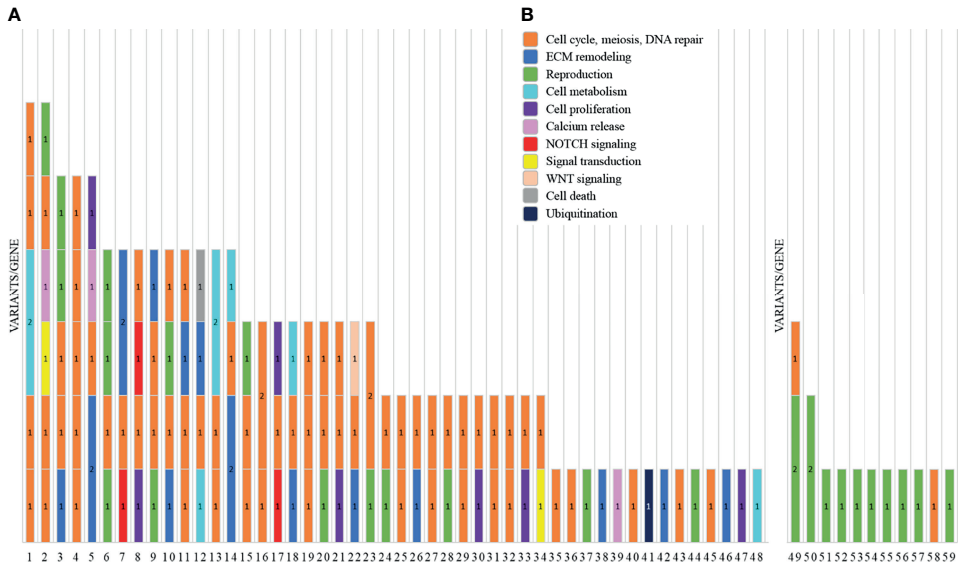

FIGURE 1 | Graph representations of the number of variants identified by NGS analysis in two subgroups of primary ovarian insufficiency (POI) patients. For each patient, indicated are the ID number (*x*-axis) and the number of variants/genes found altered (1 = 1 variant/gene; 2 = 2 variants/gene). Each color represents a different pathway: cell cycle, meiosis, and DNA repair (orange); ECM remodeling (blue); reproduction (green); cell metabolism (cyan); cell proliferation (purple); calcium release (pink); NOTCH signaling (red); signal transduction (yellow); WNT signaling (light orange); cell death (gray); and ubiquitination (dark blue). **(A)** The histogram reports the variants found by screening 295 candidate genes included in the *OVO-Array* panel. **(B)** NGS results of the analysis for diagnostic purposes of nine known POI genes.

Tables S2 and **S3**. The phenotype of each patient is also reported. Nomenclature validation was performed using Mutalyzer 2.0.33 (https://www.mutalyzer.nl/) according to HGVS nomenclature version 2.0.

Tables 1–**6** display all the genetic variations identified by *OVO-Array* by the number of variants per patient. Briefly, each table prioritizes patients depending on their phenotype severity and shows a detailed characterization (including origin, karyotype, age of menarche and POI onset, and familiarity). The frequency of each variant is then compared with those of the female general population available in the gnomAD ver.2.1.1 public dataset. The number of variants per patient is thus correlated with the VarSome predicted pathogenicity and phenotype of the patients. For each variation, VarSome criteria and its relative updated link are shown.

3.2 NGS Analysis Revealed Novel Rare Variants Affecting Pathways Involved in Ovarian Physiology

Taken together, our results uncovered 12 already known variants associated to POI: p.Arg300Leu in *REC8*; p.Arg68Trp in *BMP15*; p.Glu122Lysfs*45 in *FIGLA*; p.Pro103Ser, p.Thr121Ile, and p.Pro374Leu in *GDF9*; p.Phe543Serfs*7, p.Gly111Arg, and p.Lys371Thr in *NOBOX*; p.Val355Met in *NR5A1*; p.Arg643* in *TP63*; and p.Asn153His in *LARS2* (green colored genetic variants in **Tables S2**, **S4**). Moreover, we could identify 41 novel rare variants in genes already associated to POI etiology (red colored in **Tables S2**, **S4**) and 74 novel rare variants in additional genes participating in key steps of ovarian follicle development, which may be considered putative candidates involved in POI pathogenesis (**Table S2**). Interestingly, 74 variants have been identified in 27 recurring genes among our two POI populations, and some of these genes/variants have already been associated to POI: 9 genetic alterations and 10 genes (green and red colored in **Table 7**, respectively). Furthermore, we found 12 alterations in recurrent genes which affected more than one patient and resulted at higher frequency in our POI populations, with respect to the general female population reported in the gnomAD (ver. 2.1.1) database (**Table 7**): c.1729G>A (p.Gly577Ser) in *ADAMTS5*, c.202C>T (p.Arg68Trp) in *BMP15*, c.844G>T (p.Val282Leu) in *CYP21A2*, c.926G>C (p.Gly309Ala) in *FSHR*, c.1063G>A (p.Val355Met) in *NR5A1*, c.752C>T (p.Thr251Ile) and c.1760C>T (p.Pro587Leu) in *POLG*, c.2516G>A (p.Arg839Gln) in *RBBP8*, c.682_683insT (p.Pro228Leufs*227) in *SAMD11*, and c.4508T>C (p.Leu1503Pro) and c.4517C>T (p.Ser1506Leu) in *VWF*.

3.3 Gene Ontology Analysis

The gene ontology analysis was carried out by interrogating the Reactome pathway database and DAVID gene functional annotation tool (refer to **Tables S6**, **S7** for the statistical evaluation of the enrichment analysis). Considering both bioinformatic tools, we identified five main pathways likely affected by multiple genes found altered in our *OVO-Array* cohort and with functions in ovarian development and physiological maturation of oocytes (**Figure 1A**). Most altered genes, 34 in total, participated in meiosis, cell cycle, and DNA repair processes, whereas 15 genes were involved in reproductive

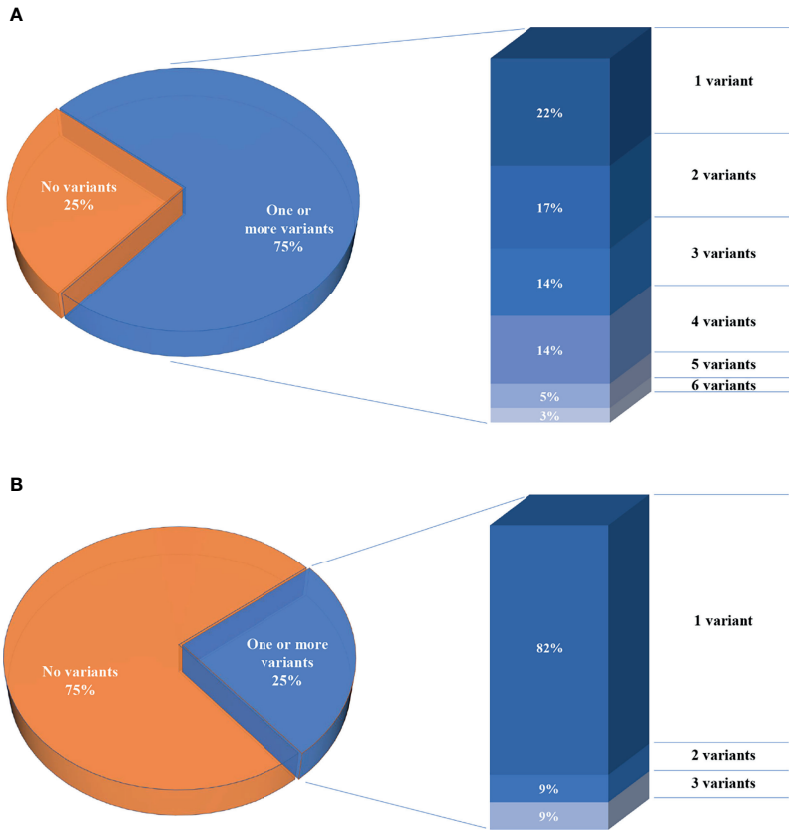

FIGURE 2 | Percentages of the number of patients per number of variants. **(A)** Seventy-five percent of patients analyzed through the *OVO-Array* panel harbor one or more variants, left. Histogram representing the percentages of patients carrying variants, right. **(B)** Twenty-five percent of patients analyzed in only nine known POI genes.

