Original Article

Whole Exome Sequencing Reveals Rare Variants in Genes Associated with Metabolic Disorders in Women with PCOS

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a complex genetic trait, the pathogenesis of which is governed by an interplay of genetic and epigenetic factors. However, the aetiology of PCOS is not fully understood. **Aims:** The objective of this study was to investigate the genetic causes of PCOS by identifying rare variants in genes implicated in its pathophysiology. **Settings and Design:** This was a hospital-based observational study. **Materials and Methods:** We used whole-exome sequencing for 52 PCOS women to identify the rare variants in genes related to PCOS pathogenesis. Subsequently, we analysed these variants using *in silico* prediction software to determine their functional effects. We then assessed the relationship between these variants and the clinical outcomes of the patients. **Statistical Analysis Used:** Student's *t*-test and Fisher's exact test were used to compare clinical parameters and frequency differences amongst PCOS patients with and without variants. **Results:** A total of four rare exonic variants in obesity- and hyperinsulinaemia-related genes including *UCP1* (p.Thr227Ile), *UCP2* (p.Arg88Cys), *IRS1* (p.Ser892Gly) and *GHRL* (p.Leu72Met) were identified in eight patients. Significant differences were observed between the patients carrying variants and those without variants. PCOS patients with identified variants exhibited significantly higher average body mass index and fasting insulin levels of PCOS subjects with identified variants compared to those without variants ($P < 0.05$). Additionally, there were significant differences in the variant frequencies of four variants when compared to the population database ($P < 0.05$). **Conclusion:** This study shows a prevalence of rare variants in obesity and hyperinsulinaemia-related genes in a cohort of PCOS women, thereby underscoring the impact of the identified rare variants on the development of obesity and associated metabolic derangements in PCOS women.

Keywords: *Hyperinsulinaemia, obesity, polycystic ovary syndrome, rare variants, whole-exome sequencing*

Introduction

Polycystic ovary syndrome (PCOS) is a prevalent endocrinopathy common amongst women of reproductive age.[1] Apart from the regional and ethnic variations, the prevalence of PCOS also greatly depends on the diagnostic criteria used. Most reports suggest that the global prevalence of PCOS ranges between 4% and 21%.[2,3] The reproductive and metabolic disruptions associated with PCOS cause the characteristic features of the syndrome, namely oligomenorrhoea/

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amenorrhoea, androgen excess and/or polycystic ovarian morphology (PCOM).[4] The pathogenesis of PCOS involves an interaction between genetic and environmental factors. The studies identifying PCOS being a familial condition[5,6] opened new avenues for the exploration of the genetic basis of the syndrome. While some clinical genetic studies are suggestive of an autosomal dominant inheritance,[5,7,8] others point at a polygenic basis of the syndrome.[9,10] Additionally, common diagnostic features of the syndrome are observed not only in affected women but also amongst their male and female family members.[11] The estimated heritability of PCOS is close to 70% as demonstrated by mono- and dizygotic twin studies.[12] Because of the association of PCOS with conditions that affect androgen biosynthesis and action, systemic inflammation and glucose metabolism, studies have focussed on mutations in genes related to these pathways including *CYP11A1, CYP17A, CYP19, CYP21, HSD17B5, HSD17B6, INS, INSR, IRS-1, IRS-2, IGF,* interleukin-6 and tumour necrosis factor-α.[13]

Despite the extensive research, none of the proposed pathophysiologic mechanisms such as hypothalamic–pituitary–gonadal pathways have been able to give a conclusive explanation for the development of the disorder. Studies suggesting a potential link between metabolic pathways and PCOS ignited a surge of interest amongst researchers, with a focus on obesity and insulin resistance with compensatory hyperinsulinaemia to understand the pathophysiology of PCOS.

