# Association of Insulin Receptor and Peroxisome Proliferator-Activated Receptor γ Gene Polymorphisms with Phenotypic Features and Insulin Resistance in Women with Polycystic Ovary Syndrome

**A Thesis Submitted to the University of Dhaka in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy (PhD) in Biochemistry and Molecular Biology**


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# DECLARATION

This is hereby declared that the research works under this Thesis, titled '**Association of Insulin Receptor and Peroxisome Proliferator-Activated Receptor γ Gene Polymorphisms with Phenotypic Features and Insulin Resistance in Women with Polycystic Ovary Syndrome**' has been based on my research work and has been carried out under the direct supervision of **Prof Yearul Kabir**, Professor, Department of Biochemistry & Molecular Biology, University of Dhaka, as partial fulfilment of the requirements for the Degree of Doctors of Philosophy (PhD).

No part of this thesis has been previously submitted for any other qualification of the University of Dhaka or any other University or Institute for any other degree.

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*Dedicated to My Beloved Mother*

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# ABSTRACT

**Introduction:** Polycystic Ovary Syndrome (PCOS), the most prevalent endocrine disorder among reproductive-aged women, is characterized by three diagnostic phenotypic features: anovulation/ oligomenorrhea (AO/OM), polycystic ovarian morphology (PCOM), and hyperandrogenism (HA). Various ethnic groups differ in the prevalence and metabolic characteristics of PCOS. Insulin resistance (IR) is an important covariate in PCOS; its prevalence is reported to vary from 44 to 70% and Asian PCOS women are known to be more prone to IR. Both PCOS and IR are under significant genetic control with substantial heterogeneity among various racial groups. Several genes of the insulin signalling pathway are targeted as candidate genes linked with PCOS, among those INSR and PPARG genes are among the major ones. His1085C/T polymorphism of the INSR gene and His447C/T polymorphism in the PPARγ gene have been found to be significantly associated with PCOS in various populations including Indian women. In facility-based studies on the Bangalee population (the 3$^{rd}$ largest ethnic group in the world after the Han Chinese and Arabs) the prevalence of PCOS has been found to vary from 6.11%-92.16%. In reality, the prevalence as well as clinical, metabolic and endocrine aspects of PCOS have not yet been well studied in this population. The prevalence of IR has varied widely from 16% to 77%, but standardized methodology was not followed in most studies. To our knowledge, no genetic analysis has also been reported to explain the various phenotypic features of PCOS in this population. Given the critical role played by insulin resistance in the pathophysiology as well as in the management and prevention of PCOS His1085C/T polymorphism of the INSR gene and His447C/T polymorphism in the PPARγ gene were chosen to be explored in this study for their possible association with phenotypic features and IR among Bangalee women with PCOS. The specific objectives were: a. to explore the proportion of IR and its covariates in PCOS; b. to find out the independent association of individual diagnostic phenotypic features of PCOS with IR; c. to compare the frequency of the two different single nucleotide polymorphisms (SNPs) between PCOS and healthy women; and d. to explore the independent association of the investigated SNPs with phenotypic features and IR on adjustment of the effects of major confounding variables.

**Methods:** Following a case-control design an observational analytic study was conducted on age-matched 111 non-PCOS and 141 PCOS subjects [age, years, median (range): 24 (19-34) in non-PCOS vs 23 (15-34) in PCOS groups], both recruited from community and hospital-based settings through purposive sampling. PCOS was diagnosed as per modified Rotterdam criteria which require the presence of at least 2 of the 3 phenotypic features: AM/PM, HA and PCOM. Subjects with pregnancy, lactation, smoking habits, and other potential modifiers of endocrine/ ovarian function (such as metabolic or other disorders and use of oral contraceptives and relevant drugs) were excluded. Hirsutism was detected and graded using the Ferriman-Gallwey score (FG-score) with a cut-off value of ≥ 8 as a marker of clinical HA. All patients undertook a standard 75-g oral glucose tolerance test (OGTT). Serum levels of hormones [c-peptide (as surrogate for insulin), total testosterone and sex hormone binding globulin (SHBG)] were assayed by chemiluminescent immunoassay (CLIA) technique; other serum parameters [Glucose (Gl) and Lipid Profile] were estimated by automation-based standard techniques. HA was defined in terms of biochemical HA, as women having free androgen index (FAI) >7.1, computed by the formula TT (nmol/L)/SHBG (nmol/l) ×100. IR was calculated by using the C-peptide-modified formula (HOMA1-IR = 1.5 + FPG × FCP / 2800) with a value > 2.4 considered to represent IR. A lower abdominal ultrasound with folliculometry was done using a Voluson E6 machine and a standard counting procedure was followed to detect PCOM. Genomic DNA was extracted from peripheral blood using a standard DNA isolation &and purification kit and PCR amplification was done with the following regions of the targeted genes:- INSR - Cytogenic location 19p13.3-p13.2, primary target rs1799817; and b. PPARγ - Cytogenic location 3q25, primary target rs3856806. The resulting amplified products (800 bp segments up- and down-streams with primary targets in the middle) were sequenced by Sanger sequencing using an automated Analyzer and the data were analyzed using bioinformatics tools. Statistical analyses were done by relevant bi- and multi-variate statistics using SPSS for Windows (v 26) software. The study was approved by the Ethics Committees of the Department of Biochemistry of the University of Dhaka and BSMMU.

**Results:** PCOS subjects had significantly higher (p=0.037) median BMI (23.98 vs 23.40) and higher (p=0.019) Waist-Hip Ratio (WHR) (0.94 vs 0.92) as compared to non-PCOS subjects. PCOS subjects also had significantly higher median 2hBG (p=0.004)], TChol (p=0.045), and TG (p=0.002) as compared to Non-PCOS subjects. PCOS subjects had significantly higher

median total Testosterone [U/l: 1.4 (0.47-5.20) vs 1.1 (0.45-2.91), p=0.001] and median HOMA-IR [%:2.5 (1.7-29.1) vs 2.2 (1.7-4.6), p<0.001] as compared to their Non-PCOS counterparts. The proportion of subjects with IR was 52% and 28% in the PCOS and Non-PCOS groups, respectively. HA was present in much higher proportion among insulin-resistant PCOS subjects (83%) as compared to the non-insulin-resistant ones (46%), with a highly significant difference ($x^2$=17.11; p <0.001). IR was found to be significantly correlated with BMI (p<0.001), TG (p=0.007), FAI (p<0.001) and 2hBG (p=0.002) in the PCOS group. On binary logistic regression analysis, BMI (p<0.001) and FAI (p=0.001) showed significant association with IR when the effects of age, WHR, TC, and TG were adjusted. Regarding His1085C/T alleles in the INSR Gene, the proportions of C and T alleles were 56% and 42% among Non-PCOS and 69% and 41% among PCOS subjects (p=ns). No significant difference in allele distribution was found for any phenotypic feature of PCOS or for IR, either in the Non-PCOS or PCOS group. No significant association of His1085C/T polymorphism of the INSR gene with individual phenotypic features of PCOS or with IR was found on binary logistic regression analysis. Regarding His 447C/T Polymorphism of the PPARγ Gene, the proportions of C and T alleles were 83% and 17% among Non-PCOS and 82% and 18% among PCOS subjects (p=ns). Except for Hirsutism, no significant difference in allele distribution was found for any phenotypic feature of PCOS or for IR, either in the Non-PCOS or PCOS group; only in the PCOS group, Hirsutism showed a significant positive association (p=0.05) with the polymorphism. No significant association of His 447C/T Polymorphism of PPARγ Gene with individual phenotypic features of PCOS or with IR was found on binary logistic regression analysis. On multiple linear regression analysis, a significant (p=0.02) association of IR with His447C/T polymorphism in the PPARγ Gene was found on adjusting the effects of age, BMI. WHR, TC, TG, and total testosterone.

**Conclusions:** The present data lead to the following conclusions: i. Overweight and obesity (both central and peripheral) are notable features of PCOS among young to early middle-aged Bangalee women; ii. Substantial proportions of non-PCOS young women are already overweight/ obese as well as hyperandrogenic signifying their potential risk of being PCOS in future; iii. Insulin resistance seems to be present among more than fifty percent of young PCOS and more than a quarter of young non-PCOS subjects; iv. Strong independent association of insulin resistance with hyperandrogenism is present in PCOS irrespective of age and lipidemic

status; v. His1085C/T polymorphism of the INSR gene does not seem to have a significant association with any of the characteristic phenotypic features of PCOS or with insulin resistance among Bangalee women; vi. Among Bangalee women, both clinical hyperandrogenism (an important phenotypic feature in diagnosing PCOS) and insulin resistance are associated with His447C/T polymorphism in the PPARγ gene; and vii. There is no independent association of biochemical hyperandrogenism with PPARG gene polymorphism among Bangalee women; however, the polymorphism contributes to clinical hyperandrogenism through the mediation of insulin resistance in these women.

# Chapter 1: INTRODUCTION

## 1.1 BACKGROUND

Polycystic Ovary Syndrome (PCOS) is the most prevalent endocrine disorder among reproductive-aged women [1]. Its prevalence is reported to vary widely (2.2%-48%) [2-3] which seems to be due to genuine racial differences and subject groups as well as due to different diagnostic criteria used in various studies [4]. Anovulation/oligomenorrhea (AO/OM), polycystic ovarian morphology (PCOM), and hyperandrogenism (HA) are the three diagnostic phenotypic features of PCOS; however, their mandatory inclusion in the clinical diagnosis of the disorder varies depending on the criteria used. Three diagnostic criteria are mainly used worldwide in the case of PCOS: National Institutes of Health (NIH) [5], modified Rotterdam Guideline 2003 [6-7], and Androgen Excess Society (AES) [8]. The NIH criteria suggests the mandatory presence of 1$^{st}$ and 3$^{rd}$ phenotypic features, the modified Rotterdam criteria suggests the presence of any two, and the AES criteria suggests the mandatory presence of the 3$^{rd}$ plus optional presence of any of the 1$^{st}$ and 2$^{nd}$ phenotypic features for the confirmed diagnosis of PCOS.

Apart from the issue of varying diagnostic criteria, genuine differences seem to exist among ethnic groups regarding the prevalence and metabolic characteristics of PCOS. From the ethnicity-specific guidelines, it is revealed that the prevalence of women with PCOS differs among Chinese, White, and Black women [9-10]. Adverse metabolic features have been found to be more common among South Asian, Asian Indian, African American, and Hispanic women with PCOS. In contrast, Middle-Eastern and Mediterranean women have been found to be more hirsute in general [9]. A multi-country study observed a maximum predisposition to metabolic syndromeamong Indian women, followed by US and Norwegian women with PCOS [11]. The influence of geographic variation on phenotypic manifestation is further highlighted by a study on Indian women from two regions of Northern India. It shows distinct phenotypes in two cities of North India - lean hyperandrogenic women from Srinagar and obese hyperinsulinemic women from Delhi [12]. A previous study demonstrated that according to Rotterdam's criteria, the pooled PCOS prevalence estimate in India is 11.34% [13]. The estimated prevalence in India ranged from 3.7 to 22.5 percent and was found to be higher or lower depending on the criteria used, which might be the obvious reason for the discrepancy in the prevalence rates among the studies [14].

Reported studies on PCOS epidemiology in Bangladesh are only facility-based. The prevalence of the disease ranged from 6.11% (among the subjects visiting the Gynecology-Obstetrics outpatient department) to 92.16% (in subjects consulted for hirsutism) [15]. The prevalence was 29.9%–46.15% among infertile women [16-20]. A study among medical students reported of 37% prevalence [21]. Moreover, two studies categorized subjects with PCOS according to phenotypic features [22-23]. Most of these studies are commonly limited by small sample sizes and selection bias, and the findings are not comparable due to inconsistencies in the diagnostic criteria for PCOS. Accordingly, studies on different subpopulations in Bangladesh, using specific diagnostic criteria, are needed to clarify the various phenotypic characteristics and metabolic abnormalities among different groups of the Bangladeshi population. Such data are required to design evidence-based, context-sensitive management and prevention policies for PCOS and to plan genotype-related studies on the disease among this population.

Insulin resistance (IR) is known to be an important covariate in PCOS both as an intermediate risk factor as well as an outcome of HA. The prevalence of IR in PCOS patients has been reported to vary from 44 to 70% [24-25]. This wide range may be due to several factors, including the heterogeneity of the diagnostic criteria for PCOS employed in these studies [24], the genetic background among the assessed population [26], and differences in the methods used for defining IR [24, 27]. Compared to European and European-American subjects, Asian PCOS women are more prone to become insulin resistant [28].

Although the pathophysiological mechanism of IR in PCOS is relatively well understood its etiological role, as well as covariates, still need to be fully clear. There is ongoing debate on whether IR is intrinsic to PCOS, related to obesity alone, or related to both factors. Several studies have indicated the presence of insulin resistance and compensatory hyperinsulinemia in approximately 80% of obese women with PCOS and 30–40% of lean women [29]. On the other hand, there is an increased prevalence of obesity and abdominal obesity in PCOS [30], which may worsen the IR-associated clinical features [31-32]. It is hypothesized that lean women with PCOS have PCOS-specific IR or intrinsic IR, which is augmented by the presence of obesity-specific IR [33]. Other authors have also suggested IR is inherent in PCOS which decreases hepatic sex hormone-binding globulin (SHBG production) and increases total as well as free androgens and LH secretion [34-35].

PCOS and insulin resistance are heritable disorders under significant genetic control [36]. Many studies have revealed abnormalities in insulin secretion and action in women with PCOS. More than a decade ago, convincing evidence showed that most women with PCOS, both obese and lean, have a degree of insulin resistance, which led to the assumption that genes involved in insulin secretion and action may play a role in the pathogenesis of PCOS. It has been found that several genes of the insulin signaling pathway are among the earliest candidate genes examined for PCOS, including insulin gene (INS), insulin receptor (INSR), insulin receptor substrate proteins (IRS1&IRS2), Peroxisome Proliferator-Activated Receptor γ (PPARγ), AKT2, GSK3B, PTP1B, PPP1R3, and SORBS1 [36-37]. This insulin action is provoked through the pathway (phosphoinositide 3-kinase/protein kinase B), which becomes active in PCOS theca cells similar to LH. A high level of insulin further enhances the synthesis of androgens [37].

Among the candidate genes, the INSR and PPARγgenes are the major ones. A study on the Indian population has shown a significant association of C/T polymorphism at His1058 of INSR with PCOS in lean rather than obese Indian women [38].Its association withinsulin resistance and hyperandrogenemia indices has also been seen in the same group of subjects.Jin et al. [39] have reported that novel T/C SNP at codon Cys1008 of INSR is associated with decreased insulin sensitivity inChinese women with PCOS and that the association is not by the change of synthesis or secretion of INSR β-subunit, but most possibly by the effects of this novel SNP on the function of INSR β-subunit. On the other hand, Lee et al. [40] have reported that C/T polymorphism in exon 17 of the INSR gene is not associated with susceptibility to PCOSin the Korean population.

The PPARγ, a gene involved in adipose tissue differentiation and thus obesity, has also been implicated in PCOS, as obesity is a frequent feature of this disorder. To further clarify the role of this transcription factor on PCOS pathogenesis, Orio and collaborators evaluated the frequency of exon 2 and six polymorphisms of the PPARγ gene [39]. Their findings showed that exon 6 T allele frequency is higher in PCOS women than in controls. In addition, the body mass index (BMI) and leptin serum concentrations were higher in PCOS patients carrying the C–T substitution than in controls. Exon 2 Pro12Ala polymorphism was unrelated to BMI and/or leptin serum concentrations in PCOS women [41]. Accordingly, Korhonern and colleagues found that the frequency of exon 2 Pro12Ala PPARγgene polymorphism is significantly

reduced in 135 PCOS patients compared with 115 controls [42]. These findings support a role for PPARγ gene polymorphism in PCOS pathogenesis, with the presence of the Ala isoform being protective against the development of PCOS.

Data on the genetic aspects of PCOS patients are invaluable in designing evidence-based therapeutic guidelines for any population, and they also have significant public health importance in preventing the disease. This is related to the fact that these genetic markers may help to precisely distinguish between the 4 main phenotypes of PCOS (according to Rotterdam Consensus: classic, NIH, ovulatory, and normoandrogenic).

## 1.2 RATIONALE

PCOS is a complex disease, whose pathophysiology is not yet fully understood. Considerable evidence, however, has already been gathered on the underlying defects of the syndrome. It is now acknowledged that insulin resistance (IR) is a major feature of this syndrome; however, there is considerable variation in the contribution of IR towards PCOS depending on various subtypes and racial backgrounds. The relative contribution of IR to PCOS is significantly higher among Asian women compared to the Caucasian population. Thus, ethnicity-specific guidelines for screening, diagnosing, managing, and preventing PCOS are necessary. This, in turn, required the generation of ethnicity-specific evidence through property-designed studies.

In Bangladesh, the prevalence is 29.9%–46.15% among infertile women [16-20]. Bangalees are the third-largest ethnic group in the world, after the Han Chinese and Arabs and, thus, they are the largest ethnic group among the Indo-Europeans [43]. The prevalence as well as clinical, metabolic, and endocrine aspects of PCOS in this population have not yet been well studied in this population. From facility-based data, the presence of IR has been reported to vary widely from 16% to 77% [23, 44-49]; however, the methodology and cut-off values of the IR assessment were not detailed in most of the studies. To our knowledge, no genetic analysis has also been reported to explain the various PCOS phenotypes in this population. Given the critical role played by insulin resistance in the pathophysiology of PCOS and, also, considering the possibility of lowering insulin resistance by drugs and lifestyle, studies on insulin receptors and insulin action-related genes are attractive targets in this field. In this context, two of the major

candidate genes for insulin resistance (the INSR and PPARγgenes) have been chosen to be explored in this study for their possible association with PCOS in our population.

## 1.3 HYPOTHESIS

The study was based on an 'alternate' hypothesis, which was as follows:

Single nucleotide polymorphism in rs1799817 of INSR and rs3856806 of PPARγ genes are associated with phenotypic features and insulin resistance among women with PCOS.

## 1.4 OBJECTIVES

### *1.4.1 General Objective*

The general objective of the study was to investigate the independent association of two SNPs in INSR and PPARγ genes with phenotypic features and insulin resistance among Bangaleewomen with PCOS**.**

### *1.4.2 Specific Objectives*

The study was undertaken with the following specific objectives:

- To explore the proportion of insulin resistance and its covariates among PCOS women of Bangladeshi racial origin;
- To find out the independent association of individual diagnostic phenotypic features of PCOS with insulin resistance among the study subjects
- To compare the frequency of polymorphism in rs1799817 of the INSR gene among PCOS subjects and healthy women;
- To compare the frequency of polymorphism in rs3856806 of PPARγ genes among PCOS subjects and healthy women;
- To explore the independent association of the investigated SNPs in the INSR and PPARγ genes with phenotypic features and insulin resistance on adjustment of the effects of major confounding variables

# Chapter 2: REVIEW OF LITERATURE

## 2.1 Polycystic Ovary Syndrome (PCOS) and its Phenotypes

### *2.1.1 Definition of PCOS*

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting women of reproductive age [50]. The condition is characterized by ovulatory dysfunction (OD), clinical or biochemical hyperandrogenism (HA) and/or a characteristic morphological change with microcysts in one or both ovaries termed 'polycystic ovarian morphology' or PCOM [51]. Heterogeneity in the clinical, biochemical and radiological features of the condition has led to the development of various guidelines for the diagnosing, grouping, and managing of the disorder.

### *2.1.2 Diagnosis and Grouping of PCOS as per Major Guidelines*

#### 2.1.2.1 National Institute of Health (NIH) Guideline:

The development of these criteria was initiated by NIH in 1990; however, as per the latest version of the criteria [Fig 2.1], any two of the three basic abnormalities (OD, HA, and PCOM) are required to diagnose PCOS. The criteria also require the identification of a phenotype as follows:- A: OD +HA +PCOM; B: HA+OD; C: HA+PCOM; D: OD+PCOM).

#### 2.1.2.2 Rotterdam Guideline:

In a meeting in Rotterdam (Netherlands) organized by the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine (ASRM) in 2003, other diagnostic criteria were suggested [6-7]. The Guideline is known as 'The Rotterdam Guideline', which requires two of the three features for the diagnosis of PCOS: (1) OD, (2) HA, and (3) PCOM. In reality, Rotterdam criteria encompass the NIH criteria, but the Guideline also broadens the definition of PCOS. It led to two new phenotypes, HA+PCOM, and OD+PCOM which were also incorporated in the latest version of the NIH criteria. This is the most widely used criterion and it is also used in Bangladesh by most clinicians.

### 2.1.2.3 AES Guideline:

This criterion was developed in 2006 by the Androgen Excess Society (now known as the Androgen Excess-PCOS Society [AE-PCOS]) through a task force of experts [54] who recommended that HA should be required for diagnosing PCOS. This limited the diagnosis of PCOS to three of the four Rotterdam phenotypes: HA+OD+PCOM, HA+OD, and HA+PCOM [8, 54]. This criterion was never widely adopted.

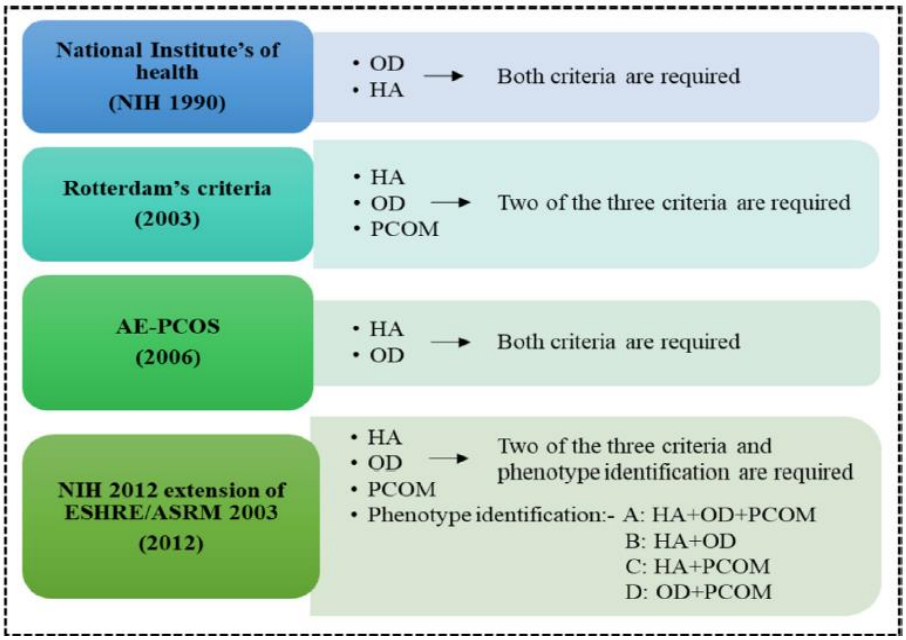


**Fig. 2.1: Summary of Diagnostic criteria of PCOS**

## 2.2 General Phenotypic Features of PCOS

### *2.2.1 Hyperandrogenism*

### 2.2.1.1 Clinical Hyperandrogenism

Hirsutism, acne, and alopecia are the major clinical signs of HA in women. Male pattern terminal hair growth (hirsutism) is a fairly reliable clinical feature of HA [56] and it is determined by the number of pilosebaceous units and their sensitivity to androgen. These are under genetic control and show racial and ethnic variations [56]. Ethnicity-specific hirsutism, assessed by Ferriman-Gallwey scores, is pathognomonic for HA; however, as many as 50% of

HA women may not develop this feature [57]. Many women also remove unwanted hair mechanically. HA also causes acne and alopecia; however, they are not considered reliable surrogate markers for androgen excess [58].

#### 2.2.1.2 Biochemical Hyperandrogenism

Elevated levels of circulating endogenousandrogens characterize biochemical HA. Testosterone (T) has specific binding to sex hormone-binding globulin (SHBG) and it is loosely associated with albumin; free T (FT) constitutes only ~1% of the circulating T [59]. Albumin-associated T and FT are biologically available [59] collectively those are referred to as non-SHBG bound T (NSB-T). Elevation of FT is found among ~70% of women with PCOS [8]. NSB-T, however, is a better index of bioavailable T [60].

A substantial minority of women with HA show isolated DHEAS elevations; thus, assessment of HA should include dehydroepiandrostenedione sulfate (DHEAS) assessment [8].

### *2.2.2 Ovulatory Dysfunction*

On biochemical assessment of ovulation, twenty to thirty per cent of women with PCOS, reporting regular menstrual cycles, are found to have anovulatory cycles [8, 54]. Oligomenorrhea (defined as <6–8 menstrual cycles per year) is virtually pathognomonic for anovulation; however, cycles with higher frequency may not necessarily indicate ovulation. Thus, a thorough clinical assessment is required to confirm this abnormality.

### *2.2.3 Polycystic Ovarian Morphology*

Infertility evaluation and treatment among women require baseline and follow-up pelvic ultrasounds to monitor follicular growth. Detection of PCOM is dependent on the sensitivity of the ultrasound equipment, the skill of the operator, the approach (vaginal vs abdominal) and the weight of the patient [57].

### *2.2.4 PCOS Phenotypes*

The Rotterdam Criteria leads to four PCOS phenotypes: - A: HA+OD+PCOM; B: HA+OD, C: HA+PCOM, and D: OD+PCOM (Fig 2.1). As per other criteria, suggested phenotypes are also shown in Table 2.1 [61].

**Table 2.1: Classification of Polycystic Ovarian Syndrome Phenotypes**

| Parameter | Phenotype A | Phenotype B | Phenotype C | Phenotype D |
|---|---|---|---|---|
| PCOS Features | HA/OD/PCOM | HA/OD | HA/PCOM | OD/PCOM |
| HA | + | + | + | - |
| OD | + | + | - | + |
| PCOM | + | - | + | + |
| NIH 1990 criteria | × | × | | |
| Rotterdam 2003 criteria | × | × | × | × |
| AE-PCOS 2006 criteria | × | × | × | |

*AE-PCOS= Androgen Exessand PCOS Society; HA= Hyperandrogenism, NIH=National Institute of Health; OD= Ovulatory Dysfunction; PCOM= Polycystic Ovarian Morphology.Source: Lizneva et al [61]*

## 2.3 Burden of PCOS

Due to inconsistency in global clinical diagnostic standards, there is a lack of comprehensive estimation of PCOS's global incidence and disability-adjusted life years (DALYs). The assessment of the disease burden for PCOS is thus difficult.

### *2.3.1 Global Burden*

The prevalence of PCOS under the National Institutes of Health (NIH), Rotterdam, and Androgen Excess and PCOS (AE-PCOS) Society criteria have been reported to be 6.1, 19.9, and 15.3%, respectively [62]. Globally, the incidence cases of PCOS grew by 54% from 1,377,924.58 in 1990 to 2,125,511.75 in 2019 [62]. Incident cases increased in most regions except Western Europe, Central Europe, Eastern Europe, and high-income Asia Pacific.

### *2.3.2 Regional Burden*

The largest increase in the age-standardized incidence rate (ASIR) of PCOS has been seen in Southeast Asia (EAPC: 2.61, 95% CI: 2.51–2.71). Also, among the changes in ASDR, the most pronounced increase was in Southeast Asia (EAPC: 2.58, 95% CI: 2.48–2.68) [62]. South Asia had the most incident cases in 2019, as India had the highest incidence cases of PCOS in the world.

### *2.3.3 Burden in Bangladesh*

The prevalence of PCOS in Bangladesh has been reported in eight studies; all but one study was single-centered [Table 2.2] [15]. The subjects in most of these studies were infertile women; one was conducted among hirsute women and another among women presenting with acne. Three studies bused revised Rotterdam criteria for PCOS diagnosis; the other five did not mention the diagnostic criteria used. Among the subjects visiting the Gynaecology outpatient departments, the prevalence of PCOS was as low as 6.11%; it ranged as high as 92.16% in subjects consulted for hirsutism. Among infertile women, the prevalence was 29.9%–46.15% [15]. In a study conducted among medical students, the prevalence was 37% [15]. To our knowledge, no population-based study for PCOS prevalence has been reported on any Bangladeshi population.

**Table 2.2: Prevalence of Polycystic Ovary Syndrome in Bangladesh** [15]

| Author, publication year | Study place | Study subjects | Age, years (Mean±SD or range) | Criteria used for PCOS diagnosis | *n* | Prevalence of PCOS (%) |
|---|---|---|---|---|---|---|
| Akhter *et al.*, 2011[9] | BSMMU, Dhaka | Patients attending infertility OPD | 34.56±6.33 | Not mentioned | 3184 | 29.9 |
| Begum *et al.*, 2012 [10] | DMCH, Dhaka | Patients with acne attending dermatology OPD | 21.5±4.8 | Not mentioned | 40 | 27.5 |
| Fatima *et al.*, 2015[11] | Eight tertiary level infertility centers in Bangladesh | New infertile patients | - | Not mentioned | 16700 | 31.7 |
| Walid *et al.*, 2018[12] | Diabetic Association Medical College, Faridpur | Patients with hirsutism attending endocrinology OPD | - | Revised Rotterdam criteria, 2003 | 51 | 92.16 |
| Mahjabeen *et al.*, 2018[13] | ZHSMCH, Dhaka | Infertile patients attending the department of obstetrics and gynecology | 25–35 | Revised Rotterdam criteria, 2003 | 1600 | 31.25 |
| Quadir *et al.*, 2020[14] | IMC, Dhaka | 3rd to 5th year medical students | 20–25 | Revised Rotterdam criteria, 2003 | 73 | 37 |
| Afreen *et al.*, 2021[15] | KGH, Dhaka | Subfertile patients attending infertility and gynecology OPD | 32.6 18–42 | Not mentioned | 140 | 46.15 |
| Fatema *et al.*, 2021[16] | BSMMU, Dhaka | Patients visiting gynecology OPD | 24.3±5.2 | Not mentioned | 3832 | 6.11 (35.39 among Infertile Women) |

## 2.4 Risk Factors of POCS

### *2.4.1 General*

The predisposing risk factors for PCOS include genetics, neuroendocrine, lifestyle/environment, and obesity. Women with predominant genes have a higher risk of developing PCOS [63] and data on genome-wide association revealed specific loci and alleles playing a major role in PCOS phenotype identification [64]. The environmental factors are equally important; those include physical exercise, lifestyle, and food, which may vary widely in different populations [65]. Endocrine-disrupting chemicals and glycotoxins are also becoming major environmental factors. They may cause genetic variance and disruption of the metabolic and reproductive pathways, leading to PCOS and related complications [66]. Impedance of the hormone levels to increase the high pulse frequency of GnRH is promoted by

androgen exposure, affecting the LH: FSH proportion and leading to follicular arrest and dysplasia [67]. These, in turn, may cause hyperinsulinemia, hyperandrogenism, oxidative stress, and irregular periods and, eventually, increase the risk of metabolic syndrome. On ultrasound examination, the characteristic multiple ovarian cysts (undeveloped follicles) of PCOS, evolve from primitive follicles, but due to disrupted ovarian function, the development ceases at an early stage.

