Polymorphism

RESEARCH

The K121Q polymorphism in the *ENPP1* gene shows association with obesity in Indian women with PCOS

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ABSTRACT

Aim: To evaluate the influence of the K121Q variant in the *ENPP1* gene on the risk of PCOS development and its associated traits of glucose tolerance, insulin resistance, hyperandrogenemia and dyslipidemia in Indian women.

Methods: Genotyping of K121Q polymorphism of *ENPP1* was performed in women with PCOS (N = 185) and controls (N =153) women, while phenotypic characterization in terms of clinical, biochemical and hormonal parameters was performed in 83 controls and 143 PCOS women. Genotype only and genotype–phenotype associations were determined by appropriate statistical tests.

Results: The K121Q polymorphism showed comparable genotypic frequency distribution between controls and women with PCOS even after BMI-based classification. Intriguingly, both lean and obese controls showed significant association of polymorphic allele with decreased total testosterone levels. Amongst women with PCOS, this polymorphism was significantly associated with lowered LH levels and LH:FSH ratios in lean women, and reduced triglyceride levels in obese women only, respectively. Importantly, the Q allele was found to be significantly associated with increased risk of obesity in women with PCOS only.

Conclusion: This is the first study to determine that even though the K121Q polymorphism of *ENPP1* does not influence PCOS risk in Indian women, it beneficially impacts hyperandrogenemia, gonadotropin levels and dyslipidemia in women in accordance with their underlying obesity and physiological status. The contribution of the Q allele to elevated tendency towards obesity may aid clinicians in suggesting appropriate therapeutic interventions to avert long term cardiometabolic complications. This warrants deeper focus on the complex genetic pathomechanisms underlying PCOS.

KEYWORDS: PCOS; *ENPP1*; obesity; genetics; polymorphism; association study.

Citation: Shaikh N et al. The K121Q polymorphism of ENPP1 shows association with obesity in Indian women with PCOS. Polymorphism 2021; 7: 36-51.

Editorial history: received: May 20, 2021; revised: June 03, 2021; accepted: June 04, 2021

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the commonest endocrine disorders to affect 5-10% of women in their child-bearing years and is linked to both gynecological as well as cardiometabolic maladies (Sagvekar et al. 2018). Typical symptoms of PCOS comprise anovulatory infertility with oligo/secondary amenorrhea, obesity and central obesity, impaired glucose tolerance, insulin resistance with compensatory hyperinsulinaemia and acanthosis nigricans, increased LH: FSH ratios, hyperandrogenism indicated by hirsutism, acne, male pattern alopecia, and polycystic ovaries on ultrasound examination (Sagvekar et al. 2018). Insulin resistance, a central factor in PCOS pathogenesis, is considered to be intrinsic to this disorder and is present in almost 50-70% of affected women independent of obesity (Mukherjee et al. 2010). PCOS is considered as a complex disorder where genetic and environmental interactions play key roles in its development.

Candidate gene approach has been utilized extensively in which genes belonging to multiple pathways involved in regulation of insulin secretion and action, ovarian and adrenal steroidogenesis, gonadotropin action and regulation, inflammation, and energy regulation have been studied for association with PCOS and its related traits (Shaikh et al. 2014). Of the insulin related pathways, a lesser explored candidate gene among various populations is the ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 (ENPP1) also known as plasma cell membrane glycoprotein (PC-1). It is a class II membrane glycoprotein that effectively binds the insulin receptor (INSR) and induces conformational changes that lead to disruption in insulin receptor auto-phosphorylation and tyrosine kinase activation, thereby impeding the insulin receptor signaling. Several lines of evidence indicate that it is an important factor affecting insulin sensitivity and has been observed to be elevated two to three-fold in muscle, adipose tissue and fibroblasts of insulin resistant subjects (Maddux et al. 1995; Goldfine et al. 2008; Pan et al. 2012; Huesa et al. 2014) and adipose tissue of women with PCOS (Corton et al. 2007).

The gene encoding for *ENPP1* has 25 exons and is located on the long arm of chromosome 6 (6q 23.2). A functional missense polymorphism, rs1044498, in exon 4 of the gene that causes amino acid changes from lysine to glutamine (K121Q), has been reported to be associated with insulin resistance, type 2 diabetes (T2D) and obesity, however with inconsistent results. *In vitro* studies have shown that the Q variant interacts more strongly with the insulin receptor than the K variant and reduces insulin receptor auto-phosphorylation (Pizzuti et al. 1999). Its indirect effects include reduction of insulin receptor substrate phosphorylation, phosphatidylinositol-3 kinase activity and glycogen synthesis. Studies in transgenic mice overexpressing Q allele of *ENPP1* in liver and muscle showed higher levels of glucose intolerance and reduced insulin uptake upon insulin infusion, further highlighting its role in insulin resistance (Maddux et al. 2006). A study by Bouhaha et al., has demonstrated the effects of both *PPARγ* and *ENPP1* polymorphisms in Tunisian diabetic subjects, and concluded that *ENPP1*-121Q confers genetic susceptibility to T2D, while the *PPARγ*-Ala allele protects against obesity (Bouhaha et al. 2008). This polymorphism has been shown to be associated with adverse metabolic outcomes of diabetes and obesity such as impaired glucose homeostasis, metabolic syndrome and cardiovascular disease (Bottcher et al. 2006; Gonzalez-Sanchez et al. 2008; Rieger et al. 2011). An important meta-analysis in European population has reported significant association of K121Q with obesity considering recessive model (Wang et al. 2011). Another meta-analysis study has also established role of the K121Q polymorphism in the *ENPP1* gene as a risk factor for coronary heart disease (CHD), more strongly seen in Caucasian but not in Chinese population (Di et al. 2018). To date, there are few studies that have investigated the association between this polymorphism with PCOS which have yielded inconsistent results (Heinonen