Obesity is a multifactorial condition, showing 40%–70% heritability.[14] The prevalence of obesity amongst PCOS women ranges between 42% and 74% which is significantly higher than in general population (25%).[15,16] Importantly, obesity is a risk factor for the development of impaired glucose tolerance (IGT), insulin resistance, hypertension, cardiovascular disease and dyslipidaemia.[17,18] Additionally, increased insulin sensitivity has been observed to be more prevalent amongst PCOS women as compared to weight-matched women in the general population.[19,20] A recent study has highlighted the prevalence of insulin resistance and hyperinsulinaemia amongst obese women with PCOS.[21] The fact that PCOS women also tend to exhibit higher rates of cardiometabolic derangements makes it clear that there is a perturbing influence of obesity on the disorder. Additionally, PCOS women with superimposed obesity may exhibit a profoundly worsening state of hyperandrogenaemia and hyperinsulinaemia.[22] Recently, it has been established that obese/overweight women are at an increased risk for type 2 diabetes (T2D).[23] The metabolic aspects of obesity and PCOS frequently run in parallel, worsening with age.[24] Given the inherent complexity of each condition, the discernment of their pathophysiology and the molecular pathways involved can be a challenging task.

To date, no candidate genes associated with PCOS pathophysiology have been attributed to or found to play a fundamental role in its aetiology. This is mainly due to studying heterogeneous groups of PCOS without phenotypic characterisation. Several novel risk loci for PCOS have been reported using genome-wide association studies (GWAS). However, the findings from these studies have only contributed to <10% of heritability in PCOS or poor associations and repeatability. Hence, it is reasonable to speculate that distinct phenotypes of PCOS may result from varied underlying aetiological factors, potentially involving rare genetic variants. Thus, through this study, we aimed to identify potentially pathogenic variants in the genes implicated in the pathogenesis of PCOS.

Materials and Methods

Subjects and ethics statement

For this study, 52 PCOS patients were recruited. Our research was approved by the Institute Ethics Committee. The patients were recruited based on the National Institutes of Health criteria, 2012, according to which two of the three characteristics are required for PCOS: hyperandrogenism, ovulatory dysfunction and/or PCOM. PCOM, examined through ultrasonography, is defined as the presence of antral follicles (≥12) measuring 2–9 mm in diameter and/or ovarian volume (>10 cm^3).[25] Clinical hyperandrogenism was diagnosed on the basis of Ferriman–Gallwey score (≥9).[26] Biochemically, hyperandrogenism was determined by elevated total testosterone (TT) levels according to laboratory criteria. The inclusion criteria were age between 18 and 35 years and hormonal analysis to study the profile of thyroid (T4 and thyroid-stimulating hormone), serum prolactin, luteinising hormone (LH), follicle-stimulating hormone (FSH), oestradiol (E2), TT and fasting insulin (FI) from morning fasting samples on D2-5 of the menstrual cycle. Exclusion criteria were based on the clinical diagnosis of congenital adrenal hyperplasia, hypothyroidism, premature ovarian failure and ovarian neoplasm. Women under medication affecting the hypothalamic–pituitary–gonadal axis were also excluded.

The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2013. Written informed consent was obtained from the subjects before sample collection. Our research was approved by the Institute Ethics Committee.

Ethics Committee approval number: IEC-730/29.12.2017.

The sample size calculation was not performed for the present study.

Anthropometric measurements

The height (cm) and weight (kg) of the patients were measured in the morning in the fasting state. Body mass index (BMI) was calculated by dividing the body weight in kilograms by the square of the height in meters. The BMI was calculated using the age, height and sex of the patients.

Fasting insulin measurement

Collection of fasting blood was done from the patients in the morning on empty stomach. Serum was separated by centrifugation at 5000 rpm for 5 min. Concentration of FI (μIU/mL) was measured using the ARCHITECT Insulin assay, Abbott (Chicago, US).

DNA extraction and whole-exome sequencing analysis

Genomic DNA was extracted from fresh peripheral blood samples of the patients using the salting-out method.[27] The patients' DNA samples were subjected to whole-exome sequencing. Targeted gene capture was performed using Agilent SureSelect V5 exome capture kit. The libraries were sequenced to mean >80-X100 coverage on Illumina sequencing platform.