### *2.4.2 Environmental Risk Factors*

In addition to genetic factors, the origin, prevalence, and modulation of PCOS are known to be affected by environmental pollutants, diet and lifestyle choices, obesity, and gut dysbiosis. These factors may cause HA, insulin resistance, partial folliculogenesis arrest, chronic low-grade release of inflammatory mediators from WBCs, and upsurging of metabolic syndrome. An increasing pool of evidence shows that environmental pollutants may lead to the development of PCOS. Endocrine Disrupting Chemicals (EDCs) are a group of ubiquitous contaminants that have been well-researched as potential environmental contributors to the pathophysiology of PCOS It was earlier thought that EDCs (including BPA and phthalates), affect mostly thyroid, estrogen, progesterone, and androgen receptors in the nuclear hormone system; however, present evidence suggests that they also affect other reproductive routes. EDCs can influence nonnuclear hormone receptors, orphan receptors, and receptors for neurotransmitters and they can also change steroidogenesis and hormonal metabolism by direct action [68]. A positive correlation between PCOS incidence, smoking, and exposure to cigarette smoke has been found [69]. Among oligo-anovulatory women with PCOS, women with normal anovulation in PCOS, and healthy controls, smoking was found to be associated with ovulatory dysfunction in a dose-dependent manner. Cigarette smoke, burnt coal, gas, wood, garbage, and meat cooked at high temperatures produce polycyclic aromatic hydrocarbons (PAHs), constituting most air pollutants. These pollutants have been shown to correlate positively with the risk of developing PCOS [70]. Other air pollutants like nitrogen oxides, sulfur dioxide, PAHs, and particulate matter (PM) alter normal steroidogenesis in exposed women leading to increased inflammatory mediators; this may be an underlying factor in the developing PCOS. Animal models also support a connection between PCOS and environmental pollutants. Direct exposure of pregnant rats to either fungicide vinclozolin or insecticide DDT is associated with

ovarian abnormalities. This, in turn, results in PCOS-like features in three subsequent generations via epigenetic processes [71].

#### *2.4.2.1 Lifestyle*

In addition to pharmacological treatments, lifestyle changes are the primary line of treatment for women with PCOS. Energy-rich diets and sedentary lifestyles are probable causes of PCOS exacerbation. Diets with high concentrations of simple sugars may lead to PCOS by altering gut flora, inducing chronic inflammation, increasing insulin resistance, and stimulating higher androgen production [72-73]. All of the defining features of this syndrome are worsened by obesity and weight gain. In line with these findings, prevention and treatment of this metabolic disorder are highly facilitated by physical exercise, maintaining a healthy body weight, adhering to healthy dietary habits, and abstaining from tobacco use.

### *2.4.3 Clinical Risk Factors*

Since the risk and presentation vary in different patient groups, due consideration is required to the demographic profile of the patient in determining the PCOS status of a patient. Cutaneous manifestations like early acne or hirsutism, persistent acne, and hirsutism for > years, persistent severe acne; frequent relapse in acne; and acne in the facial V area are known to be associated with PCOS [74]. As compared to Caucasian women, Indian PCOS women are known to have higher degrees of hirsutism, infertility, acne and they experience lower live birth rates following in vitro fertilization [75].

### *2.4.4 Biochemical Risk Factors*

BMI is a key risk factor for PCOS and higher BMI has been implicated as an important indicative marker of PCOS status [76-77]. Changes in BMI during adolescence have been found to be positively associated with changes in waist circumference, low density lipoprotein-cholesterol, triglycerides and systolic blood pressure in women with PCOS [78]. A history of weight gain often precedes the development of clinical features of PCOS in adults [79]. Factors independently associated with BMI are higher energy intake and high glycemic index food, low physical activity, smoking, and alcohol intake [80]. Even early stages of PCOS are associated with the development of IR and dysregulation of lipid metabolism [81]. Even with a normal

oral glucose tolerance test (OGTT), significantly higher IR and fasting serum insulin are observed among PCOS patients [82]. In age-matched patients with PCOS between 18-35 years, early signs of dyslipidemia (elevated serum total cholesterol, TG, and LDL-C levels) were observed [83]. Important risk factors of PCOS include a positive family history of PCOS or diabetes or an inadequate lifestyle [84].

Patients with normal menstrual cycles compared to patients with oligo/amenorrhea show significantly better metabolic parameters (BMI, fasting insulin, and Homeostasis Model Assessment- insulin resistance (HOMA-IR) [85]. Similarly, South Asians with PCOS have a higher prevalence of IR and MS compared to BMI-matched PCOS patients from other ethnic groups [86]. A rapid increase in the prevalence of PCOS-associated morbid conditions such as IR, excess body fat, adverse body fat patterning, hypertriglyceridemia, and obesity-related disease (diabetes and CVD) in Asian Indians has been noted in a recent review of literature on PCOS [87]. Thus, in patients of South Asian and specifically Indian ethnicity, regular PCOS surveillance is warranted.

### *2.4.5 Genetic Risk Factors*

A moderate heritability of PCOS was reported in studies on monozygotic and dizygotic twins. Only recently, candidate gene studies remained underpowered; thus, results were poorly reproducible in most cases. The advent of large-scale, newer molecular technologies, and advanced statistical analysis, has brought new insights into the genetic risk factors in PCOS.

Genomic data is powerful in testing the combinations of signals that indicate the potential causal influences of biological pathways. Mendelian randomization analyses of these types have suggested causal roles of higher BMI, higher IR, and lower serum SHBG concentrations in the etiology of PCOS [88]. Increasing the bioactivity of androgens or other sex steroids are the underlying mechanisms of their action.

### *2.4.6 Molecular Epidemiology*

The susceptibility of a woman to develop PCOS has been shown to be affected by certain genes, gene–gene interactions, or interactions between genes and the environment. PCOS is now known to be a polygenic and multifaceted disorder [89]. Several potential genes with single-

nucleotide polymorphisms (SNPs) or mutations are linked to a number of PCOS symptoms. Ovaries are directly or indirectly impacted by linked genes and mutations [90]. Genes encoding signalling elements related to steroidogenesis, steroid hormone action, gonadotrophin action and control, insulin action and secretion, energy metabolism, and chronic inflammation are frequent mediators in the pathophysiology of PCOS (Summary in Figure 2.2) [90]. Accordingly, exploration of significant gene variants that have the potential to alter a gene's expression or subsequent protein function is highly important to clarify the molecular risk of this complex disorder. Identification of genetic markers may facilitate the evidence-based prevention and management of this disorder with enhanced diagnosis, enabling early intervention in co-morbidities related to the syndrome as well as its phenotypes.

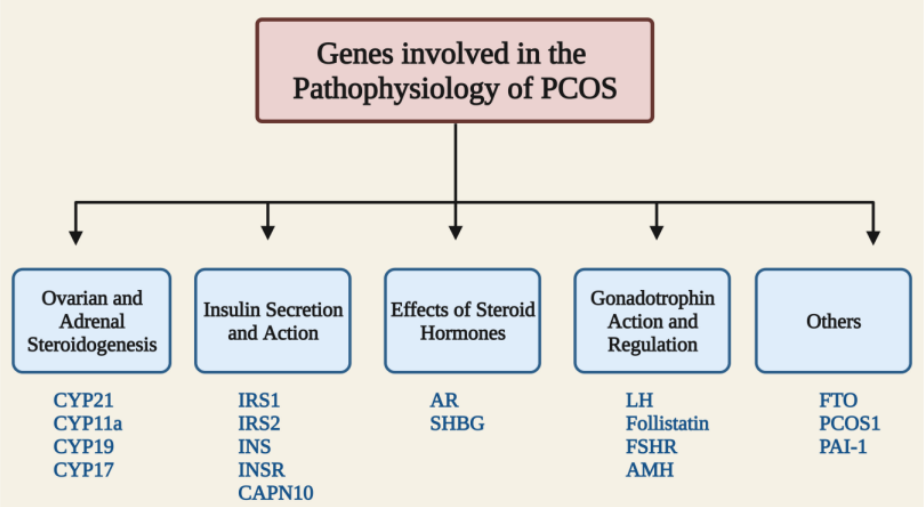


**Fig 2.2: Genes Involved in the Pathophysiology of PCOS**

## 2.5 Consequences of PCOS

PCOS women suffer from a number of risk factors for diabetes. Those include obesity, family history of type 2 diabetes (T2DM), insulin resistance and pancreatic B-cell dysfunction. PCOS women are at increased (3±7 times) risk of developing T2DM [91]. Accumulated evidence also suggests that women with PCOS are at increased risk of cardiovascular diseases (CVDs) [92]. Insulin-resistant persons are known to be more susceptible to coronary heart diseases. Women with PCOS have evidence of dyslipidemia [93] and markers of abnormal vascular function [94]. It is also thought that PCOS women are at increased risk for endometrial cancer due to

unopposed estrogen exposure to the endometrium. Supporting epidemiological evidence, however, is limited to this hypothesis [95].

## 2.6 Development of the Characteristic Phenotypic Features and their Interdependence

PCOS exerts widespread effects on physiology and metabolism some of which are detrimental and have long-term consequences. The dysfunctions are multisystem with the environment playing an important role and obesity, abnormal gonadotropin dynamics, excessive androgen production, and insulin resistance being the key features. The multisystem dysfunctions are strongly interlinked due to their pathogenesis; however, for convenience of description, they may be broadly classified as endocrine, reproductive, metabolic and biochemical dysfunction.

### *2.6.1 Endocrine Dysfunction*

The major components of the syndrome involve an array of neuroendocrine abnormalities. Anomalies of feed-forward and feedback signalling between GnRH/LH and ovarian androgens are the hallmarks of PCOS [96]. Accelerated GnRH pulsatile function, theca-stromal cell hyperactivity, LH hypersecretion, and hypofunction of the FSH-granulosa cell are the key endocrine abnormalities of the reproductive axis [97]. Fig. 2.3 illustrates the causes and effects of endocrine dysfunction in PCOS.

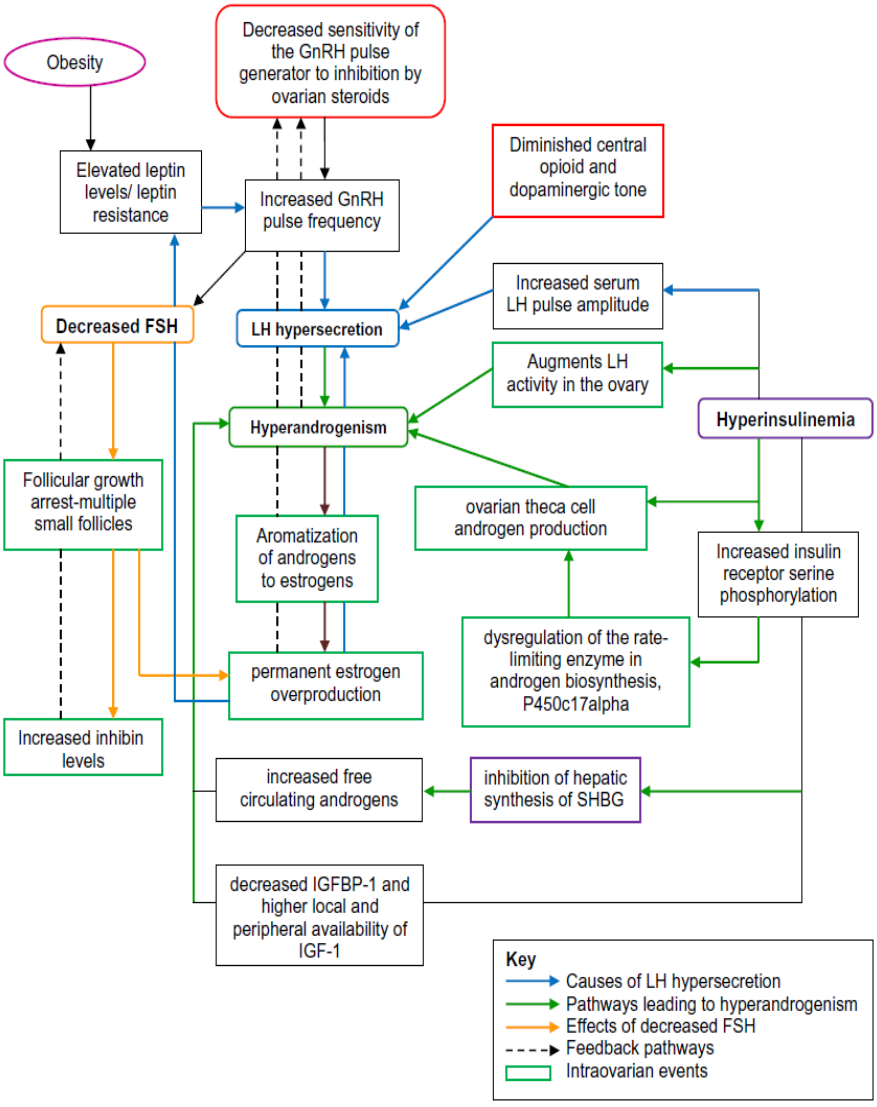


**Fig. 2.3: Causes and Effects of Endocrine Dysfunction in PCOS [96]**

### 2.6.1.1 LH Hypersecretion

LH synthesis in normal-ovulatory women is favoured by an increase in GnRH pulse frequency during the follicular phase; on the other hand, there is a decrease in GnRH pulse frequency in the luteal phase due to the progesterone effect favouring FSH synthesis [98]. In PCOS, a persistently rapid GnRH pulse frequency leads to perturbations in gonadotropin secretion, such as LH hypersecretion [99]. This is also a cause of hyperandrogenism [98]. Patients with PCOS exhibit an accelerated frequency and/or higher amplitude of LH pulses, LH secretory burst mass

augmentation, LH release with a more disorderly nature, in vitro elevation of LH bioactivity and a preponderance of basic LH isoforms [96].

#### 2.6.1.2 Decreased FSH

A decreased FSH production results from an increase in GnRH pulse frequency which has been illustrated in Fig. 2.3.

#### 2.6.1.3 Hyperandrogenism

The dysregulation of steroid production in PCOS is primarily manifested clinically through hyperandrogenism [100]. It is a result of LH hypersecretion and it is one of the most consistent expressions of PCOS traits [96]. Elevated serum 17-hydroxyprogesterone, androstenedione and testosterone concentrations are positively correlated with LH hypersecretion, and this also characterizes adolescents with PCOS. In PCOS, the pair-wise synchrony of the secretion of LH and testosterone, testosterone and androstenedione, and LH and androstenedione, are concomitantly uncoupled which points to the deterioration of both orderly uniglandular and coordinate bihormonal output [96]. The activities of steroidogenic enzymes P450c17 and 3b-hydroxysteroid dehydrogenase are increased by several hundred folds, and there is a disproportionate increase in C17, 20 lyase activities [101]. Accordingly, free testosterone assays or calculation of free androgen index are sensitive methods for assessing hyperandrogenemia [6].

The possible causes of hyperandrogenism include GnRH-mediated LH hypersecretion; increased synthesis of testosterone precursors due to a dysregulation of theca cell androgen production intrinsic to the ovary; augmentation of LH-mediated androstenedione production; and hyperinsulinemia (proposed as the primary event leading to hyperandrogenism).

Genomic variants in genes related to the regulation of androgen biosynthesis, function, availability of androgens to target tissues, insulin resistance, metabolic syndrome and proinflammatory genotypes may be involved in the genetic predisposition to functional hyperandrogenism and PCOS (Table 2.3) [35].

### 2.6.2 Diagnosis of Hyperandrogenism in PCOS

The presence of hyperandrogenism is confirmed through laboratory investigations (increased serum levels of androgens), or clinical examination, ie signs of hyperandrogenism, like hirsutism, even normal levels of androgens in the blood [102].

**Table 2.3: Genetic Etiology of PCOS [35]**

| Gene mutation | Mutation effect | Phenotypic expression |
|---|---|---|
| (i) Gene polymorphisms in insulin receptor substrate genes (IRS1 and IRS2) Impaired insulin metabolism Insulin resistance (ii) Mutations in the minisatellite in the regulatory region of the insulin gene (INS-VNTR) | Impaired insulin metabolism | Insulin resistance |
| Activating mutation of the kinase responsible for the insulin receptor (IR) serine/threonine phosphorylation | Impaired signal transduction and post-binding defect in insulin action | |
| Causes of increased androgen biosynthesis Activating mutation of the kinase responsible for the insulin receptor (IR) serine/threonine phosphorylation | Preferential hyperphosphorylation of the enzyme P450c17 Impaired post-translational regulation of 17,20-lyase activity (CYP17) | Hyperandrogenism |
| Pentanucleotide repeat polymorphisms (TTTTA)n in the regulatory region of CYP11a (encoding cytochrome P450 scc enzyme) | Cytochrome P450scc enzyme upregulation | |
| Multiple sequence variants at five susceptibility loci, especially steroidogenic enzyme genes (CYP21 heterozygosity, HSD3B2, IRS-1, GRL and ADRB3 variants) and elevated expression of the CYP11A 3BHSD2, and 17,20-lyase (CYP17) genes | Increased expression of 17a-hydroxylase | |
| Mutations in the centromeric region of insulin receptor gene (19p13.3) | Abnormal signal transduction mechanisms leading to altered expression of theca cell steroidogenesis genes | |
| Causes of increased androgen action 5a reductase gene (SRD5A1-2) mutations | Elevated 5a reductase activity | |
| 21-Hydroxylase (CYP21) gene mutation | 21-Hydroxylase (CYP21) deficiency | |
| UGT2B15 gene mutation | Disruption in androgen inactivation glucuronidation enzyme (UGT2B) mechanism | |
| Causes of increased androgen receptor activity Androgen receptor gene polymorphisms | | |
| Genetic variations in the gene receptor for AMH/BMP signaling alterations in the expression of estrogen receptors in the granulosa and theca cells | Increased AMH levels and follicle number | Disturbed folliculogenesis |
| Phosphatase and tensin homolog deletions on chromosome 10 (PTEN) induced by insulin | Increased PTEN levels in granulosa cells and proliferation of granulosa cells | |

### 2.6.2.1 Clinical Parameters

*Physical manifestations of hyperandrogenism:* In women, the skin manifestations of hyperandrogenism is seborrhea with concomitant development of acne, ipertrocosi, or hirsutism [103]. The overall impact of hyperandrogenism is manifested as hirsutism and acne in the genetically predisposed.

***Hirsutism***

This is defined as the development of male pattern terminal hair growth in women. It affects approximately 5-8% of the population [104]. A standardized system to evaluate hirsutism is suggested to be followed for clinical evaluation and diagnosis [105]. Ferriman and Gallwey scoring system is the most widely used one and it involves subjective tabulation of terminal hair growth in various body areas [106]. The modified Ferriman-Gallwey or mF-G method includes only nine body areas, excluding the forearm and lower leg (as these areas were found not to correlate with androgen excess). In the mFG scoring system, each body area is visually scored on a scale of zero to four; zero indicates no terminal hair growth, and four indicates full male pattern terminal hair growth [105].

***Acne (acne vulgaris)***

It is a cutaneous condition with pathological occlusion of the pores and skin, leading to inflammation and pus formation inside the pore itself. It is usually localized to the face and trunk area [107]. This results in the formation of comedones (point-like skin bumps made of the material that obstructs the glandular orifice).

***Acanthosis Nigricans***

It is a mucocutaneous eruption that occurs most frequently in the axillae, skin flexures, and the nape of the neck and is manifested by increased pigmentation and papillomatosis.

***Reproductive dysfunction***

It is a frequent presentation in PCOS. Ovarian function is often disturbed, with resultant abnormal folliculogenesis and steroidogenesis (Fig. 2.4). Several possible interlinked

mechanisms are involved in the pathogenesis of anovulation. These include LH hypersecretion, hyperandrogenemia, hyperinsulinemia, obesity, decreased plasminogen activator inhibitor (PAI) activity, and endothelial dysfunction [108].

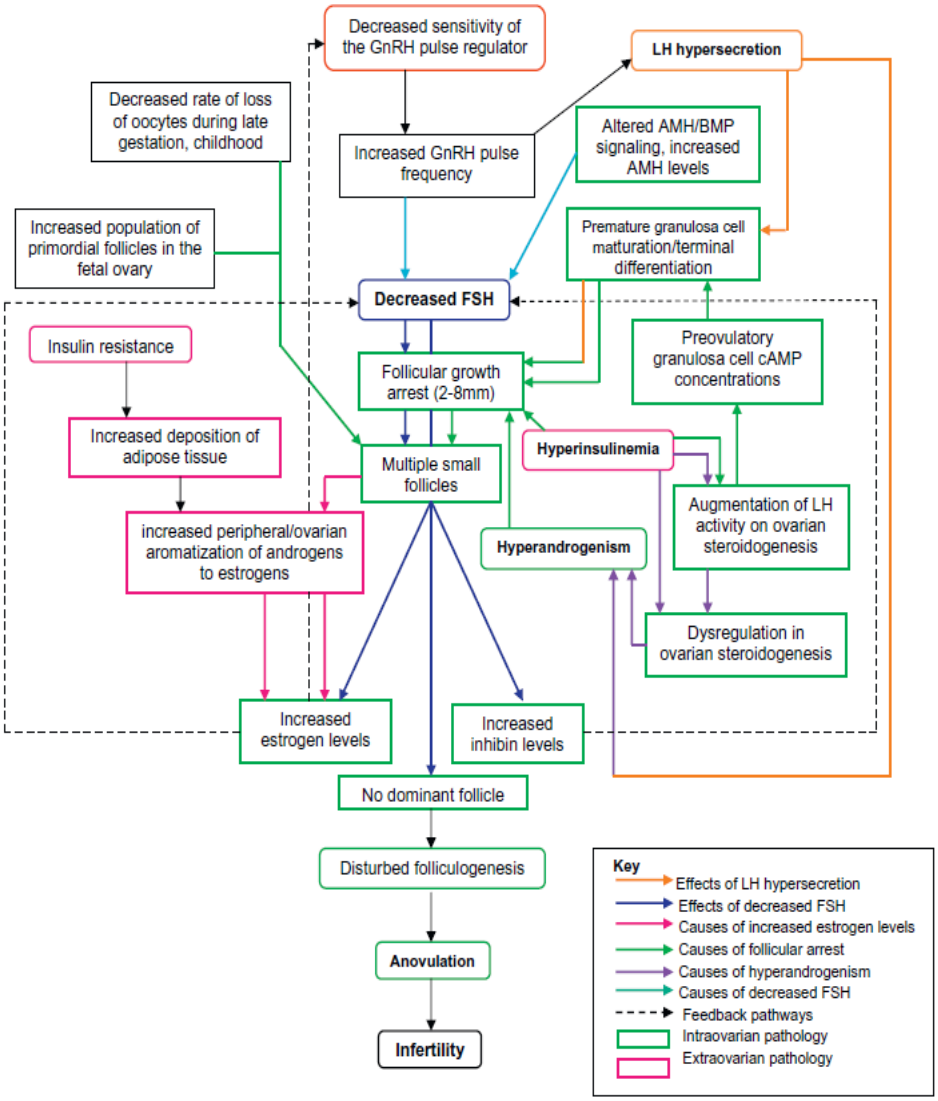


**Fig. 2.4: Reproductive Dysfunction [96]**

### *Menstrual abnormalities*

These are the most common gynaecological presentation of PCOS. Oligomenorrhea is present in approximately 85–90% of women with PCOS and 30–40% of amenorrheic patients have this disorder [109].

### *Infertility*

This is a common manifestation of PCOSand the disorder is the most common cause of anovulatory infertility [110]. There is an increased risk of miscarriage, after both spontaneous and assisted conception [95], and the development of ovarian hyperstimulation syndrome (OHSS) in assisted conception [111] is frequently associated with PCOS. First-trimester spontaneous abortions (25–73%) are higher among women with PCOS [112]. Insulin resistance (IR) and obesity are frequently associated with anovulatory infertility, due to arrested folliculogenesis in PCOS [113].

A series of intraovarian growth factors [insulin-like growth factors (IGF), AMH, growth differentiation factor 9 (GDF-9), and inhibin] and extra-ovarian factors (GH, IGF-1 and insulin) probably act together to trigger anovulation or early pregnancy loss through the impairment of folliculogenesis, oocyte maturation, steroidogenesis, and endometrial receptivity [97].

## 2.7 Biochemical Parameters

High levels of all androgens may be detected in PCOS, from the most powerful (Testosterone T; Dihydrotestosterone DHT, 5-Androstenediol D5-Adiol), to the weakest (4-Androstenediol D4-A; Dehydroepiandrosterone DHEA, Dehydroepiandrosterone sulfate DHEA-S). Estrone (E1) levels are increased, estradiol (E2) levels are normal or reduced by decreased follicular production and there is an inversion of the E2/E1 ratio. There is also a reduction in SHBG levels. The adrenal gland is the main source of DHEA and DHEAS, while the major contribution to the production of T, D4-A, and 17- hydroxyprogesterone (17-OH-P) comes from the ovary, [114]. The most sensitive method to assess hyperandrogenemia is the quantification of free testosterone (T) or the index of free T (free androgen index, FAI) [115]. Only 30-50% of patients with PCOS have elevated levels of adrenal androgens and only a small fraction can have an isolated elevation of dehydroepiandrosterone sulfate (DHEA-S), without showing, however, the suppressibility to dexamethasone and stimulation to ACTH, typical in adrenogenitalica syndrome. There is an increase in LH/FSH ratio, generally >2.5; this may be present in many but not in all women with PCOS. A normal value of this ratio does not exclude the diagnosis of this disease [116].

### 2.7.1 Diagnostic Sonographic Criteria

#### *2.7.1.1 External Aspects*

The main external aspect is the ovarian volume. As per Rotterdam Consensus criteria, PCOS is diagnosed by an ovarian volume >10cm³. Based on recent data it is more appropriate to lower the limit to 7 cm³; this increases the sensitivity of the examination. The limit of 10 cm³ provides a specificity of nearly 100%, but sensitivity lower than 50% [117].

#### *2.7.1.2 Internal Aspects*

The number of follicles constitutes the internal aspects of the ovary. As per Rotterdam Consensus criteria, a polycystic ovary contains 12 or more follicles of 2-9 mm in diameter. This distinguishes PCOS cases from multi-ovarian follicular ovaries (MFOs). The MFO is a transitory condition that is generally associated with delayed puberty, hypothalamic anovulation, amenorrhea related to weight, and hyperprolactinemia [118].

## 2.8 Insulin Resistance (IR) as a Central Biochemical Mediator of the Phenotypic Abnormalities

### *2.8.1 Epidemiology of IR in PCOS*

Assessment of IR from an epidemiologic viewpoint is typically done in relation to the prevalence of metabolic syndrome (MS) or insulin resistance syndrome (IRS). The National Cholesterol Education Program Adult Treatment Panel III (ATPIII) Criteria are usually followed in assessing these conditions. IR is found very commonly in PCOS subjects.

In a sample, the prevalence has been reported to be as high as 34.78% [119] in a study from Iraq. The prevalence of IR in another Iraqi population is 76.5% using the HOMA (Homeostasis Model Assessment) test; however, most of the women were obese [91,120]. IR has been reported to be 52.8% in North India [121-122] and 31% in Pakistani women with PCOS [123-124]. Among Japanese lean women, the prevalence has been shown as 52.9% of women [92, 125]. A study from Karachi found hyperinsulinemia among 75.32% of PCOS [121, 127]. The cut-off points for IR were found to vary among ethnic groups and these differences seem to be due to race and ethnicity [128-129].

### *2.8.2 Burden in Bangladesh*

Studies in this area are rare in Bangladesh; available facility-based data suggest more than 65% prevalence of IR in this population [15].

### *2.8.3 Risk Factors of IR*

PCOS is diagnosed based on reproductive criteria (HA, OA, and/or PCOM) [130], and the focus of management is primarily on the treatment of infertility and hirsutism [131]; however, PCOS is also grouped as a metabolic disorder. PCOS women are known to a have higher risk of presenting with impaired glucose tolerance (IGT) [24], type 2 diabetes mellitus (T2DM) [24], obesity [132], and dyslipidemia [133]. In addition to traditional risk factors for CVDs, they also show higher proportions of nontraditional, novel CVD risk factors, such as subclinical atherosclerosis [134] and an elevation in inflammatory markers [135]. Metabolic consequences seem to dominate PCOS, both as a consequence of the condition and as a vector for further complications, like CVDs, T2DM, and exacerbation of reproductive features of the disorder (like hirsutism and an/oligoovulation) [131].

Obesity is an important modulator in the expression of metabolic features and other clinical manifestations of PCOS [133-134,136]. IR may be present in normal-weight PCOS women, but its frequency and magnitude are higher in obesity [137-138]. The magnitude of IR, assessed by the insulin-to-glucose ratio, has been reported to have a strong, positive, and linear correlation with BMI among PCOS women [139]. Hepatic insulin resistance occurs in only obese PCOS women and is characterized by reduced sensitivity to insulin-mediated suppression of endogenous glucose production [140]. A 10-fold increased risk of T2 DM and a 7-fold increased risk of IGT are present among obese PCOS women as compared to their normal weight (BMI <25 $kg/m^2$) counterparts [141]. Accelerated conversion from IGT to T2DM strongly depends on BMI [142]. The CVD risk of the PCOS patients increases with obesity. Like the general population, the prevalence of metabolic syndrome among PCOS women increases with higher BMI, and it is the highest among PCOS obese women [143]. Studies suggest that PCOS, per se, increases the risk of metabolic syndrome possibly by promoting abdominal fat accumulation [144]. Obesity causes chronic low-grade inflammation [145] with an elevation of inflammatory markers (such as CRP, TNF-$\alpha$, and IL-6); these increase the risk

of CVDs [130], which is even more pronounced in PCOS. Levels of TNF-$\alpha$ correlate directly with BMI in PCOS and non-PCOS women [146-147], IL-6, and CRP [148], but overweight and obese PCOS women present with significantly higher levels of these inflammatory markers than their BMI-matched non-PCOS counterparts [136, 147].

