Epigenetic inheritance of polycystic ovary syndrome — challenges and opportunities for treatment

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Abstract | Polycystic ovary syndrome (PCOS) is the main cause of female infertility worldwide and is associated with a substantially increased lifetime risk of comorbidities, including type 2 diabetes mellitus, psychiatric disorders and gynaecological cancers. Despite its high prevalence (~15%) and substantial economic burden, the aetiology of PCOS remains elusive. The genetic loci linked to PCOS so far account for only ~10% of its heritability, which is estimated at 70%. However, growing evidence suggests that altered epigenetic and developmental programming resulting from hormonal dysregulation of the maternal uterine environment contributes to the pathogenesis of PCOS. Male as well as female relatives of women with PCOS are also at an increased risk of developing PCOS-associated reproductive and metabolic disorders. Although PCOS phenotypes are highly heterogenous, hyperandrogenism is thought to be the principal driver of this condition. Current treatments for PCOS are suboptimal as they can only alleviate some of the symptoms; preventative and targeted treatments are sorely needed. This Review presents an overview of the current understanding of the aetiology of PCOS and focuses on the developmental origin and epigenetic inheritance of this syndrome.

Polycystic ovary syndrome (PCOS) affects ~15% of women of reproductive age and is the most common endocrine disorder in women. In addition to its high economic burden, representing an annual health-care expenditure estimated at ~$5.39 billion in 2019 in the USA[1], PCOS has a detrimental effect on the long-term health of affected women[2,3]. The three main clinical features of the syndrome are clinical or biochemical hyperandrogenism, oligo-ovulation or anovulation with menstrual irregularities, and polycystic ovarian morphology (PCOM). The updated evidence-based International Guidelines for the Assessment and Management of PCOS (which strengthen the previous Rotterdam criteria) require the presence of at least two out of these three clinical features to fulfil the diagnosis[4,5] (BOX 1). Thus, four phenotypes of PCOS exist: phenotype A, comprising hyperandrogenism, ovulatory dysfunction and PCOM; phenotype B, comprising hyperandrogenism and ovulatory dysfunction only; phenotype C, comprising hyperandrogenism and PCOM; and finally phenotype D, comprising ovulatory dysfunction and PCOM. The proportion of cases accounted for by phenotypes A and B combined is 40–45%, whereas phenotype C accounts for ~35% and phenotype D for ~20% of cases in an unselected population[6]. Among these phenotypes, those that include hyperandrogenism (A–C) are considered the most severe (BOX 1).

No evidence suggests geographical or ethnic differences in the prevalence of PCOS, although the prevalence of PCOS is increased (to >25%) among women with severe obesity and the presence of comorbid obesity aggravates all PCOS symptoms[7]. The diagnosis of PCOS in adolescent girls is hampered by a lack of consistent evidence. The most recent evidence-based guidelines from the International Consortium propose that a diagnosis of PCOS should not be made until >2 years after menarche and requires the presence of clinical and/or biochemical hyperandrogenism as well as irregular or absent ovulatory menstrual cycles[8].

PCOS results in a broad spectrum of clinical outcomes. In addition to infertility, PCOS is associated with an increased risk of pregnancy-related complications, including gestational diabetes, placental dysfunction, miscarriage and neonatal complications[9–11]. Moreover, PCOS is accompanied by substantial metabolic abnormalities, including hyperinsulinaemia, insulin resistance and dyslipidaemia, which are in turn associated with increases in the risk of type 2 diabetes mellitus (T2DM), cardiovascular dysfunction and gynaecological cancers[12–18]. Women with PCOS are at an increased risk of developing neuropsychiatric disorders, including

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https://doi.org/10.1038/s41574-021-00517-x

Key points

- Polycystic ovary syndrome (PCOS) is a common heritable disorder strongly linked to hyperandrogenism and hyperinsulinaemia.
- Disentangling the genetic and non-genetic contributions to the transmission of PCOS will require further investigation.
- PCOS-like phenotypic traits are transgenerationally inherited in female offspring of androgen-exposed or anti-Müllerian hormone-exposed dams up to the F_3 generation, indicating long-lasting effects of an aberrant maternal–fetal environment.
- Studies in mouse models of PCOS demonstrate that epigenetic modulation connects early-life exposures to subsequent phenotypes and contributes to the development and familial transmission of PCOS.
- Inheritance through epigenetic mechanisms opens a path towards novel treatment strategies for PCOS-like phenotypic traits.

Mendelian randomization analyses
A genetic approach that determines the causal effects of putative risk factors in disease by using genetic variants as instrumental variables to infer whether a risk factor causally affects a health outcome.

anxiety, depression and autism spectrum disorders[19–22]. PCOS also runs in families. In a register-based study of nearly 30,000 daughters of women with or without PCOS, our group demonstrated that the daughters of women with PCOS have a fivefold increase in the risk of being diagnosed with the syndrome[23]. However, exactly how PCOS is inherited remains unclear, as genetic loci identified by genome-wide association studies (GWAS) as being associated with PCOS account for only 10% of the estimated 70% heritability of this syndrome[24].

Although hyperandrogenism is known to play a central role in the pathogenesis of PCOS, the management of PCOS is hindered by the limited understanding of its aetiology and underlying pathogenetic mechanisms. The heterogeneity of the symptoms of PCOS probably indicates a high degree of aetiological complexity. We anticipate that increasing knowledge of the aetiology of PCOS will eventually pave the way towards early diagnosis and mechanism-based treatment strategies.

This Review presents the current understanding of the developmental origin and aetiology of PCOS. Besides summarizing the genetic factors linked to PCOS, we highlight epigenetic modulation as a mechanism that bridges early-life exposures and subsequent phenotypes in contributing to the development and familial transmission of PCOS. Finally, we highlight gaps in our knowledge of PCOS that remain to be addressed by future research.

Box 1 | **The evolution of PCOS diagnostic criteria**

National Institutes of Health 1990
Hyperandrogenism and ovulatory dysfunction (either oligo-ovulation or anovulation).

Androgen Excess and Polycystic Ovary Syndrome Society 2006
Hyperandrogenism and either ovulatory dysfunction or polycystic ovarian morphology.

Rotterdam 2003
Any two or more of the following features: hyperandrogenism, ovulatory dysfunction and polycystic ovarian morphology, which results in four possible phenotypes:

- Classic PCOS: hyperandrogenism, ovulatory dysfunction and polycystic ovarian morphology.
- National Institutes of Health criteria: hyperandrogenism and ovulatory dysfunction only.
- Ovulatory PCOS: hyperandrogenism and polycystic ovarian morphology only.
- Non-hyperandrogenic PCOS: hyperandrogenism and ovulatory dysfunction only.