Bioinformatics

The sequences obtained were aligned to human reference genome (GRCH37/hg19) using Sentieon aligner and analysed using Sentieon for removing duplicates, recalibration and re-alignment of indels. Sentieon HaplotypeCaller was used to identify variants which are relevant to the clinical indication. Gene annotation of the variants was performed using VEP programme against the Ensembl release 91 human gene model. In addition to single-nucleotide variants and small indels, copy number variants were detected from targeted sequence data using the ExomeDepth (v1.1.10) method. Clinically relevant mutations were annotated using published variants in literature and a set of disease databases – ClinVar, OMIM, GWAS, HGMD (v2018.3) and SwissVar. Common variants are filtered based on allele frequency in 1000 Genomes Phase 3, Exome Aggregation Consortium (ExAC) (v1.0), gnomAD (v2.1), EVS and dbSNP (v151) databases. Non-synonymous variants effect was calculated using multiple algorithms such as PolyPhen-2, Sorting Intolerant from Tolerant (SIFT), MutationTaster2 and likelihood ratio test (LRT). Only non-synonymous and splice site variants found in the coding regions were used for clinical interpretation. Silent variations that do not result in any change in amino acid in the coding region were not reported.

Variant filtering strategy and effect prediction

Rare variants with minor allele frequency (MAF) more than 0.05% in ExAC were retained while those with higher MAF (>0.05%) were excluded from the data unless reported pathogenic or likely pathogenic in ClinVar. Variants with expected pathogenicity were selected based on their variant class followed by their functional impact prediction based on scores obtained from *in silico* tools Combined Annotation-Dependent Depletion (CADD) phred score, SIFT score, PolyPhen-2 and LRT. Variants with a low CADDphred score were excluded unless reported as pathogenic or likely pathogenic in ClinVar. Additionally, I-Mutant 2.0[28] and MUpro Tool[29] were used to assess the effect of single amino acid substitution on protein stability.

Functional annotation

Gene ontology analysis was performed on the genes harbouring rare variants, using the web-based tool genes annotation co-occurrence discovery (GeneCodis version 4.0) https://genecodis.genyo.es/.[30]

Evolutionary conservation analysis of the variants

The protein sequences from 10 different vertebrate species in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) were used to analyse the evolutionary conservation status of the identified variants. The species included *Homo sapiens, Felis catus, Canis lupus familiaris, Equus caballus, Pan troglodytes, Acinonyx jubatus, Chlorocebus sabaeus, Myotis brandtii* and *Panthera tigris altaica.* Multiple sequence alignment was performed using Clustal Omega (1.2.4) tool by EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical analysis

Continuous quantitative data are expressed as the mean ± standard deviation. Two-tailed Student's *t*-test ($P < 0.05$) was applied to compare the differences in clinical parameters amongst PCOS subjects carrying variants and those without variants. Additionally, the frequency differences of the identified variants were analysed by applying two-tailed Fisher's exact test ($P < 0.05$).

Results

Whole-exome sequencing was done to analyse the coding sequences of 52 PCOS patients [Table 1]. After examining the variants, we identified four heterozygous missense variants within the coding regions of four different genes in eight patients. The detailed analysis of the variants is given in Table 2. Notably, one of the four variants exhibited significant differences in variant

frequencies when compared with those in the ExAC database ($P < 0.05$) [Table 3].

The variants were mapped onto their respective genes and visualised on the ProteinPaint platform (https://proteinpaint.stjude.org).[31] The results revealed that of the four variants, three were present in important functional domains of their respective proteins [Figure 1].

Table 1: Clinical data of the patients

Parameter	PCOS subjects with variants (*n*=8)	PCOS subjects without variants (*n*=44)	*P*
Age (years)	25.12±2.47	23.25±5.05	0.333
BMI	28.35±4.03	24.447±4.53	0.029*
LH/FSH	1.60±0.68	1.61±0.95	0.986
T (ng/dL)	0.61±0.27	0.53±0.32	0.484
AMH (ng/mL)	10.09±5.74	11.88±5.34	0.404
FI (uIU/mL)	17.93±11.17	10.80±5.46	0.0074*

*P<0.05. Values are expressed as the mean±SD. BMI=Body mass index, LH=Luteinising hormone, FSH=Follicle-stimulating hormone, T=Testosterone, AMH=Anti-Mullerian hormone, FI=Fasting insulin, PCOS=Polycystic ovary syndrome, SD=Standard deviation

Mutational landscape in the obesity-related genes

A total of two heterozygous missense variants, *GHRL* (p.L72M) and *UCP2* (p.R88C), in genes associated with obesity, were identified in 11.53% (n = 6) of the PCOS subjects. Notably, *GHRL* (p.L72M) variant exhibited homozygosity in one of the patients. The most common of these variants was *GHRL* (p.L72M), identified in 9.6% (n = 5) of the total patients. Importantly, the most deleterious of the identified variants is the heterozygous missense variant in *UCP2* with a high CADDphred score and an extremely low allele frequency. This variant is located in the mitochondrial carrier domain of the protein [Table 3]. *GHRL*, on the other hand, was predicted to be 'probably damaging' by the PolyPhen-2 prediction tool and also has a negative effect on protein stability.