### *2.8.4 Lifestyles and DietaryFactors for IR*

PCOS is closely linked to lifestyles and dietary factors as unhealthy exposure to these factors expedites the appearance of PCOS in girls who are susceptible to PCOS. Weight gain and obesity deteriorate the characteristic features of PCOS, especially IR; on the other hand, weight reduction diminishes the characteristic features of PCOS [148].

### *2.8.5 Role of Insulin-Resistant Hyperinsulinism in PCOS Pathogenesis*

Insulin resistance (IR) is a common feature of PCOS, often excessive for the degree of adiposity and may also be found in nonobese PCOS women [149-152]. In PCOS IR is characterized by decreased sensitivity and responsiveness to insulin-mediated glucose utilization; primarily in skeletal muscle and adipose tissue [150, 153].

IR in PCOS has a typical and prominent intrinsic element; however, it may be acquired in some cases due to exogenous obesity. Constitutive, tissue-specific, postbinding defects in receptor signaling underlie IR in this disorder, which selectively affects metabolic pathways but does not lead to mitogenic or steroidogenic effects [132,152, 154-155]. Phosphorylation of the insulin receptor and insulin receptor substrate-1 is caused by intracellular serine kinases and a consequent decrease in insulin activation of the phosphatidylinositol- 3-kinase signalling pathway (which activates glucose transport); serine kinase phosphorylation also activates mitogenic pathways mediated by ERK/MAPK [24].

The tissue-selective resistance to the metabolic effects of insulin creates a paradox: the compensatory hyperinsulinemia to overcome resistance to the glucose-metabolic effect of insulin results in excess insulin action in some tissues in the presence of resistance to insulin action in others. This is known as insulin-resistant hyperinsulinism (or simply hyperinsulinism), and this constitutes a major extra-ovarian factor underlying steroidogenic dysregulation and

DM-related comorbidities of PCOS [24, 151,156]. The mitogenic signalling and protein metabolism also remain sensitive to insulin [24, 157].

Insulin-related stimulation of PCOS theca cell [158] and normal granulosa cell [159] steroidogenesis is mediated through the insulin receptor rather than through the IGF-1 receptor. This recent evidence indicates that hyperandrogenic anovulation is caused by insulin signallingthrough the theca cell insulin receptor [160]. There is also some proof that insulin signalling in the ovary is preserved in a state of resistance to the metabolic effects of insulin.

LH stimulation of ovarian androgen production is augmented by hyperinsulinemia through up-regulation of LH-binding sites and enhanced androgen production in response to LH at the level of cytochrome P450c17 (Fig. 2.3). Several other mechanisms are also involved in insulin augmentation of LH-stimulated androgen production. IGF-1 receptor, atypical IGF-1 receptors, or a hybrid receptor may also mediate insulin action [154]. Insulin may raise the bioavailable fraction of IGF-1 by lowering levels of IGF-binding protein 1 [154].

PCOS is associated with all known forms of insulin resistance [154]. These include extreme insulin resistance of hereditary insulin receptor mutations or lipodystrophy [154,161] and the IGF-1 excess state of acromegaly [162]. More modest forms of insulin resistance, including the major forms of DM, are associated with PCOS. For example, the prevalence of PCOS among premenopausal women with type 2 DM is 30%–40% [163-164]. Type 1 DM is also associated with PCOS which is probably mediated by the supraphysiologic systemic doses of insulin required to control glycemia [165].

Presently available evidence is compatible with insulin-promoting hyperandrogenism independent of hyperinsulinemic insulin resistance. It is also consistent with the concept that levels of insulin in the normal range modulate the androgenic response to LH [166].

### *2.8.6 IR as an Effect of the Abnormalities in PCOS*

On assessment by the gold-standard technique, hyperinsulinemic-euglycemic clamp, it has been estimated that up to 75% of PCOS women have impaired insulin response [167]. A large number of studies have investigated the relationship between excess weight, decreased insulin sensitivity, and hyperinsulinemia in women with PCOS; however, the understanding of this multidirectional and synergistic web of interactions has remained unclear [168]. An additional

degree of uncertainty comes from the frequent use of different surrogate estimates of IR and less convincing results for the degree of IR in lean PCOS patients compared to age- and BMI-matched controls [168-172]. IR is considered as the primary defect in PCOS and compensatory hyperinsulinemia as a consequence may maintain normoglycemia for a temporary period. Among predisposed patients persistent IR may create relative or absolute insulin deficiency leading to prediabetes or T2DM [167]. Supraphysiologic insulin levels disrupt ovarian function during a considerable period in this process [173]. Within ovarian theca cells insulin acts as a co-gonadotrophin in synergy with luteinizing hormone, by enhancing the production of androgens. Follicular development is also mediated by insulin, and thus, in hyperinsulinemic setting, the arrest of pre-antral follicle development is promoted [173-175].

Steps from pancreatic B cell deficiency to underlying mechanisms of impaired insulin action at the target cells and reduced insulin clearance are now fairly understood. The role of insulin metabolism in the pathogenesis of PCOS is summarized in Fig. 2.5; in this model, hyperinsulinemia is considered as an important factor PCOS development and progression. Two studies showed a rapid onset of IR after supraphysiological androgen administration in women [176-177], and it was also shown that IR can be partially reversed by androgen suppression or antiandrogen treatment [178-179].

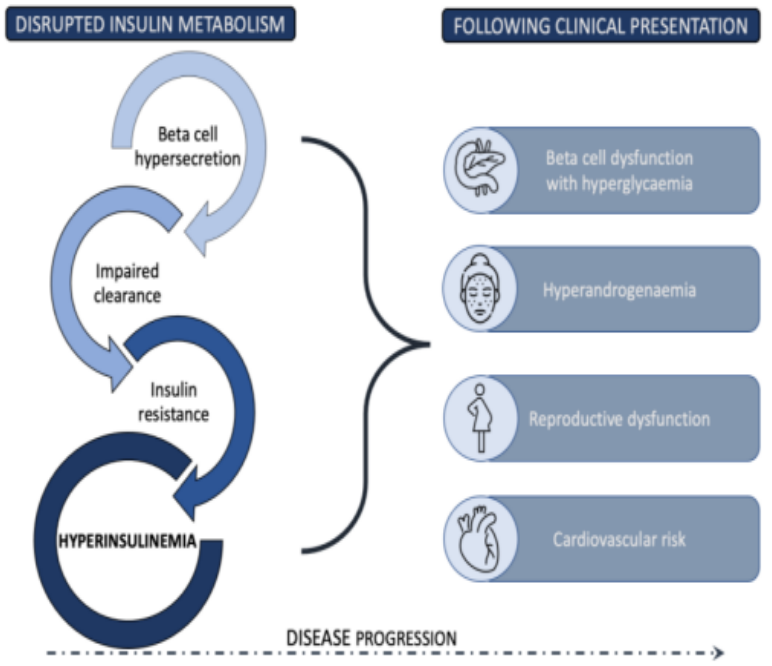


**Fig 2.5:** ***Summarized View of Insulin Metabolism in PCOS Pathogenesis*** **[144]**

***2.8.9 Pancreatic Beta Cell Function and PCOS***Findings on the pancreatic beta cell secretory fumnction in PCOS are still inconclusive. Both defective and increased insulin secretion have been proposed, and thus, various results on insulin secretion have been demonstrated. Data are stii limited in this area in general [170].

There are several reasons for difficulty in analyzing this topic. Heterogeneity in study designs including recruiting patients at different stages of PCOS, estimation of different phases of insulin release (basal, stimulated, or cumulative), and nonadjustment for the IR severity and insulin clearance seem to be the major reasons for this controversy. Further large sacle focused studies are required to explore the role of insulin secretory capacity in the development of PCOS and its clinical presentation.

## 2.9 Pathophysiology of IR

### *2.9.1 Signalling pathway of insulin*

Insulin exerts its effects through the insulin signalling pathway (Figure 2.6). Autophosphorylation of the receptor's tyrosine residues takes place after binding to its receptor (insulin receptor). As a result, phosphorylation occurs in the insulin receptor substrate (IRS). At the downstream, the PI3K pathway and the MAPK pathway convey the signals. PI3K drives the activation of protein kinase B (PKB or Akt), through interacting with various downstream proteins which are responsible for the effects of insulin on metabolic process, including GLUT4 translocation [24].

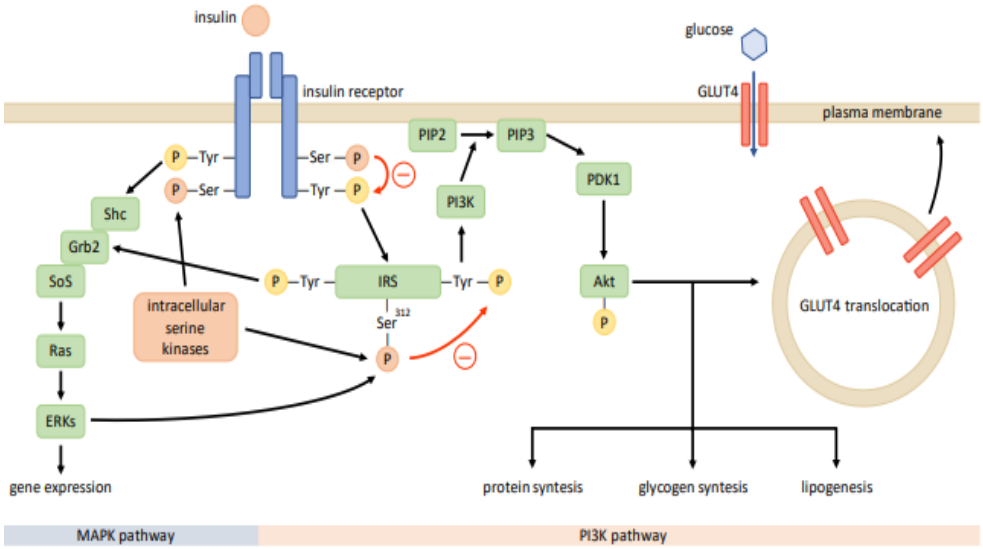


**Fig 2.6: Insulin Signaling Pathway [144]**

### *2.9.2 Basic Mechanisms of IR in PCOS*

The IR of PCOS is primarily an outcome of intrinsic factors and it is, in part, independent of obesity. There is a postbinding decrease in the phosphorylation of the tyrosine residues and, also, there is an increase in the phosphorylation of the serine residues of the intracellular domain of the insulin receptor; these leads to resistance to the metabolic actions of insulin [181]. Through elevated serine phosphorylation the responsiveness of the insulin receptor to its substrate is decreased and, at the same time, there is an increase in the activity of P450C17, the key enzyme of adrenal and ovarian steroid synthesis [182]. Therefore, both IR and hyperandrogenism may be caused by the same defect in serine phosphorylation in a subgroup of PCOS patients [181]. Increased serine phosphorylation of the adaptor protein IRS-1 is also thought to be a possible cause of insulin resistance in PCOS [181]. Serine phosphorylation of IRS-1 disrupts the intracellular signaling responsible for the translocation of GLUT4 into the plasma membrane [181]. GLUT4 expression has been reported to be reduced in the plasma membranes of adipocytes of both lean and obese PCOS patients [183]. In muscle cells of PCOS women, increased activation of ERK1/2 pathways may also be responsible for resistance to the metabolic actions of insulin [181, 184]. These pathways are usually involved in the mitogenic actions of insulin [181, 184]; however, enhanced basal activation of ERK1/2 has also been shown to inhibit the IRS-1 pathways necessary for GLUT4 translocation to the plasma membrane [184]. Hepatic insulin resistance in obese PCOS women seems to be attributed to increased lipolysis in visceral fat cells [185]. An enhanced lipolytic response to catecholamines in visceral fat cells of PCOS women has been demonstrated [185]. Fatty acid and glycerol delivery to the portal vein and liver increases due to enhanced lipolysis of visceral fat, perturbing liver function; this eventually leads to hepatic IR, as well as to hepatic inflammation and, interference with the production of SHBG [185].

### *2.9.3 Assessment of IR by Homeostatic Model Assessment (HOMA)–Based Techniques*

The homeostasis model assessment IR index (HOMA-IR) is used for the evaluation of whole-body insulin sensitivity. The HOMA-IR values are calculated using the HOMA Calculator software (Diabetes Trials Unit, University of Oxford, Oxford, United Kingdom) available in the public domain for downloading from www.dtu.ox.ac.uk/homa. The HOMA-IR is a steady-

state basal test for IR, but there is a good correlation between estimates of IR by the euglycemic clamp and the HOMA-IR (r ¼ 0.73–0.88). Nevertheless, HOMA-IR primarily explores the ability of insulin to restrain hepatic glucose production. In contrast the IR estimate, obtained by the euglycemic clamp, is a function of both hepatic and peripheral insulin sensitivity, but to a greater extent the peripheral IR. Therefore, if hepatic and peripheral IR differ, the results from the HOMA-IR and the clamp may also differ.

### *2.9.4 IR-Related Vicious Cycle in PCOS*

IR is developed by an impairment of insulin action in the target tissues (mainly liver, fat, and muscle [186]. A decreased utilization of glucose due to defective plasma membrane glucose transport is its predominant manifestation which is facilitated by defetcts in glucose transporter type 4 (GLUT4) [187]. In PCOS, disruption in signal transmission downstream from the insulin receptor, ie post-receptor abnormalities, are the major reasons for IR. Decreased responsiveness and sensitivity to insulin stimulation are found in insulin-resistant tissues, and a more pronounced effect is seen in PCOS patients as compared to obese patients [188-189]. This affects the metabolic effects of insulin (like increased protein synthesis, glucose uptake, glycogen synthesis) mediated by the phosphoinositide 3-kinase (PI3K) pathway. In addition, it mitogenic and steroidogenic effects are also exhibited, the mitogen-activated protein kinase (MAPK) pathway seems to convey the effect. In PCOS MAPK pathway has a normal function and IR affects only the PI3K pathway selectively [24, 190]. It has been suggested that the pathogenesis of IR in PCOS is unique [186,188,190,192]; this is based on findings on previous studies on the machanisms of IR in common insulin-resistant states, such as T2DM, obesity, and PCOS,

A downstream defect from the insulin receptor is suggested in PCOS as the adipocytes from patients show the number and affinity of cell surface insulin binding sites as normal [188,193]. It is observed that the constitutive phosphorylation of serine residues of the beta-subunit of the insulin receptor and IRS-1 Ser312 is higher in tissues of PCOS patients; this, on insulin stimulation, impede the tyrosine phosphorylation of both the insulin receptor and IRS-1 [194-195]. An enhancement of the tyrosine autophosphorylation of the insulin receptor has been observed on the pretreatment of fibroblasts from PCOS patients with serine kinase inhibitors [196]; this may suggest that an entity with serine kinase activity is involved in the process which

is not identified yet [188]. Also, increased activity of intracellular kinases associated with the mitogenic MAPK pathway may be the cause of the phosphorylation of Ser312 in IRS-1[190-191]. The IRS-1-associated activation of PI3K has been found to be decreased in the skeletal muscles of PCOS women in studies exploring the signaling downstream of IRS [197]. PKB is the main enzyme which propagates the translocation of GLUT4 to the cell membrane; its defect may create impaired PI3K activity which may result in reduced insulin-induced glucose uptake in the target tissues [198]. Mitochondria is the regulator of cellular ATP and reactive oxygen species levels and it has close interconnection with insulin [199]. Defect in a downregulation of nuclear-encoded genes involved in oxidative phosphorylation may create impaired mitochondrial function in PCOS and this may lead to an increased production of reactive oxygen species [200-201]. Activation of intracellular kinases may be caused by increased oxidative stress with resulting decrease in insulin sensitivity as a consequence of phosphorylation of serine residues of both the insulin receptor and IRSs [202].

### *2.9.5 Genetic Factors of IR in PCOS with Racial Heterogeneity as Derived from Population Genetics*

PCOS is an highlyheterogeneousand complex disease. The genetic basis of PCOS differs between families and within families; however, the defect is related to a common pathway. Due to complexity and heterogeneity, single gene or related genes in a single family have not been reported. The genetic susceptibility of different genes differs in patients from the same family [203]. In recent years, intrauterine programming has been hypothesized as a susceptibly factor for PCOS [204]. It is unrealistic to search for a candidate gene in a complex disease like PCOS by genome screening. In such families, linkage analysis almost invariably generates negative results. Case-control studies of larger population size and genome-wide association studies (GWAS) in such a disease are helpful in finding possible associations. Parental analysis in such diseases is often impractical; however, the known risk of disease can be estimated.

A number of studies have been conducted in various families to find the causative gene/mutation in PCOS; however, no true penetrance of a single gene mutation has been reported until now. All genes/mutations reported in familial aggregation arecompounded by low penetrance and those are associated with other covariant and hormonal or environmental factors causative to the disease. In reality, PCOS is a polygenic and multifactorial syndromic

disorder. It is a multifactorial disease caused by a host of abnormalities. All genes/mutations that affect ovaries, directly or indirectly, are associated with PCOS. An overview of the genetic scenario of PCOS is depicted in Figure 2.7.

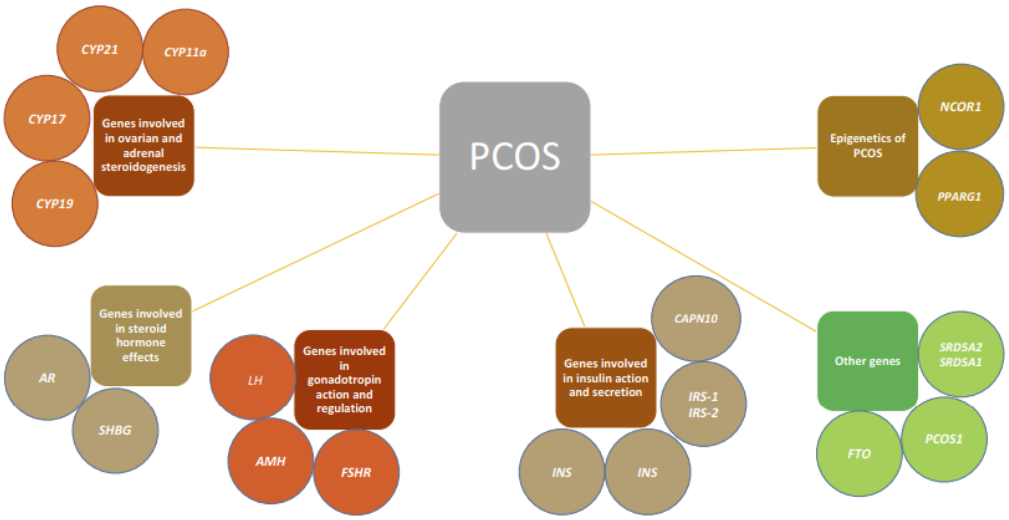


**Fig. 2.7: Summarized Genetic Scenario in PCOS [90]**

## 2.10 Genes Related to IR in PCOS

Impact of individual genes, including those related to steroidogenesis, insulin signaling pathway, and glucose metabolism were mainly targeted in the initial investigations. The genetic etiology could not be confirmed for PCOS which is highly influenced by a number of dietary, lifestyle, and environmental factors [205-206]. In recent years, it has now been shown that genes associated with gonadotropin function may be involved in the development of PCOS through genome-wide association studies [207-2012]. Also sharing of PCOS-associated genetic variants associated between ethnic groups has been found [207, 212]. It is now well acknowledged that there is a significant genetic component in PCOS as strong evidence exists to indicate that PCOS is familial [213-215]. Multiple gene variants, with each variant contributing to a small effect, are thought to contribute to this genetic component [215-218].

### *2.10.1 INSR Gene*

The INS is a sandwich gene at 11p15.5 between tyrosine hydroxylase and IGF-II [219]. This gene encodes turmeric protein composed of two alphas and two beta chains [220]. A tandem

repeat of VNTR occupies the 5′-untranslated regions of the gene [235]. VNTR polymorphism regulates the transcriptional rate of INS and IGF-II [236]. The number of repeats of VNTR varies from 26 to 200. VNTR polymorphism has been reported to be associated with PCOS [237].

One of the major molecular pathogenesis for insulin resistance in PCOS now encompasses the postbinding defects in INSR signaling [213, 221]. In a considerable proportion of PCOS women, the mechanism of insulin resistance lies in a defect in which autophosphorylation of tyrosine residues on the INSR is reduced [213, 221]. In earlier studies, a susceptibility gene for PCOS was reported to be located on chromosome 19p13.3 in the INSR region; it suggested that INSR itself is the susceptibility gene for PCOS [224]. There are 22 exons spanning 120 kilobases on chromosome 19 in the INSR gene [225]. The tyrosine kinase domain of the receptor is encoded at the region of exons 17–21 and the receptor isnecessary for insulin signal transduction. Severe insulin resistance and hyperinsulinemia have been reported to be associated with a mutation in exons 17–21 [226]. Insulin resistance among patients with PCOS is widely variable, and, thus, it is unlikely that a major mutation in INSR would lead to PCOS [227]. On the other hand, INSR polymorphisms inducing mild changes in INSR function may contribute to the development of PCOS [224].

Polymorphisms of different kinds, within the coding and noncoding regions of INSR, have been reported in patients with PCOS [224, 228]. Most of these polymorphisms were silent single-nucleotide polymorphisms (SNPs) [224, 229]; a higher frequency of SNP in exon 17 of INSR was found [227-228]. Until now, among the SNPs in exon 17 of INSR detected [224-228], the C/T SNP at His1058 in the tyrosine kinase domain of INSR has been reported to have a significant association with the development of PCOS, most possibly by the consequent effects on the autophosphorylation of the INSR function [224]. Studies of exon 17 INSR have shown that a C/T SNP at codon His1058 is associated with PCOS [224]. Since insulin resistance in PCOS is secondary to a postbinding defect in INSR signaling [221-222], the molecular pathogenesis of insulin resistance in PCOS is mainly situated at INSR β-subunits not INSR α-subunits that bind insulin. The defect is either in number or in function. Earlier in vitro and in vivo mutagenesis experiments generated this evidence where one or two critical amino acid residues (such as Lys1018, Lys1030, Tyr1162, Tyr1163, etc.), located in the tyrosine kinase domain of INSR β-subunits, were changed to one of several other amino acids. Along with the

nonresponse of these kinase-deficient INSRs to insulin, the tyrosine kinase was completely inactive, even though insulin binding to these mutant INSRs was unaffected [229-230]. The tyrosine kinase domain of INSR also hosted the T/C SNP at codon Cys1008 of INSR, precoselyat the presumed ATP binding site of the domain [231-232]. The presumed ATP binding site is a highly conserved sequence in INSR, which is functional in nature [233]. Currently, the polygenic trait of PCOS is thought to be due to the interaction of susceptibility and protective genomic variants which function under the influence of environmental factors [234].

Conway et al. studied the tyrosine kinase domain-encoding region of INSR among PCOS women [52]. The entire gene has been scanned by Talbot et al. in PCOS women [227]. An association between PCOS and INSR was not found in these studies. On searching a larger part of chromosome 19p13.2, D19S884 was reported to have the strongest association with PCOS [240]. INSR gene is contained in these regions of the chromosome.

### *2.10.2 PPARγ Gene*

Peroxisome proliferator-activated receptors (PPARs) are fatty acid-activated nuclear receptors that have three isoforms α, β/δ, and γ [241]. These isoforms have distinct metabolic regulatory activities, tissue distribution, and ligand-binding properties. These ligand-regulated transcription factors promote or inhibit the expression of their target genes by binding to specific DNA response elements within promoters [242]. Compared to other nuclear receptors the PPAR ligand binding cavity is significantly larger in size; as a result, a large number of natural and synthetic ligands can be attached and an exchange of co-repressors for co-activators can be triggered thus stimulating PPARs functions [243-244]. Diverse cellular functions are mediated by PPARs which are involved in fatty acid disposition and metabolism, energy homeostasis, cell differentiation, and immunity mechanisms [245]. In the liver, kidneys, intestine, brown adipose tissue heart, and skeletal muscles a high expression of PPARα is specifically foind. In consequence, fatty acid metabolism. PPARβ/δ is expression is influenced ubiquitously, and fatty acid oxidation as well as regulation of blood cholesterol and glucose levels are affected by these isoforms. Highest expression of the PPARγ isoform is seen in adipocytes, and, in addition to insulin sensitivity, lipoprotein metabolism lipid biosynthesis, and adipogenesis is affected significantly by this expression [243, 246].

Since PPAR isotypes co-determine the progression to nonalcoholic steatohepatitis, they have a significant impact on the metabolic syndrome. This is due to their regulatory roles in inflammation, fibrosis, and liver metabolism. Metabolic homeostasis and trace elements are linked by them, and they mediate molecular effects related to diabetic cardiomyopathy [247]. Central obesity, atherogenic dyslipidemia, insulin resistance, and hypertension are the main features of metabolic syndrome, and those are present at high proportins (up to 43%) in adult PCOS women [248]. Accordingly, the clinical features of PCOS may be grouped as metabolic syndrome [248].

### 2.10.3 Reported Genetic Polymorphisms in PPARγ Genes

A number of authors have investigated the role of diverse *PPAR* polymorphisms in PCOS. Christopoulos et al. observed that the *PPARγ* gene polymorphisms only reduce testosterone levels, but they do not increase the risk for PCOS apart from that. In contrast, elevation of fasting glucose levels has been reported im case of +294T/C polymorphism in the exon 4 of the *PPARδ* gene [249]. Knebel et al, on sequence analyses of the *PPARγ* gene, found no evidence in PCOS women for a direct correlation of polymorphism with the altered interleukin TNFα., IL-1β, IL-6, and (IL)-7 levels [250]. Antoine et al explored he relationship of the *PPARγ* Pro12Ala and silent exon 6 (His447His) polymorphisms with clinical features of PCOS; Pro12Ala and His447His did not seem to be related to increased risk of PCOS or its characteristic phenotypic features [251]. A higher frequency of the C to T substitution in exon 6 of obese PCOS patients, with the Pro12Ala polymorphism at exon 2, was suggested by Orio et al; this, however, was not found to affect BMI in PCOS women [252]. As per reports from the same group, there is no difference in adiponectin concentrations between PCOS and controls, and serum adiponectin values are not affected by Pro12Ala polymorphism [253]. Lack of differences Pro12Ala polymorphism distribution between PCOS and controls was also reported by Xita et al [254]. In contrast, association of Pro12Ala polymorphism is with risk of PCOS and abnormal metabolic parameters (like BMI, fasting triglycerides and insulin levels) was reported by Zaki et al [255].

Among relatives of PCOS patients, the relationship between the Pro12Ala polymorphism and insulin resistance was explored by Yilmaz et al; it was suggested that insulin resistance is protected by this gene polymorphism and, thus, the development of diabetes mellitus in first-

degree relatives is prevented [256]. In a later study, the group reported that the same polymorphism may also modifiy of insulin resistance among PCOS women [257]. Another study by Bidzińska-Speichert et al, to detect *PPARγ2* Pro12Ala and Pro115Gln gene polymorphism, reported the absence of the Pro115Gln polymorphism, alongside an estimated frequency of 23.15% in PCOS patients. It has been found that BMI ≥30 significantly correlates with a higher occurrence of the Ala allele [258]. Reports have been published from the same group that PCOS patients carrying Pro12Ala genotype have higher leptin levels than in those with Pro12Pro and Ala12Ala [259]. Tok et al. noted more obesity, less insulin-resistance and glucose-intolerance and lower fasting insulin levels among PCOS patients with Pro12Ala polymorphism [260]. In their genetic study, Hahn et al. concluded that higher insulin sensitivity and decreased hirsutism scores correlates with Pro12Ala polymorphism in PCOS women [261]. Koika et al suggested that reduced basic metabolic rate and laboratory hyperandrogenemia correlate with the Pro12Ala polymorphism in the *PPARγ2* gene in patients with PCOS [262]. A significant reduction in the frequency of the variant Ala isoform in PCOS patients was noted by Korhonen et al [42]. Association of PCOS risk with *PPARγ* Pro12Ala was reported by Rahimi et al and it was also shown that the variant CG genotype correlates with higher TG levels and a lower concentration of estradiol and [264]. A significantly higher expression of PPARγ splice variants in PCOS patients was suggested by Shi et al [265]. Giandalia et al underlined a similar distribution of *PPARγ* exon 2 and exon 6 variants in PCOS and non-PCOS groups. *PPARγ* exon 2 and exon 6 variants have been found to correlate with differences in the metabolic profile and/or hormonal (17-β estradiol, free testosterone levels) of PCOS women; this may indicate a protective effect on β-cell function and insulin resistance [266]. Three polymorphisms of the *PGC-1α* gene were genotyped by Reddy et al; they indicated that *PGC-1α* rs8192678 ‘Ser’ allele carriers exhibit a higher risk of developing PCOS [267]. In short, contradictory results regarding the role of PPARγ polymorphisms in PCOS have been reported by various studies.

### 2.10.4 The PPARγ Polymorphisms Depending on the Ethnic Background

The polymorphisms, as mentioned earlier, have been investigated by some research groups with a special emphasis on the ethnic backgrounds of the populations under the study.

