

Pathomechanisms of polycystic ovary syndrome: Multidimensional approaches

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Genetic approaches to unraveling PCOS pathophysiology
 - 3.1. Background
 - 3.2. GWAS in Asian populations
 - 3.3. GWAS in Caucasian populations
 - 3.4. GWAS-based Genotype-phenotype associations
 - 3.5. Going beyond GWAS
 - 3.6. Concluding remarks
4. Epigenetic approach to understand the pathophysiology of PCOS
 - 4.1. Background
 - 4.2. Theories on fetal reprogramming and developmental origins of PCOS strengthened by circumstantial epigenetic evidence
 - 4.3. Evidence on the role of epigenetic alterations in PCOS development
 - 4.3.1. Evidence based findings from drug-induced animal models of PCO
 - 4.3.2. Epigenetic studies conducted in women with PCOS
 - 4.4. Non-coding RNAs as additional epigenetic regulators in PCOS
 - 4.5. Concluding remarks
5. Proteomic attempt to unravel PCOS pathophysiology
 - 5.1. Introduction
 - 5.2. Samples used for proteomic studies in PCOS
 - 5.3. Differentially regulated common proteins identified across proteomic studies in PCOS
 - 5.4. Concluding remarks
6. Conclusion
7. Acknowledgements
8. References

1. ABSTRACT

Polycystic ovary syndrome is a complex endocrine disorder affecting numerous women of reproductive age across the globe. Characterized mainly by irregular menses, hirsutism, skewed LH: FSH ratios and bulky polycystic ovaries, this multifactorial endocrinopathy results in unfavorable reproductive and metabolic sequelae, including anovulatory infertility, type 2 diabetes, metabolic syndrome and cardiovascular disease in later years. Increasing evidence has shown that the manifestation of polycystic ovary syndrome (PCOS) is attributable to a cumulative impact of altered genetic, epigenetic and protein profiles which bring about a systemic dysfunction. While genetic approaches help ascertain role of causal variants in its etiology, tissue-specific epigenetic patterns help in deciphering the auxiliary role of environmental, nutritional

and behavioral factors. Proteomics is advantageous, linking both genotype and phenotype and contributing to biomarker discovery. Investigating molecular mechanism underlying PCOS is imperative in order to gain insight into the pathophysiology of PCOS and formulate novel diagnostic and treatment strategies. In this review we have summarized these three aspects, which have been successfully utilized to delineate the pathomechanisms of PCOS.

2. INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrinopathy associated with derangements in insulin secretion and action, androgen synthesis and

action, relative gonadotropin ratios, ovulatory function, and balance of pro- and anti-oxidant systems. PCOS is a lifelong disorder and the metabolic and reproductive ramifications of this syndrome are well documented in all stages of life. Women with PCOS are frequently obese with distinct abdominal or central obesity which in turn aggravates insulin resistance. These women are at a higher risk of developing impaired glucose tolerance (IGT), T2DM, dyslipidemia, cardiovascular diseases, hypertension and ultimately metabolic syndrome in their later lives (1). (Apart from physiological maladies, studies have suggested that women with PCOS often show symptoms of negative body image perception, low self-esteem, depression, and decreased quality of life (2-4). The elusiveness of the pathophysiology of this syndrome coupled with the substantial increase in prevalence amongst both adolescent girls and reproductive aged women which has garnered the interest of basic scientists as well as clinicians.

PCOS is a multifactorial disorder where genes individually, gene-gene interaction or gene-environment interactions have been reported to influence predisposition to PCOS development. Previous literature has reported the importance of genetic predisposition to PCOS development; however no consensus has been reached on an established genetic marker for PCOS. Identifying causal variants in genes which may alter its expression or subsequent protein function helps to delineate the genetic architecture of this multifactorial disorder (5). Tissue specific epigenetic alterations which do not affect the genetic code have been reported to be responsible for phenotypic plasticity and are mainly achieved by mechanisms involving the addition or removal of chemical groups in chromatin (6). Further, proteome profiling of pathophysiologically relevant tissues helps to elucidate the dynamic changes in the cellular proteome (7). Exploring genetic and epigenetic patterns with their consequent impact on protein profiles has contributed towards biomarker discovery and unraveling the molecular pathophysiology of PCOS. Thus, employing a multi-pronged approach and harnessing cutting-edge technologies has yielded copious and comprehensive data, which in turn has expanded and shaped the current understanding of PCOS etiology. Insight into this condition as well as its far-reaching consequences will provide both researchers and clinicians new avenues into understanding the association between the various facets of this syndrome and treating this hormonal imbalance successfully and restoring fertility. The present review seeks to summarize significant findings which have emerged from recent PCOS research in three major fields.

3. GENETIC APPROACHES TO UNRAVELING PCOS PATHOPHYSIOLOGY

3.1. Background

The progress in studies on PCOS genetics has grown in revolutionary leaps and bounds. Evidence

derived from earlier twin studies and family based association studies have substantively recognized the genetic etiology of PCOS. Chromosome anomalies were detected and autosomal dominant mode of inheritance was initially proposed based on segregation analyses (8-10). Gradually twin studies began to emerge linking genetic and environmental factors in the onset of the syndrome (11, 12) and identical twins have been reported to present with amenorrhea, polycystic ovaries and androgen overproduction with increased luteinizing hormone (LH) levels (13, 14). Familial clustering of cases (15) along with increased prevalence of PCOS-like features and co-morbidities in both male and female relatives of affected women further strengthens the basis for the genetic component of PCOS (16, 17). An early communication by Legro *et al.*, has reported that there is a strong aggregation of LH abnormalities and both ovarian and adrenal hyperandrogenemia in sisters of PCOS women (18). Furthermore both male and female first degree relatives were inclined towards hyperandrogenism and displayed adrenal or gonadal hormonal abnormalities including increased testosterone, androstenedione and dehydroepiandrosterone sulphate (DHEAS), suggesting hereditary predisposition to abnormal steroidogenesis (16). Risk of development of diabetes, metabolic syndrome and cardiovascular disease is also significantly higher in relatives of women with PCOS compared to unaffected women (19-23). PCOS is at present considered to be a multigenic disorder where genes may individually or in combination with other genes contribute to the inherited risk of this complex disorder (24). Researchers have combed the genome for an appropriate universal marker and selected genetic loci in several relevant pathophysiological pathways in order to identify susceptibility variants; however the exact mechanism of the genotype-phenotype relationship is largely unknown in scores of studies (25). Genes governing the insulin signaling pathway, insulin resistance, ovarian steroidogenesis, and inflammation have chiefly been pinpointed by many groups to be associated with risk of PCOS development (26-28). Despite the upsurge in the number of candidate gene studies, we have gained only small clues to generate bigger and more comprehensive genetic predisposition profiles. The main challenges encountered in deriving notable conclusions for genetic markers is due to lack of uniform diagnostic criteria, complex mode of inheritance, sample size variation and ethnic diversity (29). Ethnic disparities account for both genetic and phenotypic dissimilarities in PCOS pathogenetic outcomes. In this review, we focus on association studies of polymorphisms in key candidate genes which have been extricated from the expansive data generated by the application of a hypothesis free approach to establish strong genetic associations of loci with propensity to PCOS.

Advances in genotyping strategies have enabled determination of the differential frequencies of millions of gene variants between healthy and

diseased conditions on a single platform in the most time efficient manner(30) and have captured the attention of all researchers worldwide. The avant-garde technique of genome-wide association studies (GWAS) involves a de novo analysis to decipher the underlying genetic architecture of complex diseases and is not contingent on prior knowledge of the role of various genes in its pathophysiology (30-32).

3.2. GWAS in Asian populations

With the goal of elucidating genetic predisposition patterns of PCOS, a pioneering GWAS in a discovery cohort of Han Chinese population of healthy and PCOS women categorically identified three risk loci, 2p16.3, 2p21 and 9q33.3 mapping to *LHCGR*, *THADA* and *DENND1A* to be associated with PCOS susceptibility. They validated their findings in two separate, well-sized replication cohorts and further conducted a meta-analysis using data from all three studies, only to reiterate a strong association of 28 polymorphisms in these three loci with PCOS risk with no confounding effect of age and body mass index (BMI)(33). A subsequent study by the same group additionally identified eight more loci, accounting for a total of 11 risk loci for PCOS development in women of Han Chinese ancestry (34). Meticulous examination revealed that these loci mapped to the following genes: *LHCGR*, *FSHR*, *THADA*, *DENND1A*, *C9orf3*, *RAB5B*, *YAP1*, *HMGA2*, *TOX3*, *INSR* and *SUMO1P1*(34). These genes were found to be chiefly involved in steroidogenesis, gonadotropin action and regulation, follicular development, insulin signaling and type 2 diabetes mellitus (T2DM), calcium signaling and endocytosis (34). Sufficient overlap in the genes associated with both T2DM and PCOS namely the *INSR*, *HMGA2* and *THADA* genes was evident (34). In contrast, another study reported lack of association of T2DM loci with PCOS in both case-control and family based Caucasian study cohorts but these loci may contribute to the insulin resistance phenotype (35). Moreover, genes associated with cardiometabolic traits including obesity, cardiovascular, lipid and diabetes related traits were not found to be major risk factors for the genetic risk of PCOS (36). A Korean study revealed that the *GYS2* gene was associated with obesity in PCOS women rather than development of the syndrome itself (37). A novel susceptibility locus at 8q24.2 mapping to *KHDRBS3* was identified to decrease risk of PCOS development in a recent GWAS conducted in Korean population (38). This gene was reported to be associated with telomerase activity which in turn could contribute to telomere shortening. Shorter telomere lengths have been reported to be associated with early senescence of pancreatic B cells, resulting in dysregulated insulin secretion and action. The consequent hyperglycemia may in turn

inhibit telomerase activity propagating this vicious cycle to bring about adverse metabolic outcomes (39). Major reproductive anomalies including poor oocyte quality and infertility could also be attributed to short telomere length (38). Genes in other loci such as *TRIML1*, *TRIML2*, *SOX8*, *LMF1*, *SSTR5*, *GABRA4*, *GABRB1*, *COMMD8*, *PEX5L*, *USP13*, and *HIPK3* have been implicated in diverse functions such as embryo development, insulin secretion and glucose metabolism, insulin resistance, inflammation and steroidogenesis in Korean population (38); however these have not been validated in other populations.

3.3. GWAS in Caucasian populations

It has been established that geographic and ethnic variations immensely impact the genetic makeup of various populations and risk conferred in some populations may not be accurately reproduced in others. Moreover, phenotypic diversity in PCOS is common in populations of different ethnic origins(40). Hence, studies were initiated to predict whether any of these genes, previously identified in Asian populations may predispose women of European descent to PCOS onset. A large-scale GWAS was performed in a sizeable discovery cohort followed by two replication cohorts in Caucasian women of European ancestry (41). In this study, PCOS was diagnosed using the NIH criteria, allowing for exclusion of phenotypes comprising polycystic ovary (PCO) morphology, unlike the Han Chinese study which utilized Rotterdam diagnostic criteria. Hayes' group reported significant association of only three genetic loci mapping to the 8p32.1 *GATA4/NEIL2* locus, the 9q22.3.2 *c9orf3/FANCC* locus, and 11p14.1 *FSHB/ARL14EP* locus (41). Of these, only 9q22.3.2 locus corresponding to *c9orf3* intersected with previously reported GWAS loci in Han Chinese suggesting that this may be an evolutionarily conserved genetic marker. Importantly, a variant in *FSHB* showed association with PCOS in an LH-dependent manner which corroborates the role of altered gonadotropin secretion in PCOS pathophysiology in European women (41). Detection of significant association of variants in 8p32.1 involved in cholesterol biosynthesis (*FDFT1*), and regulation of steroidogenic gene transcription (*GATA4*), suggests altered testosterone biosynthesis, in European PCOS (41). Additionally, genes required for DNA damage repair (*NEIL2*), suppression of apoptosis (*FANCC*) and movement of major histocompatibility complex(MHC) Class II containing vesicles (*ARL14EP*) were found to be associated with PCOS in European women (41). This study also confirmed that polymorphisms in *THADA*, *LHCGR*, *DENND1A*, *FSHR*, *YAP1*, and *RAB5B/SUOX* were positively associated with PCOS risk in Caucasian women suggesting the presence of common genetic susceptibility loci. A subsequent