Mutational landscape in the hyperinsulinaemia-related genes

Two of the total identified variants were in the genes associated with hyperinsulinaemia. These genes and their respective variants, *IRS1* (p.S892G) and *UCP1* (p.T227I), were identified in two patients [Table 2].

Table 2: Details of the rare variants in genes associated with obesity and hyperinsulinaemia identified through whole-exome sequencing in polycystic ovary syndrome patients

DBN	BMI (kg/m^2)	Insulin (F) uIU/mL	Gene	CDNA_CHG	Variant class	Zygosity	dbSNP ID	AA change	CADDphred score	ClinVar significance	ExAc AF
82	30.4 (obese)	6.3	*GHRL*	c.214C>A	Missense	Homozygous	rs696217	p.Leu72Met	M	Pathogenic	0.085572
95	30.8 (obese)	12.2	*UCP2*	c.262C>T	Missense	Heterozygous	rs540782955	p.Arg88Cys	H	NA	2.48E-05
168	34.5 (obese)	14.9	*GHRL*	c.214C>A	Missense	Heterozygous	rs696217	p.Leu72Met	M	Pathogenic	0.07
20	30.7 (obese)	38.8	*GHRL*	c.214C>A	Missense	Heterozygous	rs696217	p.Leu72Met	M	Pathogenic	0.07
215	24.9	9.8	*GHRL*	c.214C>A	Missense	Heterozygous	rs696217	p.Leu72Met	M	Pathogenic	0.07
170	22.3	9.7	*GHRL*	c.214C>A	Missense	Heterozygous	rs696217	p.Leu72Met	M	Pathogenic	0.07
158	24.9	26.5	*UCP1*	c.680C>T	Missense	Heterozygous	rs148598275	p.Thr227Ile	M	NA	0.002
148	28.3 (overweight)	25.3	*IRS1*	c.2674A>G	Missense	Heterozygous	rs1801277	p.Ser892Gly	M	NA	0.002

BMI=Basal metabolic index, DBN=Database number, CDNA_CHG=cDNA change, AA=Amino acid, CADD=Combined Annotation-Dependent Depletion, AF=Allele frequency, ExAC=Exome Aggregation Consortium, NA=Not available

Table 3: Evaluation of the variant effect and assessment of protein stability done through *in silico* prediction tools

Gene	Variant effect				Protein stability		Catalytic domain	AF in PCOS cohort	AF (ExAC)	*P*
	Variant	LRT prediction	SIFT prediction	PolyPhen-2 prediction	I-Mutant 2.0	MUpro tool				
UCP2	p.Arg88Cys	Deleterious	Deleterious	Probably damaging	Decreased	Decreased	Mitochondrial carrier domain	1/52	3/125,000	0.0017*
IRS1	p.Ser892Gly	Deleterious	Deleterious	Probably damaging	Decreased	Decreased	-	1/52	123/50,000	0.12
GHRL	p.Leu72Met	Neutral	Tolerated	Probably damaging	Decreased	Decreased	Motilin/ghrelin-associated peptide	5/52	21,393/250,000	0.801
UCP1	p.Thr227Ile	Deleterious	Deleterious	Benign	Increased	Decreased	Mitochondrial carrier domain	1/52	4189/2,000,000	0.103

*Denotes a statistically significant difference (<0.05). The Fisher's exact test (P<0.05) showed significant differences in variant distribution between the PCOS cohort and that of ExAC database. LRT=Likelihood ratio test, SIFT=Sorting Intolerant from Tolerant, PolyPhen-2=Polymorphism phenotype v2, AF=Allele frequency, ExAC=Exome Aggregation Consortium, PCOS=Polycystic ovary syndrome