Aetiology of PCOS

The complex aetiology of PCOS is likely to be due to a combination of genetic, epigenetic and maternal–fetal environmental factors. Hyperandrogenism and hyperinsulinaemia are the two most prevalent and most important features of the syndrome and are present in about 75–95% of all women with PCOS (FIG. 1). Ovarian androgen hypersecretion is the main cause of hyperandrogenism in affected women and is also the most heritable phenotypic trait[25]. High circulating levels of luteinizing hormone (LH), occurring in response to the increased secretion of gonadotropin-releasing hormone (GnRH) and probably also the absence of negative feedback from progesterone, exacerbate ovarian hyperandrogenism by stimulating androgen production in theca cells[26,27]. Intrinsic dysfunction of theca cells also contributes to ovarian hyperandrogenism in women with PCOS[28]. Moreover, the excessive number of ovarian antral follicles seen in women with PCOS as a result of theca cell hyperandrogenism further promotes primary follicular recruitment and increases the number of gonadotropin-independent preantral and small antral follicles[29,30]. The androgen receptor (AR) is maximally expressed in preantral follicles, which indicates that the main actions of androgens occur during the early stages of folliculogenesis[31]. Furthermore, the relatively diminished levels of follicle-stimulating hormone (FSH) — that is, compared with LH levels — inhibit both follicular expansion and follicular maturation[32].

Hyperinsulinaemia resulting from insulin resistance decreases the hepatic synthesis of sex hormone-binding globulin (SHBG) and also stimulates androgen production by ovarian theca cells. These effects synergize with the stimulatory effect of LH and contribute to raised total and free circulating testosterone levels[33]. Moreover, hyperinsulinaemia upregulates LH receptors in theca cells as well as inhibiting follicular maturation and growth, thereby aggravating androgen-dependent anovulation[34–36].

However, whether PCOS is primarily caused by hyperandrogenism or hyperinsulinaemia is still under debate and further mechanistic studies are required[37]. What we do know is that treatments targeting both hyperandrogenism and hyperinsulinaemia improve reproductive and metabolic symptoms in women with PCOS. This observation highlights the importance of identifying the principal drivers or triggers of PCOS so that mechanism-based novel therapeutic strategies can be developed.

Genetic factors

Mendelian randomization analyses have demonstrated a causal effect of raised testosterone levels on the risk of PCOS (OR 1.51; 95% CI 1.33–1.72 per 1 SD increase in levels of bioavailable testosterone)[38]. Moreover, similar Mendelian randomization analyses in women have also demonstrated a causal effect of high bioavailable testosterone levels on the risk of T2DM (OR 1.37; 95% CI 1.22–1.53) and a protective effect of high SHBG levels on fasting insulin levels and on the risk of T2DM[38]. These findings demonstrate that high testosterone levels in

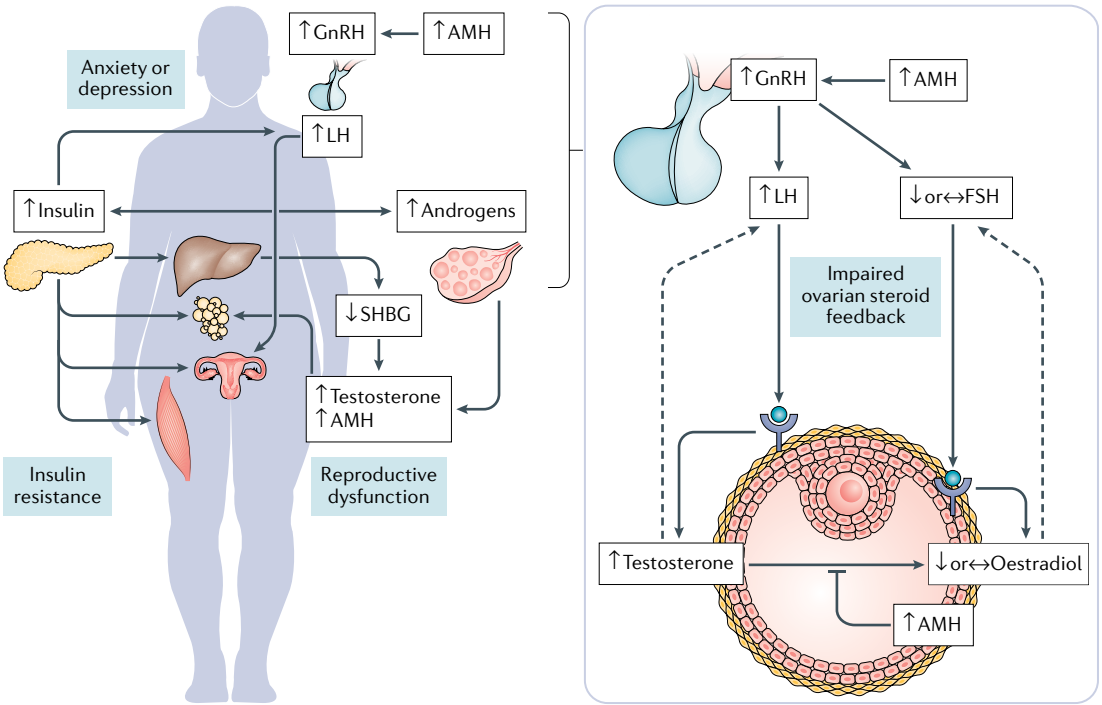

Fig. 1 | **Pathophysiology of PCOS.** Polycystic ovary syndrome (PCOS) is linked to reproductive and metabolic disturbances as well as to psychiatric conditions such as anxiety and depression. Ovarian steroidogenesis requires gonadotropin stimulation. The gonadotropin-releasing hormone (GnRH) pulse generator in women with PCOS is resistant to the negative feedback effects of ovarian steroids, which are probably mediated by androgen excess (given that they can be reversed by the androgen-receptor blocker flutamide). The resulting high GnRH pulse frequency results in hypersecretion of luteinizing hormone (LH), which stimulates theca cell hyperandrogenism and is thus a key factor in the hyperandrogenaemia of women with PCOS. Women with PCOS also have hyperinsulinaemia independent of obesity, which further stimulates theca cells to produce testosterone, exacerbates LH hypersecretion and lowers the production of sex hormone-binding globulin (SHBG) in the liver, thereby further increasing hyperandrogenaemia. The diminished secretion of follicle-stimulating hormone (FSH) inhibits the expansion of follicular size and maturation. Therefore, women with PCOS also have excessive production of anti-Müllerian hormone (AMH) as a result of the large number of preantral and small antral follicles. AMH inhibits expression of *CYP19A1*, the gene encoding aromatase, thereby preventing the conversion of androgens to oestrogens and contributing to elevated androgen levels. Elevated AMH levels also increase the activity of GnRH neurons and directly stimulate the GnRH-dependent secretion of LH, which probably further stimulates ovarian hyperandrogenism. Women with PCOS have an increased risk of obesity, which not only worsens all symptoms of this syndrome but also causes PCOS. ↓, decreased; ↑, increased; ↔, unchanged.

women could be a trigger of PCOS rather than a consequence of upstream defects in ovarian dysfunction and insulin signalling[38].