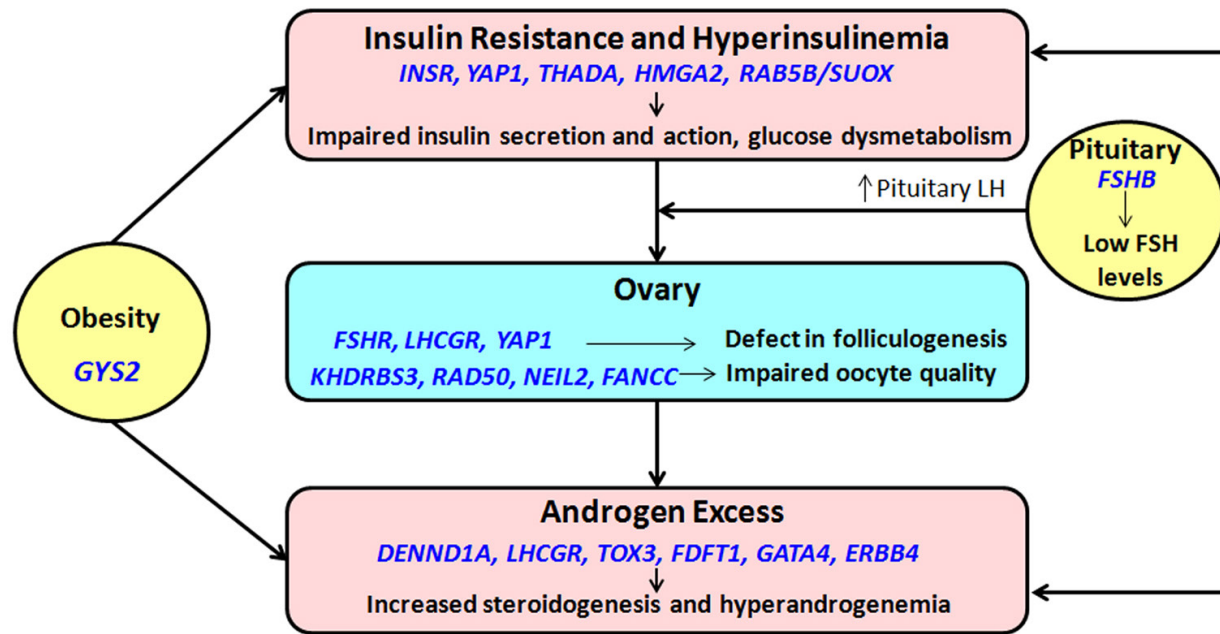


Figure 1. The schematic shows the primary underlying mechanism of PCOS pathophysiology characterized by its hallmark features of namely insulin resistance and androgen excess. This vicious cycle is exacerbated by obesity coupled with an integral role of pituitary gonadotropins resulting in disrupted physiology and functioning of the ovary. We have highlighted the genes (in blue) identified from prior GWAS whose polymorphisms have been postulated to influence these phenotypic outcomes. DENND1, DENN domain containing 1A; ERBB4, erb-b2 receptor tyrosine kinase 4; FANCC, Fanconi anemia complementation group C; FDFT1, farnesyl-diphosphate farnesyltransferase 1; FSHB, follicle stimulating hormone beta subunit; FSHR, follicle stimulating hormone receptor; GATA4, GATA binding protein 4; GWAS; Genome wide association study; GYS2, glycogen synthase 2; HMG2, high mobility group AT-hook 2; INSR, insulin receptor; KHDRBS3, KH RNA binding domain containing, signal transduction associated 3; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; NEIL2, nei like DNA glycosylase 2; PCOS, Polycystic ovary syndrome; RAB5B, member RAS oncogene family RAD50; RAD50, RAD50 double strand break repair protein; SUOX, sulfite oxidase; THADA, thyroid adenoma-associated gene ; TOX3, TOX high mobility group box family member 3; YAP1, yes-associated protein 1

study (42) in European Caucasian women achieved genome-wide significance for four novel genetic loci, in addition to confirming previously reported signals at *YAP1*, and *THADA*. However, the polymorphisms identified in the same genes were different from those reported earlier suggesting that despite common loci, PCOS susceptibility may be attributed to ancestry based changes in specific markers. The *ERBB4* gene in the region of 2q34 is a member of the epidermal growth factor receptor (EGFR) family and is reported to mediate LH-induced steroidogenesis and cancer cell proliferation and neovascularization, thereby suggesting a putative link between PCOS and cancer risk. Interestingly, strong evidence of positive correlation of *ERBB4* with PCOS was found in Chinese women has recently come to the fore, highlighting this gene to be a putative genetic link between different ethnic populations (43). The rs11031006 variant of *FSHB* gene at 11p14.1 has been previously linked to decreased follicle stimulating hormone (FSH) levels and may play a role in folliculogenesis. The *RAD50* gene in the region of 5q13.1 is actively involved in DNA double-strand break repair, whereas the *KRR1* gene in the region of 12q21.2 encodes a ribosome assembly factor (42). The causal association of these variants with increased obesity

and insulin resistance, and decreased sex hormone binding globulin (SHBG) levels by Mendelian randomization analyses has proposed the benefit of suitable treatment interventions (42). A striking result to emerge from Day's study is that certain genetic variants are associated with delayed menopause suggesting that the reproductive lifespan of affected women is longer, though they remain sub-fertile (42, 44). Collectively, GWASs from European cohorts underline the role of neuroendocrine dysfunction and altered DNA repair mechanisms in PCOS pathogenesis. However, these novel loci need to be explored thoroughly in other populations to confirm these findings.

3.4. GWAS-based genotype-phenotype associations

Upon discovery of key variants, quite a few replication studies were initiated to link these variants to PCOS development in different populations as well as to determine putative genotype-phenotype relationships in both family based and case-control association studies. Based on their association with phenotypic traits, we have summarized the role of GWAS variants in the pathophysiology of PCOS (Figure 1).

DENND1A, mapping to 9q33.3, encodes for DENN proteins which mediate Rab35 activity essential for regulating endosomal membrane trafficking pathways (45). Intense localization of a splice variant, *DENND1A* v.2 compared to *DENND1A* v.1 was observed in theca cells derived from PCOS ovaries (46). Moreover, its overexpression in normal ovaries promoted elevated steroidogenesis and consequently favoring PCOS phenotype; while knockdown or addition of antibody directed to its epitopes downregulates both *CYP11A1* and *CYP17* gene transcription and reduces unwarranted steroid production (46). These findings highlight *DENND1A*'s role in the hyperandrogenemia aspect of PCOS. Several studies have looked for association of *DENND1A* variants, mainly rs2479106, rs10818854 and rs10986105, with PCOS and its related phenotypes in ethnically diverse populations. Contradictory findings have revealed that some polymorphisms of *DENND1A* show significant association with PCOS susceptibility in both Caucasian (47-51) and Han Chinese (52) study populations, while a lack of association altogether was reported for some polymorphisms in European (49, 53), Chinese (52, 54) and Bahraini (55) women. Apart from association of *DENND1A* polymorphisms with hyperandrogenism(49), they may play a role in regulating insulin sensitivity as demonstrated by raised 2 hour insulin levels post an oral glucose tolerance test in Chinese women (56, 57). Polymorphisms are also associated with increased waist-to-hip ratios and dyslipidemia in Caucasian women (53) as well as in reproductive traits including irregular menses (49) and PCO morphology (58). A recent meta-analysis revealed significant association of both rs2479106 and rs10818854 with increased risk of PCOS development (59). On the other hand, Gao *et al.*, have reported that while rs10818854 and rs10986105 increase PCOS risk overall, rs2479106 influences PCOS susceptibility only in Asian but not in European populations (60).

The thyroid adenoma-associated gene (*THADA*), mapping to chromosome 2p21 alters pancreatic beta cell function and impacts insulin secretion (61). Contradictory reports evidencing *THADA* as a genetic factor in PCOS pathogenesis are available (47, 48, 50-52, 54, 56, 62). Transmission disequilibrium tests have revealed that the *THADA* gene may be a potentially strong susceptibility locus for PCOS risk in Chinese population (63). Multiple polymorphisms have also been reported to be associated with increased LH levels and LH:FSH ratio (56) and presence of polycystic ovaries (58) in Han Chinese women with PCOS along with increased circulating testosterone levels in both Chinese (56) and Caucasian (62) women. Association of rs12478601 polymorphism of *THADA* with increased serum low density lipoprotein (LDL) levels in Chinese women (56, 57) supports its role in influencing lipid metabolism. Recently it was observed that carriers of rs12478601 variant of *THADA* were more responsive to metformin treatment (64).

The luteinizing hormone/chorionic gonadotropin receptor (LHCGR) is a G protein-coupled receptor characterized by a large extracellular hormone binding domain found primarily in theca cells and mature granulosa cells of the adult ovary, stromal cells, and luteinized cells in females and Leydig cells of testis in males (65). Polymorphisms which alter receptor expression and activity detected in *LHCGR* gene may encourage the inherently increased LH to drive androgen production from the ovary, thereby affecting ovulation. Granulosa cells derived from women with PCOS have been reported to overexpress LHCGR coupled with significantly higher cyclic adenosine monophosphate (cAMP) accumulation which could stimulate steroidogenesis, despite no alteration in LHCGR internalization (66). An intronic variant rs13405728 was the most significant predictor of PCOS in the initial Han Chinese GWAS (34) and has been successfully replicated in other Han Chinese (52, 56) and Hui Chinese (54) cohorts. However studies in Caucasian (47, 48, 53, 67) and Bahraini Arab (68) populations did not support this association. Meta-analysis of studies conducted in Dutch, American and Chinese women revealed that rs13405728 polymorphism was significantly associated with PCOS risk (50). This polymorphism was associated with increased serum levels of testosterone, triglycerides and LDL in Hui Chinese women (54) as well as glucose and insulin metabolism (53) and ovulatory dysfunction (58), while no association with metabolic phenotypic traits was reported in Danish (48) and Han Chinese women (57). Mutharasan *et al.*, studied several polymorphisms in the first intron of the *LHCGR* gene using highly sensitive and specific Illumina custom arrays and reported association of only two variants, rs10495960 and rs7562215 with PCOS risk (67). What is more, *LHCGR* polymorphisms, rs7371084 and rs4953616 showed negative and positive association respectively with PCOS in Bahraini women (68). An exonic polymorphism, S312N, was significantly associated with increased risk of PCOS development in Sardinian (69) and Indian (70) women, but this was not observed in European women (71).

Mapping of GWAS susceptibility loci to 2p16.3 has further validated the role of *FSHR* variants in determining PCOS susceptibility and contributing to altered gonadotropin ratios. The Thr307Ala polymorphism in the extracellular domain modulates hormone binding; however only a nominal association was reported in PCOS women of European ancestry (67). This polymorphism failed to show association with PCOS in Singaporean Chinese (72), Han Chinese (73-75), Thai (76), Caucasian (77), South Korean (78) women with PCOS as well as Turkish adolescents (79). Recent meta-analyses confirmed lack of association of this polymorphism with PCOS (80, 81). Another exonic polymorphism widely studied is Asn680Ser which is located in the intracellular domain and is involved in the uncoupling of adenylyl cyclase.

This polymorphism augmented PCOS susceptibility in South Korean women (78); however, it did not show association in Singaporean Chinese (72), Thai (76), Han Chinese (73, 74), British (82) and Italian (83) women with PCOS and Turkish adolescent girls (79). A study in Han Chinese women has highlighted that regardless of non-association of individual polymorphisms with PCOS risk, the frequency of the G/A risk haplotype was found to be higher in PCOS women compared to controls (75). Women with PCOS carrying the serine allele displayed greater resistance to clomiphene citrate and were less likely to overcome the FSH threshold with clomiphene citrate despite having normal FSH levels. This possibly begets impaired folliculogenesis, ovulation defect and poor oocyte quality frequently observed in affected women (84). Recently a meta-analysis indicated association of the Asn680Ser polymorphism with reduced PCOS risk considering multiple genetic models (80). On the other hand, another meta-analysis revealed no such association in any ethnic population (34, 81). An intronic variant of *FSHR*, rs2268361 previously identified in Chinese GWAS (34) was associated with PCOS as well as decreased LH and FSH levels in women from Boston and Greece (85), but did not remain significantly associated in a cross ethnic meta-analysis study (50). Further, Chinese women with PCOS with PCO morphology showed association of rs2268361 polymorphism with lower FSH and SHBG levels with increased estrogen (86). Association of another intronic variant, rs2349415 with PCOS susceptibility was revealed in a Chinese GWAS (34), cross-ethnic meta-analysis study as well as a Chinese family trio study (63).

YAP1 (Yes-associated protein 1) in association with the Hippo pathway, is involved in regulating tissue proliferation, organ size and participates in tumor development (87). Activation of YAP1 was reported to influence follicle growth and oocyte maturation (88). Most recently, significant hypomethylation of *YAP1* promoter was reported in ovarian granulosa cells accentuating YAP1's involvement in PCOS pathophysiology (89). Intronic variants in the *YAP1* gene which is located on chromosome 11 have been identified to be significantly associated with PCOS risk in Chinese women (52, 90). Similar findings were reported in women of Northern European descent studied in Netherlands and the United States with strongest association being reported for the rs1894116 polymorphism (50) which persisted in a combined meta-analysis of studies conducted in Dutch, US and Chinese women (50). Furthermore, Chinese women who were carriers of risk alleles of rs11225161 and rs11225166 polymorphisms show impaired glucose tolerance (90) while rs11225138 polymorphism, was associated with increased LH levels (90). This suggests that *YAP1* could play a role in PCOS susceptibility as well as neuroendocrine dysfunction and glucose dysmetabolism. On the other hand another locus mapped to the *TOX3* gene which is mainly implicated

in breast cancer progression (91), was not shown to be a significant predictor of PCOS onset in both Chinese and Caucasian populations (50, 92), though one polymorphism, rs4784165, tended towards a marginally significant trend in transmission in Han Chinese families (93).

The *c9orf3* gene encodes aminopeptidase O, which in addition to modulating the synthesis of bioactive peptides, participates in the renin-angiotensin pathway by cleaving angiotensin III to angiotensin IV (94). The frequency of the A allele of rs3802457 of *c9orf3* gene was significantly higher in Han Chinese PCOS women suggesting that it contributed considerably to PCOS susceptibility (52) whereas rs4385527 polymorphism showed nominal association with PCOS in Dutch women (50). A meta-analysis of Caucasian and Chinese data confirmed the significant association of both these polymorphisms with PCOS susceptibility (50). This gene may be proposed to be an evolutionary link supported by evidence from GWAS conducted in European cohort (41). Moreover, the association of rs4385527 with hyperandrogenemia, ovulatory dysfunction and polycystic ovarian morphology, hints at its role in the pathophysiology of core characteristic traits of PCOS in Chinese women (58). Family based association study in Han Chinese family trios with PCOS also revealed that rs3802457 polymorphism showed significant difference in transmission (93), thereby providing new leads in determining new genetic risk factors in PCOS etiology.