pathways (folliculogenesis, oocyte maturation, and follicular development). Both groups resulted to be affected in all manifestations of POI, from 46,XX OD to SA, but especially in the most severe phenotypes. Twelve genes with variations were identified in the ECM remodeling pathway, which influences relevant processes during follicle development, such as cell morphology, communication, proliferation, survival, and steroidogenesis, and appeared to be mostly affected with later POI onset (SA <25 years), but also in few exceptional 46,XX OD or PA cases. Fourteen altered genes were involved in the control of specific aspects of cell metabolism with roles in ovarian function, in both PA and SA patients. A rare frameshift alteration of *SAMD11*, which was demonstrated as a promoter of cell proliferation, has been found in several patients mostly affected with SA. Four different variants of *RYR3*, coding for a calcium channel with a role in the homeostasis of calcium, have been identified among all phenotypes. Furthermore, another identified pathway, fundamental in granulosa cell differentiation and proliferation, is the NOTCH signaling, represented by *NOTCH2*, *NOTCH3*, and *NOTCH4* genes found to be altered in both PA and early SA cases. Finally, we also identified alterations in genes belonging to WNT signaling, cell death, and post-translational modifications mainly affecting SA patients (**Table S2**, **Figure 3**).

Most patients showed at least two pathways affected by variable genetic variations and a few showed several variations in genes from only one pathway (e.g., patient 27, presenting with PA and one variant in *TP63* and *FANCA* genes, both related to meiosis, cell cycle, and DNA repair processes). Among SA, we also found three patients with the involvement of several variations in a singular pathway: patient 19 with three variants and patient 31 with two variants affecting genes related to meiosis, cell cycle, and DNA repair processes and patient 23 with a variant in *BMP15* and a compound heterozygosity in *GDF9*, both key actors of folliculogenesis (**Table S3**).

4 DISCUSSION

In this study, we used the *OVO-Array* panel, a targeted NGS approach, to investigate the genetic cause of POI in a cohort of 64 patients with early onset of the disorder. The sequencing was focused on 295 candidate genes selected from literature and our previous data. Through this approach, we could identify 9 already known variants (6, 31, 34, 36–38, 42, 43) and 32 novel rare variants in genes already associated to POI etiology. Moreover, 34 novel rare variants have been found in additional genes participating in pathways important for ovarian physiology.

TABLE 1 | Correlation among severity, phenotype and pathogenicity predictions in patients with 6 variants in potentially POI genes.

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation (HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome prediction v.2.1.1	VarSome Criteria
1	Caucasian	46,XX	PA	16 (induced)	16	No	LARS2(NM_015340.4): c.1021T>G:(p.Cys341Gly)	Het	0.015625	Novel	VUS	PM2,PP2,BP4
							LARS2(NM_015340.4): c.1717T>C:(p.Phe573Leu)	Het	0.015625	Novel	VUS	PM2,PP2,PP3
							MLH3(NM_001040108.2): c.3466G>A:(p.Val1156Ile)	Het	0.015625	0.0000696	VUS	PP3,BP1
							POLE(NM_006231.4): c.4900C>T:(p.Arg1634Cys)	Het	0.015625	0.00000868	VUS	PM2,PP3,BP1
							ANAPC1(NM_022662.4): c.2279C>G:(p.Pro760Arg)	Het	0.015625	0.0000607	VUS	PM2,PP3,BP1
							ATR(NM_001184.4):c.2783A>G: (p.Gln928Arg)	Het	0.015625	Novel	VUS	PM2,BP1
2	Caucasian	46,XX	eSA	12	12 (6 month after menarche)	No	COL6A2(NM_001849.4): c.511G>A:(p.Gly171Arg)	Het	0.015625	0.00114	VUS	PM2,PP2,PP3
							ATM(NM_000051.4):c.1444A>C: (p.Lys482Gln)	Het	0.015625	0.000104	VUS	PM2,BP4
							RASAL2(NM_004841.5): c.3187G>A:(p.Glu1063Lys)	Het	0.015625	0.000338	VUS	PP3,BS1
							RYR3(NM_001036.6): c.14584C>T:(p.Arg4862Cys)	Het	0.015625	0.0000175	VUS	PM2,PP3,BP1
							TP73(NM_005427.4):c.1660T>C: (p.Tyr554His)	Het	0.015625	–	VUS	PM2,PP3
							CYP21A2(NM_000500.9): c.844G>T:(p.Val282Leu)	Hom	0.03125	–	P	PS1,PS3, PM2,PP2, PP5,BP4
							de novo microdeletion of 246kb in 1p36.33 (hg19, chr1:1.228.154-1.473.662 Mb) devoid of known POI genes	Het	0.015625	–	–	–

PA, primary amenorrhea; eSA, early secondary amenorrhea; Het, heterozygosity; Hom, homozygosity; VUS, variant of unknown significance; P, pathogenic.

TABLE 2 | Correlation among severity, phenotype and pathogenicity predictions in patients with 5 variants in potentially POI genes.

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation (HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome prediction v.2.1.1	VarSome Criteria
3	Caucasian	46,XX	OD	No	–	Yes	CYP21A2(NM_000500.9): c.1360C>T:(p.Pro454Ser)	Het	0.015625	–	P	PP5,PS3, PM2,PP2, PP3
							ATM(NM_000051.4): c.418G>C:(p.Asp140His)	Het	0.015625	Novel	VUS	PM2,PP3, BP6
							BLM(NM_000057.4): c.2333C>G:(p.Ser778Cys)	Het	0.015625	0.000156	VUS	PM1, PM2,PP3
							ADAMTS16(NM_139056.4): c.2459G>A:(p.Arg820Gln)	Het	0.015625	0.0000175	VUS	PP3,BS1
							FSHR(NM_000145.4): c.847C>T:(p.Arg283Trp)	Het	0.015625	0.000052	VUS	PM2,PP2
4	Caucasian	not available	PA	No	–	Yes	RYR3(NM_001036.6): c.393C>A:(p.Asp131Glu)	Het	0.015625	–	VUS	PP3,BP1
							NCOR2(NM_006312.6): c.3760C>T:(p.Arg1254Cys)	Het	0.015625	0.000123	VUS	PP3,BS1
							RAD52(NM_134424.4): c.761C>T:(p.Thr254Met)	Het	0.015625	0.000149	VUS	PP3
							MCM9(NM_017696.3): c.970G>T:(p.Val324Leu)	Het	0.015625	Novel	VUS	PM2,BP1
							RAD54L(NM_003579.4): c.604C>T:(p.Arg202Cys)	Het	0.015625	0.00324	LB	PM1,PP3, BS1,BP1, BP6
5	Caucasian	46,XX	SA	13	24	Yes	ERBB4(NM_005235.3): c.95C>T:(p.Thr32Met)	Het	0.015625	–	VUS	PM2,PP2, PP3
							ERBB4(NM_005235.3): c.3344T>A:(p.Val1115Glu)	Het	0.015625	Novel	VUS	PM2,PP2, BP4
							PKP1(NM_000299.3): c.883C>G:(p.Leu295Val)	Het	0.015625	0.00014	VUS	PM2,PP3, BP1
							RYR3(NM_001036.6): c.592A>G:(p.Met198Val)	Het	0.015625	–	VUS	PM2,BP1
							SAMD11(NM_152486.4): c.682_683insT: (p.Pro228LeufsTer227)	Het	0.09375	0.000183	LB	BS1

OD, ovarian dysgenesis; PA, primary amenorrhea; SA, secondary amenorrhea; Het, heterozygosity; P, pathogenic; VUS, variant of unknown significance; LB, likely benign.