In a Croatian population Baldani et al [268] conducted a molecular analysis on151 PCOS patients for the genetic polymorphism; it was found that the *PPARγ* Pro12Ala polymorphism is a non-significant determinant of PCOS. However, a positive effect on BMI and insulin sensitivity was found. Chae et al [269] conducted a genetic analysis of the *PPARγ* Pro12Ala and the *PGC-1α* Gly482Ser polymorphisms among Korean patients with 184 PCOS subjects; No variants as susceptible genes could be identified in those women; however, the concentration of serum high-density lipoprotein (HDL) levels was modulated by *PPARγ* Pro12Ala polymorphism, whereas postprandial 2h insulin levels were influenced by *PGC-1α* Gly482Ser polymorphism [269]. In contrast, correlation of PCOS with both Pro12Ala and His447His polymorphisms of *PPARγ* was shown by Gu et al in a Korean population [270]. No statistically significant differences between were found regarding *PPARγ2* Pro12Ala and *PGC-1α* Gly482Ser polymorphism between PCOS and controls in Chinese women in the study by Wang et al [271]. Yang et al [272] also reported no difference regarding *PPARγ2* Pro12Ala polymorphism distributions between Chinese women with PCOS and controls [272]. Dasgupta et al [273] sequenced 250 PCOS women and 299 controls for *PPARγ* exon 2 and 6 to identify distinct SNPS in these regions of the exons in an South Indian population; they the *PPARγ* exon 2 Ala allele and exon 6 His447His T allele were found to be significantly more common in the controls as compared to the PCOS subjects.

A reduced frequency of hyperandrogenic and metabolic features in PCOS was reported in haplotypes with wild-type alleles, and *PPARγ* haplotypes with mutations [273]. Shaikh et al. in an Indian population, investigated the associations of Pro12Ala and His447His *PPARγ* polymorphisms with PCOS susceptibility; diminished PCOS susceptibility was found to be associated with Pro12Ala polymorphism, and also, glucose metabolism (by influencing 2 h glucose, fasting insulin, or insulin resistance) was improved both polymorphisms [274]. A hospital-based, observational case–control study on PCOS and control was conducted by Thangavelu et al [275] Indian women, no significant difference in the phenotypic variables was found in the functional SNP rs3856806, which is located in exon 6 of *PPARγ*.

The management of PCOS is as complex as the condition itself. A healthy diet, regular physical activity, and medications are major components in managing and treating PCOS. PCOS management strategies mainly aim at resolving the four major components of PCOS, including

regularity of menstrual periods, control of hyperandrogenism (acne and hirsutism), management of infertility and IR along with its associated risk factors (type 2 diabetes mellitus, hyperlipidemia, and obesity). Both non-pharmacological and pharmacological management strategies are essential in the overall management of PCOS.

**Table 2.4: Examples of PPARγ Polymorphisms in Different Ethnic Groups**

| Study | Ethnic Group (Study Group Size) | *PPARγ* Polymorphism | Role in PCOS |
|---|---|---|---|
| Baldani et al. [76] | Croatian (330) | Pro12Ala | Positive effect on insulin sensitivity and BMI |
| Chae et al. [77] | Korean (440) | Pro12Ala | Modulation of HDL levels |
| Gu et al. [78] | Korean (238) | Pro12Ala, His447His | Correlation with PCOS |
| Wang et al. [79] | Chinese (348) | Pro12Ala | No significant correlation |
| Yang et al. [80] | Chinese (238) | Pro12Ala | No significant correlation |
| Dasgupta et al. [81] | South Indian (549) | Exon 2 Ala allele, Exon 6 His447His T allele | Reduced frequency of hyperandrogenic and metabolic characteristics |
| Shaikh et al. [82] | Indian (750) | Pro12Ala, His447His | Improved glucose metabolism, fasting insulin, and insulin resistance |

# Chapter 3: SUBJECTS AND METHODS

### 3.1 Study Design

It was an observational study with a case-control design.

### 3.2 Study Population

The subjects of the study were recruited based on inclusion and exclusion criteria (as mentioned below) from the relevant OPDs of selected tertiary hospitals and female student hostels in Dhaka city as shown in the flowchart for subject selection in Fig. 2.1.

The study population comprised two groups – one case group denoted as 'PCOS' and one control group denoted as 'Non-PCOS.' Subjects of the PCOS group were diagnosed following the Modified Rotterdam Criteria 2003 [276].

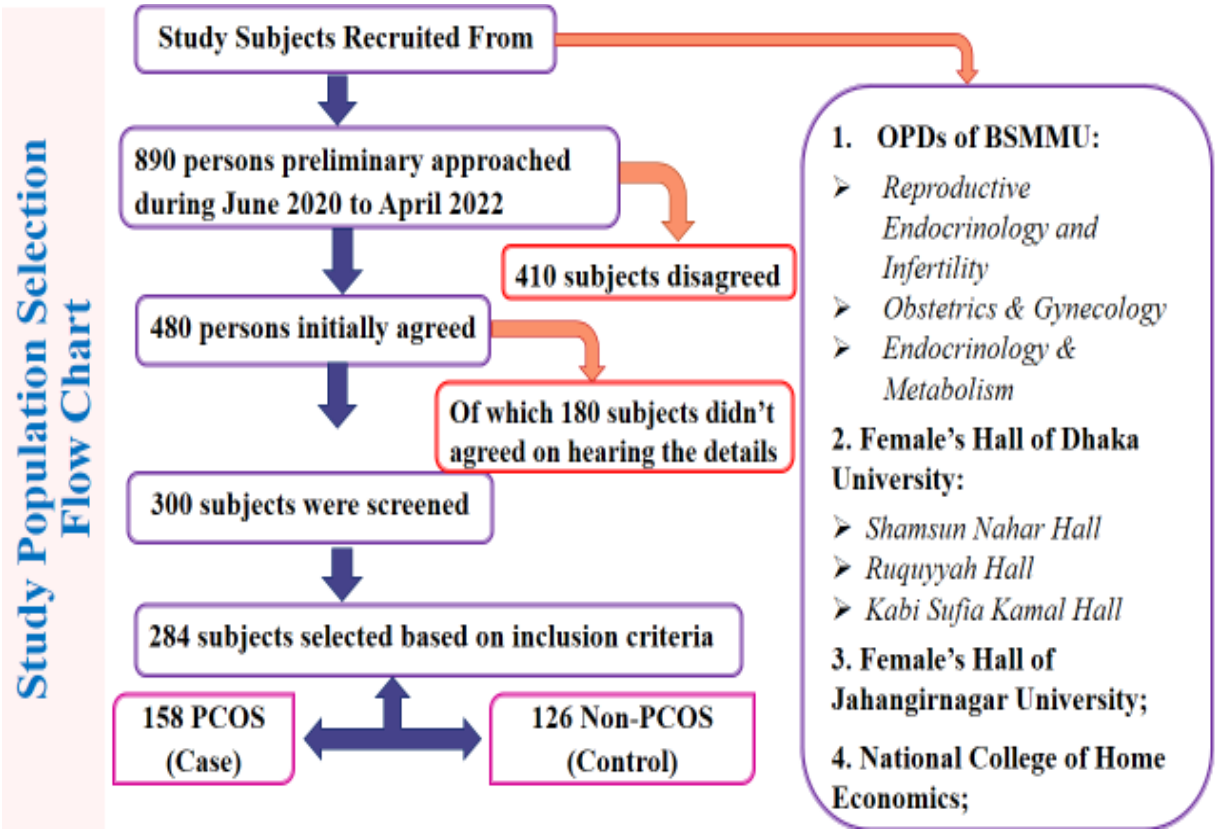


**Fig. 3.1. Flowchart for the Selection of Study Subjects in the Non-PCOS and PCOS**

### *3.2.1 Inclusion Criteria*

***Case Group:***

- Women with PCOS aged 15-49 years as per Modified Rotterdam Criteria 2003[276].

***Control Group:***

- The control women hadregular ovulatory menstrual cycles (26-35 days) with the absence of hirsutism and other manifestations of hyperandrogenism and the absence of sonographic features of PCOS.
- No sign of galactorrhea and thyroid dysfunction or personal or family history of diabetes. They had normal hormonal status and did not receive antidiabetic or antiobesity, glucocorticoids, or other hormonal therapy.

### *3.2.2 Exclusion Criteria*

***Case Group:***

- PCOS subjects using oral contraceptives ≥4 weeks;
- Using hormonal treatment or insulin-sensitizing agents≥ 2 weeks.
- Abnormal thyroid findings or nonclassical, Cushing's syndrome or androgen-secreting tumours, and adrenal hyperplasia.
- Pregnant, lactating mothers and smokers women.

***Control Group:***

- Any other notable any chronic disorder detected clinically besides the above criteria.

## 3.3 Selection of Study Subjects

### *3.3.1 Sampling Technique*

A purposive sampling technique was followed.

### *3.3.2 Calculation of Sample Size*

With a focus on exploring the independent association of insulin resistance with relevant genetic polymorphism (the primary objective), the sample size in the present study was calculated using a Binary Logistic regression-based formula with insulin resistance as the primary outcome variable, the 2 SNPs as primary input variables, and all other 7 covariates (as detailed in Section 2.6 in the conceptual framework) are confounding variables.

The sample size was calculated by following the following formula [277]:

$$n=10k/p$$

*Where, n=desired sample size; k=no of covariates; and p= prevalence of insulin resistance in PCOS patients*

With 50% prevalence (0.5) in PCOS patients and 7 covariates (independent variables), an n value of 140 was obtained from the calculation. Inflation was added for anticipated non-response (10%) = 140+10% of 140≈ 154. Hence, the required total sample for each group was 155. Accordingly, a total of 155 PCOS and 155 healthy age-matched control women were planned to be recruited in this study.

In practice, 158 PCOS and 126 Non-PCOS subjects were collected,

## 3.4 Conceptual Framework

The independent, dependent, and confounding variables of the study are enlisted in Fig. 3.2 under a Conceptual Framework.

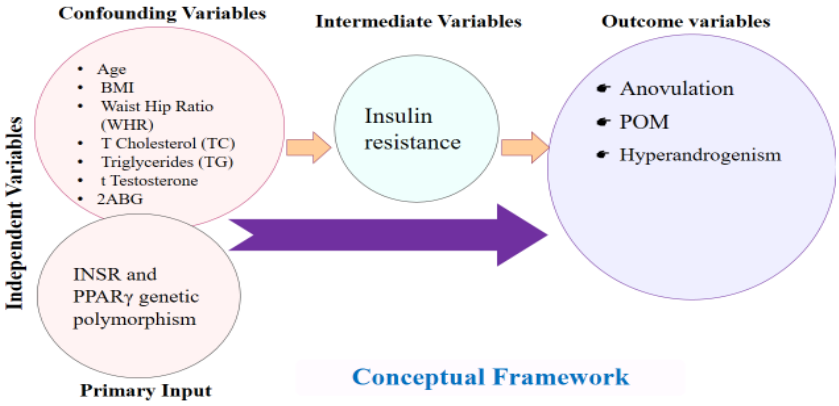


**Fig. 3.2. Study Variables under a Conceptual Framework**

## 3.5 Study Procedure

An outline of the study procedure is provided in Fig. 3.3; the details are provided in the relevant sections.

### *3.5.1 Questionnaire*

Data were collected from PCOS subjects using a pretested interviewer-administered questionnaire (Appendix II) covering sociodemographic factors and medical histories like detailed menstrual, obstetric, and gynecological histories, family history of diabetes, cardiovascular risk factors, and drug history.

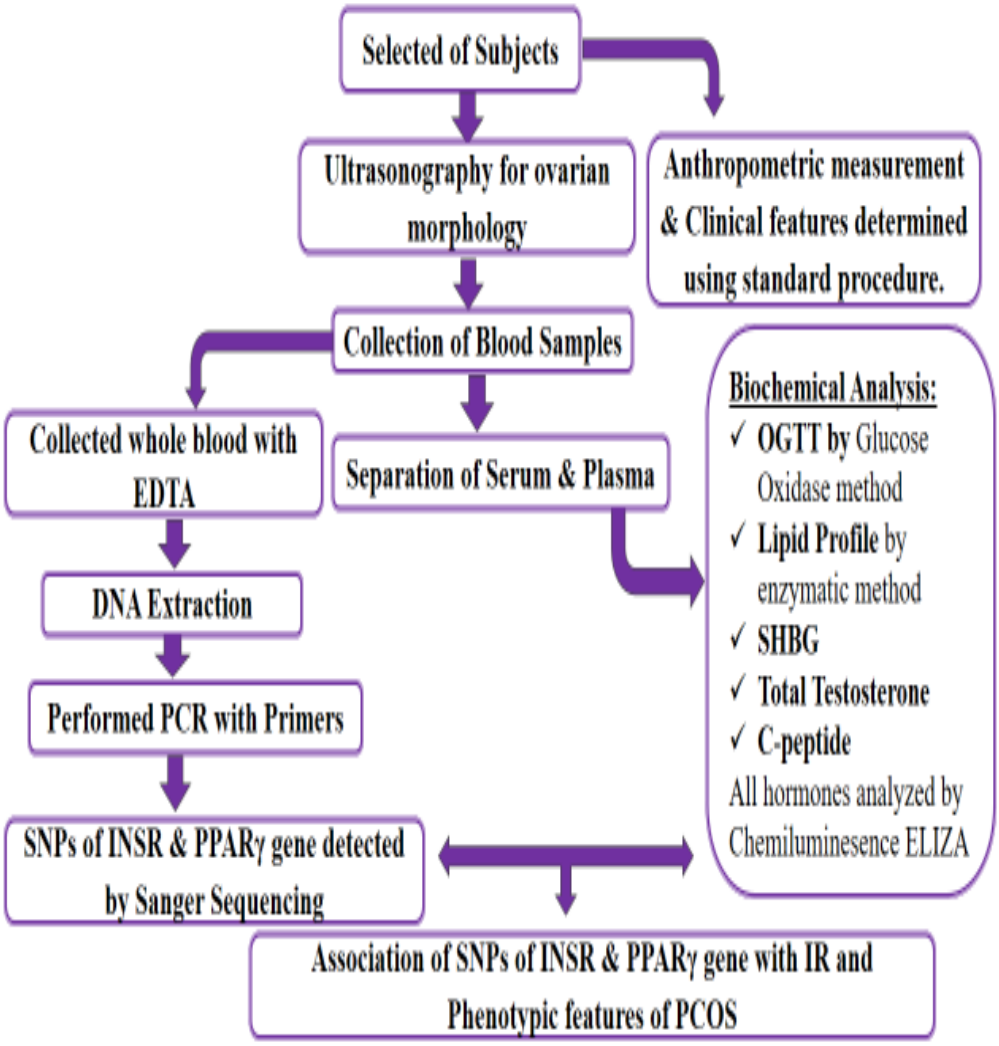


**Fig 3.3. Schematic Diagram of the Study Procedure**

### *3.5.2 Anthropometric Measurements*

#### 3.5.2.1 Height (cm)

Participants were requested to remove their shoes and braids, headbands, or anything else on their heads that may get in the way of an accurate measurement. Also, any bulky clothing was removed. The subject stood on the height-measured machine. The uttocks, heels, and occiput of the subject was placed against the back plank of the stadiometer; rotation of the head was done until the plane containing the midpoints of the ears and nose was horizontal. A wooden slide was subsequently brought down to rest on the head. The distribution of the weight ewas made even on feet, with legs straight, arms at the sides, and shoulders relaxed. The subject was asked to look at eye level and straight ahead; then recording of the measurement, from the side of the stadiometer was done to the nearest ½ inch.

#### 3.5.2.2 Weight (kg)

The scale was checked to make sure that it read zero. The participants were advised to remove heavy outer clothing and items from their pockets. They were asked to stand on the scale without moving, and the weight measurements were then recorded.

#### 3.5.2.3 BMI

BMI was calculated using the following formula:

$$\textbf{BMI= weight (kg) / [height (m)]}^2$$

The formula for BMI was weight in kilograms divided by height in meters squared. Height was measured in centimeters; it was then divided by 100 to convert to meters.

#### 3.5.2.4 Waist Circumference (cm)

The participants were asked to show the top of the hips and the bottom of the ribs. A tape was placed to measure around the middle at a point halfway between those points (just above the belly button). It was made sure that it was pulled tight, but not digging into the skin. The WC was measured in cm.

#### 3.5.2.5 Hip Circumference (cm)

To measure the circumference of the participant's hips, she was requested to stand up in a straight manner. A tape was then wrapped to measure around the widest part of their hips. The tape was not pulled too tight and the measurement was taken where the ends of the tape measure overlap.

#### 3.5.2.6 Waist-to-Hip Ratio (WHR)

The waist-hip ratio was calculated using the formula:

**WHR= waist circumference/hip circumference**

#### 3.5.2.7 Blood Pressure (mmHg)

All participants were requested to expose their upper arm to locate the brachial arterypulse of each subject by straightening her arm. Fingers were placed across the inside of the antecubital space at the elbow's inner bend, and the arrow mark on the cuff of the blood pressure equipment was placed over the brachial artery. The cuff was wrapped around the upper arm and the bulb was pumped until the pressure was 30 mmHg higher than the systolic pressure. With the stethoscope positioned over the brachial artery, the valve was released slowly by turning it counterclockwise. This allowed air to escape from the cuff slowly. The reading on the manometer was noted when the first Korotkoff sound was heard. This was the systolic pressure. The deflationof the cuff was continued, and the reading was noted until the last hearing of the Korotkoff sound. This was the diastolic pressure. The cuff was deflated completely, and it was then removed from the subject's arm.

### 3.6. Clinical Examination

#### *3.6.1 Anovulation or Oligomenorrhea*

Oligomenorrhea was defined as the presence of <9 menstrual periods per year or 3 cycleswith more than 38 days in the past year; amenorrhea was defined as cycles over 90 days (Table 3.1) [278].

**Table 3.1: Features of Oligomenorrhea**

| Type of irregular uterine bleeding | Definition of menstrual irregularity |
|---|---|
| Primary amenorrhea | Absence of menses at 15 or 3 years after breast development |
| Secondary amenorrhea | >90 days of lack of menorrhea after menarche |
| Oligomenorrhea<br>• <1 year post-menarche<br>• >1 - <3 year post menarche<br>• >3 years post menarche | • Any irregularity is normal<br>• Menses experienced <21 and >45 days after the last period or <4 cycles/year<br>• Menses experienced <21 and >35 days after the last period or <8 cycles/ year |
| Excessive anovulatory abnormal uterine bleeding | Menorrhea occurring in <21 days or having a duration of >7 days |

### *3.6.2 Hirsutism or Clinical Hyperandrogenism (HA)*

Hirsutism is the most specific sign of clinical HA, and it was defined as an increase in terminal hair in androgen-dependent areas (arms, upper and lower abdomen, anterior chest, upper lip, chin, upper and lowerback, and thighs) [279-280]. To define hirsutism, the modified Ferriman and Gallwey (FG) Score (Fig. 3.4) was used, and Score ≥ 8 was specified as clinical HA [281].

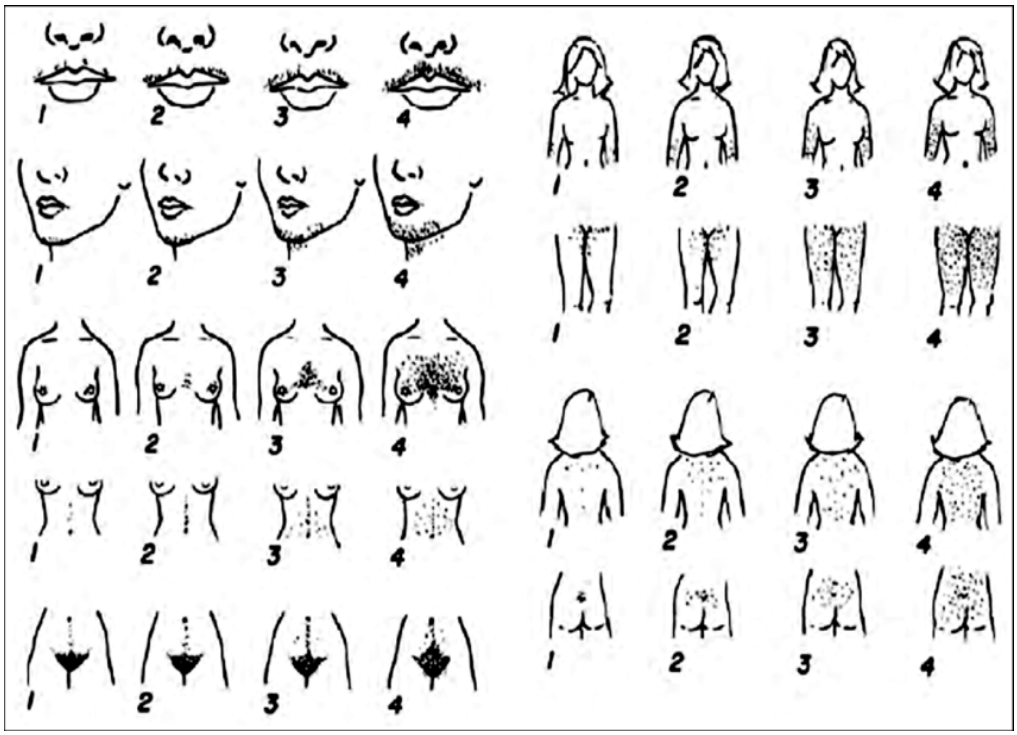

**Fig. 3.4: Modified Ferriman-Gallwey Score (mFG score) [282]**

### *3.6.3 Acne*

Acne (Fig. 3.5) was diagnosed by persistent presence of comedones in women with widespread severe acne, and in cases lacking a response to standard treatment [283].

**Fig. 3.5: Presence of acne [284]**

### *3.6.4 Alopecia*

Androgenic alopecia was assessed by using the Ludwig Scale (Fig. 3.6).

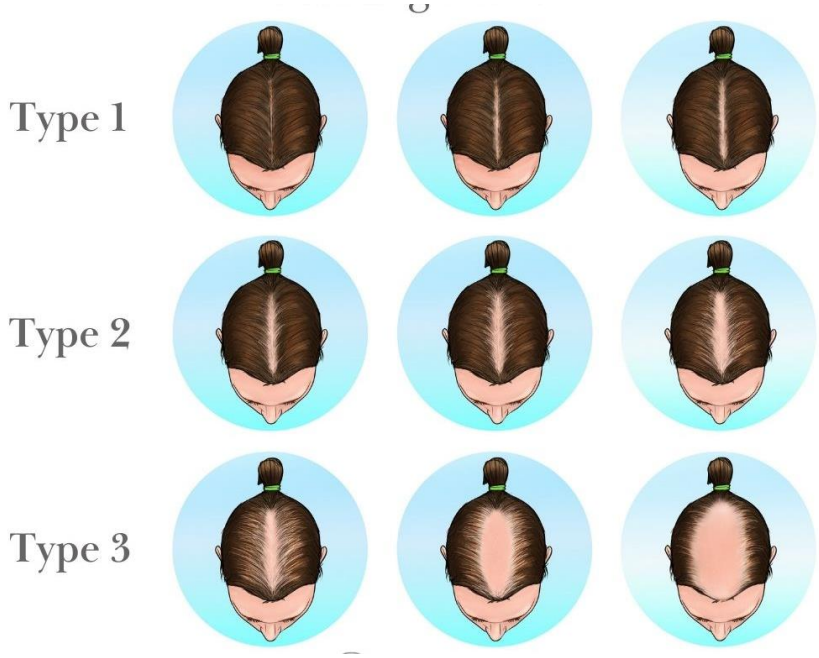


**Fig. 3.6: Ludwig Scale to Assess Androgenic Alopecia [285]**

### *3.6.5 Acanthosis Nigricans*

Acanthosis nigricans was evaluated as the presence of black velvety patches in creases and body folds and it was considered as a surrogate marker for insulin resistance (Fig. 3.7).

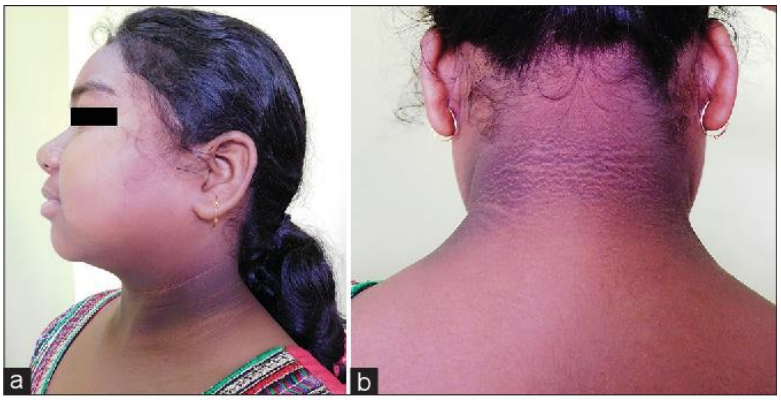


**Fig. 3.7: Presence of Acanthosis Nigricans**

## 3.7 Ultrasonographic Evidence of Polycystic Ovarian Morphology (PCOM)

Ultrasonographic examinations were performed for all participants using a Voluson E6 machine. A trained professional operated these tests. Presemce of 12 or more follicles in the ovaries, measuring 2-9 mm in diameter and/or enlarged ovarian volume (>10 $mm^3$) on abdominal ultrasonography, was considered as positive for polycystic sonography [6]. The subjective aspect of the ovaries was not considered to be necessary like their follicular distribution or the appearance of the stroma.

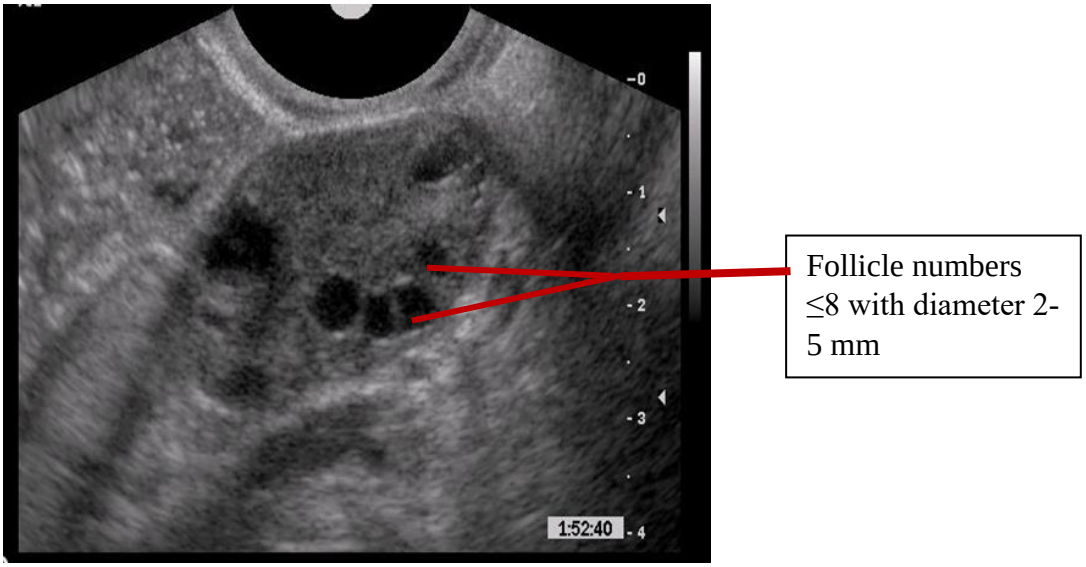


**Fig. 3.8: The Ultrasonographic View of the Ovary in a Non-PCOS Subject**

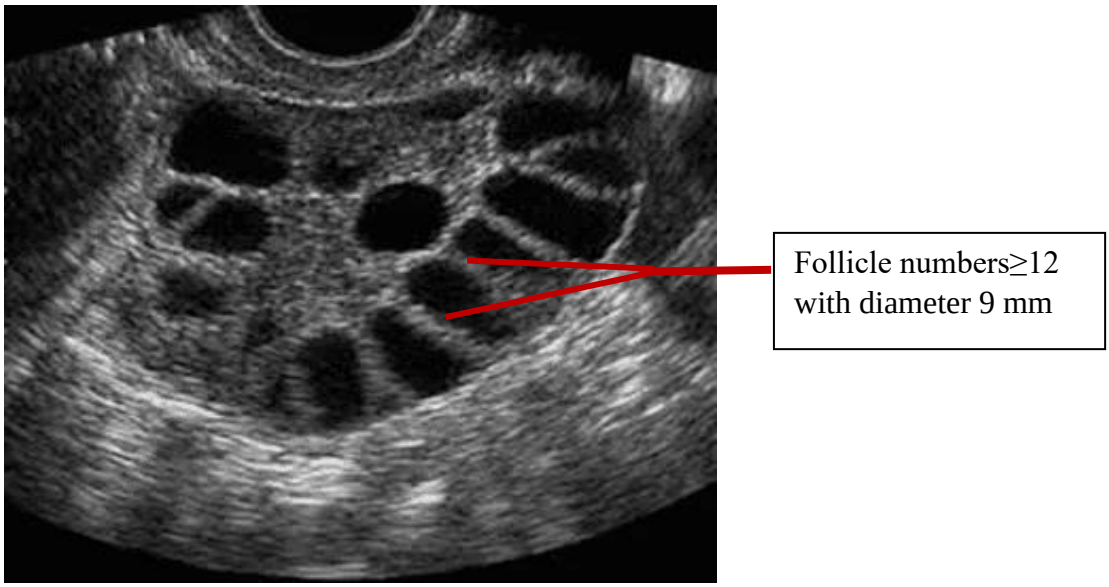


**Fig.3.9: The Ultrasonographic View of the Ovary in a PCOS Subject**

## 3.8 Biochemical Analysis

### *3.8.1 Patient Preparation*

The subjects were advised to have an average carbohydrate intake for 3 days and to fast overnight before coming for the test. The subject was requested to fast for at least 10 hours and not more than 16 hours; the test was performed in the morning to avoid the hormonal diurnal

effect on glucose. Patients refrained from exercising, eating, drinking (except water), and smoking before the blood sample was collected.

### *3.8.2 Sample Collection*

A venous blood sample was drawn immediately by venipuncture during fasting and postprandial states by a trained Phlebotomist. Ten (10) ml of blood at the fasting state and 4 ml at the postprandial state were collectedin tubes with and without EDTA using a disposable syringe. Aliquots were distributed following the aliquot distribution flow chart (Fig. 2.10). To collect the plasma or serum, samples were centrifuged at 10,000 g for 3 min. All samples were stored at -80°C for genetic analysis.