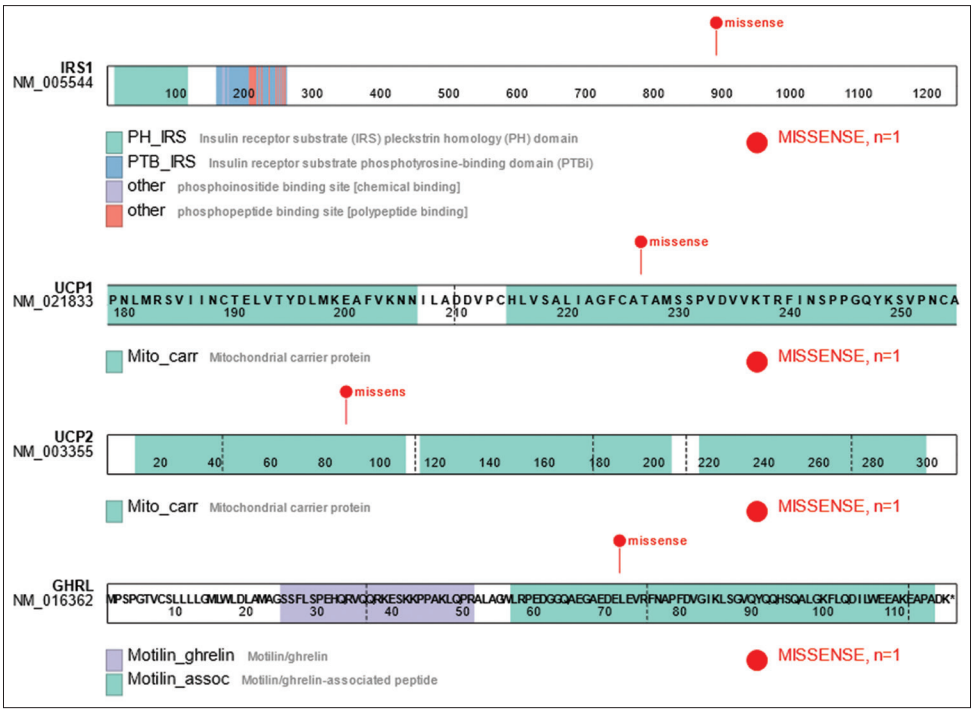

Figure 1: The precise location of the variants on their respective genes visualised by Protein Paint

Both the identified missense variants of *IRS1* and *UCP1* exhibited a medium impact on functionality of the proteins and were predicted by the *in silico* tools to be highly deleterious. Additionally, they also have a decreasing effect on the protein stability [Table 3]. Moreover, the two variants were found at an extremely low frequency (0.00246 and 0.002094, respectively) in the general population.

Association between the identified variants and patients' clinical outcomes

In the present study, 52 PCOS patients were recruited. Of these, 23.07% (n = 12) were overweight (BMI ≥25 kg/m^2), 17.3% (n = 9) were obese (BMI ≥30 kg/m^2), 1.9% (n = 1) were lean (BMI <18 kg/m^2) and the remaining 57.6% (n = 30) of the subjects had normal BMI (≥18 kg/m^2 to ≤25 kg/m^2). Abnormally high FI levels (FI >25 iui/mL) were observed in 9.61% (n = 5) of the total patients. Three of these patients fall in the overweight/obese category, while two of the PCOS patients with abnormally high FI levels had normal BMI. The average BMI of the subjects in whom the variants have been reported was 28.35 ± 4.03 kg/m^2, which is significantly higher when compared to the patients with no variants (24.447 ± 4.53 kg/m^2; P = 0.029; P < 0.05). Similarly, a significant difference was observed in FI levels in the subjects with variants (17.93 ± 11.17 uIU/mL) as compared to the ones with no variants (10.80 ± 5.46 uIU/mL; P = 0.0074; P < 0.05) [Figure 2]. No significant association of other parameters such as age, TT, anti-Mullerian hormone and LH/FSH ratio was observed between the two groups. Hyperinsulinaemia was exclusively observed in two patients with rare *IRS1* (p.Ser892Gly) and *UCP1* (p.Thr227Ile) variants. Similarly, obesity was exclusive to the patients carrying a highly deleterious rare variant in *UCP2* (p.Arg88Cys) and a common variant in *GHRL* (Leu72Met). The variant *GHRL* (p.Leu72Met) was observed in five PCOS subjects. Interestingly, four of the five patients with *GHRL* (p.Leu72Met) were obese/overweight (30.63 ± 0.191 kg/m^2), but their FI levels are highly variable (20 ± 15.6 µIU/mL). The patient with homozygosity in *GHRL* (p.L72M) was obese.