Family and twin studies have revealed that PCOS is inherited in an autosomal dominant pattern[24,25]. The phenotypic heterogeneity of the syndrome implies the presence of considerable genetic variability but a meta-analysis of GWAS data found that the genetic architecture of this syndrome is largely similar across all four PCOS phenotypes[39], indicating the absence of relevant genetic differences. Another study used biochemical and genotype data from published GWAS to identify genes associated with the different reproductive and metabolic phenotypes of PCOS. So far, ~30 PCOS risk genes have been identified in GWAS, including several genes that regulate gonadotropin secretion and action or ovarian function: *FSHB*, *FSHR*, *AMH*, *AMHR2*, *LHCGR*, *STON1*, *GTF2A1L*, *DENND1A*, *RAB5B*, *SUOX*, *HMGA2*, *C9orf3*, *YAP1*, *TOX3*, *RAD50*, *FBN3*, *PRDM2*, *KAZN*, *IQCA1* and *CDH10* as well as several others involved in metabolic and neural function: *THADA*, *GATA4*, *NEIL2*, *ERBB2*, *ERBB3*, *ERBB4*, *SUMO1P1*, *INSR*, *KRR1*, *KCNA4*, *KCNH7* and *FIGN*[39–41]. Although the applicability of these results is limited to European women with PCOS fulfilling the National Institutes of Health diagnostic criteria (that is, with hyperandrogenism and ovulatory dysfunction), these data suggest that genetic profiling might be able to distinguish between reproductive and metabolic phenotypes of PCOS[42].

However, the loci identified by GWAS account for only 10% of the observed heritability of PCOS. One explanation might be that the genetic variants identified by GWAS and common to all PCOS phenotypes have small effect sizes, whereas those identified by whole-genome or whole-exome sequencing represent rare genetic variants with large effect sizes that might perhaps be limited to specific subgroups of women with PCOS[43]. Of note, targeted sequencing together with whole-genome sequencing approaches have identified 18 rare *AMH* variants[41] and 32 rare *DENNDA1A* variants that are specific to PCOS, suggesting that these genes are implicated in the pathogenesis of this syndrome. Further

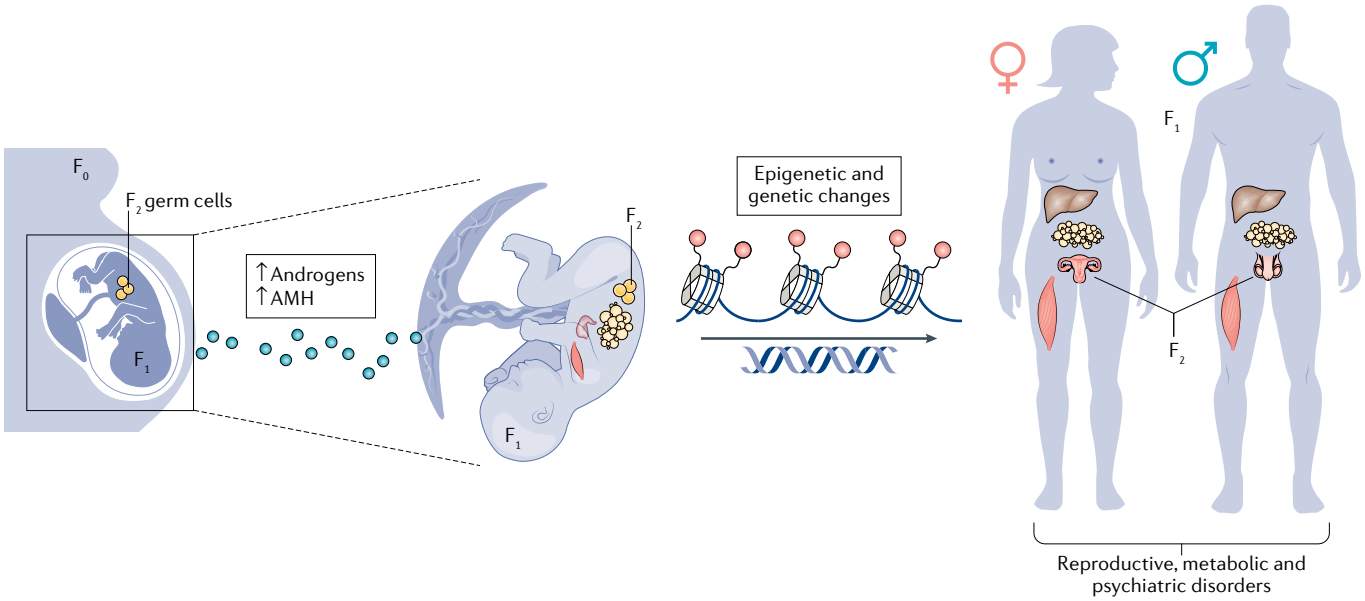

Fig. 2 | **Genetic and epigenetic mechanisms implicated in the inheritance of PCOS.** Both genetic and epigenetic factors contribute to the development of the phenotypic characteristics of polycystic ovary syndrome (PCOS). However, the PCOS loci identified in genome-wide association studies account for only 10% of its heritability and growing evidence suggests that alterations in epigenetic and developmental programming also contribute to its pathogenesis. During pregnancy, environmental factors, such as exposure to maternal androgen excess and/or anti-Müllerian hormone (AMH) excess, adversely affect placental function and could result in epigenetic changes that affect the somatic and germ cells of the growing fetus. For example, histone modifications, DNA methylation and transcriptional regulation by non-coding RNAs might predispose both female and male offspring to develop PCOS or PCOS-like phenotypic changes, including reproductive and metabolic abnormalities as well as neuropsychiatric disorders. F_0, the pregnant woman with PCOS; F_1, her first-generation offspring; F_2, her second-generation offspring.

rare genetic variants are expected to be identified; their contributions to PCOS will need to be investigated in future studies.

Given that the daughters of women with PCOS have an increased risk of developing the syndrome and that the sons of women with PCOS are also likely to have an increased risk of developing related traits (such as metabolic dysfunction), the inheritance of single-nucleotide polymorphisms associated with susceptibility to PCOS might have a phenotypic effect on the children of mothers with PCOS (FIG. 2). For example, the male children of women with PCOS who inherit PCOS-related genetic variations from their mothers have elevated markers of insulin resistance and β-cell dysfunction at ≤8 years of age and might therefore be more likely than children born to mothers without PCOS to develop metabolic dysfunction later in life[44]. In the same study, the daughters of women with PCOS had elevated circulating levels of AMH (which has been linked to the development of PCOS later in life) regardless of whether they had inherited PCOS-related genetic variants from their mothers, which suggests that non-genetic factors (such as the maternal–fetal environment) might play a role in the transmission of PCOS[44]. However, these data must be interpreted with caution owing to the fairly small sample size (172 children born to women with PCOS and 529 children born to mothers without PCOS, all of whom were conceived by assisted reproduction) and the fact that that these children might be too young to have developed overt phenotypic changes.