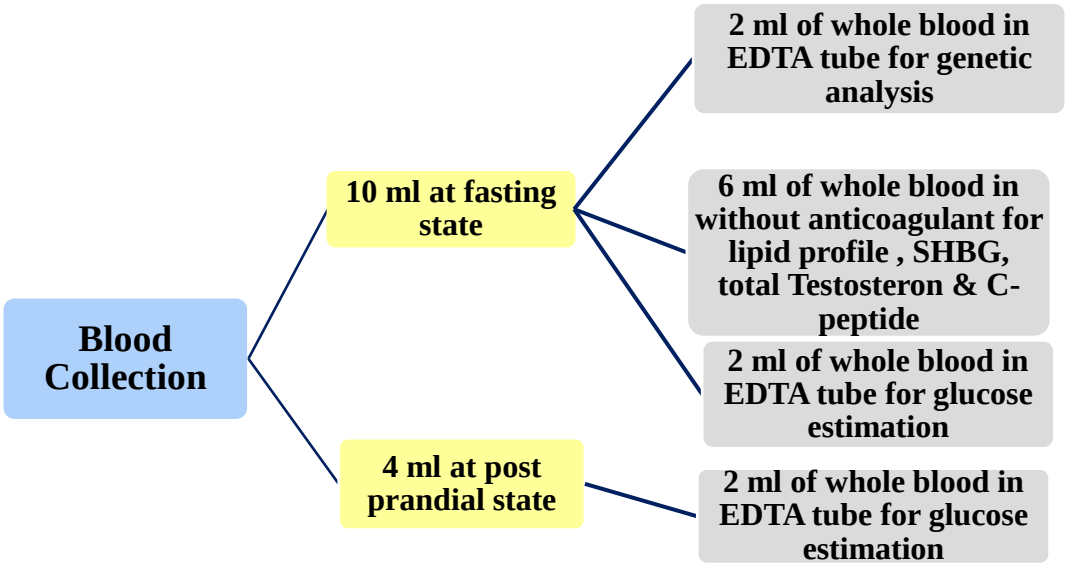


**Fig. 3.10: Flow Chart of Blood Aliquot Distribution**

### 3.8.3 Estimation of Glucose

Plasma glucose was estimated by Hexokinase/G-6-PDH method using an ARCHITECT c4000 clinical chemistry analyzer.

#### *3.8.3.1 Principle*

In the presence of adenosine triphosphate (ATP) and magnesium ions, glucose is phosphorylated by hexokinase (HK) to produce glucose-6-phosphate (G-6-P) and adenosine

diphosphate (ADP). With the concurrent reduction of nicotinamide adenine dinucleotide, Glucose-6-phosphate dehydrogenase (G-6-PDH) specifically oxidizes G-6-P to 6-phosphogluconate (NAD) to nicotinamide adenine dinucleotide reduced (NADH). For each micromole of glucose consumed One micromole of NADH is produced. The NADH produced absorbs light at 340 nm and, by spectrophotometry, can be detected as an increased absorbance.

$$Glucose + ATP \Leftrightarrow hexokinaseG6P + ADP$$

$$G6P + NADP+ \Leftrightarrow G6PDH6 - phosphoglucose + NADPH + H+$$

#### *3.8.3.2 Reagents*

**Reagents for Glucose Estimation**

| Reactive Ingredients | Concentration |
|---|---|
| NAD | 5.0 mg/mL |
| G-6-PDH | 3,000 U/L |
| Hexokinase | 15,000 U/L |
| ATP . 2Na | 9.0 mg/mL |

### 3.8.4 Estimation of Total Cholesterol

The enzymatic method measured plasma total cholesterol using an ARCHITECT c4000 clinical chemistry analyzer.

#### *3.8.4.1 Principle*

Cholesterol esterase enzymatically hydrolyzes cholesterol esters to cholesterol and free fatty acids. The originally present as well as new free cholesterol is then oxidized by cholesterol oxidase tocholest-4-ene-3-one and hydrogen peroxide. Aa chromophore (quinoneimine dye) is then formed by the combination of hydrogen peroxide, hydroxybenzoic acid (HBA) and 4-amino antipyrine to form. A measurable red quinoneimine derivative, with an absorbance at

500 nm, is formed from hydrogen peroxide (H2O2) and 4-amino-antipyrine in the presence of phenol and peroxidase.

*Cholesterol ester* —*Cholesterol esterase*→ *Cholesterol + fatty acids*

*Cholesterol* —*Cholesterol oxidase*→ *Cholest-4-en-3-one + H2O2*

*2H2O2 + Phenol + 4-Aminoantipyrine* —*Peroxidase*→ *Quinoneimine + 4H2O*

### *3.8.4.2 Reagents*

**Reagents for Total Cholesterol**

| Reactive Ingredients | Concentration |
|---|---|
| Cholesterol Oxidase (Microbial) | > 200 U/L |
| Cholesterol Esterase (Microbial) | > 500 U/L |
| Peroxidase (Horseradish) | > 300 U/L |
| 4-Aminoantipyrine | 0.25 mmol/L |
| HBA FULL | 10 mmol/L |

## 3.8.5 Estimation of Total Testosterone

### *3.8.5.1 Principle*

It is a one-step immunoassay (the ARCHITECT Testosterone assay) to determine the presence of testosterone in human serum and plasma using Chemiluminescent Microparticle Immunoassay (CMIA) technology with flexible assay protocols, referred to as Chemiflex™. The reaction mixture is created by Sample, anti-testosterone (mouse, monoclonal) coated paramagnetic microparticles, assay diluent, and testosterone acridinium-labeled conjugate. For binding, testosterone present in the sample competes with the testosterone acridinium-labeled conjugate with anti-testosterone (mouse, monoclonal) coated microparticles. This, in turn forms an antigen-antibody complex. Pre-trigger and trigger solutions are then added to the reaction mixture after washing; the resulting chemiluminescent reactionis are quantified as relative

lightunits (RLUs). There is an inverse relationship between the sample's testosterone amount and the RLUs detected by theARCHITECT i optical system.

### 3.8.6 Estimation of Sex Hormone Binding Globulin

#### *3.8.6.1 Principle*

It is a two-step immunoassay (the ARCHITECT SHBG assay) to determine the presence of SHBG in human serum and plasma using CMIA technology (Chemiflex technology) with flexible assay protocols. In the first step a combination is created with sample, anti-SHBG-coated paramagnetic microparticles, and assay diluents. There is a binding of the SHBG present in the sample with anti-SHBG-coated microparticles. It is then washed, and the SHBG binds to the anti-SHBG acridinium-labeled conjugate that is added in the second step. Pre-trigger and trigger solutions are added to the reaction mixture following another wash. The resulting chemiluminescent reaction product is measured as relative light units (RLUs). There is a direct relationship between the amount of SHBG in the sample and the RLUs detected by the ARCHITECT i System optics. The concentration of SHBG in the sample is determined by comparing the chemiluminescent signal in the reaction to the ARCHITECT SHBG calibration.

### 3.8.7 Diagnosis of Biochemical Hyperandrogenism

Hyperandrogenism was defined in terms of biochemical hyperandrogenism, estimated on the basis of free androgen index (FAI) computed by the formula as follows:

$$\textbf{FAI=TT (nmol/L)/SHBG (nmol/l) ×100}$$

The cut-off value of 7.1% of FAI was used and a value higher than the cut-off value was considered as hyperandrogenism, giving 62% sensitivity and 82% specificity [286].

### 3.8.8 Estimation of C-peptide

#### *3.8.8.1 Principle*

It is a two-step immunoassay (the ARCHITECT C-Peptide assay) for quantitatively determining C-peptide in human serum, plasma, andurine using CMIA technology (Chemiflex technology) with flexible assay protocols. In the first step a combination is created with sample,

assay diluent, and anti-human C-peptide coated paramagnetic microparticles. There is a binding of the C-peptide present in the sample with anti-human C-peptide-coated microparticles, forming an antigen-antibody complex. It is then washed, and in the second step anti-human C-peptideacridinium-labeled conjugate is added to create a reaction mixture. Pre-trigger and trigger solutions are added to the reaction mixture following another wash cycle. The resulting chemiluminescent reaction is measured as relative light units (RLUs). There is a direct relationship between the amount of C-peptide in the sample and the RLUs detected by the ARCHITECT iSysteoptics.Results were calculated automatically based on the previous establishedcalibration curve.

### 3.8.9 Insulin Resistance

For insulin resistance, plasma glucose and C-peptide at fasting were measured and IR was calculated using the C-peptide-modified formulae given by Li et al. [287]. The modified formula was:

$$\textbf{HOMA1-IR} = 1.5 + \textbf{FPG} \times \textbf{FCP} / 2800$$

HOMA of insulin sensitivity (HOMA%S) index was calculated by (1/HOMA-IR) × 100%. A HOMA-IR value >2.4 was considered to represent insulin resistance, which is the 70$^{th}$ percentile value of control women and the closest indicator value (≥2.5) of IR in Indian adults [288].

### 3.8.10 Metabolic Syndrome

Metabolic Syndrome (MS) was defined according to the modified NCEP criteria [306]. As per these criteria, the presence of any three of the following five factors is required for a diagnosis of Metabolic Syndrome: abdominal obesity, elevated blood pressure (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg or current use of antihypertensive drugs); impaired fasting glucose (fasting plasma glucose ≥5.6 mmol/l); and hypertriglyceridemia (triglycerides ≥1.7 mmol/l); low HDL cholesterol (HDL cholesterol ≤1.03 mmol/l for men and ≤1.29 mmol/l for women). The modified NCEP ATP III criteria suggested the cut-off points of waist circumference should be ethnic specific where individuals of Asian origin should use the cut-off of 90 cm in men and 80 cm in women.

## 3.9 Genetic Analysis

### 3.9.1 DNA Extraction

#### *3.9.1.1 Principle*

The kits used for the purpose are QIAamp DNA Mini and QIAamp DNA Blood Mini Kits (Qiagen, USA). These kits performs rapid purification of an average of 6 µg of total DNA (e.g., genomic, viral, mitochondrial) from 200 µl of whole human blood, and up to 50 µg of DNA from 200 µl of buffy coat,5 x 106 lymphocytes, or cultured cells that contain standard chromosomal sets.

#### *3.9.1.2 Procedure*

Into the bottom of a 1.5 ml microcentrifuge tube, 20 µl QIAGEN Protease (or proteinase K) was pipetted. Whole Blood Sample (200 µl) was added to the microcentrifuge tube. Buffer AL, 200 µl was added to the sample. It was mixed using pulse-vortexing for 15 s. Incubation was done for 10 min at 56°C in a water bath and it was centrifuged in 1.5 ml microcentrifuge tubes to remove drops from the inside of the lid. Ethanol (96–100%), 200 µl, was added to the sample, and it was mixed again by pulse-vortexing for 15 s. Without wetting the rim, the mixture was applied from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube). The cap was closed, and it was centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was then placed in a clean 2 ml collection tube and the tube was discarded containing the filtrate. To reduce noise, centrifugation was performed at 6000 x g (8000 rpm).

Without wetting the rim, the QIAamp Mini spin column was opened and added to 500 µl Buffer AW1. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spincolumn was placed in a clean 2 ml collection tube, and the collection tube was discarded containing the filtrate. The QIAamp Mini spin column was opened, and, without wetting the rim, 500 µl Buffer AW2 was added, the cap closed, and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. It was centrifuged at full speed for 1 min. The purpose of this step was to eliminate the chance of possible Buffer AW2 carryover. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube

and the filtrate collection tube was discarded. The QIAamp Mini spin column was carefully opened and Buffer AE (200 µl) was added. It was then incubated at room temperature (15–25°C) for 1 min, and centrifuged at 6000 x g (8000 rpm) for 1 min.

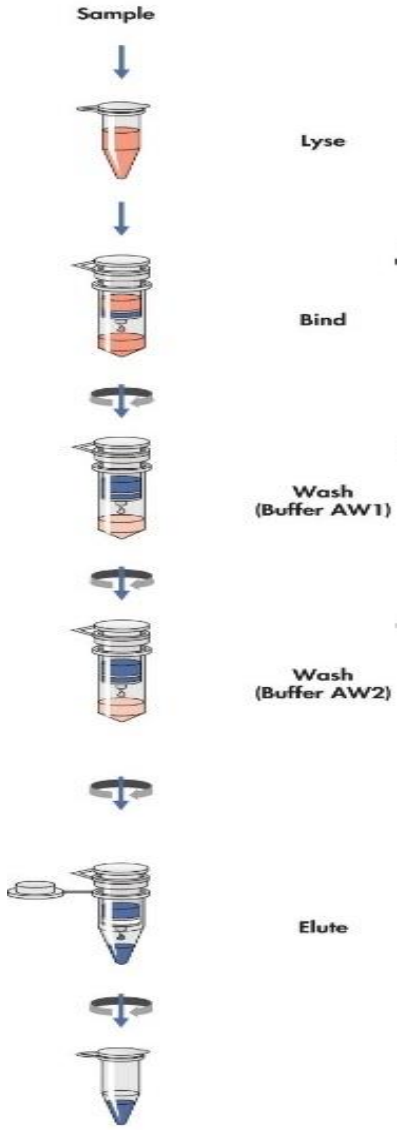


**Fig. 3.11: Steps of the DNA Extraction Procedure**

### 3.9.2 Purity Assessment and Quantification of Extracted DNA

A Nano-DropOneC Micro volume UV-V spectrophotometer (Thermo Fisher Scientific, USA) was used to quantify the extracted DNA by determining the concentration in ng/µl. A two-stage quality assessment involving measurement was used to evaluate the purity of Nucleic acid. To assess the presence of DNA and protein contamination, the purity of DNA was

determined by calculating the absorbance ratio at 260 to 280 nm (A260/280). Additionally, carbohydrates, residual salts, and phenol contaminations were identified using the 260/230 ratio.

### 3.9.3 Primer Design for Analyzing Gene SNP

The coding sequence for each gene was retrieved from NCBI. Each sequence was submitted to the NCBI Primer Blast to generate a primer for each gene. NCBI provides multiple primers for one gene, but one was screened out based on product size, specificity, Tm and GC content.

**Table 3.2: Primer Predicted by NCBI Primer Blast tool to Amplify *rs1799817* of theInsulin Receptor (INSR) Gene *(Cytogenic location: 19p13.3-p13.2)* and *rs3856806* of the Peroxisome Proliferator-Activated Receptor γ (PPARG) Gene *(Cytogenic location: 3q25)***

| Gene Name | rs No | Forward Primer Sequence | Reverse Primer Sequence |
|---|---|---|---|
| INSR | rs1799817 | CGAGAGAAGATCACCCTCCT | GGAACAAAGGAGCAGAAAGGC |
| PPARγ | rs3856806 | ATATGTGCTTCCCCAGACCG | TGTATCAGCAGTTCCACTCACA |

#### *3.9.3.1 Validation of the Primer Sequence*

The UCSC *In Silico* PCR tool was employed to verify the correctness of the chosen primers. The forward primer sequence and reverse primer sequence that had been created were submitted. The tool performed an *In Silico* PCR and predicted the sequence and size of the amplicon thereby confirming the effectiveness of the selected primers in amplifying the gene of interest.

### 3.9.4 PCR Amplification

#### *3.9.4.1 Reagents*

- GoTaq DNA Polymerase (Promega, USA)
- Primers (Oligo, South Korea)
- DMSO (Merck)
- Agarose (Sigma Chemical Co, USA)

- Ethidium Bromide (Sigma Chemical Co, USA)
- 100 bp DNA Ladder (Promega)
- Nuclease-free water

### *3.9.4.2 Quantity of Component Used in the INSR (His1085C/T) Polymorphism Detection*

The composition of each component was used for 30µl PCR reaction. The PCR product size of the primer was 688 bp.

**Table 3.3: Experimentally Derived Optimum Quantity of Each Component used in the His1085C/T Polymorphism Detection**

| Component | Volume (µl) |
|---|---|
| Nuclease free water | 6 |
| DMSO | 1 |
| Forward | 2.5 |
| Reverse | 2.5 |
| GoTaq Master Mix | 15 |
| Genomic DNA | 3 |
| **Total** | **30 µl** |

### *3.9.4.3 PCR condition for INSR Gene*

Conditions for the amplification included the initial step of denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute, and elongation at 72°C for 1 minute, and finally a step of final elongation at 72°C for 3 minutes. PCR assays were performed in a DNA thermal cycler (Applied Biosystems).

### *3.9.4.4 Evaluation of PCR*

Five (5) µl of the PCR product was checked for amplicon size on a 2% agarose gel. The size of the product was ascertained by comparing it with a 100 bp DNA ladder. The amplified DNA was stained with ethidium bromide solution and visualized under UV light (Fig.4.4).

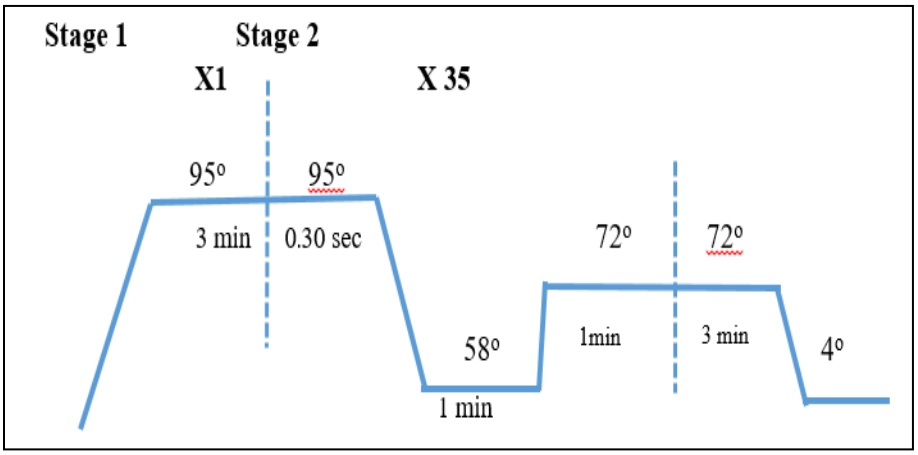


**Fig. 3.12: PCR condition for INSR Gene amplification**

### *3.9.4.5 Quantity of Component Used in the PPARγ (His 447C/T) Gene Polymorphism Detection*

The composition of each component was used for 30µl PCR reaction. The PCR product size of the primer was 662 bp.

**Table 3.4: Experimentally Derived Optimum Quantity of Each Component Used in the PPARγ (His 447C/T) Gene Polymorphism Detection**

| Component | Volume (µl) |
|---|---|
| Nuclease free water | 12 |
| DMSO | 2.0 |
| Forward | 0.5 |
| Reverse | 0.5 |
| GoTaq Master Mix | 14.0 |
| Genomic DNA | 1.0 |
| **Total** | **30 µl** |

### *3.9.4.6 PCR Condition for PPARγ Gene*

Conditions for the amplification included the initial step of denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 1 minute, and elongation at 72°C for 1 minute, and finally a step of final elongation at 72°C for 3 minutes. PCR assays were performed in a DNA thermal cycler (Applied Biosystems).

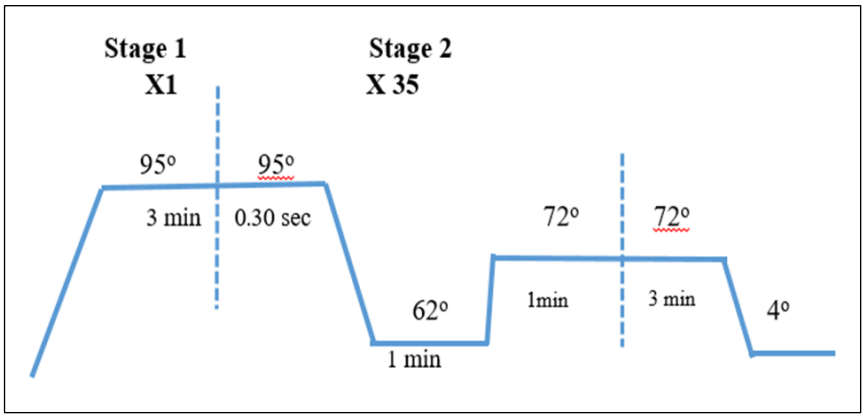


**Fig. 3.13: PCR Condition for PPARγ Gene amplification**

### *3.9.4.7 Evaluation of PCR*

5µl of the PCR product was checked for amplification on a 2% agarose gel. The size of the product was ascertained by comparing it with a 100 bp DNA ladder. The amplified DNA was stained with ethidium bromide solution and visualized under UV light (Fig.4.6).

### 3.9.5 Sanger Sequencing

This method is designed for determining the sequence of nucleotide bases in a piece of DNA (commonly less than 1,000 bp in length). Sanger sequencing with 99.99% base accuracy is considered the 'gold standard' for validating DNA sequences.

### *3.9.5.1 Principle*

A DNA primer is attached by hybridization to the template strand and deoxynucleosides triphosphates (dNTPPs) are sequentially added to the primer strand by DNA polymerase. The primer is designed for the known sequences at the 3' end of the template strand. M13 sequences are generally attached to the 3' end, and the primer of this M13 is made. The reaction mixture

also contains dideoxynucleoside triphosphate (ddNTPs) along with usual dNTPs. If, during replication, ddNTPs are incorporated instead of usual dNTPs in the growing DNA strand, then the replication stops at that nucleotide. The ddNTPs are analogues of dNTPs.ddNTPslack hydroxyl group (-OH) at C3 of a ribose sugar, so it cannotmake phosphodiester bond with nest nucleotide, thus terminating thenucleotide chain. Respective ddNTPs of dNTPs terminate the chain at their respectivesite. For example, ddATP terminates at A site. Similarly, ddCTP, ddGTP, and ddTTP terminateat the C, G, and T sites, respectively.

### *3.9.5.2 Procedure*

#### 3.9.5.2.1 Template preparation

M13 µl forward-sequence Copies of the template strand to be sequenced were prepared with short known sequences at the 3' end of the template strand. A DNA primer was essential to initiate replication of the template, so primer preparation of known sequences at 3'end is always required. For this purpose, a single-stranded cloning vector M13 was flanked with template strand at 3'end which serves as the binding site for primer.

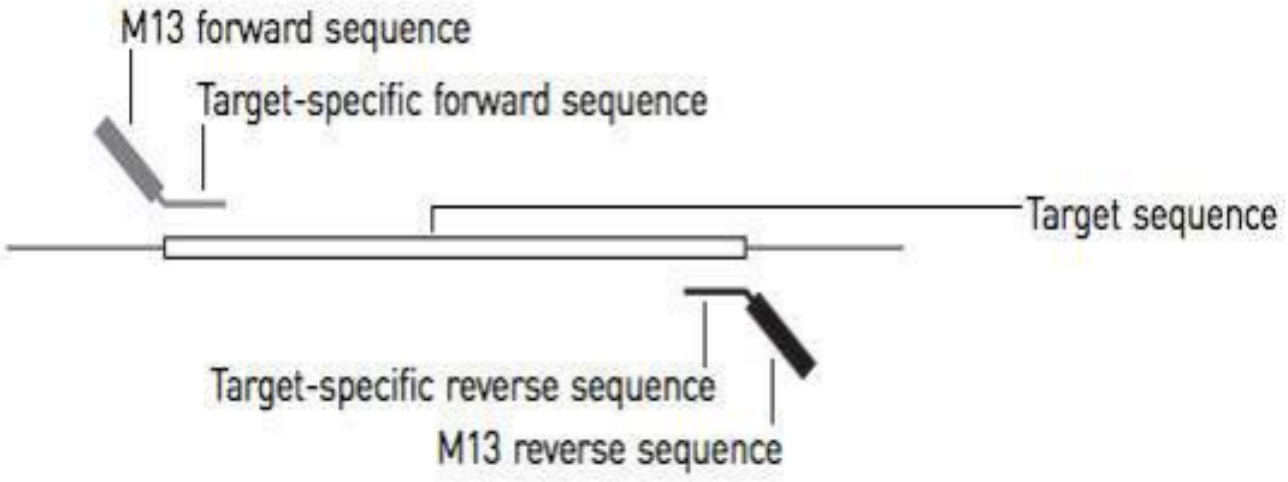


**Fig. 3.14: Template Preparation for Sanger Sequencing**

#### 3.9.5.2.2 Generation of a Nested Set of Labeled Fragments

Copies of each template were divided into four batches, and each batch was used for different replication reactions.Copies of standard primer and DNA polymerase were used in all four batches.To synthesize fragments that terminate at A, ddATP was added to the reaction mixture on batch I along with dATP, dTTP, dCTP, and dGTP, standard primer and DNA polymerase I.Similarly, to generate, all fragments that terminate at C, G, and T, the respective ddNTPs as ddCTP, ddGTP, and ddTTP were added respectively to different reaction mixtures on different batches along with usual dNTPs.

#### 3.9.5.2.3 Gel Analysis and Determination of DNA Sequence

The last step involved reading the gel to determine thesequence of the input DNA. Because DNA polymerase only synthesizesDNA in the 5’ to 3’ direction starting with a provided primer, eachterminal ddNTP corresponds to a specific nucleotide in the originalsequence. Therefore, reading the gel bands from smallest to largest determined the original DNA strand’s 5’ to 3’ sequence.

A computer reads each band of the capillary gel using fluorescence to call the identity of eachterminal ddNTP. In short, a laser-excited the fluorescent tags in eachband, and a computer detected the resulting light emitted. Each of the four ddNTPs was tagged with a different fluorescent label, and the light emitted was directly tied to the identity of the terminalddNTP.The output is called a chromatogram, which shows the fluorescentpeak of each nucleotide along the length of the template DNA.Each dNTP contains a phosphate group, a sugar group, and one of four nitrogenous bases [adenine (A), thymine (T), guanine (G), or cytosine (C)]. The dNTPs were strung together in a linear fashion by phosphodiester covalent bonds between the sugar of one dNTP and the phosphate group of the next.

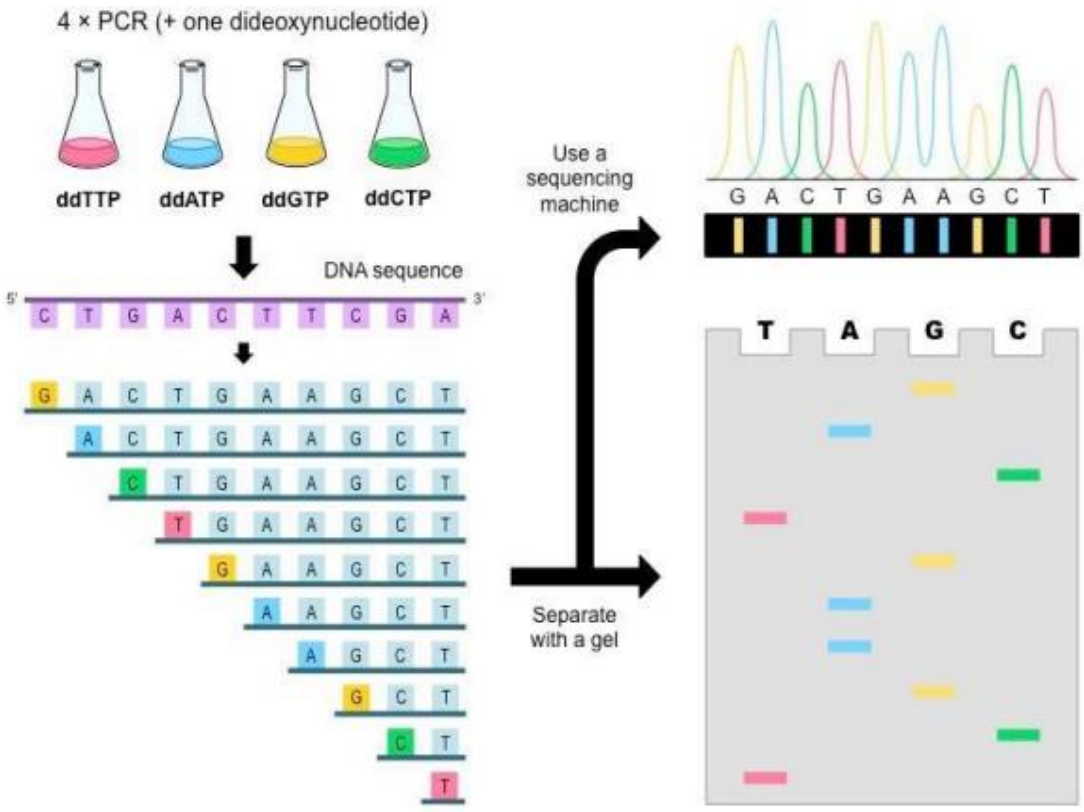


**Fig. 3.15: Flow Chart of Sanger Sequencing**

### 3.10 Statistical Analysis

Data analysis was performedusing the SPSS for Windows software (version 26). Descriptive univariate analyseswere conducted to determine the proportions of different categorical variables. Continuous variables were expressed either as means and standard deviations or median (ranges). Bivariate as well as multivariate analyses were conducted. $X^2$–test was used to to detect distribution differences among categorical variables. Mann-Whitney or t-tests were done to compare medians or means, as appropriate. Pearsons or Spearman correlation analysis was done to assess the relation between two continuous variables. Multivariate regression (binary logistic or linear) analysis was used to adjust for confounding variables. For the genetic analysis, data was tabulated in 2x2 category tables [case/control x presence/absence of variant(s)] and analyzed with Fisher's exact test because of the low frequencies of the mutations. A two-sided p-value<0.05 was considered statistically significant.

### 3.11 Ethical Considerations

The Ethical Review Committee of the Department of Biochemistry and Molecular Biology, University of Dhaka, approved the protocol. Informed written/verbal consent of the participants was obtained before data collection. Consent was conducted in the local language, Bengali. Confidentiality of data was ensured strictly. Data wasanonymously preserved in the computer and used for this study only.

# Chapter 4: RESULTS

The results of the present Case-Control study were to generate evidence on the burden of insulin resistance (IR) among Bangalee PCOS women and to explore its covariates, especially polymorphism in two selected locations (namely rs1799817 of INSR gene and rs3856806 of PPARγ gene) both of which have been reported to be associated with IR in PCOS populations including the Indian ones. Data were generated through demographic, anthropometric, clinical, biochemical, and molecular investigations. The present chapter describes the salient features of the results and observations.