Interestingly Ras-related protein *RAB5B*, another previously identified genetic locus in the Chinese GWAS involved in intracellular vesicle transport was found to be differentially expressed in PCOS adipose tissue (95, 96). Studies in Han Chinese (97) and Dutch (50) women as well as a cross-ethnic meta-analysis (50) strongly implicate the role of rs705702 polymorphism in PCOS susceptibility; however studies in women of European ethnicity demonstrated no such association with PCOS (85). *RAB5B* is instrumental in modulating glucose transporter type 4 (GLUT4) translocation and internalization, particularly endosomal trafficking of GLUT4 in combination with dynein and the cytoskeleton (98, 99). Furthermore, it has been observed that hyperinsulinemia inhibits the ability of *RAB5B* to facilitate GLUT4 redistribution (100), thus making this an important candidate gene. Saxena *et al.*, have shown that rs705702 near the *RAB5B* and *SUOX* genes was associated with 2 hour insulin and glucose levels after oral glucose testing in women with PCOS of European ethnicity (101).

The insulin resistance in PCOS is selective, where the metabolic action of insulin is affected but not its mitogenic or steroidogenic action (25). As INSR is an intricate member of insulin signaling pathway, it could be a potential candidate gene involved in

insulin resistance of PCOS. Additionally, linkage analysis studies that have found an association of the microsatellite marker D19S884 which is located on chromosome 19p13.2 and relatively close (1 cM) to *INSR* with PCOS, emphasizing it to be a candidate gene (102). The 1058C/T polymorphism has been commonly explored with T allele reported to be associated with increased PCOS risk, particularly in lean women (103-105). However, increased CC genotype frequency was recognized to be associated with PCOS risk in Japanese (106) and Iraqi (107) women. Other studies in Turkish adolescent girls (79) as well as Korean (108), Iranian Turkish (109) and Croatian (110) women failed to find any association of this polymorphism with risk of PCOS. This polymorphism impacted hyperinsulinemia, hyperandrogenemia and dyslipidemia traits in women with PCOS only (105, 107). In spite of this, a consistent negative association of this polymorphism in tandem with a previous meta-analysis (111) has been reported in a recent meta-analysis (112). Four novel single nucleotide polymorphisms (SNPs) were detected in a discovery cohort study by Goodarzi *et al.*, but only rs2252673 (G/C) showed persistent significance in the replication cohort of Caucasian women (113). The G allele was associated with PCOS and its associated dyslipidemia in Han Chinese women (114). Alternatively, one family based association study investigating a large number of Han Chinese PCOS trios failed to show any association of PCOS development with any polymorphisms in the *INSR* gene (115), while another demonstrated a weak association of rs2252673 with increased possibility of developing PCOS. Association of rs2059807 polymorphism with PCOS susceptibility in Caucasian women (51) and with only menstrual disturbances in Han Chinese women (58) has also been reported.

3.5. Going beyond GWAS

The abundant GWAS data generated has laid a foundation for a few researchers to delineate the putative underlying molecular mechanisms of PCOS pathophysiology. The genotype, methylation status and expression of several genes present in PCOS GWAS loci derived from Chinese studies were evaluated in sub-cutaneous adipose tissue (96). Lean women showed hypomethylation of CpG sites in *LHCGR* with simultaneous overexpression in adipose tissue. On the other hand, obese PCOS women showed significant hypermethylation with consequent decreased *INSR* expression, supporting earlier theory of reduced insulin sensitivity in metabolic tissues. Taken together, these findings demonstrate that there is a difference in pathway of hyperandrogenemia onset between lean and obese women with PCOS (96). Additionally CpG sites explored in other GWAS loci such as *c9orf3*, *DENND1A*, *YAP1*, *HMG2*, *TOX3* and *SUMO1P1* loci were reported to be differentially methylated in control and PCOS adipose tissue (96). Thorough analysis

of available GWAS dataset using a pathway based approach with MAGENTA has revealed enrichment of prominent biological pathways associated with PCOS including oocyte meiosis, regulation of insulin secretion by acetylcholine and free fatty acids, calcium signaling, osteoclast differentiation, cytoskeletal protein binding, acyltransferases and other kinases along with altered developmental processes (116).

When GWAS allele frequencies were investigated in women with PCOS of different populations globally, distinctive genetic patterns emerged in different ethnic populations. These groups were demarcated into genetic clusters and varying severity of metabolic and hyperandrogenic phenotypes were observed in them. Further, continental variability has been linked to random genetic drift with no effect of natural selection being noted (117).

3.6. Concluding remarks

GWASs also have certain drawbacks. The design of many commercial assays does not allow capture of rare alleles, or alleles with decreased penetrance. This may result in several important predictive allelic markers remaining undetected. Moreover, the impact of gene-environment and epigenetic alterations in addition to sequence variations may not be suitably captured (31). Previous studies have evidenced that genetic dissimilarities exist when the study groups were classified based on obesity (42, 105, 118-120) or different diagnostic criteria were used for classifying PCOS (121).

Despite its massive potential to ascertain the genetic origins of complex heterogeneous disorders such as PCOS, replication of markers in different independent populations has to be executed to enable confirmation of selected loci with PCOS susceptibility and related phenotypes and understand core biological processes underlying its etiology. In recent times, Unluturk *et al.*, have elegantly reasoned that given the similarity in PCOS susceptibility genetic loci between women of Chinese and European ancestry and the observation of comparable prevalence frequencies of PCOS across different populations, PCOS is an ancient disorder dating back nearly 50,000 years (122). However, the establishment of universal susceptibility markers consistently associated with PCOS notwithstanding ethnic and geographic disparities as well as lifestyle and environmental influences, is still lacking. Furthermore, the recent conclusion that the genetic risk score of Korean PCOS women having both oligomenorrhea and PCO morphology was higher compared to the other phenotypes and controls may bolster current diagnostic tools assessing the risk of PCOS development (123). With the implementation of GWAS in different sub-phenotypes of PCOS, it may also be possible to unlock the genetic mysteries triggering PCOS onset and it is of immense

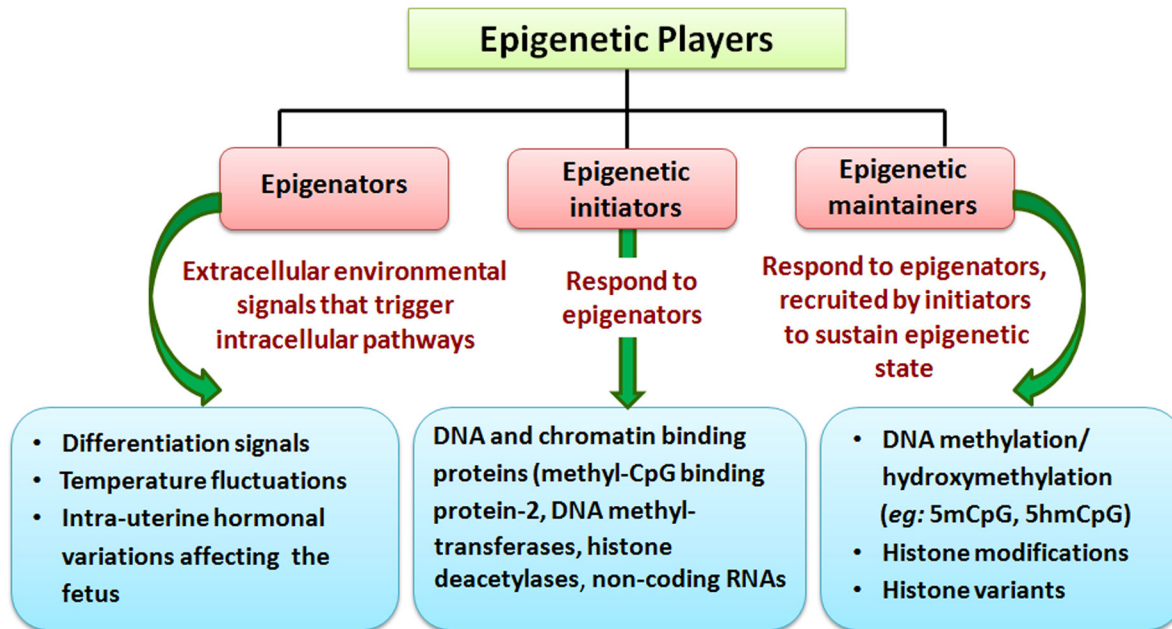


Figure 2. Schematic showing the interaction between different epigenetic components and their actions that lead to altered gene expression profiles in an individual. 5mCpG, 5'-methylcytosine-phosphodiester-guanine-3'; 5hmCpG, 5'-hydroxymethylcytosine-phosphodiester-guanine-3'.

importance to establish the genetic makeup of these phenotypically diverse patients for their management and therapy. Hence, the valuable contribution of GWAS to developing PCOS genetic predisposition profiles is undeniable.

4. EPIGENETIC APPROACH TO UNDERSTAND THE PATHOPHYSIOLOGY OF PCOS

4.1. Background

The pathophysiology of PCOS cannot be elucidated on the basis of genetic studies alone. Several indirect evidences published over the past decade using animal models, indicate an equally important role of nutritional, environmental and behavioral factors in governing the onset and aggravation of PCOS and its co-morbidities. The molecular and physiological changes sired by these factors are decoded and further translated into decipherable changes in the structure and composition of chromatin via the intrinsic epigenetic machinery of an individual. Since GWAS and candidate-gene based genetic studies undertaken till date could yet not discern any strong etiologic factors or biomarkers of PCOS, it is but evident that the cumulative impact of both genetic and epigenetic alterations would be responsible for the development of PCOS phenotypes.

Epigenetic modifiers (initiators) are vital intrinsic components of the body that decrypt molecular and physiological changes elicited in an individual in response to fluctuations in the external

environment e.g.: altered temperatures or presence of harmful constituents, which lead to the initiation of epigenetic responses in the body (epigenators). These changes are translated into chemical signatures in the chromatin (epigenetic maintainers/ modifications) (Figure 2), which can be effectively explored to distinguish between healthy and diseased individuals (124). Epigenetic alterations in the chromatin include addition or deletion of chemical moieties on DNA (without altering the nucleotide sequence) and histones. Modifications observed in the DNA include methylation of cytosine at its 5th carbon in CpG dinucleotides, i.e. 5mC (CpG denotes 5'-cytosine-phosphodiester-guanine-3'), 5'-hydroxymethylation (5hmC) and the recently discovered, 5'-formyl- and 5'-carboxyl- modifications (5fC and 5caC) on cytosine. Although 5mC has been established as the most stable and functionally the most important of all these modifications, there are several upcoming reports that have attributed high functional significance and stability to the presence of 5hmC and 5fC in several diseases (125, 126). However, due to the early discovery of 5mC and the subsequent development of numerous techniques towards its detection, 5mC remains the most widely studied modification in mammals. DNA methylation is established by DNA methyltransferases (DNMTs), i.e. the *de novo* methyltransferases DNMT3A and DNMT3B and the maintenance methyltransferase, DNMT1. Typically, the DNMTs 3A and 3B establish the methyl-signature patterns during early embryonic development, which are further maintained by the DNMT1 across subsequent somatic cell divisions by methylating the newly synthesized DNA derived from

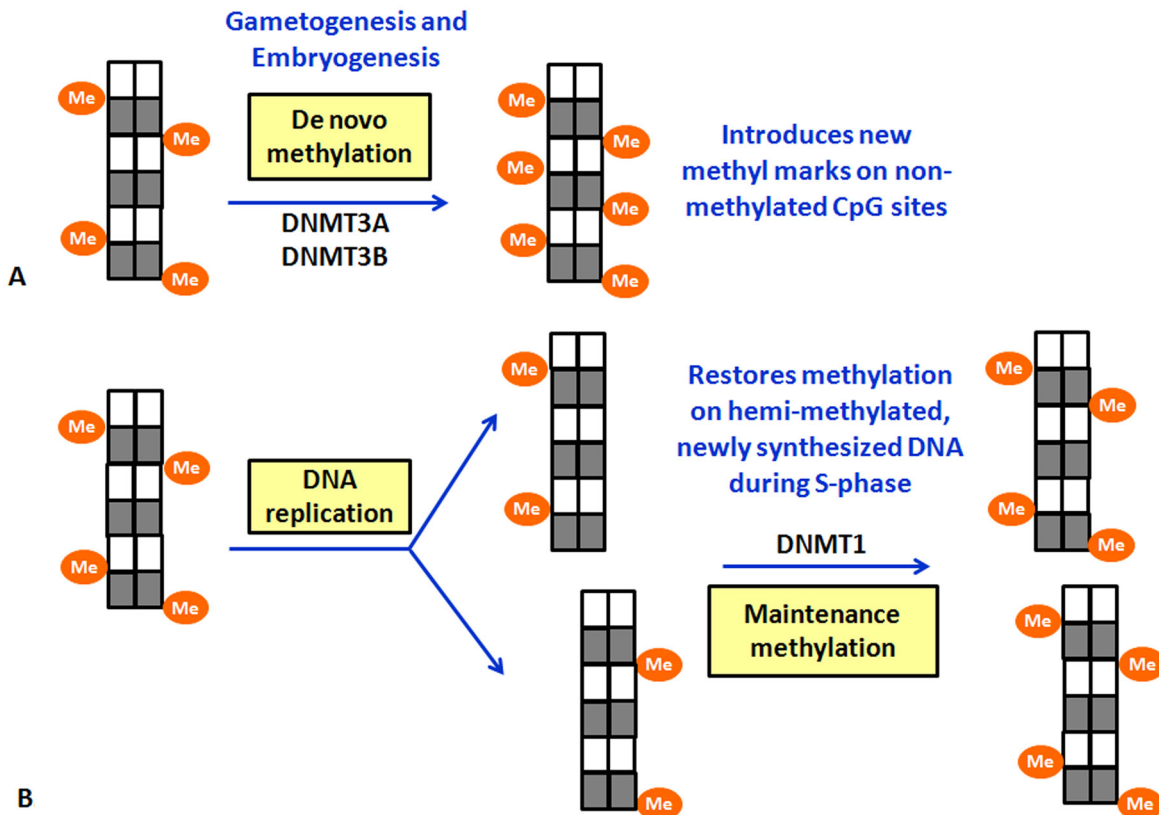


Figure 3. A snapshot depicting the classical functions of DNA methyltransferases during A) early stages of mammalian development and B) DNA synthesis. DNMTs, DNA methyltransferases; Me, 5'-methylcytosine-phosphodiester-guanine-3' or 5mCpG.

a hemimethylated template (127) (Figure 3). Recently however, it was reported that in some cells-types these DNMTs are capable of switching functions from maintenance to *de novo* methyltransferases and vice versa, depending on their relative concentrations (128, 129). Methylation in the mammalian genome mainly occurs at the CpG sites. These sites also undergo demethylation via active or passive mechanisms. Active demethylation involves the activity of ten-eleven translocation enzymes (TET 1, TET2 and TET3), which convert 5mC to 5hmC and is a crucial mechanism required for the normal development of fertilized zygotes in their early stages (130). Passive demethylation on the contrary, entails loss of maintenance methylation amidst several rounds of DNA replication, leaving the DNA strands demethylated (131). Non-CpG methylation at CpA, CpT, and CpC dinucleotides has been also reported to regulate the expression of a few genes in specific tissues or cells in mammals, though the functional importance of most of these sites is unclear (132). Histone modifications encompass a number of post-translational, covalent chemical changes such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation of specific amino acids on the tails of these proteins. Together, the combinatorial presence of specific DNA- and histone-associated molecular signatures, constitute the 'epigenetic code' of a target

cell. Aberrations incurred in the establishment of these epigenetic marks during fetal, neonatal or adult stages have resulted in gene dysregulation and phenotypic variation resembling several disease-states (133).