Epigenetic factors

The focus of research in epigenetics has gradually shifted over time from the early studies of embryonic development[45,46] and maintenance of cellular phenotype during mitosis[47] to the role of DNA methylation in gene regulation and cell differentiation[48,49]. The term epigenetics is now commonly used to refer to variations beyond those encoded by changes in the DNA sequence[50] and to include various cellular components and processes involved in transcriptional regulation, including DNA modification, chromatin structure, non-coding RNAs and nuclear architecture[51,52]. Importantly, epigenetic changes are not only heritable but also reversible in daughter cells following mitotic or meiotic division. Thus, epigenetic mechanisms could represent a bridge between early-life exposures and subsequent phenotypic variation and might contribute to disease transmission as well as to potential therapeutic interventions. Accordingly, we favour a broader definition of epigenetics as the study of molecules and mechanisms that can perpetuate alternative gene activity states in the context of an unchanged DNA sequence[53]. This definition is applicable not only to individual cells but also to organisms, a concept that is especially applicable to the study of transgenerational epigenetic inheritance[53].

DNA methylation

DNA methylation is one of the best-understood epigenetic systems and is of paramount importance in the regulation of gene expression in both health and

disease. DNA methylation can be detected at CpG islands, gene bodies (including exons and introns, that is, the gene region from transcription start to stop sites), imprinting control regions, and transposable elements and is associated with transcriptional repression and genomic imprinting.

Considerable knowledge has been acquired on the maintenance, establishment and erasure of DNA methylation. During DNA replication, the methylation pattern is accurately maintained by DNA (cytosine-5)-methyltransferase 1 (DNMT1) and the E3 ubiquitin-protein ligase UHRF1 (REFS[54,55]), which specifically binds to hemimethylated CpG dinucleotides at replication forks. In turn, UHRF1 recruits DNMT1 to release its auto-inhibition and methylate the daughter DNA strand[56]. The two major de novo DNA methylation enzymes in mammals are DNMT3A and DNMT3B[57,58]. DNMT3L is specifically expressed in the germline and is essential for maternal genomic imprinting. Although DNMT3L itself lacks any catalytic activity, the methyltransferase function is provided by its interaction with DNMT3A and DNMT3B[59]. DNMT3C also has de novo methylation activity. Its specific role in fetal spermatogenesis is silencing of the most recent and dangerous retrotransposons[60]. Essentially, DNA methylation ensures genomic stability.

Conversely, three methylcytosine dioxygenases (TET1–TET3) demethylate DNA via the oxidation of methylcytosine. In this process, 5-methylcytosine is progressively converted to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine[61–63]. Two major waves of global DNA methylation erasure occur in early embryogenesis and in germline development to remove acquired epimutations[64]. However, this erasure is incomplete and approximately 20% of methylated regions (6–8% of CpGs) remain methylated in each wave[64,65]. Thus, >116,000 genomic regions remain hypermethylated even after global demethylation in human primordial germ cells[66]. The DNA sequences that resist demethylation are largely those near transposable elements and imprinting control regions. However, some single-copy DNA sequences also remain unmethylated and might mediate epigenetic inheritance[67].

Chromatin modifications

In mammals, the majority of nucleosomes are replaced with protamines to facilitate compaction of the paternal genome during spermatogenesis[68]. However, some loci, particularly those housing important developmental genes and microRNA (miRNA) clusters, can retain nucleosomes and their associated histone modifications, which can epigenetically influence embryonic development and potentially also transmit regulatory states[69].

Both activating (H3K4me3) and repressive (H3K27me3) histone modifications are robustly passed down to embryonic cells from mammalian oocytes, which has potential implications for the regulation of embryonic development[70,71]. Transcriptional repression mediated by H3K27me3 also plays an important role in maternal imprinting, independent of DNA methylation, in mouse oocytes[70]. Constitutive heterochromatin (characterized by H3K9me3 modification) can also be epigenetically inherited in *cis* throughout multiple mitotic and meiotic cell divisions in mice and yeast[72]. Histone modifications also enable robust intergenerational inheritance through the paternal germline[69,73,74].

Non-coding RNAs

Many non-coding RNAs, especially small RNAs such as miRNAs, short interfering RNAs, transfer RNA-derived small RNAs (tsRNAs) and P-element-induced wimpy testis-interacting RNAs (piRNAs) function as heritable regulators of genomic elements in various species and contribute to both embryonic development and transmission of acquired phenotypes[67]. For example, many small RNAs act either within or outside chromatin to regulate transcriptional or post-transcriptional processes and thereby trigger long-lasting changes in gene expression in response to environmental cues[75]. Meanwhile, the abundance and composition of non-coding RNAs in germline cells are responsive to external cues that induce phenotypic changes. For example, small RNAs in mammalian sperm are increasingly recognized as another source of paternal hereditary information beyond DNA[76]. Causal evidence has been established from studies in which sperm RNAs from male mice exposed to various environmental stimuli (such as social stressors or an unhealthy diet) are microinjected into zygotes, resulting in altered metabolic and behavioural phenotypes in the offspring[77,78]. Maternal or paternal loss of miRNAs and tsRNA fragments are also implicated in the transmission of phenotypes to their offspring[79–81]. RNA modifications could also play an important role in the heritability of metabolic phenotypes[82].

Epigenetic contributions to PCOS

Although our current understanding of the pathogenesis of PCOS is incomplete, emerging evidence suggests that epigenetic modifications are strongly associated with susceptibility to PCOS and with its associated reproductive and metabolic dysfunction. Genome-wide epigenetic profiling of DNA methylation, histone modifications and non-coding RNAs has been performed in tissues derived from both animal models (BOX 2) and women with PCOS. Here, we summarize the latest research on the epigenetic alterations that accompany PCOS-related pathology.

Adipose tissue. Women with PCOS have dysfunctional adipose tissue with aberrant morphology with enlarged adipocytes, increased fat mass and decreased adiponectin production[83]. In a study that compared global DNA methylation patterns in subcutaneous adipose tissue from 64 women with PCOS and 30 healthy control individuals[84], a total of 440 sites with differential CpG methylation were identified, among which 33 were related to 30 differentially expressed genes. The 600 kb regions neighbouring these differentially expressed and differentially methylated genes (500 kb upstream and 100 kb downstream) were probed using 1,913 pairs of gene–CpG probes and found to be correlated with the transcriptional regulation of pathways involved in

Box 2 | **Animal models of PCOS**

Most animal models of polycystic ovary syndrome (PCOS) involve rodents, sheep and non-human primates, which enable the complex pathophysiology and molecular features of PCOS to be studied in multiple organs (extensively reviewed elsewhere[150]). Most models are based on maternal exposure to androgen excess during pregnancy and involve prenatal exposure of the fetus to testosterone, to the non-aromatizable androgen dihydrotestosterone (DHT, which specifically activates the androgen receptor (AR)) or to anti-Müllerian hormone (which indirectly stimulates hyperandrogenism)[150]. Other models involve continuous prepubertal exposure to testosterone, dehydroepiandrosterone or DHT[150]. These treatments all induce the development of PCOS-like traits in exposed offspring[120,150], suggesting that AR acts as a molecular gateway to PCOS pathophysiology.