## 4.1 Demographic and Anthropometric Findings of the Study Subjects

The comparative demographic and anthropometric data of the Non-PCOS and PCOS subjects are shown in Table 4.1. As per study design, the age of the two groups of subjects were purposively matched [Age in years, Median (Range): 24 (19-34) in Non-PCOS vs 23 (15-34) in PCOS groups; p=0.08)]. The greatest proportion of subjects was within the 21-30 years age group (96% in Non-PCOS and 80% in PCOS); however, a significantly higher proportion of PCOS subjects belonged to the 15-20 years age group as compared to the Non-PCOS subjects (15% vs. 1%, p <0.001).

Subjects of the PCOS group had significantly higher median BMI as compared to the Non-PCOS group [Median (Range): 23.4 (15.9-37.4) in Non-PCOS vs 23.98 (15.4-37.7) in PCOS groups; p=0.037]. The proportions of underweight, normal weight, overweight, and obese subjects did not differ significantly between the two groups (p=0.28). The Waist-Hip Ratio (WHR), however, was significantly higher in subjects of the PCOS as compared to the subjects of the Non-PCOS groups [Median (Range): 0.92(0.76-1.03) in Non-PCOS vs 0.94 (0.71-1.6) in PCOS groups; p=0.019].

**Table 4.1: Anthropometric data of the Non-PCOS and PCOS subjects**

| Characteristics | Non-PCOS (n=126) n (%) | PCOS (n=158) n (%) | p-value |
|---|---|---|---|
| **Age (yrs),** Median (Range) | 24 (19-34) | 23 (15-34) | **0.08** |
| 15-20 yrs | 1 (1) | 24 (15) | **<0.001** |
| 21-25 yrs | 97 (77) | 104 (66) | |
| 26-30 yrs | 24 (19) | 22 (14) | |
| 31-35 yrs | 4 (3) | 8 (5) | |
| **BMI**, Median (Range) | 23.4 (15.9-37.4) | 23.98 (15.4-37.7) | **0.037** |
| Under Weight (<18.5) | 9 (7) | 6 (4) | **0.28** |
| Normal Weight (18.5-24.9) | 51 (40) | 53 (33) | |
| Over Weight (25-29.9) | 26 (21) | 36 (23) | |
| Obese (≥30) | 40 (32) | 63 (40) | |
| **Waist-Hip Ratio (WHR)** Median (Range) | 0.92 (0.76-1.03) | 0.94 (0.71-1.6) | **0.019** |

*Results were presented as number(percentage) and Median (range); Data were compared using the Mann-Whitney Test. p-value<0.05 considered as significant.*

### 4.2 Clinical Characteristics of the Study Subjects

The comparative clinical characteristics of the two study groups are shown in Table 4.2. As expected from the well-known clinical features of the disorder, subjects of the PCOS group showed a much higher proportion (p, 0.03-0.001) of acne, alopecia, Acanthosis Nigricans, metabolic syndrome, right ovary volume, left ovary volume, right ovary follicles and left ovary follicles.

**Table 4.2: Clinical Characteristics of the Non-PCOS and PCOS subjects**

| Clinical Characteristics | Non-PCOS (n=126) n (%) | PCOS (n=158) n (%) | p-value |
|---|---|---|---|
| Acne | 44 (35) | 75 (48) | **0.03** |
| Alopesia | 84 (67) | 129 (82) | **0.004** |
| Acanthosis Nigricans | 1 (1) | 43 (27) | **<0.001** |
| Metabolic Syndrome | 7 (6) | 25 (16) | **0.007** |
| Right ovary volume | 4 (3) | 61 (39) | **<0.001** |
| Left ovary volume | 5 (4) | 60 (38) | **<0.001** |
| Right ovary follicles | 0 (0) | 31 (20) | **<0.001** |
| Left ovary follicles | 1 (1) | 38 (24) | **<0.001** |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test. p-value<0.05 considered significant.*

## 4.3 Glycemic, Lipidemic, Insulinemic, and Androgen Status of the Non-PCOSand PCOS Subjects (Table 4.3)

### *4.3.1 Glycemic Status*

The median value of blood glucose 2 hr after oral glucose load (2hBG) was significantly higher in the PCOS as compared to the Non-PCOS group [2hBG, mmol/l, median (range): 5.7 (4.2-16.0) in Non-PCOS vs 6.0 (4.8-12.9) in PCOS groups; p=0.004]. The fasting blood glucose, however, did not differ between the two groups (p=0.28).

### *4.3.2 Lipidemic Status*

Subjects of the PCOS group had significantly higher median Total Cholesterol [mg/l, median (range): 156.0 (105-240) in Non-PCOS vs 161.0 (105-275) in PCOS groups; p=0.045] and

higher median TG [mg/l, median (range): 84.0 (43-470) in Non-PCOS vs 102.0 (26-313 in PCOS groups; p=0.002]. LDL-c and HDL-c did not differ significantly between the two groups.

### *4.3.3 Androgen Status*

PCOS subjects had significantly higher Total Testosterone levels as compared to their Non-PCOS counterparts [Total Testosterone, U/l. Median (Range): 1.1 (0.45-2.91) in Non-PCOS vs 1.4 (0.47-5.20) in PCOS groups, p=0.001].

### *4.3.4 Insulin Resistance*

PCOS subjects showed significantly higher HOMA-IR in comparison to the Non-PCOS subjects [HOMA-IR%: 2.2 (1.7-4.6) in Non-PCOS vs 2.5 (1.7-29.1) in PCOS groups; p<0.001].

**Table 4.3: Glycemic, Lipidemic, Insulinemic, and Androgen Status of the Non-PCOS and PCOS Subjects**

| Investigations | Non-PCOS Median (Range) (n= 126) | PCOS Median (Range) (n=158) | p-value |
|---|---|---|---|
| FBG, mmol/l | 5.2 (4.0-8.20) | 5.1 (4.0-7.40) | 0.280 |
| 2hBG, mmol/l | 5.7 (4.2-16.0) | 6.0 (4.8-12.9) | **0.004** |
| Total Cholesterol (TC), mg/l | 156.0 (105-240) | 161.0 (105-275) | **0.045** |
| TG, mg/l | 84.0 (43-470) | 102.0 (26-313) | **0.002** |
| LDL-c, mg/l | 95.0 (52-157) | 101.0 (40-217) | 0.082 |
| HDL-c, mg/l | 40.0 (20-126) | 38.5 (23-246) | 0.335 |
| Total Testosterone, U/l | 1.1 (0.45-2.91) | 1.4 (0.47-5.20) | **0.001** |
| HOMA-IR, % | 2.2 (1.7-4.6) | 2.5 (1.7-29.1) | **<0.001** |

*Results were presented as Median (IQR=interquartile range); Data were compared using the Mann-Whitney Test. p-value<0.05 considered as significant.*

## 4.4 Individual Phenotypic Characteristics of PCOS among Non-PCOS and PCOS Subjects

From Table 4.4, it may be noted that, as expected, the proportion of all the individual phenotypic characteristics (anovulation, PCOM, hirsutism, and hyperandrogenism) were greater with highly significant differences ($p<0.001$) among PCOS as compared to Non-PCOS subjects. Among the Non-PCOS group, biochemical hyperandrogenism (HA) was the most prevalent (23%) feature, followed by clinical hyperandrogenism or hirsutism (14%), anovulation (5%), and PCOM (1%). On the other hand, anovulation was present in 88% of cases followed by PCOM (84%), hirsutism (69%), and HA (65%).

**Table 4.4: Presence of Phenotypic Characteristics of PCOS among Non-PCOS and PCOS Subjects**

| Phenotypic Features | Non-PCOS (n=126) n (%) | PCOS(n=158) n (%) | $\chi 2$ | p-value |
|---|---|---|---|---|
| Anovulation | 6 (5) | 139 (88) | 195.2 | **<0.001** |
| PCOM | 2 (1) | 133 (84) | 192.7 | **<0.001** |
| Hirsutism | 18 (14) | 109 (69) | 84.8 | **<0.001** |
| HA | 29 (23) | 102 (65) | 49.3 | **<0.001** |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test. p-value<0.05 considered significant.*

## 4.5 Proportion of IR among the Study Groups

Fig 4.1 depicts the proportion of IR in the two study groups. Although the proportion of subjects with IR was much higher among PCOS subjects (52%) as compared to their Non-PCOS counterparts (28%) with a highly significant ($p<0.001$) statistical difference, it needs to be noted that little more than one-fourth of the subjects without a clinical diagnosis of PCOS (i.e., Non-PCOS subjects) already have insulin resistance.

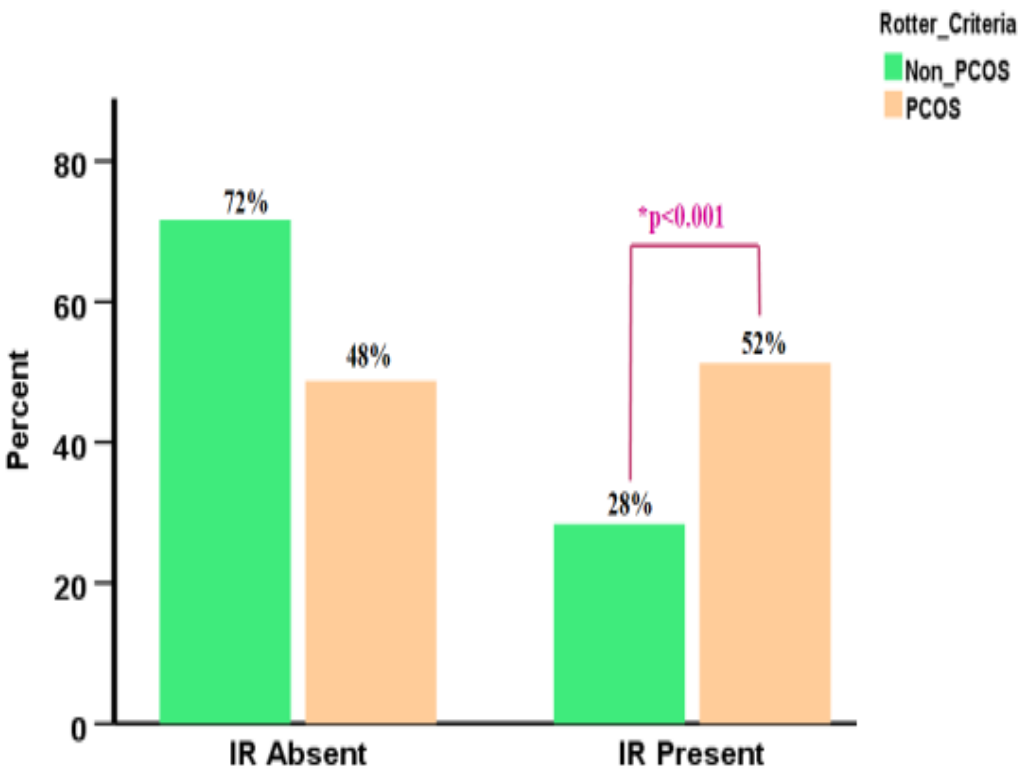


**Fig 4.1: Proportion of IR among Non-PCOS and PCOS subjects**

### 4.6. Insulin Resistance and Phenotypic Features of PCOS

The proportion of insulin-resistant subjects in the presence of different diagnostic phenotypic features of PCOS is compared between the two groups in Table 4.5. The proportions of anovulation, PCOM, and hirsutism do not differ significantly between the two groups; however, HA is present in much higher proportion among insulin-resistant PCOS subjects (83%) as compared to non-insulin resistant PCOS subjects (46%), and the difference is highly significant ($x^2$=17.11; p <0.001).

**Table 4.5: Proportion of Insulin Resistant Subjects in the Presence of Different Diagnostic Phenotypic Features of PCOS in the Case Subjects**

| Phenotypic characteristics | Insulin Resistance Absent n (%) | Insulin Resistance Present n (%) | $\chi 2$ | p-value |
|---|---|---|---|---|
| Anovulation (Yes) | 68 (88) | 71 (88) | 0.88 | 0.89 |
| PCOM (Yes) | 65 (84) | 68 (84) | 3.41 | 0.94 |
| HA (Yes) | 35 (46) | 67 (83) | 17.11 | **<0.001** |
| Hirsutism (Yes) | 56 (73) | 53 (65) | 0.98 | 0.32 |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test.* A*p-<0.05 considered significant.*

### 4.7 Insulin Resistance and Individual Phenotypic Features of PCOS

The proportion of insulin-resistant subjects in the presence of different diagnostic phenotypic features of PCOS is compared between the two groups in Table 4.6. The proportions of anovulation, PCOM, and hirsutism do not differ significantly between the two groups; however, HA is present in higher proportion among insulin resistant Non-PCOS subjects (83%) as compared to non-insulin resistant Non-PCOS subjects (46%) and the difference is highly significant.

**Table 4.6: Proportion of Insulin Resistant Subjects in the Presence of Different Diagnostic Phenotypic Features of PCOS in the Control Subjects**

| Phenotypic characteristics | Insulin Resistance Absent n (%) | Insulin Resistance Present n (%) | $\chi 2$ | p-value |
|---|---|---|---|---|
| Anovulation (Yes) | 4 (4) | 2 (5) | 0.07 | 0.78 |
| POM (Yes) | 1 (1) | 1 (1) | 0.47 | 0.49 |
| Hyperandrogenism (Yes) | 16 (18) | 13 (36) | 5.02 | **0.02** |
| Hirsutism (Yes) | 14 (15) | 4 (11) | 0.32 | 0.57 |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test. p-value<0.05 considered significant.*

### 4.8 Correlation of IR with Various Covariates

Correlation analysis was done in the individual groups with IR as the dependent variable and various covariates as independent variables. In the Non-PCOS group, IR was found to be significantly correlated with age (p=0.001), BMI (p=0.001), TC (p=0.01), Fasting Androgen Index or FAI (p<0.001) and 2hBG (p=0.04). On the other hand, in the PCOS group, IR was found to be significantly correlated with BMI (p<0.001), TG (p=0.007), FAI (p<0.001) and 2hBG (p=0.002).

**Table 4.7: Correlation of IR with Age, BMI, West-Hip Ratio (WHR), Total Cholesterol (TC) Triglycerides (TG), Free Androgen Index (FAI) and 2hBG**

| Variables | Non-PCOS (n=126) | | PCOS (n=158) | |
|---|---|---|---|---|
| | r | p-value | r | p-value |
| Age (yrs) | 0.30 | **0.001** | 0.27 | 0.74 |
| BMI | 0.28 | **0.001** | 0.31 | **<0.001** |
| WHR | 0.001 | 0.99 | 0.67 | 0.40 |
| TC, mg/dl | 0.22 | **0.01** | 0.69 | 0.39 |
| TG, mg/dl | 0.15 | 0.1 | 0.21 | **0.007** |
| FAI | 0.36 | **<0.001** | 0.59 | **<0.001** |
| 2hBG, mmol/l | 0.17 | **0.04** | 0.28 | **0.002** |

### 4.9 Independent Association of IR with Relevant Covariates among PCOS Subjects

The independent association of IR with relevant covariates in the PCOS group was explored by using binary logistic regression analysis considering IR as the dependent variable and others as the independent variables (Table 4.8). Only BMI (p<0.001) and FAI (p=0.001) showed significant association with IR when the effects of age, WHR, TC, and TG were adjusted.

**Table 4.8: Binary Logistic Regression Analysis Considering IR as Dependent Variables among PCOS Subjects**

| Variables | Standardized Coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age, yrs | -0.08 | 0.19 | 0.82 | 1.04 |
| BMI, $kg/m^2$ | 0.28 | **<0.001** | 1.17 | 1.50 |
| WHR | 0.19 | 0.92 | 0.02 | 60.7 |
| TC, mg/dl | -0.001 | 0.92 | 0.98 | 1.01 |
| TG, mg/dl | 0.008 | 0.72 | 0.99 | 1.02 |
| FAI | 0.035 | **0.001** | 1.01 | 1.05 |

### 4.10 Independent Association of IR with Relevant Covariates among Non-PCOS Subjects

The independent association of IR with relevant covariates in the Non-PCOS group was explored using binary logistic regression analysis, considering IR as the dependent variable and others as the independent variables (Table 4.9). Age (p=0.002), WHR (p=0.04) and TG (p=0.03) showed significant association with IR when the effects of age, BMT, TC and FAI were adjusted.

**Table 4.9: Binary Logistic Regression Analysis Considering IR as Dependent Variables among Non-PCOS subjects**

| Variables | Standardized Coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age, yrs | 1.32 | **0.002** | 1.11 | 1.57 |
| BMI, $kg/m^2$ | 1.10 | 0.09 | 0.98 | 1.24 |
| WHR | 0.00 | **0.04** | 0.00 | 0.57 |
| TC, mg/dl | 1.00 | 0.89 | 0.98 | 1.02 |
| TG, mg/dl | 2.89 | **0.03** | 1.11 | 7.55 |
| FAI | 1.04 | 0.43 | 0.94 | 1.16 |

### 4.11 Independent Association of IR with Relevant Covariates among PCOS Subjects

On multiple regression analysis in the PCOS group, IR was found to be significantly associated only with age (p=0.001) on adjustment of the effects of BMI, WHR, 2hBG, TG and FAI (Fig. 4.2).

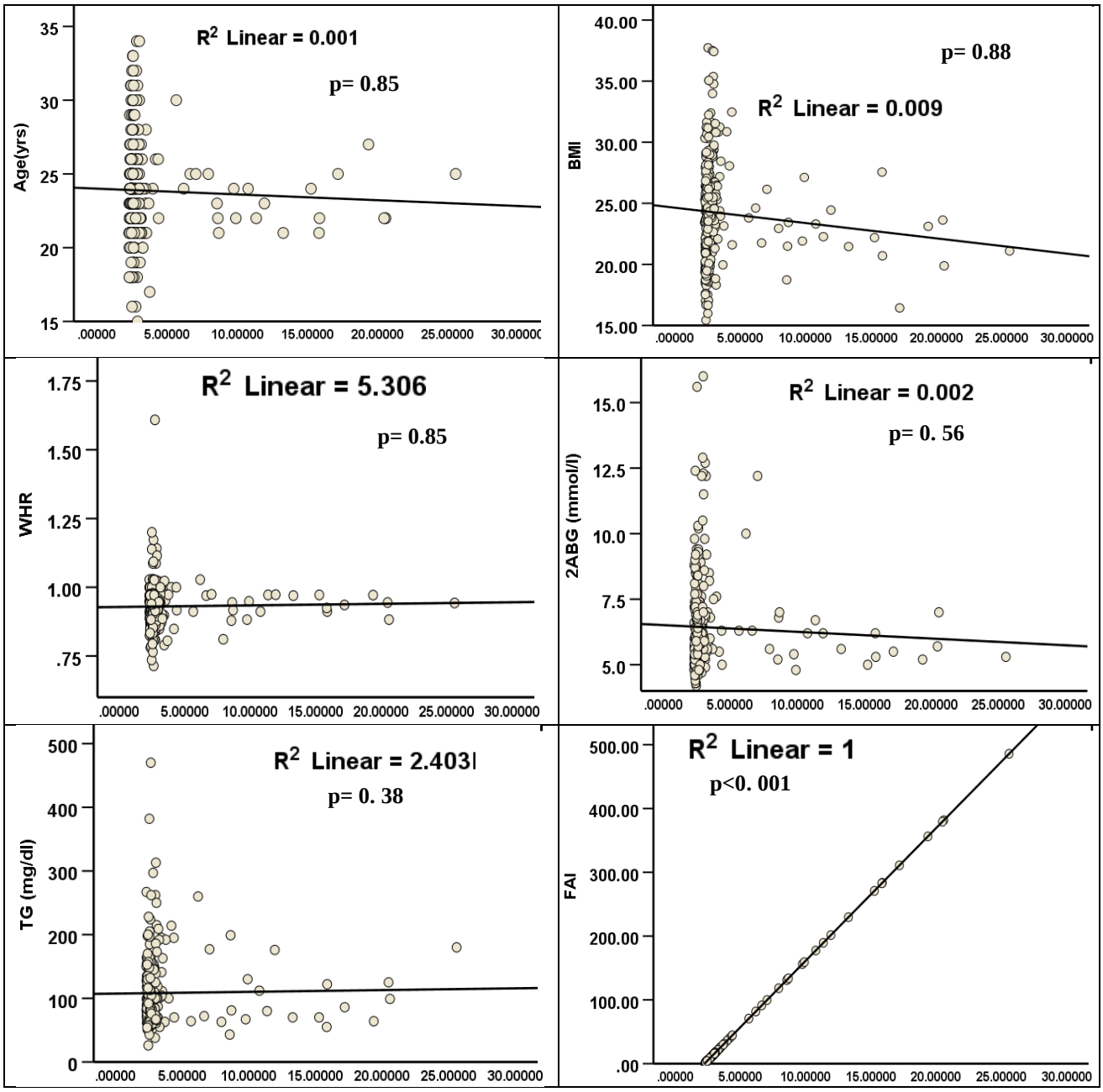


**Fig. 4.2: Multiple Linear regression analysis in the PCOS group with IR as dependent Variables and Other Relevant Covariates as Independent Variables**

### 4.12 Independent Association of IR with Relevant Covariates among Non-PCOS Subjects

On multiple regression analysis in the Non-PCOS group, IR was found to be significantly associated with BMI (p=0.001) and TG (p=0.009) on adjustment of the effects of age, WHR, TC and FAI (Fig. 4.3).

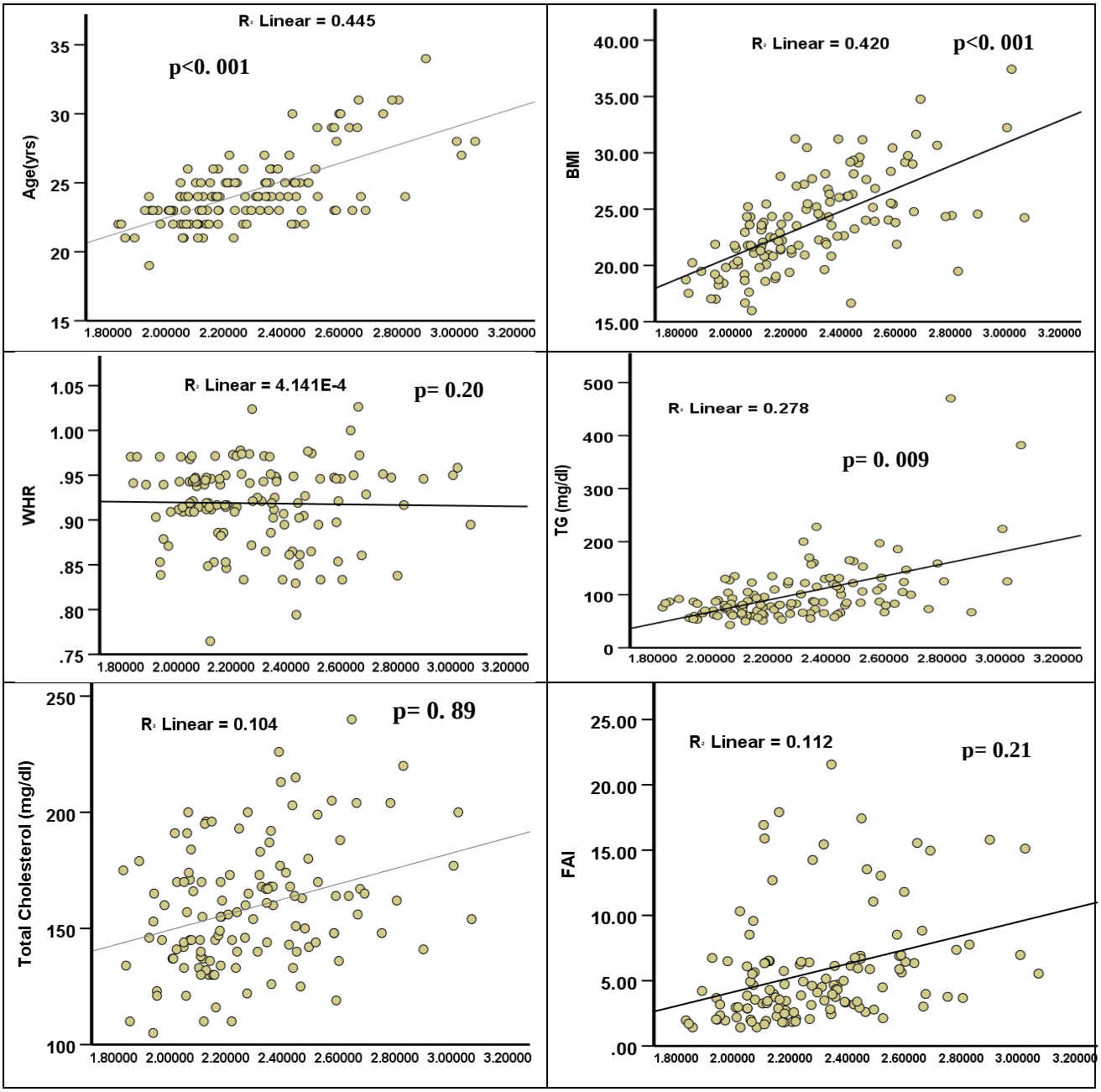


**Fig. 4.3: Multiple Linear Regression Analysis in the Non-PCOS Group with IR as Dependent Variables and Other Relevant Covariates as Independent Variables**

## 4.13 Polymorphism in INSR Gene

### *4.13.1 PCR Product for the Detection of INSR Gene Polymorphism*

Using specific primers, a segment of the INSR gene was amplified as shown in Fig. 4.5. The length of the PCR product was 688bp. The amplified PCR products were evaluated by 2% agarose gel electrophoresis, and the optimized size of the PCR product was ascertained by comparing it with a 100bp DNA ladder.

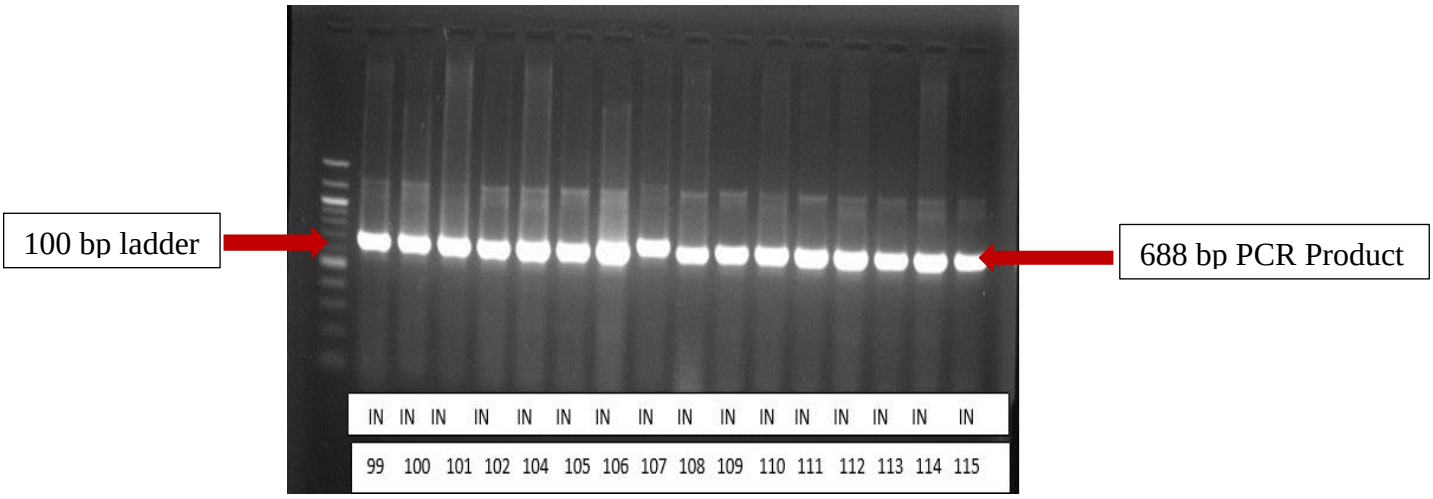


**Fig. 4.4: Representative PCR Products of INSR Gene in 2% Agarose Gel**

### *4.13.2 Detection of His1085C/T Polymorphism (rs1799817) of INSR Gene*

To determine the His1085C/T Polymorphism, the PCR products were sequenced by the automated Sanger sequencing machine by chain termination method. After aligning the fasta files, the single nucleotide polymorphism was found at 526 positions.

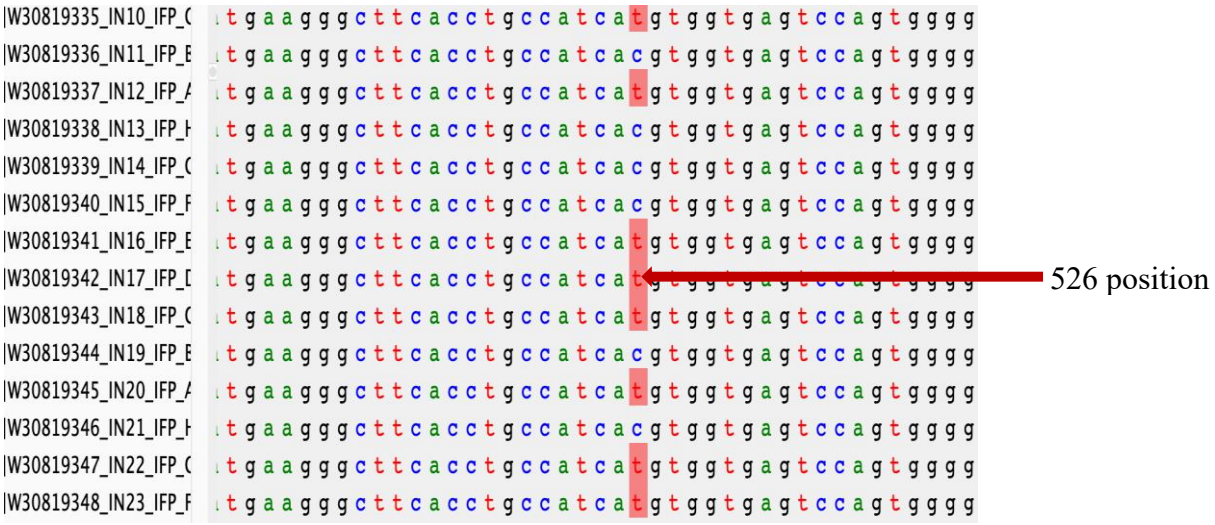


**Fig. 4.5: Aligned Sequence data of His1085C/T Polymorphism of INSR Gene**

### *4.13.3 Frequency of His1085C/T alleles in the INSR Gene among Non-PCOS and PCOS subjects*

The proportions of C and T alleles were 56% and 42% among Non-PCOS and 69% and 41% among PCOS subjects (Fig. 4.6). There were no significant differences in the distribution of alleles between the two groups.