Epigenetic modifications occur in both, somatic and germline cells although, epigenetic aberrations in somatic cells cannot be directly transferred across generations. Germline modifications on the other hand can be potentially transmitted to the subsequent progeny up to F3 generations (transgenerational epigenetic inheritance or TEI) and can effectively alter the epigenomes and transcriptomes of somatic cells and tissues (134). The reproductive organs, especially the ovaries and testes, and other tissues like kidney, prostate gland are reportedly more susceptible to epigenetic changes. Therefore the study of epigenetic changes in these tissues is important to address the pathophysiology of adult-onset diseases (135). PCOS is one such disorder that manifests tissue-specific dysfunction in ovaries and dysregulation of hypothalamo-pituitary axis, pancreas and adrenal glands of affected individuals. Tissue-specific epigenetic studies are therefore imperative to the understanding of its etiopathogenesis in women with PCOS. However, the complex and heterogeneous nature of this disease and the practical limitations involved in procuring adequate quantities of tissue-

samples from consenting individuals have posed difficulties to undertake epigenetic studies in women with PCOS. Therefore, most researchers were initially prompted to conduct epigenetic studies in drug-induced animal-models of PCO, which did not necessarily reflect the changes occurring in PCOS. Only recently, since the development of cost-efficient and high throughput techniques that were designed to detect the global and genome-wide epigenetic changes using small quantities of samples, the researchers started exploring these alterations in women with PCOS.

The involvement of an epigenetic aspect in the development of PCOS has been underscored by several theories suggesting the impact of exposure to artificially introduced toxic components in the environment, neonatal or fetal exposure to androgenic or estrogenic milieu, dietary habits and unhealthy lifestyles alongside genetic predisposition to the syndrome. These theories form the basis for the subsequent epigenetic investigations carried out in PCOS populations and are reviewed in brief in the following section. Amongst all prevailing hypotheses, the role of intra-uterine hormonal perturbations which trigger prenatal androgenization and estrogenization have been the most widely accepted and studied in the development and progression of PCOS.

4.2. Theories on fetal reprogramming and developmental origins of PCOS strengthened by circumstantial epigenetic evidence

A host of experimental evidences strongly implicate the role of intra-uterine milieu in influencing the development of PCOS phenotypes. There are two existent hypotheses to explain this phenomenon in PCOS, namely the “thrifty phenotype model” which proposes the development of insulin resistance and a compensatory hyperinsulinemia due to intra-uterine nutritional imbalance; and second, the androgen-priming of fetuses and neonates to develop a PCOS phenotype in the adult life (136). The second hypothesis is best supported by an array of animals models, in which several PCO/ PCOS features were successfully induced by the administration of androgens to the mothers of the developing offsprings(137). Similarly, the contribution of fetal or neonatal exposure to estrogens has been well documented in literature on fetal programming of PCOS (138). Additionally, animals exposed to endocrine disruptors (EDCs) in their prenatal stages such as to bisphenol-A, phthalates and androgenic EDCs have shown to develop PCOS-like features, most importantly altered postnatal growth and sexual development, formation of polycystic ovaries, hormonal alterations resulting from dysregulation of the hypothalamo-pituitary-ovarian axis (136).

Similarly, studies which were undertaken to determine the frequency and size of the androgen

receptor CAG-repeats and the role of their methylation patterns in inducing X chromosome inactivation (XCI) in women with PCOS, have provided important insights on the non-genetic influence of androgens in manifestation of PCOS (120, 139-142). Hickey *et al.* studied the association of these CAG repeats with PCOS susceptibility in an Australian population, which comprised of 83 controls and 122 infertile women with PCOS. This study showed that the longer CAG repeats were present at a higher frequency in infertile women with PCOS although their methylation patterns did not differ significantly in comparison to controls. The longer repeats were also found to be highly associated with increased levels of circulating testosterone in these women (139). The same group, later also demonstrated that 84% of sister-pairs with the same genotype but varying patterns of clinical manifestations of PCOS differed in their XCI patterns (140). The finding of higher frequency of longer CAG repeats in women with PCOS is contradicted by other studies. Assessment of these repeats in a mixed population comprised of Chinese and Indian women (141) and yet another study in German population (142) reported the presence of shorter CAG repeats in women with PCOS and the inactivation of the X chromosome harboring these repeats to be directly associated with androgen excess in these women. Similarly, a study on Indian women with PCOS showed that the mean CAG number was similar between controls and PCOS cases and that there was a higher tendency for X chromosomes bearing shorter CAG repeats (having < 19 repeats) to be activated during non-random XCI events in women with PCOS. However, the CAG repeat numbers differed between lean and obese women with PCOS, wherein the shorter CAG repeats showed up with a greater frequency in the obese-hyperandrogenic sub-type of PCOS women (120). The CAG repeat length and XCI patterns were later also investigated between the daughters of control women and women with PCOS in a Chilean cohort, but the study showed no difference between the two groups (143).

All this circumstantial information derived from studies conducted in animals exposed to altered androgenic or estrogenic milieu and endocrine disruptors *in utero* as well as XCI and other studies in women with PCOS have provided a solid basis to further explore the seemingly evident, yet a highly obscure epigenetic component in the development and manifestation of PCOS.

4.3. Evidence on the role of epigenetic alterations in PCOS development

The importance of uncovering the putative epigenetic changes associated with hereditary and chronic disease-states like PCOS is reflected by a surging interest of numerous research groups and the rising development in technology aimed at understanding disease-linked epigenetic modifications.

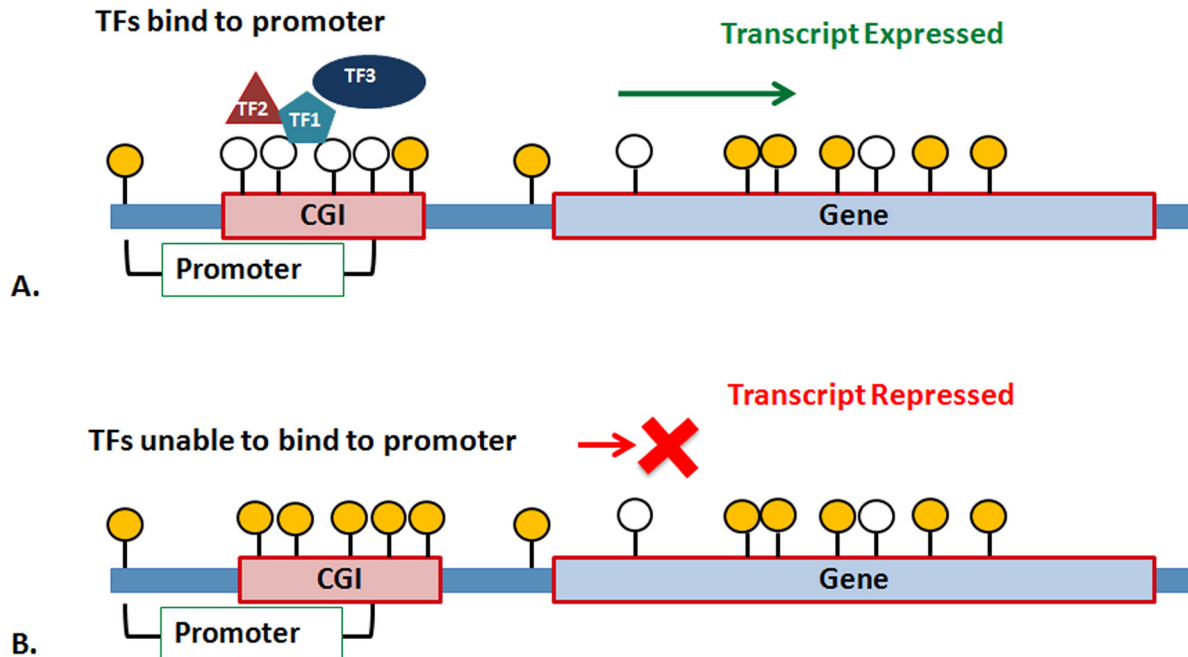


Figure 4. Mechanism underlying the activation or repression of a gene downstream to A) hypomethylated and B) hypermethylated CpG island associated with the gene promoter. Hypomethylation facilitates the transcription of a gene by providing accessibility to transcription factors that bind to the promoters while hypermethylation blocks the promoter sites, restricts the binding of transcription factors and represses transcript formation. CGI, CpG Island; TFs, transcription factors.

Most of the reports concerning epigenetic alterations in animal models of PCO/ PCOS and in women with PCOS have primarily focused on changes in DNA methylation, owing to its easy detection and direct applicability in epidemiologic studies.

DNA methylation popularly refers to the methylation of cytosines in the CpG sites/ dinucleotides. CpG sites are inadequately represented in the human genome (with an exception of CpG-rich islands i.e. CGIs), due to their tendency to get deaminated and subsequently, mutated from CpG>TpG. Reduction of CpG site numbers in non-CGI regions of the mammalian genome is attributed to increased methylation of cytosines in these regions. Also, the extent of CpG site methylation is lesser in CGIs as compared to non-CGI regions with CpG>TpG mutation rates being 7 to 10 fold lower and cytosine methylation being equally higher than in non-CGI CpG sites (144). CGIs are associated with the promoters of approximately 50-70% of all known genes and their state of hypo- or hypermethylation determines the activity of a given gene. Hypermethylation of a promoter-associated CGI usually leads to suppression of gene activity whereas hypomethylation favors increased gene expression (Figure 4). Several quantitative methods have been devised till date to measure the genomic abundance of 5mC. These include techniques based on sodium bisulfite conversion of DNA and sequencing [Sanger's method, pyrosequencing, next generation sequencing (NGS)],

high performance liquid chromatography (HPLC) and mass spectrometry based measurements to determine the percentage of 5mC over cytosine bases after enzymatic degradation of DNA into its individual bases, methylated DNA immunoprecipitation (MeDIP) and sequencing, and antibody dependent methods (ELISA, immunofluorescence, flow cytometry). Techniques employing sequencing based end-point analyses, especially NGS, are expensive but have garnered a widespread acclaim owing to the accuracy of information obtained at a single-base resolution. The following sections summarize the findings of epigenetic studies conducted in animal models of PCO/ PCOS, and women with PCOS so far.