et al. 2004; San Millan et al. 2004; Baba et al. 2007; Shi et al. 2008). Given that our earlier study has strongly associated *PPARγ* polymorphisms with PCOS susceptibility and associated insulin resistance pathophysiology (Shaikh et al. 2013), we planned to investigate the *ENPP1* gene polymorphism in women with PCOS, which is lacking in Indian population. The present study is aimed at exploring the association of K121Q polymorphism with PCOS risk and association with insulin, hyperandrogenemia, and dyslipidemia related traits in Indian women, which have further been classified into lean and obese groups. The study may help identify *ENPP1* polymorphisms as an important genetic marker that may assist in devising new diagnostic and therapeutic strategies in women with PCOS.

MATERIALS & METHODS

Subjects

A total of 185 women with PCOS visiting the Infertility Clinic of the ICMR-National Institute for Research in Reproductive Health (NIRRH), Mumbai, were recruited into the study. PCOS was diagnosed on the basis of the revised Rotterdam criteria and defined as the presence of two or more of the following characteristics: (1) clinical and/or biochemical signs of hyperandrogenism; (2) oligo- or secondary amenorrhea and (3) polycystic ovaries on ultrasound examination. Cases with thyroid disorders, Cushing's syndrome or hyperprolactinemia were excluded. We also enrolled 153 regularly cycling healthy women from the local community or those visiting the clinic due to male infertility as controls. All subjects had not taken hormonal contraceptives or any medications with insulin or lipid lowering effect for at least 3 months prior to enrolment. This study was approved by the Institutional Ethical Committee and informed written consents were obtained from all the recruited women. Genotyping was performed for all study participants and complete phenotyping of study subjects in terms of clinical, hormonal and metabolic parameters were carried out in control women and women with PCOS from whom suitable serum samples were available. Anthropometric data was documented from all participants. Study subjects were categorized as lean (BMI <23 kg/m2) and obese (BMI>23 kg/m2) as per Indian guidelines (Misra et al. 2009) to assess the influence of obesity on association of polymorphism with PCOS and its related traits.

Biochemical and hormonal estimations

Blood was drawn from the controls and oligomenorrheic women with PCOS during the follicular phase of their menstrual cycle (Day 3–Day 7) following an overnight fast, while amenorrheic PCOS women had fasting sample collected as and when available. Fasting serum was used for measuring hormonal and biochemical parameters [follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), total testosterone, insulin and sex hormone binding globulin (SHBG), total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), apolipoprotein A-1 (Apo A-1) and apolipoprotein B (Apo B)] and calculation of LDL values, indices of insulin resistance [homeostatic model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI)], and hyperandrogenism [free testosterone, bioavailable testosterone and free androgen index (FAI)] was carried out as previously described (Mukherjee et al. 2009; Shaikh et al. 2013).

Genotyping

Genomic DNA was extracted from peripheral whole blood by using the QIAamp DNA kit using manufacturer's protocol (QIAGEN Gmbh, Hilden, Germany). Previously published primers (Heinonen et al., 2004) were used to amplify 238 bp fragments containing the polymorphism of interest, namely K121Q. PCR reactions involving initial denaturation of 5 min at 94°C, followed by 35 cycles at 94°C for 40s, at 54°C for 40s, and 72°C for 40s, with a final extension of 10 min at 72°C were performed. Genotyping of all samples were carried out by restriction fragment length polymorphism (RFLP) assay. The PCR amplified products were digested

with restriction enzyme *Ava*II and the digested products were resolved in a 3% agarose gel, stained with ethidium bromide and visualized under the UV light. The nucleotide change from A to C causes a missense mutation in which the corresponding amino acid changes from lysine to glutamine. The 238 bp band corresponded to K allele and 148 and 90 bp bands corresponded to Q allele. Furthermore, presence of all 3 bands directly corresponds to heterozygosity for both alleles (Figure 1). Thirty samples of each genotype were also rechecked and validated by direct sequencing analysis using 3130 Avant Genetic Analyzer (Applied Biosystems) with Big Dye Terminator Chemistry (v. 3.1).

Statistical analysis

The Independent samples t-test was used to compare means of all the continuous variables between PCOS and control women. Chi-square tests were used to compare the genotype and allele frequencies of PCOS and control. Logistic regression analysis was used to test for association of K121Q polymorphism with PCOS risk after age and BMI adjustment. Linear regression analysis was used to evaluate association of K121Q of *ENPP1* gene with PCOS related hormonal and biochemical traits. All statistical analyses were carried out using SPSS statistical software (version 27; SPSS Inc., Chicago, IL) and $P<0.05$ was considered to be statistically significant.