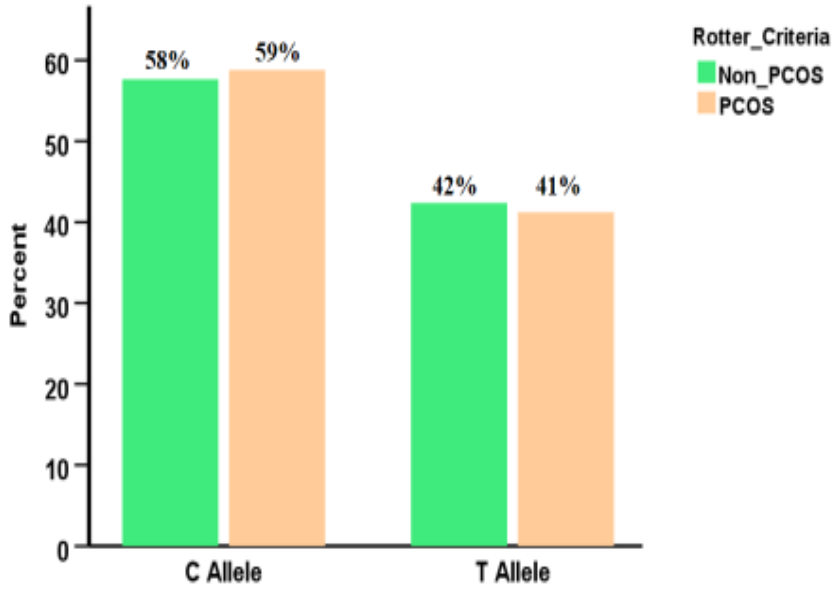


**Fig. 4.6: Allele Frequency of His1085C/T Polymorphism of INSR Gene among Non-PCOS and PCOS subjects**

### *4.13.4 Allele Frequency Distribution of His1085C/T Polymorphism and Phenotypic Features of PCOS*

The frequency distribution of the C and T alleles, in relation to the absence and presence of various phenotypic features of PCOS, were analysed in the two study groups (Table 4.10). No significant difference in allele distribution was found for any phenotypic feature either in the Non-PCOS or PCOS groups.

**Table 4.10: Allele Frequency Distribution of His1085C/T Polymorphism of INSR Gene in the Absence and Presence of Different Diagnostic Phenotypic Features of PCOS**

| Phenotypic Characteristics | Non-PCOS (n =118) | | | PCOS (n =148) | | |
|---|---|---|---|---|---|---|
| | T Allele n (%) | OR (CI) | p-value | T Allele n (%) | OR (CI) | p-value |
| Anovulation Absent | 49 (44) | 0.26 (0.02-2.27) | 0.24 | 9 (50) | 0.67 (0.25-1.79) | 0.45 |
| Anovulation Present | 1 (18) | | | 52 (40) | | |
| POM Absent | 49 (42) | 1.37 (0.08-22.40) | 1.00 | 9 (38) | 1.20 (0.49-2.96) | 0.82 |
| POM Present | 1 (50) | | | 52 (42) | | |
| Hyperandrogenism Absent | 40 (43) | 0.88 (0.36-2.17) | 0.82 | 23 (45) | 0.78 (0.39-1.56) | 0.59 |
| Hyperandrogenism Present | 10 (40) | | | 38 (39) | | |
| Hirsutism Absent | 41 (40) | 1.91 (0.66-5.54) | 0.28 | 19 (41) | 0.99 (0.49-2.02) | 1.00 |
| Hirsutism Present | 9 (56) | | | 42 (41) | | |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test. p-value<0.05 considered significant.*

### *4.13.5 Allele Frequency Distribution of His1085C/T and IR*

The frequency distribution of the C and T alleles, in relation to the absence and presence of IR, were analysed in the two study groups (Table 4.11). No significant difference in allele distribution was found for IR either in the Non-PCOS or PCOS groups.

**Table 4.11: Allele Frequencies of His1085C/T Polymorphism of INSR Gene in the Absence and Presence of IR in the Study Groups**

| Insulin Resistance | Non-PCOS Group (n=118) | | | PCOS Group (n=148) | | |
|---|---|---|---|---|---|---|
| | T Allele n (%) | OR (CI) | p-value | T Allele n (%) | OR (CI) | p-value |
| **Absent** | 37 (74%) | 0.84 (0.37-1.91) | 0.84 | 34 (56%) | 0.59 (0.30-1.14) | 0.13 |
| **Present** | 13 (26%) | | | 27 (44%) | | |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test. p-value<0.05 considered significant.*

### *4.13.6 Independent Association of Anovulation with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects on Adjustment of the Effect of Relevant Covariates*

The independent association of anovulation with His1085C/T polymorphism of the INSR gene was explored in the PCOS group by using binary logistic regression analysis considering anovulation as the dependent variable, His1085C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.12). No significant association of the polymorphism with anovulation was found. However, WHR showed a significant (p=0.02) association with anovulation in this model when the effects of age, BMT, TC, TG, and allele variations were adjusted.

**Table 4.12: Independent Association of Anovulation with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| **Age** | 0.88 | 0.135 | 0.74 | 1.04 |
| **BMI** | 1.03 | 0.70 | 0.89 | 1.18 |
| **WHR** | 7.5 | **0.02** | 3.08 | 2.1 |
| **TC** | 0.99 | 0.66 | 0.97 | 1.02 |
| **TG** | 1.01 | 0.06 | 0.99 | 1.03 |
| **His1085C/T** | 0.67 | 0.48 | 0.22 | 2.02 |

### *4.13.7 Independent Association of Anovulation with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects on Adjustment of the Effect of Relevant Covariates*

The independent association of anovulation with His1085C/T polymorphism of the INSR gene was explored in the Non-PCOS group by using binary logistic regression analysis considering anovulation as the dependent variable, His1085C/T alleles as the primary input variable and also other relevant covariates as independent variables (Table 4.13). No significant association of the polymorphism with anovulation was found.

**Table 4.13: Independent Association of Anovulation with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 1.05 | 0.75 | 0.78 | 1.41 |
| BMI | 1.20 | 0.81 | 0.97 | 1.50 |
| WHR | 0.01 | 0.66 | 0.00 | 6.10 |
| TC | 0.98 | 0.33 | 0.94 | 1.02 |
| TG | 0.99 | 0.92 | 0.98 | 1.02 |
| His1085C/T | 0.35 | 0.36 | 0.37 | 3.31 |

### *4.13.8 Independent Association of PCOM with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects on Adjustment of the Effect of Relevant Covariates*

The independent association of PCOM with His1085C/T polymorphism of the INSR gene was explored in the PCOS group by using binary logistic regression analysis considering PCOM as the dependent variable, His1085C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.14). No significant association of the polymorphism with PCOM was found.

**Table 4.14: Independent Association of PCOM with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.96 | 0.56 | 0.85 | 1.09 |
| BMI | 0.97 | 0.57 | 0.87 | 1.08 |
| WHR | 1.52 | 0.88 | 0.008 | 3.5 |
| TC | 0.99 | 0.51 | 0.98 | 1.01 |
| TG | 0.99 | 0.31 | 0.99 | 1.00 |
| His1085C/T | 1.29 | 0.59 | 0.51 | 3.24 |

### *4.13.9 Independent Association of PCOM with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects on Adjustment of the Effect of Relevant Covariates*

The independent association of PCOM with His1085C/T polymorphism of the INSR gene was explored in the Non-PCOS group by using binary logistic regression analysis considering PCOM as the dependent variable, His1085C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.15). No significant association of the polymorphism with PCOM was found.

**Table 4.15: Independent Association of PCOM with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 1.25 | 0.44 | 0.70 | 2.22 |
| BMI | 1.02 | 0.93 | 0.60 | 1.74 |
| WHR | 9.32 | 0.13 | 0.00 | 6.23 |
| TC | 0.99 | 0.86 | 0.91 | 1.08 |
| TG | 1.00 | 0.88 | 0.96 | 1.05 |
| His1085C/T | 1.52 | 0.82 | 0.03 | 62.5 |

### *4.13.10 Independent Association of Hirsutism with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of hirsutism with His1085C/T polymorphism of the INSR gene was explored in the PCOS group by using binary logistic regression analysis considering hirsutism as the dependent variable, His1085C/T alleles as the primary input variable and also other relevant covariates as independent variables (Table 4.16). No significant association of the polymorphism with hirsutism was found.

**Table 4.16: Independent Association of Hirsutism with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.96 | 0.43 | 0.86 | 1.06 |
| BMI | 1.00 | 0.93 | 0.92 | 1.10 |
| WHR | 0.16 | 0.33 | 0.00 | 6.60 |
| TC | 0.99 | 0.53 | 0.98 | 1.01 |
| TG | 1.002 | 0.66 | 0.99 | 1.01 |
| His1085C/T | 1.02 | 0.95 | 0.49 | 2.09 |

### *4.13.11 Independent Association of Hirsutism with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of hirsutism with His1085C/T polymorphism of the INSR gene was explored in the Non-PCOS group by using binary logistic regression analysis considering hirsutism as the dependent variable, His1085C/T alleles as the primary input variable and also other relevant covariates as independent variables (Table 4.17). No significant association of the polymorphism with hirsutism was found.

**Table 4.17: Independent Association of Hirsutism with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95%Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.88 | 0.31 | 0.68 | 1.13 |
| BMI | 1.12 | **0.09** | 0.98 | 1.29 |
| WHR | 0.16 | 0.78 | 0.00 | 1.90 |
| TC | 0.99 | 0.49 | 0.97 | 1.01 |
| TG | 1.00 | 0.86 | 0.99 | 1.01 |
| His1085C/T | 2.18 | 0.89 | 0.70 | 6.76 |

### *4.13.12 Independent Association of IR with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of IR with His1085C/T polymorphism of the INSR gene was explored in the PCOS group by using multiple linear regression analysis considering IR as the dependent variable, His1085C/T alleles as the primary input variable, andother relevant covariates as independent variables (Table 4.18). No significant association of the polymorphism with IR was found.

**Table 4.18: Independent Association of IR with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects**

| Variables | Unstandardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 10.7 | 0.68 | - 0.19 | 0.30 |
| BMI | 0.05 | 0.11 | - 0.40 | 0.04 |
| WHR | - 0.18 | 0.95 | - 9.64 | 9.02 |
| TC | - 0.30 | 0.33 | - 0.047 | 0.16 |
| TG | - 0.01 | 0.82 | -1.45 | 1.83 |
| His1085C/T | 0.19 | 0.22 | - 2.81 | 0.68 |

### *4.13.13 Independent Association of IR with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of IR with His1085C/T polymorphism of the INSR gene was explored in the Non-PCOS group by using multiple linear regression analysis considering IR as the dependent variable, His1085C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.19). No significant association of the polymorphism with IR was found.

**Table 4.19: Independent Association of IR with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects**

| Variables | Unstandardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.62 | **<0.001** | - 1.05 | 2.38 |
| BMI | 0.37 | **<0.001** | 0.03 | 0.09 |
| WHR | 1.05 | 0.22 | 0.01 | 0.05 |
| TC | 0.00 | 0.81 | - 2.77 | 0.65 |
| TG | 0.16 | **0.01** | - 0.003 | 0.003 |
| His1085C/T | 0.08 | 0.28 | 0.03 | 2.59 |

### *4.13.14 Independent Association of FAI with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of FAI with His1085C/T polymorphism of the INSR gene was explored in the PCOS group by using multiple linear regression analysis considering FAI as the dependent variable, His1085C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.20). No significant association of the polymorphism with FAI was found.

**Table 4.20: Independent Association of FAI with His1085C/T Polymorphism of the INSR Gene among PCOS Subjects**

| Variables | Unstandardized coefficients | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.714 | 0.72 | - 3.29 | 4.72 |
| BMI | -3.84 | 0.03 | - 7.37 | 0.31 |
| WHR | -19.76 | 0.79 | - 9.03 | 9.50 |
| TC | - 0.49 | 0.05 | -1.01 | 0.01 |
| TG | 13.39 | 0.31 | - 12.81 | 39.59 |
| His1085C/T | - 10.98 | 0.44 | - 38.93 | 16.97 |

### *4.13.15 Independent Association of FAI with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of FAI with His1085C/T polymorphism of the INSR gene was explored in the Non-PCOS group by using multiple linear regression analysis considering FAI as the dependent variable, His1085C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.21). No significant association of the polymorphism with FAI was found.

**Table 4.21: Independent Association of FAI with His1085C/T Polymorphism of the INSR Gene among Non-PCOS Subjects**

| Variables | Unstandardized coefficients | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.19 | 0.18 | - 0.09 | 0.49 |
| BMI | 0.21 | 0.03 | 0.18 | 0.39 |
| WHR | - 1.91 | 0.82 | - 18.65 | 14.83 |
| TC | 0.01 | 0.49 | - 0.20 | 0.41 |
| TG | - 0.36 | 0.56 | - 1.59 | 0.87 |
| His1085C/T | - 0.08 | 0.91 | - 1.63 | 1.46 |

### *4.14.1 PCR Product for Detection of PPARγ Gene Polymorphism*

A segment of the INSR gene was amplified using specific primers, as shown in Fig. 4.6. The length of the PCR product was 662 bp. The amplified PCR products were evaluated by 2% agarose gel electrophoresis, and the optimized size of the PCR product was ascertained by comparing it with a 100bp DNA ladder.

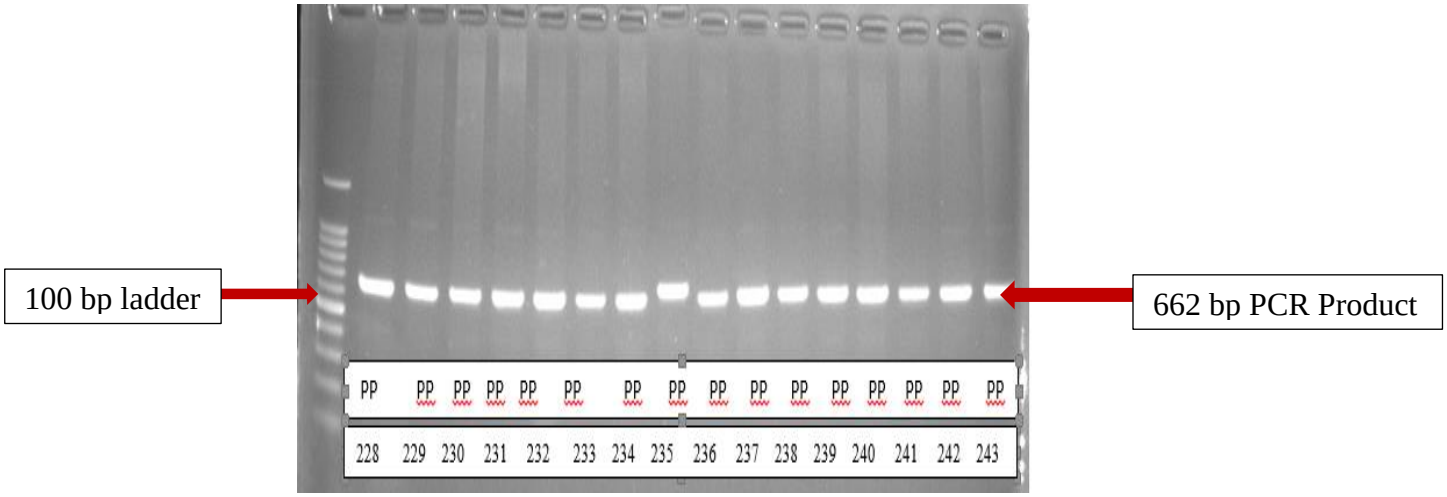


**Fig 4.6: Representative PCR Products of INSR Gene in 2% Agarose Gel**

### *4.14.2 Detection of His 447C/T Polymorphism (rs3856806) of PPARγ Gene*

To determine the His1085C/T Polymorphism, the PCR products were sequenced by the automated Sanger sequencing machine by chain termination method. After aligning the fasta files, the single nucleotide polymorphism was found at 548 position.

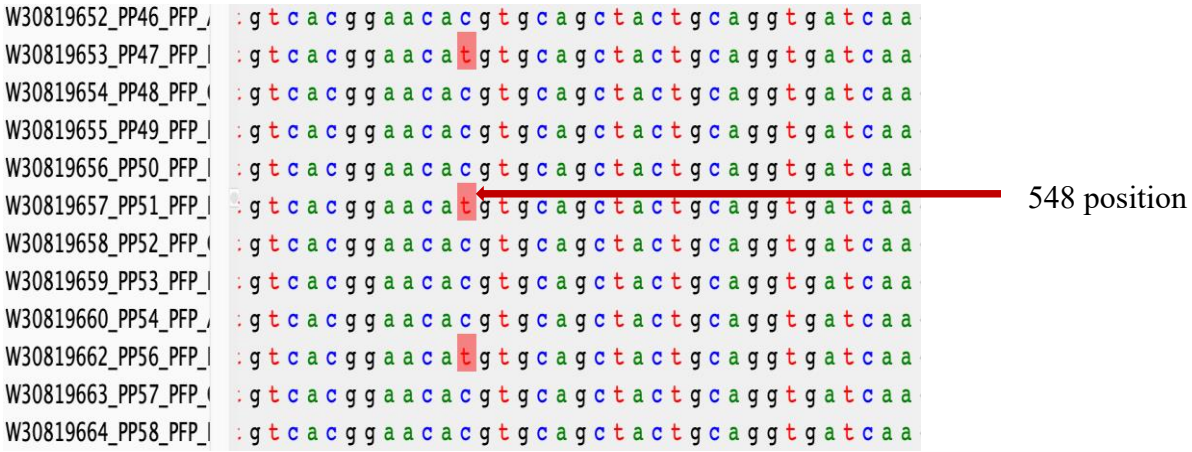


**Fig 4.7: Aligned Sequence Data of His 447C/T Polymorphism of PPARγ Gene**

### *4.14.3 Proportion of His 447C/T Polymorphism of the PPARγ Gene among Non-PCOS and PCOS Subjects*

The proportions of C and T alleles were 83% and 17% among Non-PCOS and 82% and 18% among PCOS subjects (Fig. 4.8). There were no significant differencesin the distribution of alleles between the two groups.

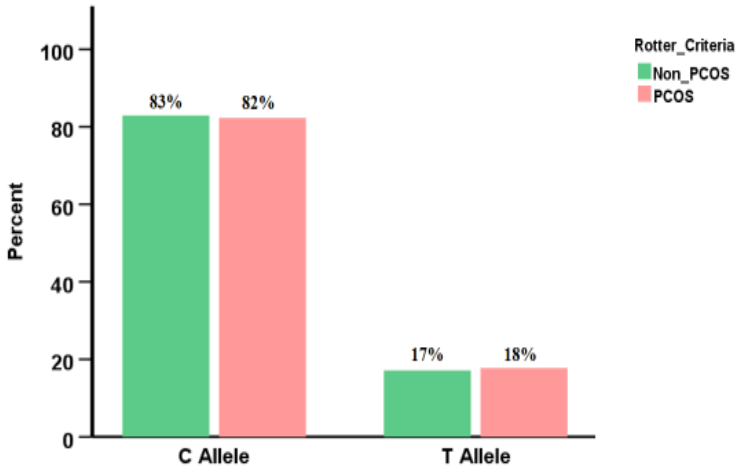


**Fig 4.8: Proportion of His 447C/T Polymorphism of the PPARγ Gene among Non-PCOS and PCOS Subjects**

### *4.14.4 Allele Frequency Distribution of His 447C/T Polymorphism of the PPARγ Gene and Phenotypic Features of PCOS*

The frequency distribution of the C and T alleles, in relation to the absence and presence of various phenotypic features of PCOS, were analyzed in the two study groups (Table 4.22). Hirsutism showed a significant positive association (p=0.05) with the polymorphism only in the PCOS group. No other significant difference in allele distribution was found for any phenotypic feature either in the Non-PCOS or PCOS groups.

**Table 4.22: Allele Frequency Distribution of His 447C/T Polymorphism of the PPARγ Gene in the Absence and Presence of Different Diagnostic Phenotypic Features of PCOS**

| Phenotypic Characteristics | Non-PCOS (n =111) | | | PCOS (n =141) | | |
|---|---|---|---|---|---|---|
| | T Allele n (%) | OR (CI) | p-value | T Allele n (%) | OR (CI) | p-value |
| Anovulation Absent | 18 (94) | 1.2 (0.13-11.5) | 1.00 | 3 (12) | 1.01 (0.26-3.80) | 1.00 |
| Anovulation Present | 1 (6) | | | 22 (88) | | |
| POM Absent | 19 (100) | 0 | - | 4 (16) | 1.09 (0.34-3.53) | 1.00 |
| POM Present | 0 (0) | | | 21 (84) | | |
| Hyperandrogenism Absent | 16 (84) | 0.56 (0.15-2.10) | 0.55 | 9 (36) | 1.05 (0.43-2.57) | 1.00 |
| Hyperandrogenism Present | 3 (16) | | | 16 (64) | | |
| Hirsutism Absent | 17 (90) | 0.65 (0.13-3.15) | 0.73 | 12 (48) | 0.39 (0.16-0.95) | **0.05** |
| Hirsutism Present | 2 (10) | | | 13 (52) | | |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test. p-value<0.05 considered significant.*

### *4.14.5 Allele Frequency Distribution of His 447C/T Polymorphism of PPARγ Gene and IR*

The frequency distribution of the C and T alleles, in relation to the absence and presence of IR, were analyzed in the two study groups (Table 4.23). No significant difference in allele distribution was found for IR either in the Non-PCOS or PCOS groups.

**Table 4.23: Allele Frequencies of His 447C/T Polymorphism of PPARγ Gene in the Absence and Presence of IR in the Study Groups**

| Insulin Resistance | Non-PCOS Group (n=111) | | | PCOS Group (n=141) | | |
|---|---|---|---|---|---|---|
| | T Allele n (%) | OR (CI) | p-value | T Allele n (%) | OR (CI) | p-value |
| Absent | 28 (87) | 0.61 (0.19-2.00) | 0.58 | 11 (44) | 1.23 (0.51-2.93) | 0.66 |
| Present | 4 (13) | | | 14 (56) | | |

*Results were presented as numbers (percentage); Data were compared using the Chi-squared Test. p-value<0.05 considered significant.*

### *4.14.6 Independent Association of Anovulation with His 447C/T Polymorphism of PPARγ Gene, among PCOS Subjects, on Adjustment of the Effect of Relevant Covariates*

The independent association of anovulation with His 447C/T Polymorphism of PPARγ Gene was explored in the PCOS group by using binary logistic regression analysis considering anovulation as the dependent variable, His 447C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.24). No significant association of the polymorphism with anovulation was found.

**Table 4.24: Independent Association of Anovulation with His 447C/T Polymorphism of PPARγ Gene among PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.86 | 0.10 | 0.71 | 1.03 |
| BMI | 1.03 | 0.68 | 0.89 | 1.18 |
| WHR | 35.3 | 0.26 | 2.97 | 8.1 |
| TC | 0.99 | 0.43 | 0.97 | 1.01 |
| TG | 1.01 | 0.14 | 0.99 | 1.03 |
| His 447C/T | 0.76 | 0.71 | 0.18 | 3.17 |

### *4.14.7 Independent Association of Anovulation with His 447C/T Polymorphism of PPARγ Gene, among Non-PCOS Subjects, on Adjustment of the Effect of Relevant Covariates*

The independent association of anovulation with His 447C/T Polymorphism of PPARγ Gene was explored in the Non-PCOS group by using binary logistic regression analysis considering anovulation as the dependent variable, His 447C/T alleles as the primary input variable and also other relevant covariates as independent variables (Table 4.25). No significant association of the polymorphism with anovulation was found.

**Table 4.25: Independent Association of Anovulation with His 447C/T Polymorphism of PPARγ Gene among Non-PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.96 | 0.86 | 0.63 | 1.5 |
| BMI | 1.24 | **0.09** | 0.96 | 1.6 |
| WHR | 0.00 | 0.21 | 0.0 | 3.9 |
| TC | 0.97 | 0.25 | 0.93 | 1.02 |
| TG | 1.00 | 0.63 | 0.98 | 1.02 |
| His 447C/T | 1.44 | 0.77 | 0.13 | 16.2 |

### *4.14.8 Independent Association of PCOM with His 447C/T Polymorphism of PPARγ Gene, among PCOS Subjects, on Adjustment of the Effect of Relevant Covariates*

The independent association of anovulation with His 447C/T Polymorphism of PPARγ Gene was explored in the PCOS group by using binary logistic regression analysis considering PCOM as the dependent variable, His 447C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.26). No significant association of the polymorphism with PCOM was found.

**Table 4.26: Independent Association of PCOM with His 447C/T Polymorphism of PPARγ Gene among PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.97 | 0.63 | 0.84 | 1.11 |
| BMI | 0.98 | 0.74 | 0.87 | 1.10 |
| WHR | 2.07 | 0.79 | 0.008 | 63.9 |
| TC | 0.99 | 0.76 | 0.98 | 1.01 |
| TG | 0.99 | 0.47 | 0.98 | 1.01 |
| His447C/T | 0.94 | 0.93 | 0.27 | 3.26 |

### *4.14.9 Independent Association of PCOM with His 447C/T Polymorphism of PPARγ Gene, among Non-PCOS Subjects, on Adjustment of the Effect of Relevant Covariates*

The independent association of PCOM with His 447C/T Polymorphism of PPARγ Gene was explored in the Non-PCOS group by using binary logistic regression analysis considering PCOM as the dependent variable, His 447C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.27). No significant association of the polymorphism with PCOM was found.

**Table 4.27: Independent Association of PCOM with His 447C/T Polymorphism of PPARγ Gene among Non-PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0 | NS | 0 | 0 |
| BMI | 0 | NS | 0 | 0 |
| WHR | 0 | NS | 0 | 0 |
| TC | 0 | NS | 0 | 0 |
| TG | 0 | NS | 0 | 0 |
| His447C/T | 0 | NS | 0 | 0 |

### *4.14.10 Independent Association of hirsutism with His 447C/T Polymorphism of PPARγ Gene, among PCOS Subjects, on Adjustment of the Effect of Relevant Covariates*

The independent association of hirsutism with His 447C/T Polymorphism of PPARγ Gene was explored in the PCOS group by using binary logistic regression analysis considering hirsutismas the dependent variable, His 447C/T alleles as the primary input variable and also other relevant covariates as independent variables (Table 4.28). A significant (p=0.04) association of the polymorphism with hirsutism was found in adjusting the effects of age, BMI, WHR, TC, and TG.

**Table 4.28: Independent Association of Hirsutism with His 447C/T Polymorphism of PPARγ Gene among PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 1.04 | 0.47 | 0.93 | 1.17 |
| BMI | 1.04 | 0.47 | 0.94 | 1.15 |
| WHR | 0.12 | 0.33 | 0.002 | 8.11 |
| TC | 0.99 | 0.49 | 0.98 | 1.01 |
| TG | 1.00 | 0.12 | 0.99 | 1.02 |
| His447C/T | 0.38 | **0.04** | 0.15 | 0.97 |

### *4.14.11 Independent Association of Hirsutism with His 447C/T Polymorphism of PPARγ Gene among Non-PCOS Subjects on Adjustment of the Effect of Relevant Covariates*

The independent association of hirsutism with His 447C/T Polymorphism of PPARγ Gene was explored in the Non-PCOS group by usinginary logistic regression analysis considering hirsutism as the dependent variable, His 447C/T alleles as the primary input variable and alsoother relevant covariates as independent variables (Table 4.29). No significant association of the polymorphism with PCOM was found. BMI, however, showed a significant association with hirsutism (p=0.03).

**Table 4.29: Independent Association of Hirsutism with His 447C/T Polymorphism of PPARγ Gene among Non-PCOS Subjects**

| Variables | Standardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.96 | 0.74 | 0.75 | 1.23 |
| BMI | 1.17 | **0.03** | 1.00 | 1.36 |
| WHR | 0.31 | 0.86 | 0.00 | 9.60 |
| TC | 0.99 | 0.78 | 0.97 | 1.02 |
| TG | 1.00 | 0.64 | 0.98 | 1.02 |
| His447C/T | 0.63 | 0.58 | 0.12 | 3.27 |

### *4.14.12 Independent Association of FAI with His 447C/T Polymorphism of PPARγ Gene, among PCOS Subjects, on Adjustment of the Effects of Relevant Covariates*

The independent association of FAI with His 447C/T Polymorphism of PPARγ Gene was explored in the PCOS group by usingmultiple linear regression analysis considering IR as the dependent variable, His 447C/T alleles as the primary input variable and also other relevant covariates as independent variables (Table 4.30). No significant association of the polymorphism with IR was found.

**Table 4.30: Independent Association of FAI with His 447C/T Polymorphism of PPARγ Gene among PCOS Subjects**

| Variables | Unstandardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 1.09 | 0.62 | - 3.29 | 5.47 |
| BMI | - 3.74 | **0.04** | - 7.41 | - 0.06 |
| WHR | - 13.51 | 0.87 | -172.9 | 5.9 |
| TC | - 0.46 | **0.09** | -1.00 | 0.08 |
| TG | 12.90 | 0.36 | - 15.2 | 41.02 |
| His447C/T | 7.04 | 0.72 | - 31.76 | 45.84 |

### *4.14.13 Independent Association of FAI with His 447C/T Polymorphism of PPARγ Gene, among Non-PCOS Subjects, on Adjustment of the Effects of Relevant Covariates*

The independent association of FAI with His 447C/T Polymorphism of PPARγ Gene was explored in the PCOS group by using multiple linear regression analysis considering IR as the dependent variable, His 447C/Talleles as the primary input variable, and other relevant covariates as independent variables (Table 4.31). No significant association of the polymorphism with IR was found.