Variation in the spectrum and severity of PCOS features in these models depend on exposure-related factors (that is, the androgen source, exposure period and stage of gestational development), suggesting the existence of discrete developmental windows during which androgen exposure might drive the pathogenesis of PCOS. This idea is in agreement with the developmental origins of health and disease hypothesis, which posits that early life represents a period of developmental plasticity that is critically important for adult metabolic health[151].

inflammation, adipogenesis, energy metabolism, sex hormone metabolism and glucose regulation[84]. Another study analysed DNA methylation at identified risk loci for PCOS and evaluated the single-nucleotide polymorphisms therein in subcutaneous adipose tissue from 23 women with PCOS and 13 control individuals, with the aim of identifying PCOS-specific alterations[85]. The researchers found decreased DNA methylation in the *LHCGR* locus and increased methylation in the *INSR* locus in the women with PCOS, together with complex patterns of methylation quantitative trait loci within these regions, indicating that local genetic variation is of importance in gene regulation. Interestingly, the DNA methylation patterns in subcutaneous adipose tissue show plasticity as they are reversible by weight loss[86] or therapeutic interventions such as low-frequency electrical stimulation[87].

The subcutaneous and visceral adipose tissue depots have different effects on metabolic function. Human studies to date have focused on subcutaneous fat owing to its ease of access, whereas visceral adipose tissue has been studied only in non-human primates exposed to maternal androgens in utero[88,89]. Only a fraction of the genes identified as differentially expressed in subcutaneous fat also exhibited differential DNA methylation in omental adipose tissue[88,89], probably owing to the stringent correlation criteria used in these two studies. The differentially methylated regions were enriched in transcription factor binding motifs and overlapped with differentially expressed genes involved in cellular metabolism and cAMP signalling[88].

In addition, 97 miRNAs are differentially expressed in women with and without PCOS, and these miRNAs are associated with impaired glucose and lipid metabolism as well as with reproductive system dysfunction[90]. This study identified the upregulation of miR-93 as a signature of PCOS. This miRNA downregulates the expression of solute carrier family 2, facilitated glucose transporter member 4 (also known as glucose transporter 4 (GLUT4)), which leads to insulin resistance. Further experiments confirmed that expression of miR-93 is negatively correlated with that of its host gene *MCM7* (encoding DNA replication licensing factor MCM7) in women with PCOS[91].

Skeletal muscle. Skeletal muscle accounts for the vast majority of glucose uptake from the circulation. Thus, insulin resistance in women with PCOS is associated with defects in insulin signalling in skeletal muscle, which are in turn a risk factor for T2DM[33].

Only one small study has investigated DNA methylation in skeletal muscle to date, in which 14 women with PCOS were compared with 11 control women without the disease[92]. Although the researchers found 85 differentially expressed transcripts, only two CpG sites remained differentially methylated after statistical correction for multiple comparisons[92]. The small number of associations detected could be related to the small sample size of this study. However, ~30% of the differentially expressed genes correlated with DNA methylation levels at CpG sites in or near skeletal muscle genes, which suggests that DNA methylation does affect the expression of these genes in women with PCOS[92]. Of note, low-frequency electrical stimulation-induced changes in DNA methylation in skeletal muscle correlated with changes in gene expression[93]. Other epigenetic mechanisms remain to be investigated in future studies.

Ovarian tissue and cumulus granulosa cells. Oligo-ovulation or anovulation, one of the key features of PCOS, is caused by the arrest of follicular maturation. As ovarian tissue from women with PCOS is scarce and difficult to obtain, only two studies to date have reported on DNA methylation profiles in human ovarian tissue[94,95] and they reached different conclusions. In one study, hypermethylated genomic regions in women with PCOS were preferably distributed on CpG island shores (that is, within 1–2 kb of a CpG island) and at promoters with a high CpG content, whereas hypomethylated genomic regions were found within gene bodies[94]. By contrast, the other study found that CpG islands and CpG island shores were hypomethylated in women with PCOS[95]. The disparate findings of these studies might be related to technical differences between the studies given that the ovarian tissue samples were obtained either by ovarian drilling[94] or via laparoscopic wedge resection[95], respectively. Moreover, the two studies also used different techniques to assess DNA methylation: targeted assays[94] or genome-wide sequencing[95]. Nevertheless, the results of these studies suggest that genes involved in hormone activity, inflammation, glucose metabolism and insulin signalling pathways are differentially methylated in women with PCOS versus those without this syndrome. Importantly, variants in *CYP19A1* (which encodes aromatase, the rate-limiting enzyme in oestrogen biosynthesis) have been linked to PCOS in GWAS and increased DNA methylation in the *CYP19A1* promoter correlates with the disturbed metabolism of androgens observed in women with PCOS. However, changes in DNA methylation only account for a small fraction of the differential gene expression observed in ovarian tissue from women with PCOS, an observation that is also true of other tissues[95,96].

Several studies of DNA methylation have been conducted specifically in ovarian granulosa cells, which have a fundamental role in steroidogenesis and folliculogenesis. Compared with other ovarian cell types, cumulus granulosa cells are relatively easy to access because they can often be stripped off from metaphase II (MII) oocytes that have been matured in vitro during in vitro fertilization. Targeted analyses of DNA methylation at genomic loci that include key genes in granulosa cells have identified hypomethylation in the promoters of *YAP1*, *LHCGR* (which encodes the LH receptor), *NCOR1* and *HDAC3* that correlates with ovarian pathology, including the proliferation of granulosa cells, overexpression of LH receptor and dysregulated hormonal signalling[97,98]. In addition, hypomethylation of CpG sites in the 5′ untranslated region of L1 (also known as long interspersed nucleotide element 1 (LINE1)) transposable elements has also been reported and is suggested to be an indication of overall DNA hypomethylation in granulosa cells[99]. By contrast, the promoters of *CYP19A1* and *PPARG* are hypermethylated, which indicates suppression of androgen metabolism[97].

In two studies of genome-wide DNA methylation in granulosa cells[100,101], women with both PCOS and obesity had 5,202 (versus women with PCOS but without obesity) and 6,936 (versus healthy control women) differentially methylated CpG sites. Intriguingly, twice as many differentially methylated CpG sites were identified when women of normal weight and with PCOS were compared with control women of normal weight[100]. This finding suggests that obesity affects the epigenetic programming of granulosa cells, which could be relevant to the pathophysiology of PCOS. In another study that used high-throughput, next-generation bisulfite sequencing (a more powerful technique than traditional (probe-based) microarrays), hypomethylation was noted in 977 CpG sites representing 2,063 genes, whereas hypermethylation was noted in 2,509 CpG sites within 1,777 genes[101]. These differentially methylated genes are associated with ovarian morphology, function and hormonal regulation.