4.3.1. Evidence-based findings from drug-induced animal models of PCO

Several candidate-gene based studies have been carried out in animal models of which, a study by Zhu *et al.*, investigated the promoter methylation patterns of luteinizing hormone receptor (*Lhr*), follicle stimulating hormone receptor (*Fshr*), androgen receptor (*Ar*) and *H19* genes in DHEAS treated murine model of PCO by bisulfite sequencing and restriction-enzyme based methylation analyses. This study showed that the promoter of *Lhr* in treated animals was significantly hypomethylated relative to control groups (145). Another group has investigated the epigenetic alterations in the promoters of peroxisome proliferator-activated receptor- γ (*Ppar- γ*) and its co-repressor,

the nuclear receptor co-repressor 1 (*Ncor1*) in a testosterone propionate (TP) induced model of PCO (146). PPAR- γ plays an important role in adipogenesis and lipid uptake by the fat cells and has been widely implicated in the pathogenesis of metabolic abnormalities like obesity, T2DM, atherosclerosis and cardiovascular disease (CVDs)(147, 148). In this PCO model, the *Ppar- γ* gene promoter was found to be hypermethylated in concurrence with attenuated expression of its mRNA in the ovaries of hyperandrogenic PCO animals compared to controls. Additionally, hypomethylation of the *Ncor1* promoter with increase in its gene expression and increased expression of *Hdac3* transcript (another co-repressor of *Ppar- γ*) were also reported (146). Jia *et al.* have investigated the association of altered one-carbon metabolism pathway with oocyte mitochondrial DNA (mtDNA) methylation and the quality of oocytes in gilts with polycystic ovaries ($n = 147$). In this model, aberrant stimulation of one-carbon metabolism and mtDNA hypermethylation, correlated with mitochondrial dysfunction, hyperhomocysteinemia (one of the recently established features of PCOS) and poor oocyte-quality (149). This study proposes that mtDNA methylation and function may also be altered in PCOS. Another study assessed the transgenerational effects of testosterone and dihydrotestosterone on global DNA methylation in zebra-fish models of PCO. This study demonstrated that the exposure of neonates (F0 generation) to excess androgens during appearance and migration of gonadotropin releasing hormone(GnRH) neurons to the brain (26 to 56 h post fertilization), sired global methylation changes in the ovaries of these fish. These changes, which were detected in the mature ovaries of the exposed F0 generation, were found to persist in the ovaries of unexposed F1 adults. These findings conform to the theory of fetal androgen priming in the development of PCOS (150). Xia *et al.* investigated the methylation differences in the promoters of *Ar*, *Cyp11a1* and *Cyp17a1* genes in circulating lymphocytes and ovarian tissues of prenatally androgenized rat PCO models. Here, 3 CpG sites in the *Ar* and 1 CpG site in the *Cyp11a1* promoter were hypomethylated in lymphocytes; whereas 5 CpG sites in *Ar* and 1 CpG site in *Cyp11a1* were found to be hypomethylated in the ovary of PCO animals (151). Amongst genome-wide methylation studies, the study conducted by Abbott's group on visceral adipose tissue samples of prenatally androgenized (PA) infant and adult Rhesus monkey models of PCOS, yielded a total of 163 differentially methylated loci in PA infant monkeys ($n = 7$) and 325 loci in PA adult animals ($n = 8$) compared to controls ($n = 10$) (152). Analysis showed association between high circulating androgen levels and differential methylation in genes such as OACT2, SEPT9, RAB6A and SUV39H2 in the adult PA monkeys, which are vital to the maintenance of cell-membrane integrity and function and participate in the TGF- β signaling pathways. Further, CpG loci within the genes that

partake in functions associated with axonal guidance, tight junction and Wnt/ β -catenin pathway signaling were found to be differentially methylated in adult PA monkeys, whereas in the infant PA animals an entirely different set of pathways including transducer of ERBB2 (TOB)-mediated T cell signaling, vitamin D receptor-retinoid X receptor(VDR/RXR) activation, methionine metabolism, complement and nucleotide excision repair pathway were annotated to the differentially methylated loci. This study provided evidence-based data in support of gestational androgen-excess-mediated epigenetic reprogramming in offsprings of hyperandrogenic mothers. Another genome-wide analysis of aberrantly methylated promoters using a MeDIP approach in testosterone propionate induced, prenatally androgenized rat model revealed differential methylation of 2497 genes. Of these, a total of 528 genes, involved in pathways related to postembryonic and *in utero* embryonic development, mammary gland development, positive regulation of anti-apoptotic processes and response to peptide hormone stimulus were reported to be hypermethylated in the PCO rat model (153).

4.3.2. Epigenetic studies conducted in women with PCOS

Assessment of global DNA methylation is highly essential to understand the principal molecular defects underlying a disease, especially in tissues that are functionally dysregulated in a disease condition. Although XCI and CAG repeat polymorphism studies mark the beginning of epigenetic studies in humans, the first study to investigate any change in global DNA methylation in humans came from Xuet *et al.* in 2010, in which global DNA methylation levels were measured in peripheral blood leukocytes (PBLs) of a subset of American Caucasian women comprised of 20 controls and 20 women with PCOS, using an ELISA based approach (154). This study revealed no change in total genomic 5mC content of PBLs in women with PCOS, stating that the plausible factors for these non-significant findings were a) its experimental design, which could not enable any CpG-site directed analysis, b) small sample-size of study groups and c) lack of tissue-specific, targeted gene-based global methylation analysis in ovaries, adrenals or adipose tissues, which are highly affected in PCOS. Recently, our group carried out a global DNA methylation study in Indian women with PCOS, where most of these limitations were addressed (with the exception of sample size, which was also a limiting factor in our study). We investigated the methylation differences in, a) cumulus granulosa cells (CGCs) obtained from the mature ovarian follicles of controls ($n = 21$) and women with PCOS ($n = 20$) undergoing controlled ovarian hyperstimulation i.e. COH for assisted reproduction (COH group), b) PBLs obtained from women in this COH group and c) PBLs obtained from women not undergoing assisted reproduction (non-COH group)

having 20 controls, and 41 women with PCOS. These differences were investigated using two experimental approaches *i.e.* ELISA to measure the total content of 5mC in the genome and direct bisulfite sequencing of the 5'-untranslated region (5'-UTR) of long interspersed nuclear element-1 (LINE1/ L1). Repeat elements interspersed in the genome [LINEs, SINEs (eg: Alu elements)] occupy nearly 50% of the human genome and are hugely responsible for the build-up of high frequency frameshift mutations and the transposition of retrogenes that lead to either the activation or repression of downstream genes. Active transposition of these elements also causes an increase in the gene copy numbers, thereby mediating the overexpression several genes and promoting disease development. These elements have a high prognostic value in disease prediction and serve as important surrogate indicators of altered global methylation in disease states. Our studies show no change in total genomic 5mC or L1 5'-UTR methylation levels in PBLs or CGCs of women with PCOS compared to healthy controls; though a single CpG site (CpG -4) out of 22 CpG sites that were analyzed was found to be consistently hypomethylated and strongly associated with PCOS susceptibility and its traits of systemic and ovarian androgen-excess (155). We also noted that CGCs exhibited a hypomethylated status relative to PBLs (global as well as CpG-site specific hypomethylation), perhaps to facilitate the expression of ovary-specific genes that are otherwise not functional in PBLs. In contrast to our observations on global methylation levels in CGCs of mature follicles, the studies by Pruksananonda *et al.*, report higher L1 methylation in women with PCOS (n = 19), as compared to controls (n = 22) although their studies in CGCs from immature oocytes show no difference between these groups (156). Also, an ELISA based global DNA methylation analysis between floating granulosa cells (GCs) performed by Xuet *et al.* alongside a genome-wide methylation study showed hypermethylation in GCs of PCOS women compared to controls (n = 9, per group) (157). This was also the first report to study 5hmC levels in floating GCs of women with PCOS and controls, although no difference was detected between these groups.

Barring these four reports, the rest of the studies have either targeted candidate genes or have performed high-throughput techniques for analysis of methylation differences in PCOS. Amongst the candidate gene-based study reports, Huang *et al.* showed increased methylation of 2 CpG sites in the PPARG1 promoter and decreased methylation of 5 sites in the NCOR1 promoter in the granulosa cells (GCs) of hyperandrogenic (HA) sub-group of women with PCOS (HA = free androgen index > 30.02). These observations also correlated with low expression levels of PPARG1 and high expression levels of NCOR1 transcripts in these cells and also in

DHT treated cultured GCs (146). Sang *et al.* screened for differences in the promoter and 5'-UTR regions of the follistatin gene (FST) in PBLs (controls, n = 120; PCOS, n = 120) and endometrial tissues of 24 controls and women with PCOS each, using a mass-array based quantitation method (158). No change was observed in CpG-site methylation levels between control and PCOS groups in either tissue-samples and also the expression levels of FST transcripts remained unchanged in the endometrial samples of these groups. The same group has also investigated CpG methylation patterns of epoxide hydrolase 1 (EPHX1), steroid 5-alpha-reductase 1 (SRD5A1) and cytochrome P450 family 11 subfamily A member 1 (CYP11A1) genes in PBLs in 2 independent cohorts (*cohort 1*: controls and PCOS women (n = 32, each); *cohort 2*: controls, (n = 67); PCOS, (n = 49). While no difference was observed at any CpG sites in SRD5A1 and CYP11A1 promoter regions, EPHX1 showed significant differences in methylation levels of the CpG clusters, 13-14 and 19-24, in both these cohorts (159). EPHX1 is one of the key enzymes that regulate steroid synthesis and plays a critical role in susceptibility to spontaneous abortions, preeclampsia and ovarian cancers. We also have found upregulation of EPHX1 in follicular fluid of women with PCOS compared to controls in a differential proteomics study (160). EPHX1 may therefore be one of the important candidate genes associated with PCOS. LHCGR, which was one of the candidate genes identified in the first GWAS study, was explored for novel polymorphisms (discussed in the GWAS section) in PBLs as well differences in its promoter methylation levels in both, PBLs and GCs of women with PCOS by Wang *et al.* (161). While PBLs showed hypomethylation at 2 CpG sites, a total of 8 CpG sites were found to be hypomethylated in GCs, which also correlated with an increase in its mRNA expression at the ovarian level. Lamin A/C (LMNA) was another candidate gene explored by the same group, which revealed hypermethylation of 12 CpG sites in the promoter region in PCOS (n = 24), compared to controls (n = 24). These CpG sites were found to be strongly associated with susceptibility to insulin resistance (IR) in women with PCOS (162). A study comparing the methylation differences in INSR gene promoter in endometrial biopsies of IR and non-IR women with PCOS (n = 18, each), detected no change in methylation in these groups (163). In another candidate-gene study in the Chinese population, the promoter methylation levels of CYP19A1 gene, which plays a key role in estrogen biosynthesis in ovarian GCs, were found to be high in PCOS as compared to controls (n = 10, each) and were consistent with its decreased transcript and protein levels (164). Promoter methylation levels of YAP1 (yes-associated protein 1) gene assessed in GCs of Chinese women with PCOS (n = 36) revealed hypomethylation of 12 CpG sites, which was concurrent with overexpression of its transcript and protein in these cells relative

to controls (n = 72) (89). Zhao *et al.* assessed the PPARGC1A promoter methylation and mtDNA content in PBLs of 175 women with PCOS and 127 controls. In this study, women with PCOS showed lowered mtDNA content and hypermethylation at this gene promoter, which was associated with PCOS-related traits of hyperandrogenism and IR (165). Also, the methylation levels at a CpG site of the promoter of traf2- and Nck-interacting kinase (TNIK) gene were observed to be increased in the ovaries of PCOS women; although the transcript levels failed to show a corresponding decrease with this methylation pattern (166).

A total of 6 high-throughput methylation studies conducted in women with PCOS have been published till date. The first study was conducted by Shen *et al.* using a MeDIP approach in PBLs of controls (n = 5) and women with PCOS (n = 10), which were further subcategorized as IR and non-IR PCOS. In this study a total of 40 genes were found to be differentially methylated between controls and all PCOS women, whereas 79 genes were differentially methylated between IR-PCOS and non-IR PCOS women (167). The gene ontology pathways which were significantly enriched between IR and non-IR PCOS women were those involved in several cancers (e.g.: chronic myeloid leukemia, prostate cancer, pancreatic cancer), whereas those enriched between controls and all PCOS women were the MAPK, Wnt, ErbB signaling pathways, and pathways related to cancer and focal adhesion. Another genome-wide study used a MeDIP-microarray based approach to investigate differential methylation between the ovarian tissues of controls and women with PCOS (n = 10, per group) (168). Of the several hundreds of genes that were differentially methylated between the two groups, the promoters of the genes viz. SLC2A8, NRIP1, IGF2BP2, CYP19A1, and AMHR2 were found to be hypermethylated in the PCOS ovary while those of INSR and AMH were found to be hypomethylated upon further validation of the MeDIP results. Wang *et al.* combined microarray-based DNA methylation and transcriptome profiling analyses to investigate methylation differences between normal and PCOS ovaries (n = 3, each group) and observed differential methylation 7929 CpG sites with differential expression of 650 transcripts (169). Of these, 16 genes that were detected as hypomethylated, showed upregulation at the transcript level and 17 hypermethylated genes showed downregulation. The pathways enriched in transcriptome analysis included those for transcriptional regulation, cell proliferation, apoptosis and response to stress whereas differential methylation was mainly detected for processed involved in cell-adhesion and small GTPase activity. A methylation microarray based study carried out by Li *et al.* in PBLs of Chinese women (n = 30 for controls and women with PCOS each) showed differential methylation at 52 CpG sites (FDR < 0.0.5) (170). A total of 22 functional pathways were found to be enriched,

amongst which, the most prominent ones were those involved in immune and inflammatory processes, and metabolism of proteins and carbohydrates. The genome-wide DNA methylation study by Xu *et al.* (157) compared the methylation differences in the GCs of controls, and obese and non-obese women with PCOS (n = 8, for all 3 groups). Here, a total of 6936, 12245 and 5202 differentially methylated CpG sites were identified between controls and obese-PCOS, controls and non-obese-PCOS, and obese and non-obese PCOS women respectively. Pathways for transcriptional regulation and co-factor activity, cell-cell signaling and adhesion, cellular morphogenesis and embryonic development were differentially enriched between these groups. Further, validation of the differentially methylated, single CpG sites detected in MATN4, MGAT5B and DLGAP2 genes in the genome-wide analysis revealed hypermethylation of these sites in obese and non-obese PCOS women, in relation to controls. Apart from PBLs and ovarian cells/ tissues, genome-wide methylation differences have also been evaluated in the adipose tissue of women with PCOS (95). In this study on 64 women with PCOS and 30 controls, a methylation-microarray analysis was performed in association with gene expression arrays. A total of 63,213 CpG sites were found to be differentially methylated while 1720 unique transcripts were differentially expressed in the PCOS adipose tissue. Amongst these genes, differential expression observed in 30 genes corresponded with altered methylation at 33 different DNA methylation sites. This study has by far, yielded the highest number of differentially methylated genes corresponding to the pathways that have been strongly implicated in the pathophysiology of PCOS (eg. androgen signaling, oxidative stress, TGF- β signaling pathways and so on).