TABLE 3 | Correlation among severity, phenotype and pathogenicity predictions in patients with 4 variants in potentially POI genes.

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation(HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome predictionv.2.1.1	VarSome Criteria
6	Caucasian	not available	OD	No	–	Yes	FIGLA(NM_001004311.3): c.364del:(p.Glu122LysfsTer45)	Het	0.015625	Novel	P	PVS1, PM2,PP5, PP3
							NOBOX(NM_001080413.3): c.1626del:(p.Phe543SerfsTer7)	Het	0.015625	Novel	P	PVS1, PM2,PP5
							NR5A1(NM_004959.5): c.1063G>A:(p.Val355Met)	Het	0.03125	0.000122	LP	PM1, PM2,PP5, PP2,PP3
							NCOR2(NM_006312.6): c.3755C>A:(p.Pro1252Gln)	Het	0.015625	Novel	VUS	PM2,PP3
7	Caucasian	not available	PA	No	–	No	VWF(NM_000552.5):c.4517C>T: (p.Ser1506Leu)	Het	0.046875	–	P	PP5,PM1, PM2,PP2, PP3
							VWF(NM_000552.5):c.4508T>C: (p.Leu1503Pro)	Het	0.03125	Novel	LP	PM1, PM2, PM5,PP2, PP3
							NOTCH3(NM_000435.3): c.2791A>G:(p.Ser931Gly)	Het	0.015625	Novel	LP	PM1, PM2,PP2, PP3
							TRRAP(NM_001244580.1): c.10171A>G:(p.Met3391Val)	Het	0.015625	Novel	VUS	PM2,PP2
8	Caucasian	not available	eSA	16	18	No	SAMD11(NM_152486.4): c.682_683insT: (p.Pro228LeufsTer227)	Het	0.09375	0.000183	LB	BS1
							ATG4C(NM_032852.4): c.607dup: (p.Trp203LeufsTer4)	Het	0.015625	0.000659	VUS	PP3,BS1
							NOTCH4(NM_004557.4): c.2945C>T:(p.Thr982Ile)	Het	0.015625	Novel	VUS	PM2,PP3, BP1
							RMI1(NM_024945.3):c.746C>T: (p.Ala249Val)	Het	0.015625	Novel	VUS	PM2,PP3
9	Caucasian	not available	eSA	14	14	No	NR5A1(NM_004959.5): c.502G>C:(p.Ala168Pro)	Het	0.015625	Novel	VUS	PM2,PP2, BP4
							SYNE1(NM_182961.4): c.16709A>G:(p.Gln5570Arg)	Het	0.015625	Novel	LP	PP3,PM2
							RAD50(NM_005732.4): c.2165dup:(p.Glu723GlyfsTer5)	Het	0.015625	0.000301	P	PVS1, PP5,PM2, PP3
							ADAMTS5(NM_007038.5): c.1729G>A:(p.Gly577Ser)	Het	0.03125	0.0151	VUS	PP3,BS1
10	Caucasian	46,XX	SA	13	25	–	ADAMTS4(NM_005099.6): c.803G>A:(p.Arg268Gln)	Het	0.015625	–	VUS	PM2,BP4
							DHCR24(NM_014762.4): c.1046C>G:(p.Pro349Arg)	Het	0.015625	0.0000087	VUS	PM2,PP2, PP3
								Het	0.015625	Novel	VUS	

(Continued)

TABLE 3 | Continued

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation(HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome predictionv.2.1.1	VarSome Criteria
							FSHR(NM_000145.4):c.491C>T:(p.Ser164Phe)					PM2,PP2,PP3
							RAD52(NM_134424.4):c.175G>A:(p.Gly59Arg)	Het	0.015625	0.000699	VUS	PP3
11	Caucasian	46,XX	SA	11	14	No	APC2(NM_005883.3):c.2887C>T:(p.Arg963Trp)	Het	0.015625	0.0000135	VUS	PM2,PP3,BP1
							ATG2A(NM_015104.3):c.4414G>C:(p.Gly1472Arg)	Het	0.015625	0.0000174	VUS	PM2,PP3,BP1
							COL6A1(NM_001848.3):c.350T>C:(p.Val117Ala)	Het	0.015625	0.000982	VUS	PM2,PP2,PP3
							KPNA2(NM_002266.4):c.445T>C:(p.Ser149Pro)	Het	0.015625	Novel	VUS	PM2.PP3
12	Caucasian	not available	SA	11	25	No	LARS2(NM_015340.4):c.2192A>T:(p.Tyr731Phe)	Het	0.015625	Novel	VUS	PM2,PP2,BP4
							RBBP8(NM_002894.3):c.2516G>A(p.Arg839Gln)	Het	0.03125	0.000415	VUS	PM2,PP3,BP1
							THBS2(NM_003247.5):c.1183G>A:(p.Val395Met)	Het	0.015625	0.0000528	VUS	PP3,BS1,BP1
							RIPK1(NM_003804.6):c.700G>A:(p.Glu234Lys)	Het	0.015625	0.000684	VUS	PM2,PP2,BP4,BP6
13	Caucasian	not available	SA	12	21	No	ATM(NM_000051.4):c.7375C>T:(p.Arg2459Cys)	Het	0.015625	0.0000433	VUS	PM1,PM2,PP3
							NCOR2(NM_006312.6):c.3709G>A:(p.Val1237Ile)	Het	0.015625	0.00000886	VUS	PP3
							POLG(NM_001126131.2):c.752C>T:(p.Thr251Ile)	Het	0.03125	0.00147	LP	PS3,PM2,PP2,PP5
							POLG(NM_001126131.2):c.1760C>T:(p.Pro587Leu)	Het	0.03125	0.0015	P	PP5,PS3,PM2,PP2,PP3
14	Caucasian	46,XX	SA	12	16	Yes	VWF(NM_000552.5):c.4517C>T:(p.Ser1506Leu)	Het	0.046875	Novel	P	PP5,PM1,PM2,PP2,PP3
							VWF(NM_000552.5):c.5641G>A:(p.Asp1881Asn)	Het	0.015625	Novel	VUS	PM2,PP2,PP3
							LGR4(NM_018490.5):c.2531A>G:(p.Asp844Gly)	Het	0.015625	0.0124	VUS	PP3,BS1,BP1,BP6
							HK3(NM_002115.3):c.2389G>A:(p.Glu797Lys)	Het	0.015625	0.000113	VUS	PP3,BS1,BP1

OD, ovarian dysgenesis; PA, primary amenorrhea; eSA, early secondary amenorrhea; SA, secondary amenorrhea; Het, heterozygosity; P, pathogenic; VUS, variant of unknown significance; LP, likely pathogenic; LB, likely benign.

TABLE 4 | Correlation among severity, phenotype and pathogenicity predictions in patients with 3 variants in potentially POI genes.