Folliculogenesis and steroid synthesis are both regulated by miRNAs in granulosa cells[102]. In mice, loss of function of Dicer1 in these cells leads to infertility and multiple reproductive defects[103], including the accelerated recruitment of primordial follicles that fail to mature and eventually degenerate[104]. Specifically, the expression of miR-15a and miR-182 (which regulate proliferation, apoptosis and steroidogenesis in granulosa cells) is markedly decreased[104,105], whereas that of miR-93 and miR-21 (androgen-responsive factors in granulosa cells) is markedly increased in women with PCOS[106]. Members of the TGFβ family of growth factors are also implicated in granulosa cell growth and differentiation. The expression of miR-24 (which decreases TGFβ signalling and thereby inhibits oestradiol secretion) is elevated in women with PCOS. Furthermore, the expression of miR-27a-3p is implicated in hormonal dysfunction and apoptosis of granulosa cells and might be involved in the pathophysiology of PCOS[107].

Despite this growing circumstantial evidence, the role of epigenetic regulation in the pathogenesis of PCOS remains to be fully defined. Further studies are needed to identify the underlying molecular mechanisms and to investigate the functional consequences of epigenetic changes, both common and unique, across tissues. Crosstalk between these mechanisms might also be linked to the pathophysiology of PCOS.

Developmental drivers of PCOS

In the early 1980s, a series of epidemiological observations linking low birthweight and metabolic disease in adulthood among people living in socioeconomically deprived neighbourhoods led to the proposal that an adverse fetal environment followed by overnutrition in adulthood seems to result in chronic metabolic disease in adulthood, a phenomenon now termed the 'thrifty phenotype' hypothesis[108]. Further evidence in support of this theory came from a series of studies involving children conceived during the Dutch famine of 1944–1945 (known as the Hunger Winter), which affected 4.5 million people, directly caused at least 18,000 deaths and contributed to the deaths of many more individuals[109–113]. The results showed that maternal undernutrition during fetal development had long-term adverse effects on their children's metabolism and cardiovascular health, which persisted into adulthood and exacerbated the age-associated decline of cognitive function[109–111]. Moreover, studies in the Överkalix cohort (comprising 317 individuals from 277 families in north Sweden whose grandparents were exposed to sharp fluctuations in food availability) show that changes in food availability during pregnancy were linked to an increased risk of cardiovascular mortality and sudden death in paternal grand-offspring[112]. The results of these studies, together with many others that investigated the developmental origins of health and disease, revealed that critical time windows associated with organ development are sensitive to prenatal insults and are associated with the risk of disease in later life[11,23,114,115].

Maternal–fetal environment

Accumulating evidence supports the increased susceptibility of not only the daughters[23,116] but also the sons[117,118] of women with PCOS to this complex disorder and its comorbidities. Pregnant women with PCOS have more obesity than their counterparts without PCOS and also gain more weight early in pregnancy[119]. PCOS per se is an independent risk factor for both gestational diabetes and hypertension[9]. Moreover, women with PCOS retain hyperandrogenism (that is, high circulating levels of both androgens and AMH) throughout pregnancy[11,120], which diminishes placental aromatase activity[10,11]. All these features have detrimental effects on fetal development and thereby predispose the offspring of women with PCOS to a spectrum of reproductive, metabolic and psychiatric disorders[11,23] (FIG. 2).

The daughters of women with PCOS have an elongated anogenital distance[121], which is a strong marker of in utero androgen excess. Moreover, female fetuses and daughters of women with PCOS also have elevated AMH levels[122,123] and the increased density of small antral follicles in these individuals suggests intrinsic abnormalities in the ovaries that could be due to an increased

population of fetal ovary germ cells or to a reduced rate of oocyte loss occurring during late gestation or before the onset of puberty[30]. These observations suggest that the consequences of in utero androgen excess are apparent even at an early stage of ovarian development and oogenesis. These clinical features, together with the observation that the daughters of women with PCOS are five times more likely than their peers to be diagnosed as having PCOS[23] strengthen the hypothesis that PCOS has a developmental origin. Notably, the aberrant intrauterine environment of women with PCOS also affects male fetuses, albeit to a lesser extent than it does female fetuses. As a result, sons of women with PCOS have been far less thoroughly studied than daughters. The available studies include only limited numbers of male offspring and further investigation is warranted to define sexually dimorphic effects among the offspring of women with PCOS[16].

AR signalling is likely to be involved in the developmental origin of PCOS, which suggests that direct or indirect targeting of AR-driven pathways could be of therapeutic benefit. Interestingly, treatment with flutamide ameliorates reproductive and cardiometabolic phenotypes in mouse models of prepubertal DHT exposure and prenatal testosterone exposure. These findings support the notion that AR signalling pathways are molecular gateways to PCOS pathophysiology[124,125]. Moreover, treatment of AMH-exposed pregnant mice with cetrorelix acetate, a GnRH antagonist, prevented the development of increased anogenital distance and reproductive phenotypes resembling PCOS in the female offspring of these mice, by normalizing their circulating testosterone levels[120]. Neither antiandrogens nor GnRH antagonists are used for the treatment of pregnant women with PCOS owing to safety issues: antiandrogen treatment induces feminization of male fetuses and GnRH antagonist treatment can cause miscarriage or abnormalities in the developing fetus. However, both types of agents are useful in animal experiments, for example, to investigate whether the adverse effects of androgen or AMH exposure are triggered by direct activation of AR or whether they are mediated via neuroendocrine pathways as well as to identify potential druggable targets downstream of AR.

Transgenerational transmission

Transgenerational phenotype transmission through the germline has the potential to be either detrimental or adaptive, both of which have implications for evolution. One possible mechanism that might relay persistent phenotypic changes is germline transmission of epigenetic markers. Intergenerational and transgenerational epigenetic inheritance (also known as meiotic epigenetic inheritance) is well documented in plants, yeast, and the nematode *Caenorhabditis elegans* and involves miRNAs and chromatin modifications (reviewed elsewhere[126–128]). However, in mammals, resetting of the epigenome occurs during gametogenesis, which explains why meiotic epigenetic inheritance has been demonstrated in mammals only in very few, exceptional situations such as at the agouti viable yellow (A^{vy}) locus, in which ectopic expression of agouti protein is controlled by epigenetic activation of a retrotransposon[129].

Functional gametes in mammals are gradually developed from primordial germ cells (PGCs). PGCs are initially derived from pluripotent epiblast cells under the regulation of WNT and BMP signalling pathways[130,131]. One unique feature of germ cell development is that PGCs undergo extensive epigenetic reprogramming, including genome-wide passive and active loss of 5-methylcytosine, as they migrate to the gonads and become gonocytes[132]. Prior to sex determination, germ cells reach the lowest DNA methylation level[133], at which only ~10% of 5-methylcytosines remain. Following sex determination, de novo methylation in germ cells occurs in a sex-specific manner, such that the methylation pattern is fully established before birth in the male germline and shortly after birth (during the oocyte growth phase) in the female germline[133]. In contrast to pre-implantation, demethylation in PGCs is pervasive and occurs in sequential stages, which eventually result in demethylation in imprinting control regions, endogenous retrovirus intracisternal A particle regions, and the promoters of genes involved in meiosis and gamete generation[134,135]. The evolutionary advantage of first removing and then re-establishing the epigenome is that it ensures the erasure of any unwanted modification of the parental genome imposed by the environment, thereby faithfully transmitting the original genetic blueprint for the next generation. However, although demethylation occurs over most of the genome, not all genomic sequences are processed with the same efficiency[136,137]. Moreover, DNA methylation is maintained at some sites, including at potentially deleterious retroelements and intracisternal A particle retrotransposons and at some repetitive elements and regions of the genome that have been associated with metabolic and neurological diseases. The retention of DNA methylation represents a potential route to transgenerational inheritance through the germline[53,138]. In addition, histone modifications and small non-coding RNAs have also been suggested as potential routes for meiotic epigenetic inheritance[67,75].