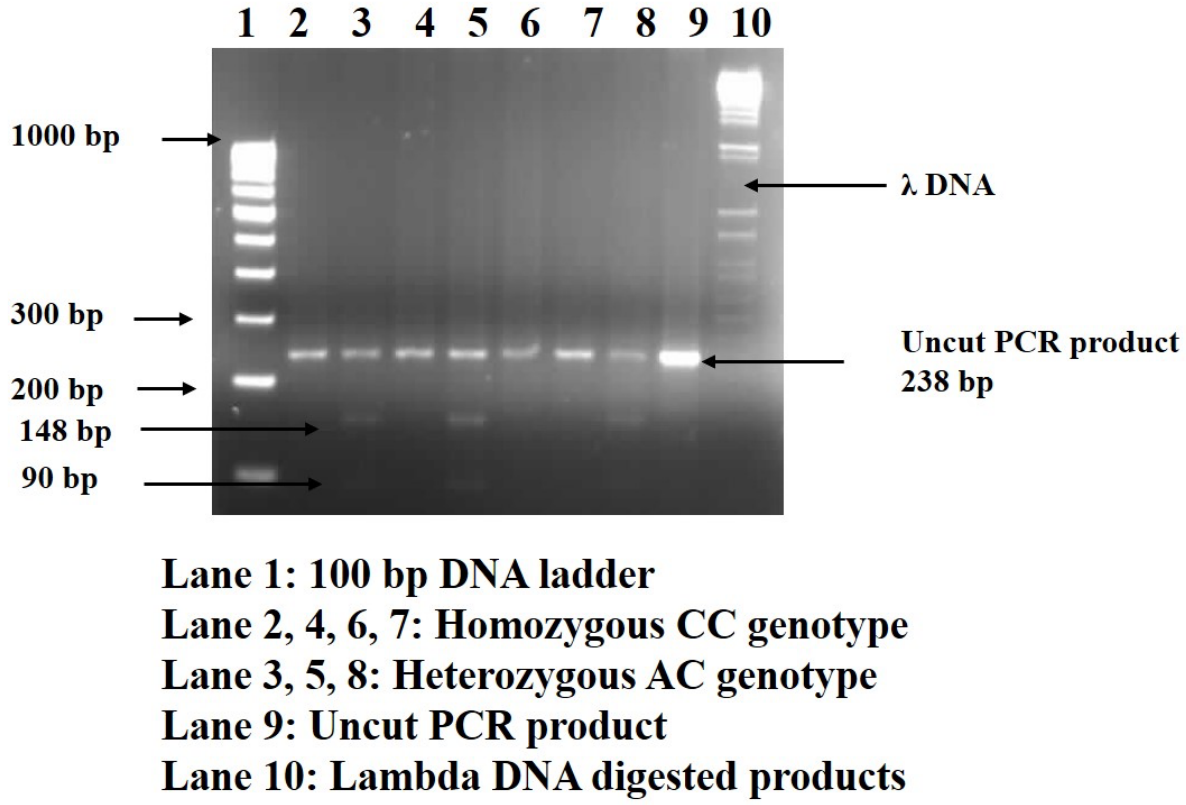

Fig 1. Representative gel image for genotyping of K121Q polymorphism of *ENPP1* by RFLP assay. The image above shows the *Ava*II restriction enzyme digested PCR products analyzed on 3% agarose gel.

RESULTS

Clinical and biochemical characteristics of study subjects

The strong link between insulin resistance and obesity and their simultaneous correlation to PCOS development and progression prompted us to investigate the impact of this polymorphism after classification of study group into lean and obese groups and their clinical, hormonal, and biochemical profiles are compared in Table 1. No significant differences in age, fasting sugar, total cholesterol, HDL-C, ApoA-1 and ApoB levels were observed between controls and PCOS women in both lean and obese groups. Women with PCOS presented with impaired glucose metabolism, increased insulin resistance, hyperandrogenism and hypertriglyceridemia compared to controls regardless of BMI as demonstrated by higher levels of 2-hour glucose, fasting insulin, total, free and bioavailable testosterone, and triglycerides, increased HOMA-IR and FAI along with decreased QUICKI and SHBG levels. Only lean women with PCOS showed significantly higher LDL-C levels while raised ApoB: ApoA-1 ratios were observed only in obese PCOS women compared to corresponding BMI matched controls.

Table 1 Comparison of clinical, hormonal and metabolic characteristics among study participants.