4.4. Non-coding RNAs as additional epigenetic regulators in PCOS

Non-coding RNAs (ncRNAs) are seminal regulators of gene expression and are currently under focus for understanding novel molecular mechanisms in various disease states. Amongst several classes of ncRNAs, micro-ribonucleic acids (miRNAs) and a few long non-coding RNAs (lncRNAs) have been studied as the putative determinants of altered gene activity in PCOS. It has been recently established that the expression of several miRNAs and lncRNAs is regulated by DNA methylation and histone modifications.

miRNAs (miRs), which are approximately 21-24 nucleotide-long RNA molecules, negatively regulate gene expression and are transcribed by the RNA Pol II enzyme. They are responsible for post-transcriptional regulation of genes. The binding of miRs to target mRNAs, mainly in the 3'-UTR regions causes interference in the process of translation,

causing downregulation of target gene expression. It has been recently demonstrated that the expression of several species of miRNAs is regulated by DNA methylation and histone modifications, which further modulate the expression of other target genes as well as other miRNAs in several mammalian species including humans(171-173). Interestingly, few miRNAs such as hsa-miR-29, hsa-miR-148, hsa-miR-449, hsa-miR-1 have also been recognized as the regulators of epigenetic modifiers such as DNMTs, histone deacetylases (HDACs) and methyl-CpG binding protein 2 (MeCP2)(171). Thus, miRNAs have been attributed important epigenetic functions in the pathophysiology of numerous disease-states.

The abundance of several miRs in tissues such as peripheral blood, adipose tissue, the ovary, its GCs and oocytes; and in follicular fluid of women with PCOS has been found to differ in comparison to control subjects. In peripheral blood, decreased expression of circulating hsa-miRs namely hsa-miR-21, hsa-miR-27b, hsa-miR-103 and hsa-miR-155, which have previously shown association with obesity and diabetes, were also associated with androgen-excess and risk of PCOS (174). Amongst other circulating miRs identified in serum of women with PCOS, hsa-miRs-23a/b were associated with androgens and obesity (175), hsa-miR-200b and hsa-miR-429 with anovulation (176), hsa-miR-6767-5p, hsa-miR-21, hsa-miR-222, hsa-miR-146a and hsa-miR-30c with risk of PCOS development (177-179), and lastly hsa-miR-122, hsa-miR-193b and hsa-miR-194 with altered glucose metabolism (180). High levels of hsa-miR-93, hsa-miR-133, and hsa-miR-223 have been reported in adipose tissues of women with PCOS having elevated insulin-excess indices and reduced GLUT4 levels (181). hsa-miR-223 further demonstrated the potential for predicting ovarian hyperstimulation syndrome (OHSS) in PCOS (182). Additionally, hsa-miR-25, hsa-miR-106b have been reported to be downregulated in adipose tissues of PCOS women (183). Several studies on differential expression of miRNAs in follicular fluid of women with PCOS have identified a number of miRNAs (hsa-miR-32, hsa-miR-34c, hsa-miR-135a, hsa-miR-18b, and hsa-miR-9) that are important in regulating processes related to reproductive, endocrine and metabolic functions in the ovary (184). Also, amongst other miRs found to be differentially expressed in PCOS follicular fluid, hsa-miR-132, hsa-miR-320, hsa-miR-520c-3p, hsa-miR-24, and hsa-miR-222 controlled estradiol concentrations while hsa-miR-24, hsa-miR-193b, and hsa-miR-483-5p were involved in regulation of progesterone (185). Additionally, decreased levels of hsa-miR-30a, hsa-miR-140 and hsa-let-7b in follicular fluid of PCOS also showed correlation with clinical pregnancy outcomes in women undergoing IVF (186). At the ovarian level, hsa-miRs-92a and 92b were downregulated in ovarian tissues of women with PCOS (187). Also, a recent study indicated that low expressions of hsa- 126-5p

and hsa- miR-29a-5p, insulin growth factor 1, Wnt family member 1 and decreased Akt phosphorylation, coupled with increased Klotho protein expression could contribute to GC apoptosis and influence cystic follicle development (188). Also, in GCs, the miRs, hsa-miR-320a (189), hsa-miR-145 (190), hsa-miR-509-3p (191), hsa-miR-483-5p and 486-5p (192) and hsa-miR-93(193) have shown to be associated with steroidogenic dysregulation, impaired estradiol secretion, impaired follicular growth and increased GC proliferation.

LncRNAs are ncRNAs that are >200 nucleotides in length; and are generated by the same machinery that encodes the RNA pol II dependent mRNAs (194). LncRNAs also act as epigenetic modulators, the functional inventory of which include, interacting with chromatin-modifying proteins, targeting the catalytic activity of proteins to a specific site on chromatin to alter chromatin assembly and binding to target mRNAs to alter their expression levels (195). In PCOS, some of the earlier studies on X-chromosome inactivation mediated by the *Xist* and *Tsix* lncRNAs have provided important leads on the plausible involvement of lncRNA in its pathophysiology. A recent study on lncRNAs in cumulus cells derived from ovaries of PCOS women reported that lncRNAs were mostly upregulated in these cells (196). Interestingly, several of these differentially regulated lncRNAs were the ones transcribed from chromosome 2, which is an established genetic locus for PCOS. Another group reported that an lncRNA steroid receptor activator was significantly increased in PCOS and showed positive association with BMI and obesity in these women (197). Also a novel lncRNA, namely C-Terminal binding protein 1 antisense(CTBP1-AS), which is known to be a regulator of androgen receptor expression was found to be increased in peripheral blood leukocytes of PCOS women. Its overexpression was correlated with high testosterone and HOMA-IR values(198).

Although studies on non-coding RNAs have identified several miRs and lncRNAs that are differentially expressed in PCOS, these findings are restricted only towards discerning their expression levels in different tissues. Hence there is a pressing need to expand the available information towards dissection of molecular mechanisms and physiological alterations that would increase the risk of PCOS development.

4.5. Concluding remarks

Epigenome-wide data derived from clinical studies in disease states is anticipated to provide promising avenues in the formulation of novel epigenetic therapies by identification of novel susceptibility genes in affected tissues. Also, the reversible nature of epigenetic modifications ensures a wide range of prospects for targeted therapy with the use of inhibitors

such as 5-azacytidine (azacitidine) that targets DNMTs and 5-aza-2'-deoxycytidine (decitabine), which inhibits histone deacetylases (HDACs). However, this translation from bench to bedside aimed towards the management of PCOS is still in primitive stages of development. This is principally due to heterogeneity of the condition and high screening costs associated with high throughput technology, which leads to either inadequate coverage of genomic regions with sufficient number of samples or in-depth coverage of a specific region with adequate sample size. This limitation therefore makes the undertaking of candidate-gene and single gene methylation studies imperative for cost-effective screening of genomic loci in larger populations. Additionally, alteration of epigenetic signatures is largely a tissue-specific phenomenon. Hence unlike genetic studies which make use of peripheral blood leukocytes that are easily obtained from larger sample sets, epigenetic investigations have to rely on data obtained from smaller cohorts. There is another possibility of defects in gene imprinting, which is the silencing of specific maternal or paternal alleles in the germ line cells, which can also contribute to the pathophysiology of PCOS. While spermatocyte imprinting signatures can be easily investigated in relation to male factor infertility, the invasive nature of techniques associated with retrieval of oocytes imposes significant constraint on imprinting studies in women. Thus, there is an urgent need for inter-regional collaboration between IVF centers and hospitals, preferably adhering to dietary and ethnic uniformity to screen larger populations for tissue-specific epigenome-wide alterations. Such tie-ups can enable both, comprehensive epigenetic profiling of the entire population and findings based on phenotypic and ethnic sub-grouping of individuals under study. Thus large scale epigenome-wide studies may provide compelling evidence on involvement of non-genetic determinants in the development of PCOS.

5. PROTEOMIC ATTEMPT TO UNRAVEL PCOS PATHOPHYSIOLOGY

5.1. Introduction

The flow of information begins with the transcription of genes into mRNAs and their translation into protein that are vital to the maintenance of structural and functional integrity of a cell. Proteomics provides the platform to study comprehensive changes in protein expression, post-translational modifications, and protein-protein interactions in a wide array of biological samples, such as primary cells, whole tissues, tissue biopsies and explants and tissue and body fluids such as saliva, serum, plasma and follicular fluid. The extensive profiling of such cell or tissue based proteomes helps in elucidating complex cellular processes, and their alterations pertaining to disease conditions. Thus, proteomics makes a robust

tool for the validation of molecular perturbations and complex pathways alongside genotype-phenotype correlations in conjunction with disease pathogenesis. Proteomics can be further exploited as a means to link the findings from genomics and epigenomic studies. A great advantage of proteomics is in discovery of biomarkers, which aids in improved diagnostics and targeted molecular therapy.

PCOS being a complex disorder of unknown etiology, many researchers have utilized a wide range of proteomic techniques combined with mass spectrometry (MS) such as 2 dimensional gel electrophoresis (2DE), fluorescent labeled dyes in 2D- difference in gel electrophoresis (2D-DIGE), pre-fractionation technique like reversed-phase solid-phase-extraction (RP-SPE), surface-enhanced laser adsorption/ ionization (SELDI) have been exploited so far to dissect systemic and tissue-specific changes in the expression levels of various proteins. MS operates by converting analyte molecule to charged species and their detection on the basis of m/z ratio. Several different ionization and detection system employed with MS technique helps in reduction of complexity of proteins and thereby generating vast data sets. Identification of proteins with the use of liquid chromatography-MS (LC-MS) is now a widely used technique, providing robust and simple interphase. Gel-free and high throughput screening technologies such as isobaric tagging for relative and absolute quantitation (iTRAQ) developed in the recent years have also been employed with LC-MS/MS analysis to address the differential abundance of proteins in normal and disease conditions. The use of diverse protein detection techniques and proteomic platforms including classical to high throughput approaches, different types of samples compounded with complexity of disorder and different diagnosis criteria for PCOS have resulted in the heterogeneity of the proteomic outcome in these studies (199). Results obtained across different studies are rarely overlapping due to the differences in sensitivity and accuracy of these techniques, thus rendering it difficult to interpret, analyze and select a unanimous biomarker for diagnosis of PCOS (199).

5.2. Samples used for proteomic studies in PCOS

A variety of tissues/cells have been used for proteomic analyses in PCOS including the circulating bio-fluids such as serum and plasma, which have been widely used to reflect the systemic changes in a pathophysiological condition. A brief overview of published proteomics studies in women with PCOS have been listed in Table 1. Zhao *et al.*, have used SELDI-protein chip technology for the discovery of biomarkers from serum samples of women with PCOS (both, insulin resistant (IR) and non-insulin resistant (non-IR)). They found, a total of 27 differential protein peaks between IR women with PCOS and controls,

Table 1. Summary of published proteomic studies in PCOS

Biological sample	Samples Size		Analytical method	Detection method	Total differentially regulated proteins	Validation technique			PCOS selection criteria	Ref
	PCOS	Control				WB	ELISA	IHC		
Ovarian tissue	3	3	2D-PAGE	MALDI-TOF/MS	69	HSP27		HSP27	Irregular menstrual cycles, chronic anovulation, polycystic ovaries and hyperandrogenism	222
					Up-54	HSP-10		HSP 10		
					Down-15	HSP47		HSP47		
						ANXA2				
					hnRNPA1					
Serum	5	6	2D PAGE 2D PAGE RP-SPE	MALDI-TOF/MS	4	complement component C4a			Rotterdam consensus criteria 2003	201
					Up-3					
					Down-1					
Serum	30	30		SELDI-TOF/MS	PCOS IR- 27				Rotterdam consensus criteria 2003	200
					Up-17					
					Down-10					
Visceral adipose tissue	10	9	2D-DIGE	MALDI-TOF/MS	PCOS non- IR 17				Presence of oligo-ovulation, clinical and/or biochemical hyperandrogenism	210
					Up-16					
					Down-1					
					9					
Granulosa cells	14	11	2D-PAGE	LC-MS/MS	20				Rotterdam consensus criteria 2003	208
					Up-12					
					Down-8					
T lymphocytes	20	20	2D-PAGE	MALDI-TOF/MS	10				Rotterdam consensus criteria 2003 and NIH criteria	212
					Up-2					
					Down-8					

Blood	12	12	2D-DIGE	MALDI-TOF/MS	4	HP	Rotterdam consensus criteria 2003	202
						Up-2 Down-2		
Follicular fluid	30	30	2D-PAGE	MALDI-TOF/MS	20	SERPINA1	Rotterdam consensus criteria 2003	204
						APOA1		
						TF		
Follicular fluid	5	5	2D-PAGE	MALDI-TOF/MS	6	APOA4	Rotterdam consensus criteria 2003	205
						A1BG		
						KNG1		
						KRT9		
						AT-B		
Visceral and subcutaneous adipose tissue	7	7	2D-DIGE	MALDI-TOF/MS	13Up-1 (between control and PCOS women)	FGG	National Institute of Child Health and Human Development (NICHD) conference criteria 1990	209
						Up-6		
						LC-MS/MS		
Follicular fluid	26	26	ITRAQ	LC-MS/MS	186	SERPINA1	Rotterdam consensus criteria 2003	160
						Up-99		
						Down-87		
Peripheral T lymphocytes	10	10	2D-PAGE	MALDI-TOF/MS	11	Up-8	Hyperandrogenic PCOS without metabolic and cardiovascular symptoms	211
						Down-3		

Abbreviations: 2D-DIGE, 2D gel electrophoresis, fluorescent labelled dyes in 2D- difference in gel electrophoresis; 2D-PAGE, 2D gel electrophoresis, polyacrylamide gel electrophoresis; A1BG, alpha-1-B-glycoprotein; ANXA2, annexin A2; APOA1, apolipoprotein 4; AT-B, antithrombin chain B; ELISA, enzyme linked immunosorbent assay; FGG, fibrinogen gamma chain; FN1, fibronectin 1; hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; HSP 10, heat shock protein 10; HSP27, heat shock protein 27; HSP47, heat shock protein 47; HSPG2, heparin sulphate proteoglycan 2; HC, immunohistochemistry; ITRAQ, isobaric tagging for relative and absolute quantitation; KNG1, kininogen-1; KRT9, keratin 9; LC-MS/MS, nano liquid chromatography-tandem mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RP-SPE, reversed-phase solid-phase extraction; SELDI, surface-enhanced laser adsorption/ionization; SERPINA1, serpin peptidase inhibitor, clade A (alpha-1 antitrypsin), member 1; TF, transferrin; WB, western blot

17 between non IR PCOS women and controls, and 19 proteins between IR and non-IR women with PCOS. However the limitations on reproducibility and reliability associated with the SELDI technique have compelled the researchers to explore other proteomic approaches (200). Similarly, Matharoo-Ball *et al.* also tried to identify some differentially expressed proteins in the serum of women with PCOS and controls. To achieve this, the samples were first pre-fractionated either by RP-SPE or by 2DE followed by SDS-PAGE and bands / spots were identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) technique. This group suggested that the complement proteins C4 α 3c and C4 γ , and haptoglobin (HP) α and β chains may be considered as potential biomarkers for PCOS (201). Another study reported the down-regulation of HP β chain levels in plasma using 2D-DIGE combined with MALDI-TOF/TOF, which was subsequently confirmed by Western blotting and ELISA (202). Though serum/plasma protein is very important for discovery of biomarkers, but many such proteins expected to have low abundance in serum/plasma making their detection difficult by best of the available techniques. Therefore it is critical to use tissue samples or fluids from the diseased tissue/organ for protein biomarker discovery.