Evolutionary conservation analysis

Evolutionary conservation analysis of the identified exonic variants done in 10 vertebrate species revealed that the four variants, *GHRL* (p.Leu72Met), *UCP2* (p.Arg88Cys), *IRS1* (p.Ser892Gly) and *UCP1* (p.Thr227Ile), were highly conserved amongst the different species [Figure 3]. *IRS1* (p.Ser892Gly) was, however, not identified in *P. t. altaica*.

Discussion

A disproportionately large number of PCOS women suffer from metabolic derangements such as obesity, hyperinsulinaemia and insulin resistance,[32] which translate into a heightened risk of obesity-related comorbidities, namely T2D and possibly hypertension,[33]

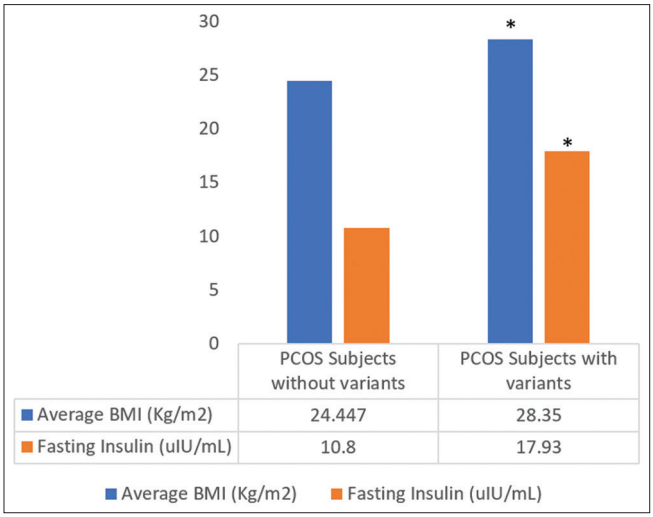

Figure 2: Differences in the average body mass index (kg/m^2) and fasting insulin (uIU/mL) levels between polycystic ovary syndrome subjects with variants and those without variants. The patients with variants had significantly higher BMI and fasting insulin levels than those without variants (*$P < 0.05$). PCOS: Polycystic ovary syndrome, BMI = Body mass index

making these pathways useful targets for exploring potential candidate genes for PCOS. A causal link between obesity and PCOS was established through recent large-scale genome-wide meta-analysis.[34] With this background information, we considered a possible role of genetic variants on the susceptibility for the development of polygenic obesity and hyperinsulinaemia in PCOS patients.

In the present study, we screened rare exonic variants in genes associated with the pathogenesis of PCOS. In the cohort of 52 PCOS patients, 60.78% (n = 31) of the patients had BMI <25 kg/m^2, while the remaining 40.38 (n = 21) were overweight/obese. We reported four rare variants, of which one (*GHRL* p.Leu72Met) has been reported previously as pathogenic. The genes are significantly enriched in biological processes pertaining to metabolism and thermogenesis, and a majority of them are localised in the mitochondria of the cells, suggestive of their role in energy-related pathways. The four variants exhibited heterozygosity, while one of the obese patients with *GHRL* p.Leu72Met was homozygous. Of the eight patients with variants, four were obese, one was overweight and two had normal BMI (BMI = 24.9 kg/m^2). Nevertheless, we found a significant difference in the BMI of patients with variants versus the patients without variants. High insulin levels (≥25 uIU/mL), suggestive of hyperinsulinaemia, were observed in four overweight patients and one patient with normal BMI. This points towards a positive association of BMI with hyperinsulinaemia. Additionally, FI levels were

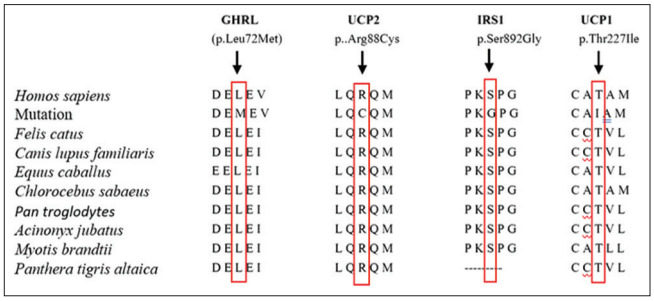

Figure 3: Evolutionary conservation analysis of the identified mutations in 10 different vertebrate species

significantly higher in patients with variants compared to patients without variants ($P < 0.05$).