	Lean			Obese		
Variable	Controls (n=51)	PCOS (n=76)	P	Controls (n=32)	PCOS (n=67)	P
Age (years)	23.04±3.85	23.83±4.13	0.280	27.69±5.53	25.75±5.59	0.108
BMI (kg/m^2)	19.07±1.93	20.16±2.05	0.003	26±2.55	28.70±4.39	<0.0001
WHR	0.75±0.05	0.79±0.06	<0.0001	0.78±0.05	0.83±0.06	<0.0001
FBS (mg/dl)	84.84±6.41	86.03±7.60	0.360	88.28±6.51	90.17±9.71	0.256
2h glucose (mg/dl)	88.86±14.65	94.96±17.64	0.043	95.31±16.66	106.64±18.66	0.004
Insulin (µIU/ml)	8.58±4.24	11.05±6.23	0.009	9.45±3.58	17.11±8.84	<0.0001
HOMA-IR	1.80±0.94	2.38±1.43	0.006	2.05±0.78	3.85±2.14	<0.0001
QUICKI	0.36±0.03	0.35±0.03	0.034	0.35±0.02	0.32±0.03	<0.0001
FSH (µU/ml)	7.67±2.70	6.48±2.04	0.006	6.95±1.38	6.56±1.67	0.255
LH (µU/ml)	5.10±1.98	11.71±5.31	<0.0001	4.44±1.69	11.35±6.06	<0.0001
LH:FSH	0.71±0.28	1.96±1.02	<0.0001	0.66±0.27	1.79±0.98	<0.0001
TT (ng/dl)	44.22±20.04	65.80±27.97	<0.0001	43.11±15.31	60.93±25.28	<0.0001
SHBG (nmol/l)	86.16±37.48	75.21±37.43	0.109	86.88±41.44	44.10±29.40	<0.0001
Free-T (pmol/l)	15.68±8.45	26.88±15.03	<0.0001	15.46±7.68	35.55±18.04	<0.0001
Bio-T (nmol/l)	0.37±0.20	0.63±0.35	<0.0001	0.36±0.18	0.85±0.42	<0.0001
FAI	2.15±1.35	3.90±2.62	<0.0001	2.17±1.38	6.29±4.03	<0.0001
Cholesterol (mg/dl)	142.36±25.03	150.85±29.27	0.092	148.69±26.71	152.63±28.09	0.510
HDL-C (mg/dl)	50.96±18.03	48.40±17.09	0.419	47.98±16.82	44.08±15.64	0.260
TG (mg/dl)	67.79±18.60	85.37±38.93	0.001	87.17±26.32	104.57±39.21	0.011
LDL-C (mg/dl)	76.55±21.44	85.38±24.45	0.038	83.28±26.42	86.58±24.22	0.539

ApoA-1 (mg/dl)	114.63±41.34	109.97±44.05	0.550	97.92±30.20	90.41±28.95	0.237
ApoB (mg/dl)	57.31±12.87	61.64±21.14	0.154	61.10±16.67	67.40±25.60	0.146
ApoB: ApoA-1	0.57±0.23	0.62±0.27	0.279	0.65±0.23	0.79±0.30	0.027
Hirsutism	-	49 (64.5)		-	37 (55.2)	
Acne	-	37 (48.7)		-	30 (44.8)	
Acanthosis nigricans	-	17 (22.4)		-	36 (53.7)	
Oligomenorrhea	0 (0)	31 (40.8)		0 (0)	29 (43.3)	
Secondary amenorrhea	0 (0)	41 (53.9)		0 (0)	34 (50.7)	
Regular cycle	51 (100)	4 (5.3)		32 (100)	4 (6)	

Note: Data are represented as mean ± SD or n(%), P= P values obtained by comparison of variables between controls and PCOS by independent samples t-test BMI= body mass index, WHR= waist to hip ratio, FBS= fasting glucose, HOMA-IR= homeostasis model assessment for insulin resistance, QUICKI = quantitative insulin sensitivity check index, TT= total testosterone, Free-T= free testosterone, Bio-T= bioavailable testosterone, SHBG= sex hormone binding globulin, FAI= free androgen index, HDL-C= high density lipoprotein cholesterol, TG= triglycerides, LDL-C= low density lipoprotein, ApoA-1= apolipoprotein A-1, ApoB= apolipoprotein B.

Allelic and genotypic frequencies of K121Q polymorphism of *ENPP1*

We analyzed K121Q polymorphism of *ENPP1* in 185 women with PCOS and 153 controls. The polymorphism distribution in both women with PCOS ($\chi 2$ = 15.47, P= <0.0001) and controls ($\chi 2$ = 9.10, P= 0.003) women were not consistent with Hardy Weinberg Equilibrium. This is mainly due to the absence of the homozygous QQ genotype in our study population in both PCOS and controls. Due to absence of the minor allele homozygotes, comparison was done between wildtype homozygotes (coded as 0) and the heterozygotes (coded as 1) using additive model. We observed no significant difference in genotypic and allelic frequencies in the entire study population and even after classification into lean and obese groups (Table 2). Logistic regression with age and BMI as covariates further revealed no significant association of this polymorphism with PCOS susceptibility, regardless of BMI (Table 2).

Table 2 K121Q genotype and allelic distribution of *ENPP1* in controls and women with PCOS

Genotype	Controls (n=153) n (%)	PCOS (n=185) n (%)	χ^2 (P)	B	S.E	OR 95% C.I	P
KK	93 (60.8)	102 (55.1)	1.095 (0.295)	0.232	0.222	1.261 (0.816-1.949)	0.296[a]
KQ	60 (39.2)	83 (44.9)					
QQ	0 (0)	0 (0)					
K	246 (80.4)	287 (77.6)	0.641 (0.423)				
Q	60 (19.6)	83 (22.4)					

	Lean Controls (n=51) n (%)	Lean PCOS (n=76) n (%)	χ^2 (P)	B	S.E	OR 95% C.I	P
KK	35 (68.6)	51 (67.1)	0.032 (0.857)	0.223	0.413	1.250 (0.556-2.809)	0.590[a]
KQ	16 (31.4)	25 (32.9)					
QQ	0 (0)	0 (0)					
K	86 (84.3)	127 (83.6)	0.026 (0.872)				
Q	16 (15.7)	25 (16.4)					
	Obese Controls (n=32) n(%)	Obese PCOS (n=67) n(%)	χ^2 (P)	B	S.E	OR 95% C.I	P
KK	17 (53.1)	31 (46.3)	0.408 (0.523)	0.077	0.478	1.080 (0.423-2.757)	0.873[a]
KQ	15 (46.9)	36 (53.7)					
QQ	0 (0)	0 (0)					
K	49 (76.6)	98 (73.1)	0.117 (0.732)				
Q	15 (23.4)	36 (26.9)					

Note: P<0.05 is considered significant. B= Estimate, S.E. Standard Error, OR= Odds Ratio, CI= Confidence Interval; [a]= adjusted for age and BMI as covariates; χ^2 values for comparing genotype frequencies were performed by 2x3 analysis; χ^2 values for comparing allelic frequencies were performed by 2x2 analysis.