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation (HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome predictionv.2.1.1	VarSome Criteria
15	Caucasian	not available	PA	9	9	Yes	TEX15(NM_001350162.2): c.6511C>T:(p.Arg2171Ter)	Het	0.015625	0.0000175	p	PVS1,PM2,PP3
							TUBA8(NM_018943.3): c.967G>A:(p.Val323Met)	Het	0.015625	0.000243	VUS	PM2,PP3,BP1
							ID1(NM_002165.4): c.458_460del:(p.Leu153del)	Het	0.015625	0.00000886	VUS	PM2,PM4,BP4
16	Caucasian	not available	PA	–	–	No	ATR(NM_001184.4): c.4610T>A:(p.Leu1537Ter)	Het	0.015625	Novel	P	PVS1,PM2,PP3
							POLG(NM_001126131.2): c.1760C>T:(p.Pro587Leu)	Het	0.03125	0.0015	P	PP5,PS3,PM2, PP2,PP3
							POLG(NM_001126131.2): c.752C>T:(p.Thr251Ile)	Het	0.03125	0.00147	LP	PS3,PM2,PP2, PP5
17	Caucasian	46,XX	eSA	12	12	No	NOTCH2(NM_024408.4): c.2084C>A:(p.Ala695Glu)	Het	0.015625	Novel	VUS	PM2,PP3
							TP53(NM_001126114.2): c.475G>A:(p.Ala159Thr)	Het	0.015625	–	P	PM1,PM2, PM5,PP2,PP3
							SAMD11(NM_152486.4): c.682_683insT: (p.Pro228LeufsTer227)	Het	0.09375	0.000183	LB	BS1
18	Caucasian	46,XX	SA	13	19	No	ERBB3(NM_001982.4): c.2269dup: (p.Thr757AsnfsTer70)	Het	0.015625	Novel	P	PVS1,PM2,PP3
							POLG(NM_001126131.2): c.803G>C:(p.Gly268Ala)	Het	0.015625	0.00339	VUS	PP2,PP3,PP5, BS2
							LARS2(NM_015340.4): c.457A>C:(p.Asn153His)	Het	0.015625	–	LP	PP3,PM2,PP2, PP5
19	Caucasian	46,XX	SA	14	23	No	RAD52(NM_134424.4): c.388G>A:(p.Glu130Lys)	Het	0.015625	0.000138	VUS	PP3
							RBBP8(NM_002894.3): c.2516G>A:(p.Arg839Gln)	Het	0.03125	0.000415	VUS	PM2,PP3,BP1
							PLEC(NM_201380.4): c.5801G>A:(p.Arg1934His)	Het	0.015625	0.000128	VUS	PM2,PP3,BP1
20	Caucasian	46,XX	SA	13	22	No	ATG4C(NM_032852.4): c.774_777del: (p.Ile258MetfsTer13)	Het	0.015625	0.0000546	VUS	PP3,BS1
							ATM(NM_000051.4): c.4829G>C:(p.Arg1610Thr)	Het	0.015625	Novel	VUS	PM2,PP3
							PRIM1(NM_000946.3): c.911G>T:(p.Arg304Leu)	Het	0.015625	Novel	VUS	PM2,PP3
21	Caucasian	46,XX	SA	not available	19	No	SAMD11(NM_152486.4): c.682_683insT: (p.Pro228LeufsTer227)	Het	0.09375	0.000183	LB	BS1
							KMT2D(NM_003482.4): c.10876C>T:(p.Arg3626Trp)	Het	0.015625	–	VUS	PM2,PP3
							NBN(NM_002485.5): c.596C>G:(p.Pro199Arg)	Het	0.015625	–	VUS	PM2,PP3

(Continued)

TABLE 4 | Continued

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation (HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome predictionv.2.1.1	VarSome Criteria
22	Caucasian	46,XX	SA	not available	20	No	COL6A2(NM_001849.4): c.343C>T:(p.Arg115Trp)	Het	0.015625	0.00000873	VUS	PM2,PP2,PP3
							LRP5(NM_002335.4): c.4511C>T:(p.Pro1504Leu)	Het	0.015625	0.000389	VUS	PM2,PP2,PP3, BP6
							APC2(NM_005883.3): c.932C>T:(p.Ser311Leu)	Het	0.015625	–	VUS	PM2,PP3,BP1
23	Caucasian	46,XX	SA	14	16	not available	BMP15(NM_005448.2): c.202C>T:(p.Arg68Trp)	Het	0.015625	0.000708	B	PM1,PP3,PP5, BS1,BS2,BP1
							GDF9(NM_005260.5): c.1121C>T:(p.Pro374Leu)	Het	0.015625	0.0000346	VUS	PM2,PP3,BP6
							GDF9(NM_005260.5): c.278A>G:(p.Tyr93Cys)	Het	0.015625	Novel	VUS	PM1,PM2,PP3

PA, primary amenorrhea; eSA, early secondary amenorrhea; SA, secondary amenorrhea; Het, heterozygosity; P, pathogenic; VUS, variant of unknown significance; LP, likely pathogenic; LB, likely benign; B, benign.

4.1 The Oligogenic Nature of POI Is Suggested by Multilocus Analysis

Our data are consistent with several lines of evidence recently emerging pointing to an oligogenic architecture for POI, according to which the presence of multiple genetic variants may partially explain the heterogenicity of phenotypes observed in POI patients. Indeed, we observed at least two variants in 35 patients, 5% of the cohort screened with our *OVO-Array* panel. Noteworthy, two of these patients harbored six variants. On the contrary, the screening of nine POI genes for diagnostic routine permitted to identify only 2 out of the 11 patients with alterations (18%) carrying either two or three variants.

We observed that almost all patients screened by the *OVO-Array*, who carried the major number of variants, presented with a severe phenotype (PA or early SA onset just after menarche), whereas the majority of women carrying only one or two alterations (15 out of 25) were affected by SA. However, we also found that some patients harboring fewer pathogenic or likely pathogenic variants displayed a severe phenotype. These findings suggest that POI could emerge either by the disruption of a single fundamental genetic function or as a result of multilocus variations in genes interacting within different pathways, as proposed by other authors for oligogenic diseases (44). On this line, patient 6 of this cohort was previously reported for the potential synergic detrimental effect of a complex pattern of multiple inherited genetic variants in *FIGLA*, *NOBOX*, and *NR5A1* (36). In the present study, we included this patient into the *OVO-Array* analysis, and by sequencing additional genes, we could identify another alteration (c.3755C>A, p.Pro1252Gln) in *NCOR2*. This gene encodes for a nuclear receptor corepressor that, together with its paralog NCOR1, mediates the retinoic acid-dependent repression of Fgf8 in mouse during organogenesis and, if this complex is mutated, exhibits increased Fgf8 expression, similar to retinoic acid deficiency (45). On one hand, retinoic acid is critical for the entrance in meiosis of ovarian germ cells (46); on the other hand, FGF8 cooperates with BMP15 to promote glycolysis in cumulus cells (47). Therefore, we could hypothesize that an alteration affecting these fundamental pathways might combine with the other three already described variants and play a synergic effect leading to the severe phenotype of the patient. This evidence supports multilocus analysis as a fundamental tool to explain the full phenotypic spectrum of women with POI.

Since NGS has demonstrated to be a powerful tool for the identification of new molecular players and pathways in POI onset, the information derived from the analysis of large NGS panels, such as the *OVO-Array*, might increase the diagnostic power up to 75% of POI cases, in contrast to the current 25% of positive diagnosis obtained by screening few POI genes. The actual limit of this approach is the necessity of validating new putative candidates by further functional testing and sequencing in larger cohorts. Nonetheless, in this study, we could observe both the presence of the same variant in more than one patient at an increased frequency in women with POI with respect to the general female population and different alterations affecting the same genes in several patients. Although the fertility history of

TABLE 5 | Correlation among severity, phenotype and pathogenicity predictions in patients with 2 variants in potentially POI genes.