A number of studies have provided conclusive evidence to establish the association of polymorphisms in the genes identified in our study with obesity, hyperinsulinaemia, insulin resistance and/or T2D mellitus (T2DM). These results are consistent with the outcomes observed in various knockout/knockdown studies of these genes and their associated phenotypes. Polymorphisms in these genes and their association with PCOS have also been examined in multiple studies. Consistent with these reports, we present cases where rare variants in these genes are linked to obesity and hyperinsulinaemia in women with PCOS.

Uncoupling protein-1, *UCP1* (p.Thr227Ile)

As a mitochondrial-resident protein, UCP1 is expressed in brown adipose tissue (BAT) and is involved in thermogenesis regulation of energy metabolism.[35] It has been observed that mice deficient in *UCP1* develop diet-induced obesity with age.[36] However, the obesity phenotype in this case does not manifest at the young-adult stage. Kontani *et al*. also observed that the diet-induced obesity in *UCP1*-deficient mice was notably higher in females than in males, making it clear that *UCP1* deficiency will have a more dramatic effect on females. However, the study did not report the effect of *UCP1* deletion on insulin sensitivity. Nevertheless, many subsequent studies have proved the hypothesis that BAT activation through cold exposure, β3-agonist or thyroid treatment improves glucose tolerance and insulin resistance.[37-39] In line with these reports, we found *UCP1* (p.T227I) variant in a PCOS patient (database number [DBN] 158) who was borderline overweight and had severe hyperinsulinaemia. It seems plausible that increased insulin levels in this patient were secondary to increased resistance to insulin action and was, in fact, a case of compensatory hyperinsulinaemia which itself is induced as a result of low thermogenesis due to the dysfunctional consequences of *UCP1* (p.Thr227Ile) variant. Increased energy expenditure as a result of

increased *UCP1* expression was observed in mice which were rendered genetically incapable of high-fat diet-induced fasting hyperinsulinaemia.[40] Therefore, increasing the BAT activity by increasing the repertoire of UCP1-positive cells will therefore prove to be useful targets for the treatment of obesity, T2DM resulting from compensatory hyperinsulinaemia and insulin resistance in PCOS women. Additionally, the patient also exhibited abnormally high testosterone and DHEA-S levels, typical of PCOS. In a recent study, androgen treatment was reported to reduce the β-adrenoreceptor-stimulated increase in *UCP1* expression in PCOS mice, establishing a negative correlation between BAT thermogenesis and androgen levels.[41] Thus, high levels of androgens can have a compounding effect on BMI by downregulating the genes other than *UCP1*, leading to further decline in thermogenesis. All in all, considering that the threonine at position 227 is highly conserved amongst the different vertebrate species [Figure 3], the Thr227Ile substitution might have a functional significance.

Uncoupling protein 2, *UCP2* (p.Arg88Cys)

The properties of another uncoupling protein, UCP2, are consistent with roles both in obesity and hyperinsulinaemia.[42] The expression of *UCP2* takes place at varying levels in a wide range of cells and tissues including pancreatic β-cells.[43] Increase in adenosine triphosphate (ATP) as a result of glucose metabolism is sensed by pancreatic β-cells which promotes insulin secretion.[44,45] It has been established through *UCP2* gene knockout experiment that *UCP2* decreases ATP production and thus negatively regulates insulin secretion.[43] This indicates that *UCP2* is critical in the development of obesity and hyperinsulinaemia. Additionally, the polymorphism (rs660339) in *UCP2* puts the patient at an increased risk of obesity and T2DM.[46] Transcriptional profiling of *UCP2* in PCOS women has revealed that decreased expression of *UCP2* in the skeletal muscles is associated with insulin resistance.[47] We have presented the case of an obese PCOS woman (DBN 95; BMI = 30.8 kg/m^2) with *UCP2* (p.Arg88Cys), having normal serum levels of FI (12.2 μIU/mL), suggesting that the variant *UCP2* (p. Arg88Cys). No other metabolic complications were reported in the patient. Since *UCP2* (p.Arg88Cys) has been predicted to be deleterious and decrease protein stability, it suggests that the variant confers a phenotype of obesity.