Genetic influence of K121Q polymorphism on PCOS associated traits

Linear regression analysis with age and BMI adjustment carried out separately in the control and PCOS groups, considering the additive model, revealed that this polymorphism was only significantly associated with decreased total testosterone levels in both lean and obese control women but not in women with PCOS. In women with PCOS, variant genotype carriers showed significant association with diminished LH levels and LH:FSH ratios in lean women with PCOS only. On the other hand, this polymorphism was significantly associated with reduced triglyceride levels only in obese women with PCOS. Strikingly, no association of polymorphism with metabolic traits was observed in controls, or with hyperandrogenic traits in PCOS women, was noted (Tables 3 and 4).

Genetic influence of K121Q *ENPP1* polymorphism on obesity

Given the mounting evidence from previous studies demonstrating relationship of this polymorphism with obesity, we investigated its influence in our study subjects (Table 5). Using logistic regression, we found that when the entire study population was analyzed, the Q allele was associated with increased risk of obesity.

Table 3 Association of K121Q polymorphism of *ENPP1* with PCOS related traits in lean control and PCOS women.

Variable	K121Q					
	Lean Controls (N=51)			Lean PCOS (N=76)		
	KK (N=35)	KQ (N=16)	β (P)	KK (N=51)	KQ (N=25)	β (P)
WHR	0.75±0.05	0.76±0.04	0.092 (0.515)	0.79±0.06	0.79±0.05	-0.027 (0.822)
FBS (mg/dl)	83.89±4.89	86.94±8.71	0.188 (0.184)	86.75±7	84.56±8.67	-0.137 (0.261)
2h glucose (mg/dl)	88.29±16.43	90.13±10.07	0.066 (0.648)	92.80±17.38	99.36±17.69	0.188 (0.117)
Insulin (µIU/ml)	8.74±4.46	8.22±3.82	-0.038 (0.795)	11.27±6.42	10.62±5.93	0.005 (0.966)
HOMA	1.82±1.01	1.74±0.79	-0.027 (0.856)	2.43±1.41	2.28±1.47	0.004 (0.975)
QUICKI	0.36±0.03	0.36±0.03	0.010 (0.945)	0.34±0.03	0.35±0.03	0.051 (0.674)
FSH (µU/ml)	7.71±2.78	7.56±2.62	-0.033 (0.824)	6.49±2.10	6.45±1.96	-0.006 (0.960)
LH (µU/ml)	5.14±1.96	5.01±2.07	-0.053 (0.717)	12.57±4.78	9.94±5.98	-0.261 (0.031)[a]
LH:FSH	0.73±0.29	0.68±0.28	-0.075 (0.610)	2.13±1.01	1.62±0.97	-0.272 (0.024)[a]
TT (ng/ml)	48.58±21.12	34.67±13.66	-0.324 (0.024)[a]	66.92±29.88	63.51±24.00	-0.050 (0.682)
SHBG (nmol/l)	89.39±34.88	79.09±42.98	-0.158 (0.247)	73.28±34.22	79.15±43.76	0.053 (0.667)
Free-T (pmol/l)	16.38±9.01	14.15±7.09	-0.102 (0.469)	27.09±15.09	26.46±15.21	0.015 (0.903)
Bio-T (nmol/l)	0.38±0.21	0.33±0.17	-0.103 (0.464)	0.64±0.35	0.62±0.36	0.015 (0.902)
FAI	2.19±1.39	2.07±1.29	-0.016 (0.908)	3.89±2.49	3.91±2.93	0.037 (0.759)
Cholesterol (mg/dl)	142.28±25.11	142.53±25.67	-0.025 (0.863)	150.42±29.74	151.73±28.89	0.051 (0.672)
HDL-C (mg/dl)	50.45±20.13	52.09±12.76	0.013 (0.928)	48.25±16.48	48.68±18.61	0.059 (0.620)
TG (mg/dl)	67.53±18.09	68.34±20.27	0.003 (0.986)	86.96±41.36	82.14±34	-0.027 (0.825)
LDL-C (mg/dl)	76.44±23.21	76.78±17.64	-0.007 (0.963)	84.77±24.70	86.62±24.39	0.028 (0.804)
ApoA-1 (mg/dl)	116.32±44.69	110.94±33.87	-0.054 (0.710)	113.33±45.49	103.11±40.99	-0.066 (0.583)

ApoB (mg/dl)	55.58±12.38	61.11±13.51	0.170 (0.236)	62.15±22.66	60.60±18.02	-0.017 (0.886)
ApoB:ApoA-1	0.54±0.23	0.63±0.25	0.146 (0.306)	0.60±0.27	0.65±0.28	0.062 (0.611)

Note: The beta coefficients after adjustment for age and BMI are given in the table. The beta coefficients with their directions with P-value for testing their significance given in parentheses are provided above. [a] P<0.05 is considered significant.