Like serum, follicular fluid (FF) which provides a nutrient rich microenvironment to the growing oocytes, has also been subjected to proteomic analysis. FF, is a reservoir of intra-ovarian factors as well as blood plasma components, imparts developmental competence to the oocytes and facilitates ovulation (203). Variation in FF milieu is commonly observed in ovarian pathologies therefore, proteomic profiling of FF derived from women with PCOS has been carried out by few groups to elucidate the pathophysiology of PCOS. FF is collected from the ovarian follicles during oocyte retrieval from women undergoing *in vitro* fertilization. FF proteome, like serum or plasma is also complex and contains dynamic ranges of proteins, thereby necessitating the use of advanced techniques which employ a gel-free approach to identify large number of proteins. Until the advent of iTRAQ based comparative approach, a few researchers used 2DE combined with MALDI-TOF/MS or nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) to study the FF proteomes (204, 205), and identified twenty differentially expressed proteins which are involved in lipid metabolism and inflammation in PCOS. Our group has employed iTRAQ-based quantitative proteomic approach to compare the proteome of FF from women with PCOS with that of control women (160). We have identified a total 770 proteins in the FF, which is the largest protein dataset identified in FF from a single study to date of which 186 proteins were differentially regulated. The altered proteins are involved in angiogenesis, complement coagulation cascade, lipid transport and metabolism, expansion of

the cumulus oocyte complex matrix and basal lamina matrix (160), which are important for growth and maturation of the follicle. It provided us information on the biological processes which are dysregulated in PCOS and probably leads to the cessation of follicle growth in them. Although proteomic studies using body fluids could prove to be highly effective for biomarker discovery, the high abundance of certain proteins and complexity of these samples renders it cumbersome to detect low-abundance proteins and analyze the data.

Apart from body fluids, proteomic studies have also been carried out in a large variety of cells and tissues obtained from women with PCOS. Analysis of the tissues/cells obtained from the organ which are directly affected in a specific disease can provide insights in the tissue specific pathophysiology of that disorder. Ovary is the main organ implicated in PCOS, but it is difficult to obtain ovarian samples for research studies. Only one group compared the proteome of the ovary from women with PCOS collected during wedge resection surgery with normal ovarian tissues, which were collected from ovaries of cadavers (206). Using 2DE-MALDI TOF/TOF technique a total of 69 differentially expressed proteins were identified, of which most of the proteins were involved in regulation of cellular physiological processes and metabolism. The limitation of this study wasa relatively smaller sample size (n=3) and no detailed information about the ovarian biopsies mentioned. These drawbacks however, can be partially addressed by the use of homogeneous cells (cumulus or mural granulosa cells) obtained from women undergoing controlled ovarian hyperstimulation. Granulosa cells (GCs) which envelope the growing oocyte, play an essential role in the maturation and release of oocytes. GCs from women with PCOS often exhibit alterations in their responses to gonadotropins and their steroidogenic capacities (207). A comparative proteomic study on GCs from women with PCOS has been carried out by Choi *et al.* by using 2DE and liquid chromatography, coupled with mass spectrometry (LC-MS/MS). Apolipoprotein A1 (ApoA1) was found to be down-regulated and putatively involved in altered steroidogenesis observed in PCOS (208).

Obesity plays a major role in the development of PCOS and is a well-established precursor for obesity-induced insulin resistance and hyperinsulinemia. Adipose tissue secretes steroid hormones, their metabolites and growth factors which are crucial for maintenance of energy homeostasis. Comparative proteomic studies have been carried out using 2D-DIGE combined with MALDI-TOF in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) (209, 210) obtained from women undergoing bariatric surgery. proteins involved in oxidative stress, toxicity and several metabolic processes were found to be dysregulated in omental fat tissue obtained from

Table 2. Summary of published proteomic studies in PCOS

Protein name	Gene symbol	Differential regulation	References
3-hydroxyisobutyrate dehydrogenase mitochondrial	HIBADH	Down	(211, 202)
Serpin family A member 1	SERPINA1	Up	(160, 204)
Aconitase 2, mitochondrial	ACO2	Up	(160, 222)
Albumin	ALB	Up	(209)
Albumin	ALB	Down	(160, 210)
Aplha-1-B-glycoprotein	A1BG	Up	(204, 205)
Aplha-1-B-glycoprotein	A1BG	Down	(160)
Alpha-enolase	ENO1	Down	(211, 212)
Annexin 5	ANXA5	Up	(208, 210)
Apolipoprotein A1	APOA1	Up	(160, 204)
Apolipoprotein A1	APOA1	Down	(208, 210, 222)
Apolipoprotein A4 precursor	APOA4	Up	(204, 205)
Apolipoprotein C1	APOC1	Up	(200)
Apolipoprotein C1	APOC1	Down	(160)
Cathepsin D	CTSD	Down	(211, 212)
Cofilin 1	CFL1	Down	(211, 212)
F-actin capping protein alpha-1 subunit	CAPZA1	Down	(211, 212)
Fibrinogen b chain precursor	FGB	Up	(204)
Fibrinogen gamma chain precursor	FGG	Up	(205, 222)
Fibrinogen, alpha chain, isoform alpha preproprotein	FGA	Up	(222)
Glutathione S-transferase Mu 2	GSTM2	Up	(222)
Glutathione S-transferase omega 1	GSTO1	Up	(160)
Glutathione S-Transferase Mu 3	GSTM3	Up	(210)
Haptoglobin	HP	Up	(160, 201, 204)
Haptoglobin	HP	Down	(202)
Heat shock 27 kDa protein (HSP 27)	HSPB1	Down	(222)
Heat shock 27 kDa protein (HSP 27)	HSPB1	Up	(209)
Kininogen 1	KNG1	Up	(160, 205)
Platelet basic protein (precursor)	PPBP	Up	(211, 212)
Protein disulfideisomerase A3	PDIA3	Down	(211, 212)
Reticulocalbin 1 precursor	RCN1	Up	(208)
Reticulocalbin 1 precursor	RCN1	Down	(160)
Rho GDP- Dissociation Inhibitor	ARHGDI A	Down	(211, 212)
Superoxide dismutase (Mn), mitochondrial (precursor)	SOD2	Up	(211, 212)
The Raf kinase inhibitor protein	RAF1	Down	(211, 212)
Cytokeratin 9	KRT9	Up	(205)
Cytokeratin 9	KRT9	Down	(204)
Transferrin	TF	Up	(202, 204)

morbidity obese women with and without PCOS (209, 210). The same group compared the VAT and SAT of PCOS women and found upregulation of peroxiredoxin 6, creatine kinase, selenium-binding protein 1, albumin etc. in VAT compared with SAT (209).

Circulating T lymphocytes and their subpopulations are extremely sensitive to environmental modifications, physiological alterations and the molecular signals elicited by these changes

and can serve as molecular sensors to reflect normal or pathological conditions. These are easily available in large numbers and therefore make a good sample for studying immunological alterations in PCOS. The proteome of T lymphocytes compared in controls and PCOS, using comparative 2D-PAGE and MALDI-TOF and showed differentially expressed proteins involved in various pathways like regulation of the cytoskeleton structure, apoptosis, angiogenesis, insulin resistance, cell proliferation and oxidative stress (211, 212).

5.3. Differentially regulated common proteins identified across proteomic studies in PCOS

Although proteomic studies in PCOS have not yet been successful in establishing biomarkers, many proteins showed differential abundance across two or more studies which are discussed below (Table 2).

Oxidative stress is increased in women with PCOS leading to molecular damage and altered cellular function at a systemic as well as tissue-specific level (213, 214). Increased oxidative stress in ovary may contribute to abnormal follicular development, poor oocyte quality and infertility (215, 216). Several proteins involved in maintaining the balance between reactive oxygen species/antioxidants were found to be differentially regulated in PCOS. Three enzymes of glutathione-S-transferase (GST) family have detected to be upregulated in ovarian tissue, VAT and FF of women with PCOS (160, 206, 210). In our study, we found altered expression of GST family members, upregulated GST omega-1 isoform 2, downregulated GST alpha1 and unchanged levels of GST mu 1 and mu 2 (160). These enzymes are involved in detoxification of hydrophobic electrophiles compounds, including carcinogens, drugs, toxins and products of oxidative stress, by conjugation with reduced glutathione. Higher levels of annexin 5 (208, 210) are present in GCs and VAT of women with PCOS. The functions of annexin5 have been attributed to cellular responses to interferon gamma (IFN- γ) (a pro-inflammatory molecule), by attenuation of signaling mediated by the Jak-Stat1 pathway (217), and in reduction of oxidative stress induced chronic inflammation in the adipose tissue (210).

Several circulating proteins have been observed to be dysregulated, including serpin family A member 1 (SERPINA1), transferrin and HP, which are dysregulated in blood and/ or FF of women with PCOS (160, 201, 202, 204) which are predominantly synthesized and secreted by hepatocytes and their expression increases during inflammation (204, 218). HP is involved in reduction of oxidative stress while transferrin, a beta-globulin molecule, plays a role in the transport of iron to various organs and tissues in the body. High levels of transferrin can reportedly block the binding of FSH with its receptors on granulosa cells and also decrease cAMP production (204). SERPINA1 is a serine protease inhibitor, which inhibits the plasminogen activator protein. Plasminogen activator produces a proteolytic enzyme, plasmin that cleaves off from plasminogen which is involved in clot lysis. We have found reduced expression of plasminogen and overexpression of SERPINA1 in FF of PCOS (160), which may limit plasmin production and thus impair fibrinolysis. Plasminogen and plasminogen activator inhibitor plays a role in follicle wall breakdown during ovulation, extra cellular matrix remodeling, coagulation (219). Dysregulation of these proteins may be one of the reasons for increased inflammation, oxidative

stress, dysregulation of iron metabolism and failure of oocyte maturation and ovulation, failed implantation and miscarriage (160, 202, 204) observed in PCOS. Fibrinogen, a blood-borne glycoprotein which is cleaved by thrombin to form fibrin following vascular injury, was found to be upregulated in the ovary and FF of women with PCOS (204-206). It is involved in regulation of cell adhesion, vasoconstriction, chemotactic activities, cell metabolism, homeostasis and proliferation. Higher expression of fibrinogen in ovarian tissue and FF might contribute to impaired vascular permeability, abnormal fibrinogenesis and thrombosis in women with PCOS (204-206). Higher spontaneous abortion rates and complications in pregnancy are often observed in PCOS women because of hypofibrinolysis and thrombophilia which may be due to altered fibrinogen and lowered plasmin levels in them and impaired fibrinolysis have been proposed to contribute to cardiovascular disease risk in PCOS (206).

APOA1 is the major structural protein component of high density lipoprotein (HDL) cholesterol and has anti-inflammatory properties. This enzyme is synthesized in liver and small intestine and promotes cholesterol efflux and regulates lipid metabolism. Decreased APOA1 levels have been reported in GCs, VAT and ovarian tissue (206, 208, 210) which may be related to altered synthesis of steroid hormones reported in PCOS (208). On the contrary APOA1 levels are increased in serum and FF (160, 204) of women with PCOS which may be associated with increased cardiovascular risk (220). Apolipoprotein A4 (APOA4) is a glycoprotein, expressed in response to fatty meals in small intestine, plays an important role in lipid transport, metabolism of lipoproteins and gastric function. In FF of women with PCOS, APOA4 is significantly higher compared to normal women, which may cause defects in lipoprotein metabolism, transport and ovulation contributing to PCOS pathophysiology (204, 205). However, we have not observed any difference in the APOA4 levels in our study (160). On the other hand, apolipoprotein C1 (APOC1) concentrations in serum are high in PCOS but low in FF of these women, an observation that still necessitates validation in PCOS (200), as APOC1 acts by inhibiting lipoprotein metabolism in the liver and increases LDL, thus increasing the risk of cardiovascular disease. Also, there are a few contradictory reports about albumin and cytokeratin levels in adipose tissue (209, 210) and FF (204, 205) of women with PCOS. In FF, after immunodepletion of the 14 most abundant proteins, albumin levels were found to be down regulated (160).