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation(HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome predictionv.2.1.1	VarSome Criteria
24	African	46,XX	OD	No	–	Yes	NOBOX(NM_001080413.3): c.1112A>C:(p.Lys371Thr)	Het	0.015625	–	B	BS1,BS2, BP1,BP4
							STAG3(NM_012447.4): c.1079G>A:(p.Arg360His)	Het	0.015625	–	LP	PM1,PM2, PP2,PP3
25	African	not available	PA	No	–	No	REC8(NM_005132): c.899G>T:(p.Arg300Leu)	Het	0.015625	0.00234	VUS	PPP3,BS1
							LHCGR(NM_000233): c.C568A:(p.Gln190Lys)	Het	0.015625	0.000563	VUS	PM2,PP2, BP6
26	Caucasian	46,XX	PA	No	–	Yes	AGRN(NM_198576.4): c.2860G>A:(p.Ala954Thr)	Het	0.015625	0.000026	VUS	PM2,PP3, BP1
							VLDLR(NM_003383.5): c.902G>A:(p.Arg301Gln)	Het	0.015625	0.00192	VUS	PM2,BP1
27	Caucasian	46,XX	PA	No	–	Yes	TP63(NM_003722.5): c.1927C>T:(p.Arg643Ter)	Het	0.015625	Novel	P	PVS1,PM2, PP3,PP5
							FANCA(NM_000135.4): c.1340C>T:(p.Ser447Leu)	Het	0.015625	0.000486	VUS	PM2,PP2, PP3
28	Caucasian	46,XX	eSA	12	13	No	DMRT3(NM_021240.4): c.897dup:(p.Ala300ArgfsTer4)	Het	0.015625	–	P	PVS1,PM2, PP3
							RELN(NM_005045.4): c.2015C>T:(p.Pro672Leu)	Het	0.015625	0.000139	LP	PM1,PM2, PP3,PP5,BP1
29	Caucasian	46,XX	eSA	13	13	No	HDAC5(NM_001015053.2): c.446A>G:(p.Glu149Gly)	Het	0.015625	–	VUS	PM2,PP3, BP1
							AKAP9(NM_005751.5): c.4351A>G:(p.Met1451Val)	Het	0.015625	–	VUS	PM2,PP3, BP1
30	Caucasian	46,XX	SA	9	22	Yes	SAMD11(NM_152486.4): c.682_683insT: (p.Pro228LeufsTer227)	Het	0.09375	0.000183	LB	BS1
							HK3(NM_002115.3): c.2077A>C:(p.Met693Leu)	Het	0.015625	0.000165	LB	PP3,BS1,BP1
31	Caucasian	46,XX	SA	12	22	not available	CCNB1IP1(NM_182852.3): c.454G>A:(p.Glu152Lys)	Het	0.015625	Novel	VUS	PM2,BP4
							MLH3(NM_001040108.2): c.3943G>A:(p.Glu1315Lys)	Het	0.015625	–	VUS	PM2,PP3, BP1
32	Caucasian	46,XX	SA	13	15	No	MSH4(NM_002440.4): c.1286A>T:(p.Glu429Val)	Het	0.015625	Novel	VUS	PM2,PP3
							CYP21A2(NM_000500.9): c.844G>T:(p.Val282Leu)	Het	0.03125	Novel	VUS	PP2,PP3
33	Caucasian	46,XX	SA	10	17	No	SAMD11(NM_152486.4): c.628C>T:(p.Arg210Cys)	Het	0.015625	0.000536	LB	PP3,BS1, BP1,BP6
							COL6A2(NM_001849.4): c.2575G>A:(p.Val859Met)	Het	0.015625	0.000241	VUS	PM2,PP2
34	Caucasian	46,XX	SA	10	25	No	GPR137C(NM_001099652.2): c.1211A>G:(p.Asp404Gly)	Het	0.015625	Novel	VUS	PM2,BP4
							RELN(NM_005045.4): c.3651C>G:(p.Ile1217Met)	Het	0.015625	0.00255	VUS	PM2,PP3, BP1

OD, ovarian dysgenesis; PA, primary amenorrhea; eSA, early secondary amenorrhea; SA, secondary amenorrhea; Het, heterozygosity; P, pathogenic; VUS, variant of unknown significance; LP, likely pathogenic; LB, likely benign; B, benign.

TABLE 6 | Correlation among severity, phenotype, and pathogenicity predictions in patients with only 1 variant in potentially POI genes.

Patients ID	Origin	Karyotype	Phenotype	Menarche (yrs)	POI onset (yrs)	Familiarity	Variation (HGVS)	Zigosity	OVO-Array patients' frequency (n = 64)	gnomAD ver. 2.1.1 female population frequency	VarSome predictionv.2.1.1	VarSome Criteria
35	Caucasian	not available	PA	No	–	No	RAD54L(NM_003579.4): c.2209C>A:(p.Gln737Lys)	Het	0.015625	Novel	VUS	PM2,PP3,BP1
36	Caucasian	not available	eSA	14	15	No	BRCA1(NM_007294.4): c.902A>G:(p.Lys301Arg)	Het	0.015625	Novel	VUS	PM2,PP3
37	Caucasian	46,XX	eSA	16	16	No	AR(NM_000044.6): c.2395C>G:(p.Gln799Glu)	Het	0.015625	0.00147	LP	PM1,PP2,PP3, PP5,BS2
38	Caucasian	46,XX	eSA	not available	Only menarche	No	COL6A2(NM_001849.4): c.2308G>C:(p.Glu770Gln)	Het	0.015625	Novel	VUS	PM2,PP2,PP3
39	Caucasian	not available	SA	12	25	Yes	RYR3(NM_001036.6): c.13709G>A:(p.Arg4570His)	Het	0.015625	0.00000877	VUS	PM2,PP3,BP1
40	Caucasian	not available	SA	12	18	No	CHEK2(NM_007194.4): c.1039G>A:(p.Asp347Asn)	Het	0.015625	0.0000259	LP	PM1,PM2,PM5, PP2,PP3,BP6
41	Caucasian	46,XX	SA	not available	15.5	Yes	USP35(NM_020798.4): c.1963dup: (p.Thr655AsnfsTer74)	Het	0.015625	0.0000267	VUS	PVS1,BS1
42	Caucasian	46,XX	SA	13	24	No	PKP1(NM_000299.3): c.2096A>T:(p.Lys699Met)	Het	0.015625	0.000156	VUS	PM2,PP3,BP1
43	Caucasian	not available	SA	12	18	No	KMT5A(NM_020382.7): c.287_289del:(p.Glu97del)	Het	0.015625	–	VUS	PM2,PM4,PP3
44	Caucasian	46,XX	SA	11	14	No	NR5A1(NM_004959.5): c.1063G>A:(p.Val355Met)	Het	0.03125	0.000122	LP	PM1,PM2,PP5, PP2,PP3
45	Caucasian	46,XX	SA	13	25	No	NCOA6(NM_014071.5): c.1250C>G:(p.Pro417Arg)	Het	0.015625	Novel	VUS	PM2,PP3,BP1
46	Caucasian	not available	SA	not available	18	Yes	ADAMTS5(NM_007038.5): c.1729G>A:(p.Gly577Ser)	Het	0.03125	0.0151	VUS	PP3,BS1
47	Caucasian	46,XX	SA	12	14	No	SAMD11(NM_152486.4): c.682_683insT: (p.Pro228LeufsTer227)	Het	0.09375	0.000183	LB	BS1
48	Caucasian	46,XX	SA	11	19	No	POLG(NM_001126131.2): c.3436C>T:(p.Arg1146Cys)	Het	0.015625	0.000182	VUS	PM2,PP2,PP3

PA, primary amenorrhea; eSA, early secondary amenorrhea; SA, secondary amenorrhea; Het, heterozygosity; VUS, variant of unknown significance; LP, likely pathogenic; LB, likely benign.

TABLE 7 | Summary of the recurring variants in our POI cohorts.