**Table 4.31: Independent Association of FAI with His 447C/T Polymorphism of PPARγ Gene among Non-PCOS Subjects**

| Variables | Unstandardized coefficients (β) | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.16 | 0.32 | - 0.16 | 0.49 |
| BMI | 0.22 | **0.03** | 0.02 | 0.43 |
| WHR | 1.56 | 0.87 | - 16.81 | 19.95 |
| TC | 0.10 | 0.58 | -.0.02 | 0.04 |
| TG | - 0.47 | 0.49 | - 1.83 | 0.89 |
| His447C/T | 0.03 | 0.97 | - 2.16 | 2.24 |

### *4.14.14 Independent Association of IR with His 447C/T Polymorphism of PPARγ Gene among PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of IR with His 447C/T Polymorphism of PPARγ Gene was explored in the PCOS group by using multiple linear regression analysis considering IR as the dependent variable, His 447C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.32). A significant (p=0.02) association of the polymorphism with IR was found in adjusting the effects of age, BMI, WHR, TC, TG, and total testosterone.

**Table 4.32: Independent Association of IR with His 447C/T Polymorphism of PPARγ Gene among PCOS Subjects**

| Variables | Unstandardized coefficients | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.05 | 0.64 | - 0.17 | 0.27 |
| BMI | - 0.13 | 0.15 | - 0.32 | 0.05 |
| WHR | 1.74 | 0.67 | - 6.41 | 9.89 |
| TC | - 0.005 | 0.69 | - 0.03 | 0.02 |
| TG | - 0.005 | 0.53 | - 0.02 | 0.01 |
| t Testosterone | 2.90 | **<0.001** | 2.15 | 3.64 |
| His447C/T | 2.35 | **0.02** | 0.38 | 4.33 |

### *4.14.15 Independent Association of IR with His 447C/T Polymorphism of PPARγ Gene among Non-PCOS Subjects on Adjustment of the Effects of Relevant Covariates*

The independent association of IR with His 447C/T Polymorphism of PPARγ Gene was explored in the PCOS group by using multiple linear regression analysis considering IR as the dependent variable, His 447C/T alleles as the primary input variable, and other relevant covariates as independent variables (Table 4.33). No significant association of the polymorphism with IR was found.

**Table 4.33: Independent Association of HOMA-IR with His 447C/T Polymorphism of PPARγ Gene among Non-PCOS Subjects**

| Variables | Unstandardized coefficients | p-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower bound | Upper bound |
| Age | 0.58 | **<0.001** | 0.03 | 0.09 |
| BMI | 0.04 | **<0.001** | 0.02 | 0.06 |
| WHR | -1.29 | 0.15 | - 3.08 | 0.50 |
| TC | - 0.001 | 0.52 | - 0.004 | 0.002 |
| TG | 0.002 | **0.01** | 0.00 | 0.003 |
| t Testosterone | 0.01 | **0.07** | -0.016 | 0.32 |
| His447C/T | - 0.005 | 0.96 | -0.22 | 0.21 |

# Chapter 5: DISCUSSION

## 5.1 Insulin Resistance and its Covariates among Bangalee Non-PCOS and PCOS Women

### *5.1.1 Burden of Insulin Resistance among PCOS Subjects*

In the present Case-Control study evidence was generated on the burden of insulin resistance among Bangalee PCOS women, and exploration was also done on its covariates, especially polymorphism in two selected genetic locations (namely rs1799817 of INSR gene and rs3856806 of PPARγ gene). Single nucleotide polymorphism (SNP) in both of these locations have been reported to be associated with IR in PCOS populations [38 &252] including the Indian ones. Insulin resistance has also been reported to be more common among people of Indian subcontinent origin [290-291]. Data in the present study are generally in line with these previous findings since the proportion of insulin-resistant subjects and median value of HOMA-IR in the PCOS group are significantly higher as compared to those in the Non-PCOS group. Compared to the median value of 2.5% HOMA-IR among the PCOS subjects, the corresponding value of HOMA-IR was 2.2% among the non-PCOS counterparts ($p<0.001$, Table 4.3). These values, in controls as well as in patients, are nearly parallel to those reported for the South Indian population [13]. In Bangladesh, the values of HOMA-IR have been variably reported, and the assessment techniques used have not always been mentioned [15]. Shah et al. [293] reported a mean HOMA-IR of 1.40% among control and 4.44% among PCOS subjects with a similar age range. The WHR of their subjects was lower than the present one (mean values of 0.83 among PCOS and 0.80 among controlsin contrast to median values of 0.94 and 0.92 among their counterparts in the present study). The median values of 3.98 and 3.34 have been reported by Hurjahan-Banu et al [47] among hyperandrogenemic and normoandrogenemic subjects, respectively. Zamila et al [48] reported the median values of HOMA-IR% as 4.38, 3.70, and 3.05 among amenorrheic, oligomenorheic, and eumenorrheic subjects, respectively; in the control group, they found a median value of 1.64. Differences in age, BMI, WHR and variation in laboratory techniques seem to explain these HOMA-IR values among various studies.

The proportion of insulin-resistant subjects among the PCOS population has been shown to vary from 44 to 70% [9-10]. In Indian subjects, the proportion has been claimed to be higher, [24]. The cut-off value of HOMA-IR% for IR positivity (to define an insulin-resistant subject)

in this study (2.4%) was derived from the 75$^{th}$ percentile value of the parameter among the control (Non-PCOS group). The value is very close to the cut-off value used for the Indian population [13], which was derived through a different technique. Using this criterion, a 52% value for IR positivity among PCOS subjects was obtained in the present study (Fig 4.1), which was somewhat lower than that reported by other Bangladeshi Authors [15].

In Bangladesh, from facility-based data, the presence of IR has been reported to vary widely from 16% to 77% among PCOS subjects [15]; however, the methodology and cut-off values of the IR assessment were not detailed in most of the studies [15]. Methods, as well as HOMA%IR cut-off value similar to the methods of the present study, were followed in three studies. In the study of Shah et al [45], 65% of the PCOS subjects were insulin resistant using a cut-off value of HOMA-IR as 2.6%. Using the same cut-off value of HOMA%IR, Hurjahan-Banu et al [47] reported a 70% prevalence of IR among Bangladeshi women. Zamila et al [48] have reported that IR is present among 78% eumenorrheic, 72% oligomenorrheic, and 63% amenorrheic subjects. In all these studies, the PCOS subjects had higher degrees of overweight/obesity than their counterparts in the present study. This, along with differences in subject characteristics and laboratory techniques, may partly explain the lower proportion (52%) of insulin resistance among the present group of PCOS women. Since the cut-off value of HOMA%IR in the present study was 2.4, this value, by itself, cannot explain the variations; instead, it can be postulated that the present proportion would be a little lower if the cut-off value of 2.6 was used. Despite these differences, it is apparent that insulin resistance is present among more than fifty percent of young PCOS women of Bangalee ethnicity. Thus, in general, the current data demonstrate the existence of a high burden of IR among Bangalee PCOS women.

### *5.1.2 Burden of Insulin Resistance among Non-PCOS Subjects*

The existence of insulin resistance among more than a quarter (28%, Fig 4.1) needs to be specially noted as the finding has significant public health importance. Only one study [45] from Bangladesh has reported the proportion of HOMA%IR among Non-PCOS control subjects, and it is substantially lower [only 5%] in comparison to that in the present one (28%). As mentioned previously, the cut-off value of HOMA%IR in that study was closely similar to that of the present one. The reasons for the difference in proportions of insulin-resistant Non-PCOS subjects in the two studies are unclear. However, the sample size in the earlier study

was very small (only 40) in contrast to the present study, where data from 126 subjects have been analyzed. This, and variations in patient characteristics and laboratory techniques, may partly explain the difference in proportions.

The finding of IR among more than one-fourth of the Non-PCOS women raises a public health concern as the condition is known to create substantial risk for PCOS itself as well as a number of chronic cardiometabolic disorders and other NCDs like T2DM, hypertension, chronic liver diseases (CLDs) and chronic obstructive pulmonary diseases (COPD). It needs to be noted that the Non-PCOS subjects in the present study are relatively young (age range 19-34 years, 96% within 21-30 years, Table 4.1), and public health interventions targeted to dietary practices, physical exercise, and other lifestyle related issues can help them to avoid the potentially serious consequences of IR.

### *5.1.3 Presence of Individual Phenotypic Features of PCOS among the Study Subjects*

As implicit in the different guidelines for diagnosing and managing PCOS, not all phenotypic features of the disease are present in every subject. As per Rotterdam Modified Criteria 2003 [6-7] presence of the two of any three of the features (anovulation, PCOM, and HA) is diagnostic for the disease. Among the present PCOS subjects, anovulation is the commonest characteristic phenotypic feature (present in 88% of cases, Table 4.4), followed by PCOM (84%). In a recent review, Kamrul-Hassan et al [15] reported that menstrual irregularities are present among more than 80% of PCOS subjects in most of the studies conducted on the Bangladeshi population. It is noteworthy that clinical HA (hirsutism) and biochemical HA are evident in a much lower proportion of cases (69% and 65% cases). In the previously mentioned review [15], the proportion of hirsutism showed high variation (44% to 88.9%). The proportion of PCOM showed even greater variations (42% to 96%); use of different subject populations, diversity in imaging techniques by operators with varying levels of expertise, and use of nonuniform criteria seem to be the reason underlying this variation in the proportion of PCOM. The sample sizes were also relatively small. With an adequate number of subjects, standardized techniques, and well-trained experts, the proportion of PCOM among PCOS subjects is 84% in the present study.

It needs to be noted that the presence of anovulation and PCOM among Non-PCOS subjects are very low in proportion (5% and 1%, respectively, Table 4.4); however, a relatively higher proportion of them show biochemical (23%) and even clinical (hirsutism, 14%) HA. Since IR and HA create a vicious cycle [55], it may be postulated that HA may be associated with IR (as discussed in the previous section) among these subjects. Again, this should be considered as a public health issue. About one-fourth of Bangalee young and early middle-aged women seem vulnerable to HA and IR-related disorders, many of which are preventable by simple lifestyle and pharmacological intervention.

### *5.1.4 Anthropometric, Clinical and Biochemical Covariates of Insulin Resistance in Relation to Specific Phenotypic Features*

On chi-square analysis, IR was found to be strongly associated with biochemical hyperandrogenism among PCOS subjects ($p<0.001$, Table 4.5), and it also had some significance in the Non-PCOS group ($p=0.02$, Table 4.6). On the other hand, there was no significant association with anovulation, PCOM, and hirsutism in either of the subject groups. On correlation analysis, the association differed between the two study groups, with BMI, FAI, and 2hBG being the common covariates in both groups, age being significant only in the Non-PCOS group and TG being significant only in the PCOS group (Table 4.7). On binary logistic regression analysis, age and FAI showed significant ($p=0.001$) association with IR, and in the Non-PCOS group, a different set of variables, namely age ($p= 0.002$), WHR ($p=0.04$) and TG ($p=0.03$) showed significant association with IR. The pattern of association remained almost the same on multiple regression analysis, and 2hBG was found to be independently associated with IR ($p=0.001$). In this analysis, BMI and TG were found to be to be independently associated with IR. These findings indicate that age, obesity, and dyslipidemia are important covariates of insulin resistance among the young and early middle-aged Bangalee women with variable degrees of involvement among Non-PCOS and PCOS subjects. The importance of these factors in the development and severity of insulin-resistance is well known [2] and, again, these findings have significant clinical and public health importance as the conditions can be managed or even prevented through appropriate lifestyle and/or minimum public health interventions.

To our knowledge, no study has been conducted in Bangladesh with a specific target to explore the association of IR in PCOS with its probable covariates. Even then, from descriptive data in various studies, some preliminary ideas can be generated. Overweight and obesity are well-known covariates of IR and their combined presence has been reported with a high degree of variation (42%-74%) [15]. Again, a diverse subject population, unstandardized assessment techniques, and lack of adequate sample size are probably the causes of such variation. The variability is substantially less in the case of central obesity (77.3%-84.6%). Dyslipidemia was assessed in only 5 studies [15-20]; the disorder was found to be present among >90% of cases in 4 of the studies. These findings, in general, conform to the findings in the present study.

## 5.2 Polymorphism in the INSR Gene among PCOS and Non-PCOS Subjects

### *5.2.1 Proportion of C/T Alleles in the INSR Gene*

The His1085C/T polymorphism (ie, T allele) at the rs1799817 location of the INSR gene was found to be present among 42% of Non-PCOS and 41% of PCOS subjects. Thus, there was no significant difference in SNP between the two groups. A similar lack of association under case-control design has been reported in UK [52], Chinese [191], and Korean [180] studies. In contrast, association of the SNP with PCOS has been reported in an Indian [2] and Chinese populations [24]. The study in India [2] was conducted on a mixed Indian lean population (collected in a South Indian setting) with a relatively adequate number of subjects (180 cases, 120 controls). However, our findings (with an almost similar number of subjects), suggests that no association of PCOS with His1085C/T polymorphism (at rs1799817) of the INSR gene seems to exist in the Bangalee ethnic group.

### *5.2.2 Association of His1085C/T Polymorphism in the INSR Gene with Phenotypic Features of PCOS*

On extensive analysis using bivariate and multivariate (binary logistic as well as multiple linear regression analysis, as applicable, Tables 4.9 and 4.11-4.16), no significant association of the His1085C/T polymorphism (at rs1799817 location) of the INSR gene with any of the PCOS specific phenotypic features was found either in the Non-PCOS or in the PCOS group. The data suggest that this particular polymorphism is probably not associated with PCOS or any of the

characteristic phenotypic features of PCOS. Mukherjee et al. [53] have reported the association of this polymorphism with hyperandrogenemia in lean PCOS subjects. The present findings emphasize the heterogenic nature of PCOS.

### *5.2.3 Association of His1085C/T Polymorphism in the INSR Gene Polymorphism with IR*

Again, on extensive analysis using bivariate and multivariate (binary logistic as well as multiple linear regression analysis, as applicable, Tables 4.10, 4.17-4.18), no significant association of the His1085C/T polymorphism (at rs1799817 location) of the INSR gene with insulin resistance was found either in the Non-PCOS or in the PCOS group. The data suggest that this specific polymorphism is probably not associated with insulin resistance among Bangalee women. Mukherjee et al. [53] have reported the association of this polymorphism with IR in a mixed Indian population in a South Indian setting. The present findings emphasize the heterogenic nature of insulin resistance among various populations.

## 5.3 Polymorphism in the PPARγ Gene among Non-PCOS Subjects

### *5.3.1 Proportion of C/T Alleles in the INSR Gene*

The His447C/T polymorphism (ie, T allele) at rs3856806 location of the PPARγgene was found to be present among 17% of Non-PCOS and 18% of PCOS subjects (Fig. 4.8). Thus, there was no significant difference in SNP between the two groups. The findings conform to the results of Antoine et al [251], who found no association of this specific polymorphism with PCOS in the US population. Our findings contradict the claims of Dasgupta et al [273] regarding a positive association of this polymorphism with PCOS in a South Indian population.

### *5.3.2 Association of PPARγ Gene Polymorphism with Phenotypic Features of PCOS*

On extensive analysis using bivariate and multivariate (binary logistic as well as multiple linear regression analysis, as applicable, Tables 4.21, 4.23-4.29), no significant association of the His447C/T polymorphism at (rs3856806 location) of the PPARγ gene with any of the PCOS specific phenotypic features was found either in the Non-PCOS or in the PCOS group. The data suggest that this particular polymorphism is probably not associated with PCOS or any of the characteristic phenotypic features of PCOS among the Bangalee population. To the best of our

knowledge, no report has yet been published specifically addressing the association of PPARγ gene polymorphism with individual phenotypes of PCOS in any population of Indian origin.

### *5.3.3 Association of PPARγ Gene Polymorphism with IR*

Again, on extensive analysis using bivariate and binary logistic analysis (Tables 4.22), no significant association of the His447C/T polymorphism (rs3856806 location) of the PPARγ gene with IR was found either in the Non-PCOS or in the PCOS group. However, a significant (p=0.02) positive association of the polymorphism with IR was revealed on multiple linear analyses (Table 4.30) on adjusting the effects of age, BMI, TC, TG, and tTestosterone. The data suggest that this specific polymorphism seems to be associated with insulin resistance among Bangalee women. Dasgupta et al [273] have reported the association of this polymorphism with IR in a South Indian population. Antoine et al [251], in contrast, reported no association in the US population. The present findings emphasize the heterogenic nature of insulin resistance among populations. It may be noted that a highly significant independent association of IR exists with tTestosterone in the same analysis (Table 4.30). Accordingly, IR seems to play a major role in developingPCOS among Bangalee women through the mediation of hyperandrogenemia.

# Chapter 6: CONCLUSIONS, LIMITATIONS & RECOMMENDATIONS

## 6.1 CONCLUSIONS

The present data lead to the following conclusions:

- Overweight and obesity (both central and peripheral) - notable features in PCOS among young to early middle-aged Bangalee women;
- Anthropometric risk factors - present among a substantial proportion of young Non-PCOS women;
- A substantially high proportion (23%) of Non-PCOS young women already hyperandrogenic which means that there is a potential risk of PCOS among a large number of females;
- Insulin resistance is present among 52% of young PCOS cases; 28% of Non-PCOS subjects are also insulin resistant;
- Strong independent association of insulin resistance with hyperandrogenism is present in PCOS irrespective of age and lipidemic status;
- His1085C/T Polymorphism (at rs1799817 location) in the INSR Gene does not seem to have a significant association with any of the characteristic phenotypic features of PCOS among Bangalee women;
- Insulin resistance in PCOS does not seem to have a significant association with His1085C/T Polymorphism (at rs1799817 location) in the INSR Gene;
- Clinical hyperandrogenism, an important phenotypic feature in diagnosing PCOS, is independently associated with His447C/T polymorphism (at rs3856806 location) of the PPARγ gene among Bangalee women;
- Insulin resistance is independently associated with His447C/T polymorphism (at rs3856806 location) of the PPARγ gene among Bangalee women;
- There is no independent association of biochemical hyperandrogenism with His447C/T polymorphism (at rs3856806 location) of the PPARγ gene among Bangalee women; however, the polymorphism contributes to clinical hyperandrogenism through the mediation of insulin resistance which has a strong independent association with the biochemical markers of hyperandrogenism.

## 6.2 LIMITATIONS

- The subjects are mainly from university/college hostels, not spread in a community;
- A few subjects were from above 30 years age range which may influence the distribution of the measured variables;
- Some other confounding variables (like relevant hormones) were not included.

## 6.3 RECOMMENDATIONS

The following recommendations can be made from the present study:

- Management and prevention of overweight and obesity could be a component of the strategy to combat the rapidly growing burden of PCOS in Bangladesh.
- The contribution of insulin resistance in developing diagnostic features should be investigated among different PCOS phenotypes for more targeted interventions.
- Genes associated with insulin resistance and PCOS should be investigated in detail to understand the etiopathogenesis of this disorder in our population.

# Chapter 7: REFERENCES

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# APPENDIX I

## বাঙ্গালী পলিসিস্টিক ওভারি সিন্ড্রোম রোগীদেও ফেনোটাইপিক বৈশিষ্ট্য ও ইনসুলিন রেজিস্ট্যান্সের সংগে ইনসুলিন রিসিপ্টর এবং ইনসুলিন রিসিপ্টরের সাবস্ট্রেট জিন এর সর্ম্পক

### তথ্যপত্র

পিসিওএস বন্ধ্যাত্বেও প্রধান কারন যা দৈহিক, সামাজিক ও মানসিক সমস্যা সৃষ্টিকরে। জাতি ভেদে এর জিন ও পরিবেশগত কিছু পার্থক্য রয়েছে অথচ বাঙ্গালী নারীদেও মধ্যে এ সর্ম্পকে মৌলিক গবেষণা নেই বললেই চলে। এ ধরনের গবেষণার তথ্য পিসিওএস চিকিৎসা ও প্রতিরোধের জন্য অপরিহার্য।

উপরোক্ত পটভূমিতে, এই গবেষণায় বাংলাদেশের পিসিওএস রোগীদেও বিভিন্ন ফেনোটাইপিক বৈশিষ্ট্যেও আনুপাতিক হার নির্ণয় করা হবে। প্রতিটি ফেনোটাইপিক নির্দিষ্ট বৈশিষ্টগুলোর সাথে ইনসুলিন রেজিস্ট্যান্সের জন্য কোনকারন গুলো প্রভাব ফেলছে এবং ইনসুলিনরিসিপ্টর ও ইনসুলিন রিসিপ্টরের সাবস্ট্রেট জিনের সাথে কোন সর্ম্পক আছে কিনা তা জানা যাবে। এই গবেষনার ফলাফল পরবর্তিতে পিসিওএস জনিত জটিলতা প্রতিরোধের জন্য প্রমান ভিত্তিক গাইডলাইন তৈরীতে সাহায্য করবে।

এই গবেষণায় শুধুমাত্র কিছু ব্যক্তিগত তথ্য ও রক্তের নমুনা (ডি এন এ সহ) সংগ্রহ করা হবে এবং ল্যাবরেটরী পরীক্ষার ফলাফল প্রদান করা হবে। আপনি যে কোনসময় এই গবেষণা থেকে নিজেকে প্রত্যাহার করতে পারবেন এবং তা আপনার চিকিৎসায় কোন রকম প্রভাব ফেলবে না।গবেষনায় প্রদত্ত তথ্য শুধুমাত্র উপরোক্ত গবেষণার কাজে ব্যবহৃত হবে এবং তথ্য সমূহের গোপনীয়তারক্ষা করা হবে।

### সম্মতি পত্র

আমি ----------‘বাঙ্গালী পলিসিস্টিক ওভারি সিন্ড্রোম রোগীদের ফেনোটাইপিক বৈশিষ্ট্য ও ইনসুলিন রেজিস্ট্যান্সের সংগে ইনসুলিন রিসিপ্টর এবং ইনসুলিন রিসিপ্টরের সাবস্ট্রেট জিন এর সর্ম্পক’ বিষয়ক গবেষণায় অংশগ্রহনকারী হিসাবে তথ্য প্রদানে আমার সম্মতি প্রদান করিতেছি।

আমার দেওয়াসকল তথ্য পিসিওএস সম্পর্কিত গবেষণার কাজে ব্যবহৃত হবে এবং বাংলাদেশে পিসিওএস জনিত জটিলতা প্রতিরোধের ক্ষেত্রে গুরুত্বপূর্ণ অবদান রাখবে।

(স্বাক্ষর/ টিপসই)

তারিখঃ-

ঠিকানাঃ-

ফোন নম্বরঃ-

# APPENDIX II

# QUESTIONNAIRE

| | | | |
|---|---|---|---|
| আইডি নং | | তারিখ | |
| রোগীরউৎস: | | বিভাগ: | |
| **Particulars of the Patients: -** | | | |
| রোগীরনাম | | বয়স | |
| বর্তমানঠিকানা | | | |
| স্থায়ীঠিকানা | | | |
| নিজস্ব মোবাইল নং: | | জরুরী মোবাইল নং: | |
| মাসিকআয় (টাকায়) | | | |
| **1. Physical / Clinical Examination:** | | | |
| a. উচ্চতা (সেঃ মিঃ): | | b. ওজন (কেজি): | |
| c. হিপেরপরিধি (সেঃ মিঃ): | | d. কোমড়েরপরিধি (সেঃ মিঃ): | |
| e. BMI: | | f. WHR: | |
| **g.** BP (in mmHg)/ SBP: | | h. DBP: | |
| বৈবাহিকঅবস্থা: | | | |
| বিবাহিত ☐ | অবিবাহিত ☐ | তালাকপ্রাপ্ত ☐ | বিধবা ☐ |
| বিবাহেরবয়স: ☐ | | | |
| **2. Medical History:** | | | |
| **2.1 Oligomenorrhea:** | | | |
| a. ১ম মাসিক কত বছর বয়সে হয়েছিল yrs | | | |
| b. ২১ দিনেরআগেঅথবা ৪৫ দিন পর পরকিমাসিক হয়? | উপস্থিত ☐ | অনুপস্থিত ☐ | |

| | | |
|---|---|---|
| প. মাসিক ১ বছরের বেশিবিরতিতে হয় কিনাঅথবা কোনমাসিকতিনমাসের বেমিসময় পর হয় কিনা? | উপস্থিত ☐ | অনুপস্থিত ☐ |
| ফ. মাসিককি ৭ দিনের বেশি থাকে? | উপস্থিত ☐ | অনুপস্থিত ☐ |
| ব. বছরে ৮ বারমাসিক হয় কিনা? | উপস্থিত ☐ | অনুপস্থিত ☐ |
| f. অন্যান্য: | | |

**2.2 Status of Fertility:**

| | | |
|---|---|---|
| a. আপনারা কি গর্ভধারনের চেষ্টা করছেন?? হ্যাঁনা | ☐ | ☐ |
| b. বন্ধাত্ব: উপস্থিত | ☐ | অনুপস্থিত ☐ |
| c. প্রাইমারী বন্ধাত: উপস্থিত | ☐ | অনুপস্থিত ☐ |
| d. সেকেন্ডারী বন্ধাত: উপস্থিত | ☐ | অনুপস্থিত ☐ |
| e. বাচ্চার সংখ্যা: | | f. শেষ মাসিকের সময়কাল (LMP): |
| g. কত বছরবয়সে ১ম গর্ভধারন করেছিলেন? ☐ | | h. কত বছরবয়সে শেষ গর্ভধারন করেছেন? |
| i. গর্ভপাতেরসংখ্যা: | | |
| **2.3** গর্ভাবস্থায়ডায়াবেটিস এর ইতিহাস: হ্যাঁনা | ☐ | ☐ |

**3 Clinical Features:**

| | | |
|---|---|---|
| a. ব্রণ: | হ্যাঁনা ☐ | ☐ b. আগেব্রণছিল: |
| c.অবাঞ্ছিত চুল (F&G Scale): | উপস্থিত ☐ | অনুপস্থিত ☐ |
| | ঠোঁট ☐ | থুতনি ☐ |
| | বুক ☐ | উপরের পেট ☐ |
| | নীচের পেট ☐ | বাহুরউপরেরঅংশে ☐ |
| | হাত ☐ | ঊরু ☐ |
| | পায়েরনীচেরঅংশে ☐ | পিঠেরউপরেরঅংশে ☐ |
| | পিঠেরনীচেরঅংশে ☐ | সম্পূর্ণ ফলাফল: |
| | ≥৮ ☐ | ≤৮ ☐ |
| কেশলুপ্তি/টাক: | হ্যাঁ ☐ | না ☐ |
| | *Grade I* ☐ | *Grade II* ☐ *Grade III* ☐ |
| e. ঘাড়েরকাল দাগ: | উপস্থিত ☐ | অনুপস্থিত ☐ |

**4 Biochemical Hyperandrogenism:**

| Name of Test | Results with Unit | Reference Range |
|---|---|---|
| a. Serum free testosterone | | |
| b. Sex Hormone Binding Globulin (SHBG) | | |
| c. Fasting blood glucose | | 4.1-5.9 mmol/l |
| d. 2 hr after of glucose | | <7.8 mmol/l |
| e. Serum C-peptide or Fasting Insulin | | |
| f. Total Cholesterol: | | <200mg/dl |
| g. TG | | <150 mg/dl |
| h. LDL: | | <100 mg/dl |
| i. HDL: | | <40 mg/dl |

**6. Ultrasonography report for POM:**

| | |
|---|---|
| Last menstrual period: | Features of PCOS**:** Yes ☐ No ☐ |
| Enlarged Right ovary: Yes ☐ No ☐ | Enlarged Left ovary: Yes ☐ No ☐ |
| Finding of Right Ovary: | Finding of Left Ovary: |
| Right Ovary Size: cm | Left Ovary Size: cm |
| Ovarian volume of right ovary: cc | Ovarian volume of left ovary: cc |
| Presence of Cyst in right ovary: Yes ☐ No ☐ | Presence of Cyst in left ovary: Yes ☐ No ☐ |
| Total follicle number of right ovary: | Total follicle number of left ovary: |

| | | |
|---|---|---|
| Follicle distribution pattern | Right: | Left: |
| Presence of stromal echogenicity | Right: | Left: |

| | |
|---|---|
| **7. Co-Morbidities:** | |
| **7.1.** a. উচ্চ রক্তচাপ: হ্যাঁ ☐ না ☐ | |
| *If yes go Question No 4& if No go Question No 6* | |
| b. কত বছর ধরে: ……. year | c. ঔষুধের নাম: |
| **7.2. Diabetes Mellitus (DM):** | |
| a. ডায়বেটিসমেলিটাস (DM): হ্যাঁ ☐ না ☐ | |
| b. IFG: হ্যাঁ ☐ না ☐ | c. IGT: হ্যাঁ ☐ না ☐ |
| d.কত বছর ধরেডায়বেটিস: | e. Any Relatives: |
| f. ডায়বেটিসেরধরন: DM1 ☐ DM2 ☐ | g. ঔষুধের ধরন: OAD ☐ Insulin ☐ OAD +Insulin ☐ |
| h. ঔষুধের নাম OHA/ Insulin: | |
| **7.3.a.থাইরয়েড ব্যাধি:** হ্যাঁ ☐ না ☐<br>*If yes go next Q. if no go variable(Other Biochemical Investigations)* | |
| কত বছর ধরে: | Results of TSH:……. nmol/l |
| FT3: ……..nmol/l | Thyroxine / $FT_4$:..... nmol/l |
| Hyper thyroid ☐ | Hypo thyroid ☐ |

**8. Existed Biochemical Investigations:**

| SI No | Investigations | Results | Unit |
|---|---|---|---|
| a. | Luteinizing Hormone (LH): | | |
| b. | Follicle Stimulating Hormone (FSH): | | |
| c. | Prolactin: | | |
| d. | Serum Creatinine: | | |
| e. | SGPT: | | |
| f. | Anti Mullerian Hormone (AMH) | | |
| g. | Blood Group | | |