Ghrelin, *GHRL* p.Leu72Met

Our study reveals a significant enrichment of a common variant of *GHRL* gene in PCOS patients. Ghrelin, coded by the *GHRL* gene, is a peptide involved in the food intake process.[48] A study done on rodents suggested that administration of ghrelin causes weight gain by attenuating food utilisation and increasing food intake;[49] however, obese subjects have low levels of circulating ghrelin in their plasma.[50] Additionally, there is a negative correlation between FI and ghrelin concentration in obese subjects.[51,52] The association of *GHRL* p.Leu72Met with insulin resistance and T2DM risk has remained controversial.[53-55] However, a positive association between *GHRL* p.Leu72Met and early onset of obesity was found amongst Italian obese children.[56] In our cohort, we have identified *GHRL* p.Leu72Met in five PCOS patients, of which three were obese and two subjects had normal BMI. One of the obese patients also had abnormally high FI. Four patients were heterozygous for the variants, while an obese patient was homozygous. The genotype–phenotype correlation of *GHRL* p.Leu72Met can moderately substantiate that the variant is associated with obesity in PCOS subjects. Furthermore, the association of this common variant (MAF = 0.08) with PCOS was significantly high frequency in our cohort compared to its overall allele frequency in the ExAC database (P = 0.0005).

Insulin receptor substrate, *IRS1* p.Ser892Gly

IRS1 modulates tissue response to insulin by acting as a docking protein between insulin receptor and multiple Src homology-2 (SH2) in the insulin signalling cascade.[57,58] Targeted disruption of *IRS1* gene in mice causes the development of insulin resistance and IGT.[59] Two *IRS1* polymorphisms in codon 513 and 972 were found to be associated with a 50% reduction in insulin sensitivity.[60] *IRS1* p.Gly972Arg polymorphism has been found to be significantly associated with PCOS risk.[61] The present study highlights a missense variant in *IRS1* (p.Ser892Gly) identified in an overweight PCOS patient with hyperinsulinaemia. The high FI levels imply compensatory hyperinsulinaemia linked to insulin resistance. Notably, the variant is uncommon in the population, as evidenced by a low allele frequency (MAF = 0.00246). The utilisation of *in silico* tools predicts a functional consequence of the variant, indicating its likely role in influencing the observed phenotype. This highlights the significance of genetic factors in the intricate development of PCOS and its related metabolic characteristics.

Taken together, *UCP2* (p.Arg88Cys) and *GHRL* (p.Leu72M) variants impact obesity, while *IRS1* (p.Ser892Gly) and *UCP1* (p.Thr227Ile) variants result in hyperinsulinaemia. The other three variants, although they have been implicated in obesity phenotype, were observed in overweight patients. The identified

missense variants were considered to be causing obesity and hyperinsulinaemia manifestations in PCOS patients. While it is unlikely that these variants could individually serve as common causes of polygenic traits such as obesity and hyperinsulinaemia, there is a possibility that they contribute to anticipating the onset of these disorders and, in turn, the pathogenesis of PCOS.

Conclusion

We identified four rare variants in genes associated with obesity and hyperinsulinaemia in PCOS patients. The patients with these variants presented with either high BMI (suggestive of overweight condition or obesity) or hyperinsulinaemia or had these phenotypes in combination. Taken together, both *in silico* and genotype–phenotype correlations suggest that amongst the identified variants, *IRS1* (p.Ser892Gly) and *UCP1* (Thr227Ile) are associated with hyperinsulinaemia, while *UCP2* (p.Arg88Cys) and *GHRL* (p.Leu72Met) are associated with obesity. Our study supports the hypothesis that exomic rare variants in genes related to obesity and hyperinsulinaemia drive the progression of PCOS.

Author's Contributions

PS - Clinical analysis, laboratory work and data analysis, secondary bioinformatic analysis and manuscript writing. AH - Conceived, designed the experiments, clinical analysis, made critical revisions to manuscript and arranged funding; MJ - Laboratory work and data analysis; MT - Secondary bioinformatic analysis. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

WES data supporting the results reported in this article can be obtained from the authors on request.

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