Table 4 Association of K121Q polymorphism of *ENPP1* with PCOS related traits in obese control and PCOS women.

Variable	K121Q					
	Obese Controls (N= 32)			Obese PCOS (N=67)		
	KK (N=17)	KQ (N=15)	β (P)	KK (N=31)	KQ (N=36)	β (P)
WHR	0.79±0.03	0.78±0.06	-0.047 (0.770)	0.84±0.05	0.82±0.06	-0.139 (0.281)
FBS (mg/dl)	88.59±6.70	87.93±6.50	-0.130 (0.493)	90.23±9.25	90.12±10.22	0.086 (0.497)
2h glucose (mg/dl)	90.71±13.36	100.53±18.86	0.165 (0.313)	110.71±20.94	103.14±15.94	-0.119 (0.339)
Insulin (µIU/ml)	10.42±4.14	8.34±2.51	-0.292 (0.131)	17.70±9.07	16.61±8.73	0.035 (0.779)
HOMA	2.27±0.89	1.81±0.56	-0.315 (0.102)	3.97±2.15	3.75±2.15	0.057 (0.646)
QUICKI	0.34±0.02	0.35±0.02	0.229 (0.239)	0.32±0.03	0.32±0.03	-0.056 (0.646)
FSH (µU/ml)	7.17±1.43	6.70±1.32	-0.171 (0.379)	6.21±1.41	6.86±1.82	0.234 (0.070)
LH (µU/ml)	4.88±1.85	3.94±1.38	-0.280 (0.144)	10.05±5.28	12.46±6.53	0.144 (0.261)
LH:FSH	0.71±0.32	0.61±0.20	-0.180 (0.361)	1.66±0.92	1.90±1.02	0.058 (0.654)
TT (ng/ml)	48.06±16.80	37.49±11.52	-0.460 (0.010)[a]	54.26±28.25	66.68±21.15	0.190 (0.134)
SHBG (nmol/l)	85.48±39.67	88.47±44.72	0.036 (0.857)	39.39±33.85	48.16±24.73	0.122 (0.334)
Free-T (pmol/l)	17.43±8.44	13.23±6.24	-0.326 (0.091)	35.24±21.43	35.82±14.84	0.014 (0.916)
Bio-T (nmol/l)	0.41±0.20	0.31±0.15	-0.328 (0.089)	0.85±0.50	0.84±0.35	-0.022 (0.867)
FAI	2.41±1.40	1.90±1.34	-0.217 (0.268)	6.70±4.64	5.94±3.44	-0.072 (0.571)
Cholesterol (mg/dl)	153.06±26.08	143.75±27.45	-0.104 (0.583)	154.12±29.04	151.34±27.60	-0.077 (0.559)

HDL-C (mg/dl)	49.20±18.01	46.60±15.88	-0.056 (0.777)	44.50±16.48	43.71±15.10	-0.026 (0.846)
TG (mg/dl)	79.22±29.27	96.18±19.78	0.335 (0.081)	116.75±43.01	94.08±32.69	-0.259 (0.035)[a]
LDL-C (mg/dl)	88.01±28.12	77.92±24.17	-0.136 (0.474)	83.99±27.15	88.81±21.53	0.065 (0.623)
ApoA-1 (mg/dl)	103.23±36.29	91.90±21	-0.138 (0.468)	91.60±32.16	89.39±26.30	-0.031 (0.813)
ApoB (mg/dl)	62.29±15.19	59.75±18.66	-0.098 (0.622)	71.42±24.07	63.95±26.69	-0.148 (0.259)
ApoB:ApoA-1	0.66±0.22	0.64±0.24	-0.122 (0.522)	0.84±0.29	0.75±0.32	-0.148 (0.244)

Note: The beta coefficients after adjustment for age and BMI are given in the table. The beta coefficients with their directions with P-value for testing their significance given in parentheses are provided above. [a] P<0.05 is considered significant.

Table 5 Logistic regression results for association of K121Q polymorphism of *ENPP1* with obesity independently as well as combined control and PCOS groups

Study Group	B	S.E	OR (95% C.I)	P
Total Controls and PCOS	0.801	0.277	2.229 (1.296-3.832)	0.004[a]
Total Controls	0.658	0.465	1.930 (0.775-4.805)	0.158
Total PCOS	0.862	0.346	2.369 (1.203-4.667)	0.013[a]
Total PCOS (adjustment with essential covariates	1.287	0.466	3.623 (1.453-9.037)	0.006[a,b]

Note: [a]P<0.05 is considered significant; [b] indicates p<0.05 after adjustment with covariates in the final logit model as log (π/1−π)=β0+β1 WHR+β2 SHBG+β3ApoA-1+β4 2h glucose+β5 Fasting Insulin; where π is the probability of being affected.

Interestingly, when we separately sought to see its effect on obesity outcome in controls and PCOS women, we found that significance remained only in PCOS group. As BMI may be influenced in turn by many other phenotypic features, we performed stepwise forward regression analysis with all variables in our PCOS study group and found that WHR, SHBG, ApoA-1, 2h glucose and fasting insulin were significant and these were included in another independent logistic regression association test. Analysis revealed that even after adjusting for all the above-mentioned parameters, the K121Q polymorphism showed significant association with obesity ascertained as increased BMI.