Nonetheless, epigenetic changes in gene expression during early mammalian development occurring in response to intrauterine exposures can result in persistent functional changes. In mouse models, the sperm of the first generation (F_1) of male offspring born to starved dams showed locus-specific hypomethylation of genes associated with metabolic functions. Second generation (F_2) male offspring derived from these F_1 male mice also manifested a metabolic phenotype and an aberrant methylation pattern in regulatory genomic regions[139]. In humans, a comprehensive analysis of genes showing differential DNA methylation in whole blood from individuals (then aged in their late 60s) with and without early gestational exposure to the Dutch famine identified key regulatory regions within promoters, open chromatin and enhancers with a critical role in early development[140]. Genes regulated by these regions were involved in pathways related to growth and metabolism[140]. These results also suggested that prenatal famine exposure had a long-term effect on the phenotypes of these individuals.

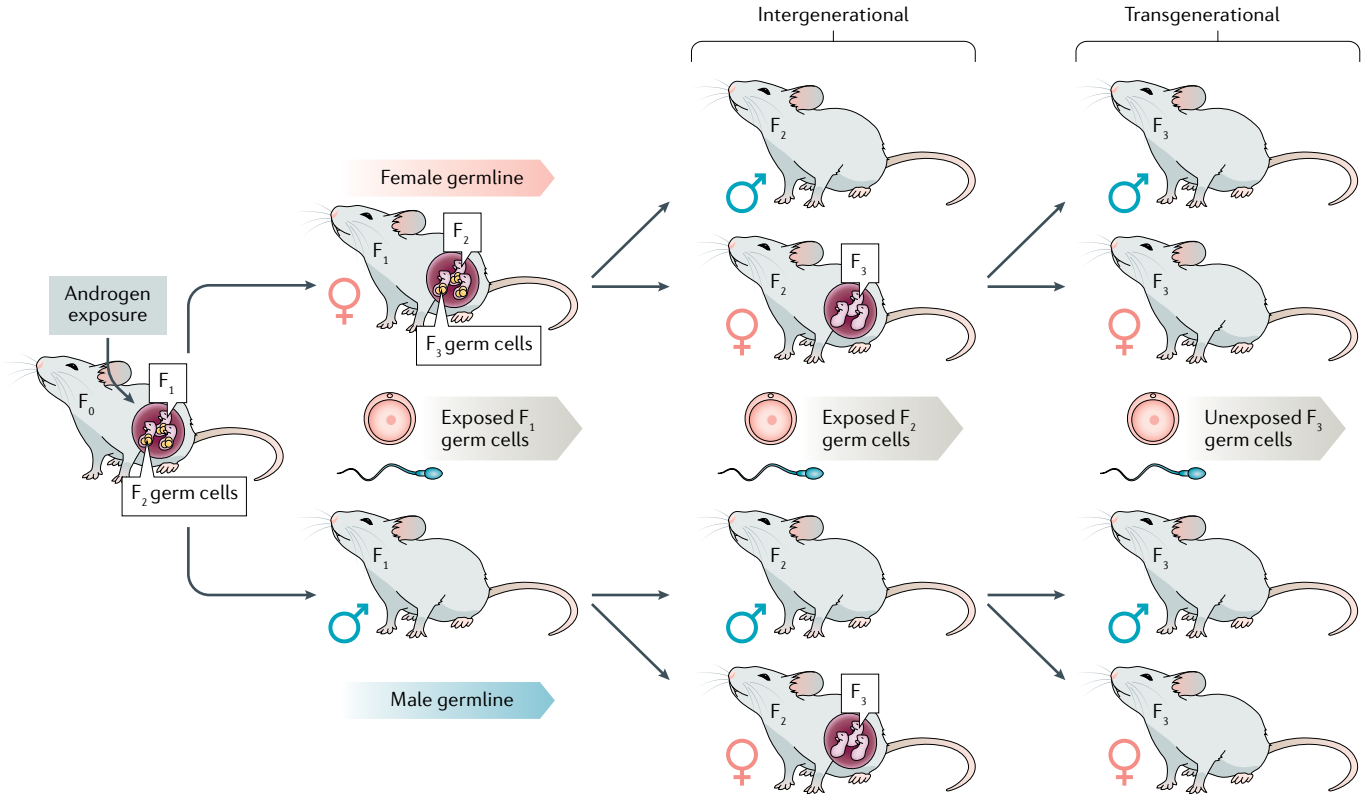

Fig. 3 | **Distinguishing between the effects of in utero environment and germline transmission.** The direct effects of in utero environment on germline development are a confounding factor in the study of epigenetic inheritance via the maternal germline. As F_1 fetus and F_2 germ cells are both exposed to the initial adverse uterine environment that occurred in the F_0 generation, the F_3 generation is the first to be free from that initial exposure. Hence, transgenerational inheritance can only be concluded if the phenotypes of interest persist into the F_3 generation. Whether the F_2 sons of F_1 daughters with polycystic ovary syndrome (PCOS) or the F_2 daughters of F_1 sons of mothers with PCOS are at an increased risk of developing reproductive, metabolic and psychiatric disorders remains unclear.

Epidemiological studies have found that the grandchildren of individuals who experienced the Dutch famine also share their increased susceptibility to cardiovascular and metabolic diseases[141,142].

Genuinely transgenerational effects refer to the first generation for which phenotypic traits cannot be ascribed to direct exposure of the affected offspring[143]. As exposure of a pregnant female (F_0) also exposes the fetus (F_1) and the fetus' germ cells (future F_2) to the same event in utero (FIG. 3), only phenotypic traits occurring in F_3 female (or F_2 male) offspring can be attributed to true transgenerational exposures transmitted via the germline (FIG. 3). However, it is nearly impossible to perform longitudinal studies in humans spanning multiple generations to investigate whether the granddaughters and great-granddaughters of women with PCOS have an increased propensity to develop the syndrome. By contrast, many studies have been published on the transgenerational effects of exposures to environmental factors in mice and rats[53,144,145]. These factors include endocrine disruptors[146], nutrition[147], trauma and stress[148]. Animal models of PCOS (BOX 2) have similarly facilitated longitudinal studies of the effects of this syndrome over multiple generations (FIG. 3). Our group has demonstrated that PCOS-like reproductive and metabolic traits can be identified in the female offspring of pregnant dams exposed to androgens, up to the third generation — that is, in the daughters (F_1), granddaughters (F_2) and even great-granddaughters (F_3) of exposed dams. This effect was not observed with in utero exposure to obesity on its own[23]. Of interest, a study published in 2021 that employed a similar experimental design but used a different mouse model of PCOS (involving prenatal AMH exposure) also showed that the development of PCOS-like traits can be transgenerationally passed on to the F_3 female offspring of exposed dams[149].