Another protein, Cofilin 1, a F-actin capping protein, which regulates mitosis, cytokinesis, cytoskeletal rearrangement and actin dynamics in T cell migration and activation (221) was found to be down regulated in T lymphocytes of women with

PCOS (211, 212). The lower expression of this protein might reflect the different functional state of the cellular immunity in PCOS. Similarly, the expression levels of proteins such as 3-hydroxyisobutyrate dehydrogenase mitochondrial, protein disulphideisomerase A3, Raf kinase inhibitor protein were found to be decreased in T lymphocytes of women with PCOS while that of superoxide dismutase (SOD) was increased in these cells. These proteins are involved in insulin resistance and in adipocyte differentiation, which is altered in PCOS (211, 212). Also, cathepsin D, an aspartyllysosomal protease involved in apoptosis, angiogenesis and proliferation processes were found to be down-regulated in peripheral T cells (211, 212).

Several novel proteins were identified in ovary in proteomics studies, however, these proteins need to be studied further to understand their role in ovarian physiology and pathophysiology. Reticulocalbin 1 (RCN1) belongs to the family of Cab45, reticulocalbin, ERC-55 and calumenin (CREC) proteins that were characterized as Ca²⁺-binding proteins in the endoplasmic reticulum with EF hands. It is found to be upregulated in granulosa cells and down regulated in FF of women with PCOS (160, 208). Proteins involved in coagulation cascade like alpha enolase, platelet basic protein and kininogen 1 are observed to be dysregulated in peripheral T cells and FF (211, 212). Alpha-1-B-glycoprotein is a plasma protein that belongs to the immunoglobulin superfamily is identified in FF of PCOS women by three independent studies, however results are controversial (160, 204, 205). Aconitase and heat shock protein 27 (HSP27) are also dysregulated in FF and ovarian tissue of women with PCOS (160, 209, 222).

Post-translation modification (PTM) of proteins plays an important role in biological processes. The widely studied PTM is protein phosphorylation, which regulates critical cellular processes such as cell signaling, enzymatic activity, cell cycle etc. Altered phosphorylation is often associated with diseases; therefore analysis of the phosphoproteome is important to understand disease mechanism. However phosphoproteome analysis has lot of challenges. More than 50% of proteins are glycosylated in human body, which provides more structural stability or function to the native protein structure. Altered protein glycosylations have been associated with many diseases. Therefore, it is necessary to study phosphoproteome and glycoproteome as well as protein-protein interaction by gel-free MS/MS quantitative techniques, which will give in depth information of dynamic changes in different forms of proteins in diseased condition like PCOS.

5.4. Concluding remarks

Proteomic approaches have identified proteins known to participate in several metabolic and cellular pathways including oxidative stress, cell proliferation,

inflammation and insulin resistance that might be involved in the pathogenesis of PCOS. These proteins, in particular those that have been detected more frequently in different studies could be possibly considered as candidates markers in future studies addressing the molecular phenotypes of this syndrome. Although, there is a dire need to validate the reproducibility of such data in larger populations. As PCOS is a complex disorder many more low abundant proteins related to disorder may still not have discovered. Recent advances in proteomics technology using gel free approaches for quantitative proteomics using in-vivo methods (SILAC, stable isotope labelling by amino acids in cell culture, CDIT, culture derived isotope tags), or in-vitro methods (ICAT, isotope coded affinity tag VICAT, visible isotope coded affinity tag) with high end mass spectrometry may help to identify promising biomarkers and unravel disease pathophysiology.

6. CONCLUSION

In the current review we have summarized the salient findings emerging from multi-omic studies previously carried out with a focus towards dissecting the pathophysiology of PCOS. We can thus conclude that integrating the data from GWAS regarding susceptibility variants, epigenetic studies highlighting key methylation sites and differentially regulated from proteome profiles could unravel complex underlying networks, which may otherwise be missed by applying a single approach. As can be observed from the extensive review above, advancement in high throughput platforms with superior resolution as well as statistical techniques facilitates novel biomarker discovery. What is interesting is that although these studies have revealed several known and novel markers, there is not as much of overlap observed across all three approaches. We have summarized a few key markers which have commonly emerged from GWAS and epigenetic studies (Table 3). This highlights that genetic variation coupled with altered expression could perpetuate PCOS pathogenesis. Collectively, these molecular biomarkers trigger several biological processes which may be tapped to develop integrated pathophysiology and offer suitable targets for extending existing, or altogether creating new, therapeutic regimes.

Currently most PCOS management strategies are directed towards ameliorating the clinical symptoms of PCOS mainly hirsutism and acne and irregular menses. Management protocols involving lifestyle and dietary modifications directed towards weight loss in overweight women with PCOS have shown that even small amounts of weight reduction ameliorate androgen and insulin levels, restore menstrual irregularities and improve fertility (223). However, the success of medical interventions employed may vary depending on the genetic makeup of the individual. Prominently,

Table 3. ANOVA for the experimental results of the central composite design (Biomass)

Gene	GWAS Findings	DNA Methylation Based Findings	Function
LHCGR	Several polymorphisms across the gene strongly associated with risk of PCOS development and metabolic and reproductive traits	Promoter CpG hypomethylation in human and rat ovarian granulosa cells and human peripheral blood leukocytes	Binds LH and stimulates steroidogenesis via cAMP signaling, participates in follicular development, atresia, ovulation, luteinization, oocyte maturation
INSR	Important candidate gene and polymorphisms have been reported to be associated with PCOS susceptibility and its related traits	Partial promoter methylation in the human endometrial tissue, no association with insulin resistance	Activates insulin signaling pathways, mainly the PI3K-AKT/PKB pathway responsible for metabolic actions of insulin; and the Ras-MAPK pathway which regulates gene expression. Regulates glucose uptake, release and utilization
YAP1	rs11225161 (A/G) allele frequency was altered in PCOS and correlated with increased glucose tolerance in PCOS	Decreased promoter methylation in ovarian granulosa cells of PCOS women correlating with increased transcript and protein level	Transcriptional regulator of Hippo signaling pathway, mediates tumour suppression and promotes apoptosis. Regulates tissue proliferation, gene expression, induces epithelial mesenchymal transition in presence of TEAD transcription factors
TOX3	rs4784165 polymorphism showed a marginally significant trend in transmission in Han Chinese families	TOX3 promoter hypomethylated in DNA isolated from serum and ovarian granulosa cells of women with PCOS, increased level of protein in PCOS	Participates in LH driven steroidogenesis, transcriptionally coactivates p300/CBP-mediated transcription complex, mediates transactivation via the cAMP response element (CRE) sites, imparts protection against cell death via anti apoptotic mechanisms, and stimulates transcription from estrogen-responsive or BCL-2 promoters

Abbreviations:cAMP, Cyclic adenosine monophosphate; CpG, 5'-cytosine-phosphodiester-guanine-3; INSR, insulin receptor; LH, luteinizing hormone; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; Ras-MAPK, Ras/mitogen activated protein kinase; TEAD, TEA domain family member TOX3, TOX high mobility group box family member 3; YAP1, yes-associated protein 1

the action of pharmacologic agents may be altered depending on modified expression or action of its target downstream signaling molecules, which in turn may hinge on genetic and epigenetic variations. For instance, metformin, an insulin sensitizer, widely used in medical management of PCOS, has been shown to be more effective in stimulating ovulation and restoring menstrual cycles in lean compared to obese women with PCOS(224, 225) with the highest rate of beneficial endocrine changes being observed in hyperinsulinemichyperandrogenic lean women with PCOS(226). PPAR γ , another widely studied candidate marker in both genetic and epigenetic studies, is targeted by the thiazolidinedione (TZDs) group of drugs, commonly used to treat hyperinsulinemia and insulin resistance in PCOS(227). The use of gonadotropins, aromatase inhibitors, anti-androgens for inducing ovulation and managing hyperandrogenism in women with PCOS(228) would also rely upon the sensitivity of the respective receptors, which again may be altered in expression and activation due to molecular changes.

The effective selection and simultaneous assessment of molecular markers in determining key aspects of PCOS pathophysiology may impact clinical approaches to patient care. Biological significance of key markers along with their association with heterogeneous phenotypes can be suitably assessed. Thus, combining tissue specific markers to decipher overall systemic biology changes, novel and effective models for diagnosis, prognosis and therapy may be shaped.

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Abbreviations: 2D-DIGE, 2D gel electrophoresis, fluorescent labelled dyes in 2D- difference in gel electrophoresis; 2DE, 2 dimensional gel electrophoresis; 5'-UTR, 5'-untranslated region; 5caC, 5'-carboxylcytosine; 5fC, 5'-formylcytosine; 5hmC, 5'-hydroxymethylcytosine; 5mC, 5'-methylcytosine; A1AT, alpha 1- antitrypsin, A1BG, alpha-1-B-glycoprotein; AMH, anti Mullerian hormone; ANXA2, annexin A2; AT-B, antithrombin chain B, ApoA1, apolipoprotein A1; APOA4, apolipoprotein 4; ApoC1, apolipoprotein C1; AR/ Ar, androgen receptor; BMI, body mass index; C9orf3, chromosome 9 open reading frame 3; cAMP, Cyclic adenosine monophosphate; CDIT, culture derived isotope tags; CGCs, cumulus granulosa cells; CGI, CpG island; COH, controlled ovarian hyperstimulation; CpG, 5'-cytosine-phosphodiester-guanine-3; CREC, Cab45, reticulocalbin, ERC-55 and calumenin; CVD, cardiovascular disease; Cyp11a1, cytochrome P450 Family 11 subfamily A member 1; Cyp17a1, cytochrome P450 Family 17 subfamily A member 1; Cyp19a1, cytochrome P450 Family 19 subfamily A member 1; DENN, differentially expressed in normal and neoplastic cells; CTBP1-AS, C-terminal binding protein 1 antisense; DENND1, DENN domain containing 1A; DHEAS, dehydroepiandrosteronesulphate; DNA, deoxyribonucleic acid; DNMT, DNA

methyltransferase; EDC, endocrine disruptor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; EPHX1, epoxide hydrolase 1; ERBB2, ERB-B2 receptor tyrosine kinase 2; FDR, false discovery rate; FF, follicular fluid; FGG, fibrinogen gamma chain; FN1, fibronectin 1; FSH, follicle stimulating hormone; FSHR, follicle stimulating hormone receptor; GC, granulosa cells; GCs, granulosa cells; GLUT4, glucose transporter type 4; GnRH, gonadotropin releasing hormone; GSTM3, glutathione S-transferase M3; GWAS, genome-wide association study; GYS2, glycogen synthase 2; HA, hyperandrogenic PCOS; HDAC, histone deacetylase; HDL, high density lipoprotein; HMGA2, high mobility group AT-hook 2; HNRNPA1, heterogeneous nuclear ribonucleoprotein A1; HSPG2, heparin sulphate proteoglycan 2; HP, haptoglobin; HPLC, high performance liquid chromatography; hsa-mir, homo sapien micro-ribonucleic acid; HSP 10, heat shock protein 10 HSP27, heat shock protein 27; HSP47, heat shock protein 47 ICAT, isotope coded affinity tag; IFN γ , interferon gamma; IGFBP2, Insulin Like Growth Factor Binding Protein 2; IHC, immunohistochemistry; INSR, insulin receptor; IR, insulin resistant; iTRAQ, isobaric tagging for relative and absolute quantitation; KHDRBS3, KH RNA binding domain containing, signal transduction associated 3; LC-MS/MS, nano liquid chromatography-tandem mass spectrometry; LDL, low density lipoprotein; LH, luteinizing hormone; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; Lhr, luteinizing hormone receptor; LINE1, long interspersed nuclear element; LncRNA, long non-coding ribonucleic acid; KNG, kininogen-1, KRT1, keratin 1; KRT9, keratin 9 MAGENTA, meta-analysis gene-set enrichment of variant associations; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight/ mass spectrometry; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight; MeDIP, methylated DNA immunoprecipitation; MHC, major histocompatibility complex; mtDNA, mitochondrial DNA; ncRNA, non-coding ribonucleic acid, NGS, next generation sequencing; NIH, National Institutes of Health; NRIP1, nuclear receptor interacting protein-1; OHSS, ovarian hyperstimulation syndrome; OACT2, membrane bound O-acyltransferase domain containing 2; PA, prenatally androgenized; PBL, peripheral blood leukocytes; PCO, polycystic ovary; PCOS, polycystic ovary syndrome; PPARG/ γ Pparg, peroxisome proliferator-activated receptor gamma PTM, post translational modifications; RAB5B, member RAS oncogene family; RAB6A, member RAS oncogene family; RCN1, reticulocalbin 1; RNA Pol II, ribonucleic acid

polymerase II; RP-SPE, reversed-phase solid-phase-extraction; SAT, subcutaneous adipose tissue; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SELDI, surface-enhanced laser adsorption/ ionization; SERPINA1, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1; SEPT9, septin-9; SHBG, sex hormone binding globulin; SILAC, stable isotope labelling by amino acids in cell culture; SINE, short interspersed nuclear element; SNP, single nucleotide polymorphism; SLC2A8, solute carrier family 2 member 8; SOD, superoxide dismutase; SRD5A1, steroid 5 alpha-reductase 1; SUMO1, small ubiquitin-like modifier 1; SUMO1P1, SUMO1 pseudogene 1; SUOX, sulfite oxidase; SUV39H2, suppressor of variegation 3-9 homolog 2; T2DM, type-2 Diabetes Mellitus; TEI, transgenerational epigenetic inheritance; TET, ten eleven translocation enzymes; TF, transferrin; TGF- β , transforming growth factor beta; THADA, thyroid adenoma-associated gene; TNIK, TRAF2 and NCK interacting kinase; TOB, transducer of ERBB2; TOX3, TOX high mobility group box family member 3; TP, testosterone propionate; VAT, visceral adipose tissue; VDR-RXR, vitamin D receptor-retinoid X receptor; VICAT, visible isotope coded affinity tag; XCI, X-chromosome inactivation; YAP1, yes-associated protein 1

Key Words: Polycystic ovary syndrome, Genetics, Epigenetics, Proteomics, Review

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