Gene	Transcript	cDNA Variation	Protein Variation	PatientID	Pheno-type	POI group freq. (N=107)	gnomAD exomes (female) freq.	Varsome link	HGMD	Ref.
ADAMTS5	NM_007038	c.1729G>A	p.Gly577Ser	9	eSA	0.0186916	0.0151	varso.me/RpWE		
				46	SA					
APC2	NM_005883	c.2887C>T	p.Arg963Trp	11	SA	0.0093458	0.0000135	varso.me/TdbK		
		c.932C>T	p.Ser311Leu	22	SA	0.0093458	–	varso.me/Tdbc		
ATG4C	NM_032852	c.607dupT	p.Trp203Leufs*4	8	eSA	0.0093458	0.000659	varso.me/T0VM		
		c.774_777del	p.Ile258Metfs*13	20	SA	0.0093458	0.0000546	varso.me/T0Vd		
ATM	NM_000051	c.418G>C	p.Asp140His	3	46,XX OD	0.0093458	–	varso.me/LMjL		(29)
		c.1444A>C	p.Lys482Gln	2	eSA	0.0093458	0.000104	varso.me/FFvg		
		c.4829G>C	p.Arg1610Thr	20	SA	0.0093458	not available	varso.me/T0Bg		
		c.7375C>T	p.Arg2459Cys	13	SA	0.0093458	0.0000433	varso.me/SIfh		
ATR	NM_001184	c.4610T>A	p.Leu1537*	16	PA	0.0093458	not available	varso.me/TZxD		(30)
		c.2783A>G	p.Gln928Arg	1	PA	0.0093458	not available	varso.me/TZxU		
BMP15	NM_005448	c.202C>T	p.Arg68Trp	23	SA	0.0186916	0.000708	varso.me/Ne2w		(31)
				54	PA					
		c.406G>C	p.Val136Leu	55	PA	0.0093458	0.00000866	varso.me/TZn5		
COL6A2	NM_001849	c.343C>T	p.Arg115Trp	22	SA	0.0093458	0.00000873	varso.me/N8DA		
		c.511G>A	p.Gly171Arg	2	eSA	0.0093458	0.00114	varso.me/Orla		
		c.2308G>C	p.Glu770Gln	38	eSA	0.0093458	not available	varso.me/TZnl		
		c.2575G>A	p.Val859Met	33	SA	0.0093458	0.000241	varso.me/RelP		
CYP21A2	NM_000500	c.844G>T	p.Val282Leu	2	eSA	0.0186916	not available	varso.me/N9iB	CM880022	
				32	SA					
		c.1360C>T	p.Pro454Ser	3	46,XX OD	0.0093458	not available	varso.me/Jl3r	CM920233	
ERBB4	NM_005235	c.3344T>A	p.Val1115Glu	5	SA	0.0093458	not available	varso.me/T1xM		(32)
		c.95C>T	p.Thr32Met			0.0093458	-	varso.me/T1x2		
FSHR	NM_000145	c.491C>T	p.Ser164Phe	10	SA	0.0093458	not available	varso.me/T1wg		(33)
		c.847C>T	p.Arg283Trp	3	46,XX OD	0.0093458	0.000052	varso.me/SWBI		
		c.926G>C	p.Gly309Ala	49	SA	0.0186916	not available	varso.me/S1eC		
				56	PA					
		c.1964G>C	p.Arg655Thr	49	SA	0.0093458	not available	varso.me/S1eT		
		c.909-917del	p.Tyr303*	50	SA	0.0093458	–	varso.me/TZcz		
		c.1118G>A	p.Ser373Asn			0.0093458	not available	varso.me/T1ug		
GDF9	NM_005260	c.362C>T	p.Thr121Ile	52	PA	0.0093458	0.000277	varso.me/TZ8s		(6, 34)
		c.566C>T	p.Thr189Ile	53	PA	0.0093458	not available	varso.me/TZ8M		
		c.307C>T	p.Pro103Ser	57	PA	0.0093458	0.0028	varso.me/TZ99		
		c.278A>G	p.Tyr93Cys	23	SA	0.0093458	not available	varso.me/Tjdt		
		c.1121C>T	p.Pro374Leu			0.0093458	0.0000346	varso.me/Tjdi	CM066831	
HK3	NM_002115	c.2077A>C	p.Met693Leu	30	SA	0.0093458	0.000165	varso.me/TjkN		(23)
		c.2389G>A	p.Glu797Lys	14	SA	0.0093458	0.000113	varso.me/TjkG		
LARS2	NM_015340	c.1021T>G	p.Cys341Gly	1	PA	0.0093458	not available	varso.me/TdCz		(35)
		c.1717T>C	p.Phe573Leu			0.0093458	not available	varso.me/TdEN		
		c.2192A>T	p.Tyr731Phe	12	SA	0.0093458	not available	varso.me/TdGx		
		c.457A>C	p.Asn153His	18	SA	0.0093458	not available	varso.me/R5je		
MLH3	NM_001040108	c.3466G>A	p.Val1156Ile	1	PA	0.0093458	0.0000696	varso.me/SR8H	CM1410381	
		c.3943G>A	p.Glu1315Lys	31	SA	0.0093458	–	varso.me/TjgS		
NCOR2	NM_006312	c.3755C>A	p.Pro1252Gln	6	46,XX OD	0.0093458	not available	varso.me/T2Av		
		c.3709G>A	p.Val1237Ile	13	SA	0.0093458	0.00000886	varso.me/T2BI		
		c.3760C>T	p.Arg1254Cys	4	PA	0.0093458	0.000123	varso.me/T2BR		
NOBOX	NM_001080413	c.331G>A	p.Gly111Arg	59	SA	0.0093458	0.0000131	varso.me/TZr6	CM152396	(36–38)

(Continued)