DISCUSSION

Several candidate genes related to insulin resistance have been reported to be significantly associated to the risk of PCOS development. In the current study, intriguingly, our population was marked by both, absence of the homozygous K121Q polymorphic genotype and lack of association of this polymorphism with PCOS risk. However, genotype phenotype analysis revealed significant association of polymorphic genotype with reduced testosterone in controls, regardless of BMI and alleviated gonadotropins and triglycerides in lean and obese women with PCOS respectively. Moreover, the association of Q allele with

increasing BMI in women with PCOS highlighted the influence of this polymorphism in determining their propensity towards obesity development. Thus, our study is the first to show that K121Q polymorphism of *ENPP1* showed differential associations with hyperandrogenemia, gonadotropin, and dyslipidemia related parameters in controls and women with PCOS which was further influenced by the underlying obesity status.

Elevated ENPP1 glycoprotein is related to impaired insulin receptor associated tyrosine kinase activity in skeletal muscle, adipose tissue, and cultured skin fibroblasts of insulin resistant subjects (Frittitta et al. 1999). Subsequent study involving suppression of ENPP1 in hepatoma cell culture and ENPP1 knockdown db/db mice has shown marked improvement in insulin-stimulated Akt phosphorylation and improved glucose tolerance (Zhou et al. 2009). On the contrary, adipose tissue from streptozotocin induced diabetic rats and Zucker fatty rats showed increased ENPP1 activity with lower glucose uptake (Sakoda et al. 1999; Barrett et al. 2006; Goldfine et al. 2008). Inconsistent reports on obesity and ENPP1 expression warrants studies to dissect the complex relationship between ENPP1 and obesity, however certain *ENPP1* polymorphisms including the K121Q polymorphism, are associated with the risk of developing obesity suggesting that the relationship between ENPP1 expression and obesity may involve the interaction of both genetic and acquired factors (Goldfine et al. 2008).

ENPP1 was considered as a candidate gene based on its function of suppression of insulin receptor signaling leading to insulin resistance. The *ENPP1* Q variant had a stronger binding affinity to insulin receptor and is a strong inhibitor of insulin-stimulated insulin receptor auto-phosphorylation, consequently leading to markedly reduced downstream insulin stimulation of IRS-1 phosphorylation, phosphatidylinositol 3-kinase activation, and glycogen synthesis compared with the K variant (Costanzo et al. 2001; Goldfine et al. 2008). A global diverse study across three different cohorts has demonstrated the ability of K121Q to predict genetic susceptibility to T2D in South Asians and Caucasians (Abate et al. 2005). It is now well established from several studies that K121Q variants in the *ENPP1* gene increase the susceptibility to many cardiometabolic disorders including obesity (Wan et al. 2006), abdominal obesity, hyperglycemia and glucose homeostasis (Meyre et al. 2007; Stolerman et al. 2008; Maranghi et al. 2013), T2D (Li 2012; Tang et al. 2014) and its related complications (Sortica et al. 2015; Neamati et al. 2017; Gohari-Lasaki et al. 2020), and cardiovascular diseases (Bacci et al. 2011; Sumi et al. 2017). This polymorphism has been found to be associated with T2D in Korean (Lee et al. 2010), Chinese (Li 2012), Italian (Pizzuti et al. 1999; Bacci et al. 2005; Baratta et al. 2008), Iranian (Sharafshah et al. 2018), Ukrainian (Marchenko et al. 2018), and Taiwanese (Hsiao et al. 2016) populations. In contrast, other studies have not been able to replicate this positive association in North Indian (Bhatti et al. 2010), Chinese (Chen et al. 2006; Shi et al. 2011; Zhao et al. 2011), German (Gouni-Berthold et al. 2006), Iranian (Saberi et al. 2011), Korean (Seo et al. 2008), Japanese (Keshavarz et al. 2006), Tunisian (Ezzidi et al. 2009), Moroccan (El Achhab et al. 2009) and Danish (Grarup et al. 2006) populations.

Despite its crucial role in influencing insulin resistance, the *ENPP1* gene has not been studied in much detail in PCOS context and this is, to our knowledge, the first study in Indian women to investigate association of this polymorphism with PCOS and its related traits. In our study we found comparable genotypic as well as allelic frequency distributions of K121Q polymorphism of *ENPP1* between the women with PCOS and controls and failed to find any significant association with risk of PCOS development despite BMI based classification. What is interesting, is the complete absence of the QQ genotype in our study population, which is along similar lines to another T2D study in North Indian population wherein they also reported no subjects with QQ genotype (Bhatti

et al. 2010). Previous studies carried out to investigate the association of this polymorphism with PCOS have been few and inconclusive. An early study strongly implicated the role of this polymorphism in PCOS susceptibility in Finnish population (Heinonen et al. 2004). In contrast, ensuing studies in Japanese (Baba et al. 2007), Chinese (Shi et al. 2008) and Spanish (San Millan et al. 2004) women demonstrated similar distribution of the K121Q polymorphism among PCOS and controls and no association with metabolic or hormonal traits in these women. Saxena et al., reported nominal association of this variant with PCOS in European women, which did not persist on multiple testing comparisons (Saxena et al. 2013). These findings reiterate the impact of ethnic and geographic differences in determining association of polymorphisms with PCOS risk in various populations. A relatively recent study by Pappalardo et al., genotyping both G972R of *IRS-1* and K121Q of *ENPP1* in Italian women showed that although allelic and genotypic frequencies did not differ between women with PCOS and controls, QQ genotype carriers, albeit very rare, were only found in the PCOS group (Pappalardo et al. 2017). Transmission disequilibrium tests (TDT) in family-based association study on T2D genes in families with either one or two daughters having PCOS were also unsuccessful in reporting any such association of this variant with PCOS (Ewens et al. 2010).