As a result of factors affecting the intrauterine environment, the germ cells inside the developing fetus could be either genetically or epigenetically modified and pass on these modifications to future generations. To define the molecular mechanisms driving transgenerational transmission, our group has conducted ultrastructural and molecular analysis of mature MII mouse oocytes from F_1 to F_3 generations. Common alterations identified in the transcriptome of MII oocytes affect genes involved in RNA binding, DNA repair, germ cell development and signalling pathways related to reproductive processes[23]. Although we did not conduct epigenetic profiling in this study, this altered gene expression is probably the result of epigenetic modification in the genome. Potential molecular pathways underlying the transmission of PCOS-like phenotypes to F_3 offspring have also been

Box 3 | **Outstanding research questions**

To further advance the understanding of polycystic ovary syndrome (PCOS), several critical research questions remain unexplored:

- To understand the causal effect of genetic factors in PCOS, for example, by investigating the functional consequences of rare genetic variants on PCOS aetiology and pathophysiology in mice.
- To understand the genetic and epigenetic mechanisms underlying the heterogenous phenotypes of PCOS and the causal association of the PCOS diagnosis with these phenotypes.
- To reveal the sex-specific effects of PCOS on the offspring of affected mothers.
- To define the key trigger(s) that cause the transmission of PCOS phenotypes and how female and male individuals are affected.
- To determine whether the sons of women with PCOS can transmit the physiological dysfunction to their offspring.
- To examine the comprehensive molecular signatures, including gene expression and epigenetic modification of the germline, and somatic tissues involved in the transmission of a PCOS-like phenotype.
- To identify novel predictive and diagnostic biomarkers, such as circulating molecules, that enable the early identification of daughters and sons at risk of developing PCOS.
- To develop and investigate treatments based on targeting epigenetic modulation.
- To develop preventive strategies that might decrease the prevalence of PCOS and associated health-care costs in future generations.

investigated by comparing whole ovaries of F_1 control mice to those of F_3 offspring of an AMH-exposed dam using RNA sequencing and methylated DNA immunoprecipitation combined with sequencing[149]. The results showed that 4 of 102 differentially expressed genes were directly regulated by differential methylation and that these genes were implicated in Slit–Robo signalling, Notch signalling, inhibition of cell proliferation and inflammation. The gene loci with altered DNA methylation included *Tet1* and *Uhrf1*, which suggests that transmission of PCOS-like phenotypes involves epigenetic mechanisms[149]. However, as the whole ovary was sequenced in this study, whether these effects result from epigenetic programming of somatic cells, germ cells or both remains to be determined. Furthermore, 15 days of treatment with the universal methyl group donor *S*-adenosylmethionine partially rescued the reproductive and metabolic traits observed in 6-month-old F_3 female offspring[149], although methylation of the targeted genes and others was not examined to evaluate the specificity of this treatment. These first pieces of evidence in support of the epigenetic inheritance of PCOS across generations provides an intriguing explanation for the increasing prevalence of PCOS.

Current studies have focused on female offspring and the extent to which male offspring are also affected is unclear. If male and female offspring are both equally affected, the effects of PCOS might be potentially much more widespread than is currently appreciated. Moreover, if germ cells are able to drive the transmission of PCOS and related disorders, both women and men could transmit PCOS to future generations.

Conclusions and future directions

Women with PCOS have abnormally high levels of circulating androgens and AMH throughout pregnancy[11,120] and this adverse intrauterine environment has a negative influence on fetal development. PCOS is known to be inherited, but further investigation is required to distinguish transgenerational epigenetic inheritance through germ cells from the direct effects of the adverse intrauterine milieu on children born to mothers with PCOS. Importantly, growing evidence suggests that a male form of PCOS might also exist.

Susceptibility to PCOS is inherited not only by genetic alleles but also via epigenetic changes and developmental programming, which might contribute to its increasing prevalence. Moreover, the PCOS-like traits induced by androgen exposure during pregnancy in mice are passed on from mothers (F_0) to daughters (F_1), granddaughters (F_2), and great-granddaughters (F_3) and are accompanied by transcriptional and mitochondrial perturbations of oocytes[23]. Importantly, several of the identified candidate genes in mouse ovaries were hypomethylated and several of the oocyte gene signatures that accompanied transgenerational transmission in mice were also detectable in sera from daughters of women with PCOS and in the adipose tissue of unrelated women with PCOS[23,149], indicating communication between germ cells, serum, and somatic cells or tissues and supporting the translational relevance of the mouse findings. Thus, the PCOS-induced adverse intrauterine environment during pregnancy affects not only the developing fetus itself but also the germ cells of that fetus, which could undergo genetic and/or epigenetic changes that can be passed on to future generations. Whether germ cells per se can drive disease transmission across generations in the absence of direct developmental programming in the fetus requires further investigation. Cross-comparisons of epigenetic modifications across various disruptors accompanied by epigenetic inheritance to future generations would be valuable to define common or unique features. Moreover, the different androgen triggers and molecular pathways involved in the inheritance of PCOS-like phenotypes need to be delineated in female and male mice to determine whether AR is the gateway to transmission.

Current management of PCOS is symptom based and hindered by a lack of insight into the origin of and pathogenetic mechanisms underlying this syndrome (BOX 3). Accordingly, of greatest importance is the identification of the molecules that mediate the transmission of PCOS (including small non-coding RNAs, histone modifications and DNA methylation), which could provide new avenues for treatments, including those based on epigenetic modulation. Future research aimed at the fundamental understanding of disease transmission and identification of candidate biomarkers of transmission will ultimately transform the diagnosis and management of PCOS by facilitating the early detection of susceptibility to PCOS and associated disorders. In turn, these approaches raise the possibility of preventing this syndrome and its associated comorbidities, which could markedly reduce the substantial socioeconomic burden of PCOS.

Published online 7 July 2021

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Acknowledgements
The authors' research is supported by the Swedish Medical Research Council (Project No. 2018-02435: E.S.V., 2018-02557: Q.D.), Novo Nordisk Foundation (NNF19OC0056647: E.S.V.), the Strategic Research Programme in Diabetes at Karolinska Institutet (E.S.V.), Adlerbert Research Foundation (E.S.V.), Åke Wibergs Stiftelse (Q.D.) and faculty funding at Karolinska Institutet (Q.D.).

Author contributions
E.S.V. and Q.D. contributed equally to all aspects of the article.

Competing interests
The authors declare no competing interests.

Peer review information
Nature Reviews Endocrinology thanks E. Diamanti-Kandarakis, H. Teede, who co-reviewed with A. Johan, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.