TABLE 7 | Continued

Gene	Transcript	cDNA Variation	Protein Variation	PatientID	Pheno-type	POI group freq. (N=107)	gnomAD exomes (female) freq.	Varsome link	HGMD	Ref.
		c.1626delC	p.Phe543Serfs*7	6	46,XX OD	0.0093458	–	varso.me/T1kg		
		c.1112A>C	p.Lys371Thr	24	46,XX OD	0.0093458	0.000238	varso.me/SWXS		
NR5A1	NM_004959	c.1063G>A	p.Val355Met	6	46,XX OD	0.0186916	0.000122	varso.me/TdLE	CM076367	(36)
				44	SA					
		c.502G>C	p.Ala168Pro	9	eSA	0.0093458	-	varso.me/Tdlj		
PKP1	NM_000299	c.883C>G	p.Leu295Val	5	SA	0.0093458	0.00014	varso.me/T2AC		
		c.2096A>T	p.Lys699Met	42	SA	0.0093458	0.000156	varso.me/T2Ao		
POLG	NM_001126131	c.752C>T	p.Thr251Ile	16	PA	0.0186916	0.00147	varso.me/FCE6	CM021660	(40)
				13	SA					
		c.803G>C	p.Gly268Ala	18	SA	0.0092592	0.00339	varso.me/IbzD	CM033442	
		c.1760C>T	p.Pro587Leu	16	PA	0.0186916	0.0015	varso.me/Ez4d	CM03133	
				13	SA					
		c.3436C>T	p.Arg1146Cys	48	SA	0.0093458	0.000182	varso.me/N8U8	CM060433	
RAD52	NM_134424	c.175G>A	p.Gly59Arg	10	SA	0.0093458	0.000699	varso.me/Td7L		
		c.761C>T	p.Thr254Met	4	PA	0.0093458	0.000149	varso.me/Td7a		
		c.388G>A	p.Glu130Lys	19	SA	0.0093458	0.000138	varso.me/Td7m		
RAD54L	NM_003579	c.604C>T	p.Arg202Cys	4	PA	0.0093458	0.00324	varso.me/FIC4		
		c.2209C>A	p.Gln737Lys	35	PA	0.0093458	not available	varso.me/TZvx		
RBBP8	NM_002894	c.2516G>A	p.Arg839Gln	12	SA	0.0186916	0.000415	varso.me/Fdvl		
				19	SA					
RELN	NM_005045	c.2015C>T	p.Pro672Leu	28	eSA	0.0093458	0.000139	varso.me/GFVk		
		c.3651C>G	p.Ile1217Met	34	SA	0.0093458	0.00255	varso.me/TCd9		
RYR3	NM_001036	c.393C>A	p.Asp131Glu	4	PA	0.0093458	–	varso.me/T1hi		
		c.592A>G	p.Met198Val	5	SA	0.0093458	–	varso.me/T1gq		
		c.14584C>T	p.Arg4862Cys	2	eSA	0.0093458	0.0000175	varso.me/T1fv		
		c.13709 G>A	p.Arg4570His	39	SA	0.0093458	0.00000877	varso.me/T1hS		
SAMD11	NM_152486	c.628C>T	p.Arg210Cys	33	SA	0.0093458	0.000536	varso.me/N60l		
		c.682_683insT	p.Pro228Leufs*227	8	eSA	0.0560748	0.000183	varso.me/N7qi		
				5	SA					
				17	eSA					
				47	SA					
				21	SA					
				30	SA					
STAG3	NM_012447	c.1079G>A	p.Arg360His	24	46,XX OD	0.0093458	–	varso.me/SWXv		(41)
		c.3433G>A	p.Glu1145Lys	49	SA	0.0093458	0.0000346	varso.me/S1e5		
		c.1678-10_2228del	p.?	58	PA	0.0093458	not available	varso.me/TZuL		
VWF	NM_000552	c.4508T>C	p.Leu1503Pro	7	PA	0.0093458	not available	varso.me/TYo1	CM095108	
		c.4517C>T	p.Ser1506Leu	7	PA	0.0186916	-	varso.me/TYnR	CM930731	
				14	SA					
		c.5641G>A	p.Asp1881Asn	14	SA	0.0093458	not available	varso.me/T1dI		

PA, Primary Amenorrhea; SA, Secondary Amenorrhea; eSA, early Secondary Amenorrhea; 46,XX OD, Ovarian Dysgenesis with normal karyotype (46,XX).

Gene variations are shown, together with the patients' phenotype. Genetic alterations genes and variants already associated to POI are shown in green and red characters, respectively. The frequency in our POI women (n= 107) and in the female population are given, together with the HGMD, if available.

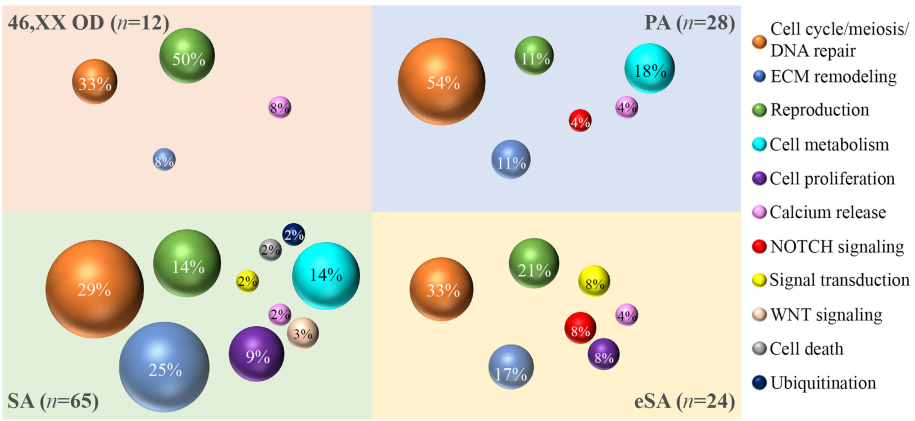

FIGURE 3 | Bubble chart of the pathways identified by the gene ontology analysis. The sample numerosity (*n*) is reported for each phenotype (46,XX OD, light red; PA, light blue; eSA, light yellow; and SA <25 years, light green). Pathways are represented by colored bubbles (refer to the legend on the right). The size of the bubbles depends on the number of variants identified in each pathway for each phenotype. The percentages within the bubbles indicate the ratio between the number of variants per pathway associated to a phenotype and the total of variants associated to that phenotype. 46,XX OD = ovarian dysgenesis with normal karyotype (46,XX); PA = primary amenorrhea with functional ovarian defect, eSA = secondary amenorrhea onset few months after menarche; SA = secondary amenorrhea <25 years of age.

the controls reported in public datasets is unknown, thus introducing a potential bias in the analysis, our findings support the relevance of these candidates in POI pathogenesis and deserve further investigations.

4.2 Large-Scale Genetic Analysis Identified Novel Variants in Genes Participating in Pathways Relevant for Ovarian Function

A first hint pointing at the possible involvement of the newly identified candidates derives from gene ontology, which highlighted pathways fundamental for the reproductive success in mammals.

Meiotic recombination is a complex process, which requires the combination of a large plethora of factors (48). Furthermore, during the arrest at meiotic prophase I, primordial follicle oocytes are more vulnerable to DNA double stand breaks emanating from endogenous and exogenous sources (49); thus, pathogenic variations in one or more of their encoding genes might explain the onset of POI, as previously proposed (50). Noteworthy, we found 34 altered genes participating in meiosis, cell cycle, and DNA repair as possible candidates for POI. This pathway resulted to be mainly affected in patients with the most severe phenotypes, and in several cases, we found variants in at least two genes encoding DNA repair and meiotic factors. Moreover, this was the only pathway affected by genetic variants in some patients. Our data indicate that, in line with its oligogenic nature, POI might also result from alterations in more loci belonging to the same pathway.

The next major pathway in which we have found several altered genes was folliculogenesis, encompassing all stages of ovarian follicle development. In one case of SA (patient 23), we found variations in two major factors of folliculogenesis: a variant of *BMP15* (p.Arg68Trp) with deleterious functional effects (31), together with a compound heterozygosity in *GDF9* [p.Tyr93Cys and the previously reported p.Pro374Leu (34)], supporting the possibility that the disruption of a unique pathway at variable levels can also cause the disorder. Besides the known actors (i.e., *FIGLA*, *NOBOX*, *BMP15*, *GDF9*, *FSHR*, *LHCGR*, *NR5A1*, *AR*), we found variants in two genes involved in autophagosome assembly (*ATG2A* and *ATG4C*). Their role may deserve further attention because autophagy is an important mechanism in mammalian ovarian development, regulating follicular atresia (51). We also identified genes causing disorders of sex development or with roles in spermatogenesis. Indeed, *CYP21A2* alterations are responsible for congenital adrenal hyperplasia, caused by diminished aldosterone and cortisol production that result in ambiguous genitalia in female affected (52). *DMRT3* encodes for a transcription factor with evolutionary conserved roles in sex development, whose haploinsufficiency leads to 46,XY male to female sex reversal (53). *TUBA8* encodes an isoform of α-tubulin highly expressed in ovarian follicle (www.proteinatlas.org). This gene has an ortholog in mouse which is expressed in the brain and testis, with a role in spermatid development (54), and can cause asthenozoospermia in men (55). *ID1* has been described as potential AMH downstream target genes (56, 57), and since it is involved in the regulation of follicular growth, further characterization of its molecular function in this pathway would be needed. *KMT2D* alterations are the main cause of Kabuki syndrome, a congenital intellectual disability with genitourinary anomalies among other additional features. Interestingly, a reduced number of dominant families was reported in a Kabuki cohort (58), thus supporting a possible role of *KMT2D* variations in female fertility.