Strikingly, the genotype-phenotype association analysis revealed that the K121Q polymorphism of *ENPP1* had beneficial effects on lowering testosterone levels in control women only. Additionally, Q allele carriers show lowered LH levels with concomitant lower LH:FSH ratios in lean, and reduced triglycerides in obese women with PCOS respectively. This finding is unanticipated as Q allele being a gain of function mutation, is expected to be associated with greater suppression of insulin signaling and subsequently higher insulin resistance, which could lead to detrimental effects on gonadotropin, lipid and testosterone levels. Along similar lines, other studies reported association of Q allele with lower triglycerides in European women with PCOS (Saxena et al. 2013; Pappalardo et al. 2017). Furthermore, Italian women with PCOS carrying the Q allele also showed significantly higher FSH levels (Pappalardo et al. 2017), indicating that this polymorphism may have effects on modulating gonadotropin levels, which is still unexplored. Another study has shown that the Q allele had independent and additive effects with obesity on modulating insulin resistance (Frittitta et al. 2001). The observation of protective effects may indicate that this polymorphism may be influenced by other polymorphisms with which it may be in linkage disequilibrium or via other as yet unknown gene-gene interactions and requires further investigation. Interestingly, Pappalardo et al., have shown that Q allele carriers in combination with *IRS-1* Gly972Arg polymorphism wildtype carriers had lower free testosterone, fasting insulin, HOMA-IR and Matsuda index (Pappalardo et al. 2017). Thus, this study substantiates that polymorphisms in different genes may work in tandem, with other and have mixed effects on metabolic and androgen profiles. By contrast, Baba et al., reported no association of this polymorphism with any metabolic or hormonal traits in these women (Baba et al. 2007).

The association of ENPP1 with obesity has been well documented in several studies with contrasting results being reported. Our study highlights that the Q allele may augment predisposition to obesity in women with PCOS only and could be used as a genetic marker to identify at risk women and recommend early interventions for maintaining healthy weight to facilitate favorable treatment outcomes. An early meta-analysis showed significant association of Q allele with increased risk of obesity considering the recessive model in European populations (Wang et al. 2011), while another study showed that *ENPP1* haplotype comprising three alleles including K121Q could contribute to childhood obesity onset (Meyre et al. 2005). Opposing studies have shown association of this polymorphism with obesity in a gender specific

manner, being higher in Turkish males but not female subjects (Tanyolac et al. 2008), while Han Chinese women were at higher risk of obesity and hyperinsulinemia (Wan et al. 2006). In contrast, one study reported lower incidence of obesity in QQ genotype carriers in recessive model (Prudente et al. 2007), whilst some other studies have failed to show any association (Lyon et al. 2006; Peeters et al. 2009; Zhao et al. 2011).

Major limitations of our study include the smaller size; however, our study was sufficiently powered to strongly observe the differential influence of K121Q polymorphism on PCOS related phenotypes affecting testosterone, gonadotropins and the triglyceride levels among the study cohort. Another limitation we observed was the deviation from Hardy Weinberg equilibrium which could possibly be due to lack of recessive genotypes observed in our study population. Our results highlight an indirect influence of *ENPP1* on PCOS related traits which may be due to epistatic relationship with other related genes that may play a strong role in PCOS pathophysiology, but this investigation is beyond the scope of the current study.

CONCLUSION

To the best of our knowledge, this is the first study from India investigating the plausible association between K121Q polymorphism of *ENPP1* with PCOS susceptibility and its related traits. Despite lack of association with risk of PCOS, few traits, particularly related to androgen, gonadotropin and triglyceride levels were differentially associated with controls and PCOS women which in turn were dependent on the underlying obesity and physiological state of the woman. Additionally, we did find strong association of presence of Q allele with increased obesity only in women with PCOS, indicating its role in modulating propensity to obesity in them. Thus, the genetic pathomechanisms of PCOS related traits are deeply influenced by obesity and could be attributed to unique epistatic relationships which are required to be explored further.

Acknowledgements

The authors acknowledge NIRRH and ICMR for providing necessary support (NIRRH/RA/1067/05-2021). The authors are grateful to all the study participants, clinicians, and collaborators. We also thank Ms. Nanda Joshi (DNA sequencing core facility, NIRRH) for her help in DNA sequencing and all technical support from Ms. Sushma Khavale and Ms. Gayatri Shinde for the study.

Author's contribution

The study design was conceived by SM. NS and RD collected the patient samples, processed the samples, carried out all the experiments, performed data interpretation, and wrote the paper. AP is the clinical collaborator on this manuscript who supervised the recruitment of study participants. SM critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript. NS and RD have carried out this work during the tenure of their doctoral studies at ICMR-National Institute for Research in Reproductive Health, Mumbai, India

Conflict of interest

The authors declare that no competing or conflict of interest exists.

Source of Funding

This study was supported partially by the Department of Science and Technology (DST), Government of India [SR/SO/HS-60/2005] and the Indian Council of Medical Research (ICMR).

Declaration of originality

The data represented in this paper is original and appropriate citation has been provided for all sources.

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