We also identified alterations in genes involved in ECM remodeling in the *OVO-Array* POI cohort, such as *ADAMTS4*, *ADAMTS5*, *ADAMTS16*, *AGRN*, *COL6A1*, *COL6A2*, *ERBB3*, *ERBB4*, *PKP1*, *RELN*, *THBS2*, and *VWF*. Given the ECM

contribution to granulosa cell survival and proliferation, the ECM is required for maintaining the follicular cell morphology in all phases of ovarian follicle function (59). This ECM role is supposed to be mediated, at least in part, by the distinct ADAMTS subtypes and collagens. The ECM regulates cell aggregation and intracellular communication between the oocyte, granulosa, and theca cells within the follicle. The communication of small metabolites, ions, and second messengers between each cell type is made possible by a network of gap junctions, such as desmosomes containing among others the accessory plaque protein plakophilin 1. The ECM proteins are also necessary to support granulosa cell survival and steroidogenesis; for example, reelin contributes to follicular stability. Factors within the ECM are important in the control of follicular development and atresia. Included in this group of ECM proteins are thrombospondins, which are thought to contribute to the regulation of angiogenesis, and von Willebrand factor (59).

Several elements of Notch signaling appear to be involved in the POI pathogenesis in the *OVO-Array* cohort (*NOTCH2*, *NOTCH3*, *NOTCH4*). The Notch pathway is a contact-dependent signaling system active in the mammalian developing ovary which has multiple functions in follicle assembly, maturation, development, and meiotic entry (60). Notch proteins (NOTCH1, NOTCH2, NOTCH3, and NOTCH4) function as transmembrane receptors for one of the membrane-bound ligands (JAG1, JAG2, DLL1, DLL3, and DLL4) and their binding causes a conformational change of the Notch protein starting a series of sequential proteolytic cleavages at the receptor juxtamembrane region allowing the release of the Notch intracellular domain that is eventually free to translocate to the nucleus. Within the nucleus, it activates the transcription of Notch target genes. Receptors, ligands, modulators, and activated genes belonging to this system are expressed and finely regulated during folliculogenesis. Although Notch1 and Notch4 expression is limited to the ovarian vasculature, Notch2 and Notch3 are expressed in the granulosa cells of developing follicles and mediate follicle assembly and growth in mammals. Consistent with its role within the mammalian ovarian follicle, genetic alterations in *NOTCH2* were previously identified *via* whole-exome sequencing and functional evidence demonstrated the correlation of *NOTCH2* missense variations in POI (23, 61).

Several rare variants have been identified in genes regulating different aspects of follicular cell metabolism. Hexokinase 3 (HK3) converts glucose to glucose-6-phosphate in the first step of glucose metabolism, exerting a protective effect against oxidative stress (62), and it was associated with age at natural menopause (63). A recent transcriptomic study in primates demonstrated that genes most regulated in aged oocytes and granulosa cells are related to antioxidant defenses (64), further supporting the importance of cellular damage in infertility. *POLG* and *LARS2* play roles in mitochondrial DNA replication, gene expression, and protein synthesis and degradation (65). Mutations in *POLG* can cause among others the neurological conditions Alpers syndrome and progressive external ophthalmoplegia (PEO), and the latter can associate with POI (35). Four *POLG* variants emerged by our analysis: a PA (patient 23, see 3.1.1 section) and a SA patient with c. c.752C>T (p.Thr251Ile) and c.1760C>T (p.Pro587Leu) in compound heterozygosity; c.803G>C (p.Gly268Ala) and c.3436C>T (p.Arg1146Cys) in two patients with SA. Unfortunately, no further neurological information was available for these patients. Mutations in *LARS2*, encoding mitochondrial leucyl-tRNA synthetase, lead to POI and hearing loss in Perrault syndrome (66). We identified the c.457A>C (p.Asn153His) variant (ClinVar VCV000191173.2) of *LARS2* in a patient with SA, who was not previously associated with Perrault syndrome, and other three missense variants in two SA patients. Unfortunately, we have no further clinical information also in these patients. The cholesterol synthetase DHCR24 belongs to the pathway of steroid biosynthesis which regulates many physiological processes (i.e., stress response, ovarian cycle, and endocrine system) (67), and this is the first report of a variant in *DHCR24* in a patient with POI. The *VLDLR* gene in humans is relevant for steroidogenesis as its expression in granulosa cells of pre-ovulatory follicles keeps a relevant role in lipoprotein endocytosis during follicular growth (19). Evidence in hens correlates the VLDLR function with fertility (68) and its haploinsufficiency in women has been associated to impaired folliculogenesis (69). Here, we confirm previous findings by describing a novel missense variant in a patient with PA.

WNT signaling has essential functions in ovary differentiation, follicle development, and hormone synthesis (70). In this study, we identified missense variants in *LGR4*, one of the receptors for R-spondins, which augment the WNT signaling pathway (71), and *LRP5*, a co-receptor for the canonical Wnt–β-catenin signaling with a known role in the regulation of bone mineral density (72), but we could not find any further evidence in the ovary.

Cell proliferation and death pathways were represented by the identification in our cohort of variants in *SAMD11* and *RIPK1*, respectively. *SAMD11* has never been associated with ovarian phenotypes before. This gene encodes for a transcriptional modulator relatively uncharacterized but phylogenetically conserved from zebrafish to humans, widely expressed in various tissues (73), where it might promote cell proliferation. Conversely, *RIPK1* can mediate apoptosis during embryonic development (74). This is the first report of variants in these genes associated with an ovarian phenotype, and their role in the ovary should be investigated in further studies. Another gene involved in mitosis control that we found altered in POI was *USP35*, encoding a deubiquitinating enzyme which regulates the stability and function of Aurora B kinase and whose depletion results in inhibited metaphase chromosome alignment (75).

In mammalian oocytes, calcium is one of the major signal molecules involved in meiotic cell cycle resumption, arrest, and apoptosis. *RYR3* encodes a calcium channel that mediates the intracellular release of Ca^{2+}, and the ryanodine receptors (RyR) family has been identified in mammalian oocytes (76). Here, we reported several *RYR3* variants with predicted pathogenic effect,

which might dysregulate the homeostasis of calcium within oocytes, thus supporting a role for this gene in the disorder.

5 CONCLUSIONS

We propose novel candidate gene-disease variants likely causative or conferring susceptibility to POI onset. Our findings, by supporting the oligogenic nature of POI, suggest that in the presence of the most severe forms of ovarian insufficiency, it is necessary to screen multiple candidates or to perform WES analysis, since more alterations in different genes may synergize for the determination of the phenotype. Moreover, we show that multilocus analysis could increase the diagnostic power and the accuracy of POI diagnosis, thus ameliorating genetic counseling in patients. The systematic application of the *OVO-Array* multilocus analysis shall improve the management of POI including a personalized approach to the fertility defect and to the associated extra-ovarian abnormalities that can frequently anticipate or follow the POI onset.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material** and online at https://doi.org/10.5281/zenodo.4543275 (Digital Object Identifier 10.5281/zenodo.4543275). Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRCCS Istituto Auxologico Italiano Ethics Committee. Written informed consent to participate in this study was provided by the patients or, if minors, by the legal guardian/next of kin of the participants.

AUTHOR CONTRIBUTIONS

RR was responsible for the conception and design, *OVO-Array* experiment, and interpretation of data and drafted the manuscript. SM was responsible for the sequencing analysis, carried out the *in silico* predictions, and helped in drafting the manuscript. FG was responsible for NGS for diagnostic screening. DG was responsible for the bioinformatic NGS analysis. LL contributed to the *OVO-Array* experiment. AM, CM, FB, and MB were responsible for the enrollment of patients and critical revision of the manuscript. LP designed and supervised the research and revised the manuscript critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.664645/full#supplementary-material

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