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Research article

Assessment of *THADA* gene polymorphisms in a sample of Colombian women with polycystic ovary syndrome: A pilot study

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ABSTRACT

Polycystic ovary syndrome (PCOS) is a multifactorial and polygenic endocrine-metabolic disorder in women of reproductive age. SNPs in the *THADA* gene have been identified as PCOS risk loci. In this study, we evaluated the frequency of five polymorphisms in a sample of Colombian women with PCOS, and their association with clinical and endocrine-metabolic parameters. Forty-nine women with PCOS and forty-nine healthy women were included. Allelic discrimination was performed in the *THADA* gene by iPLEX and the MassARRAY system (Agena Bioscience). Haploview software was conducted to analyze the linkage disequilibrium (LD) and haplotypes of polymorphisms. There was an association between the genotypes TT of rs12468394, CC + AA of rs12468394, and GG of rs6544661 and an increase in the levels of free testosterone. The CC + AA of rs12468394 genotype also was associated with an increase of androstenedione levels. *THADA* gene SNPs were not associated with PCOS risk. There was very strong LD among the SNPs. No significant differences in the frequency of haplotypes between groups were observed. The statistical power of this analysis is low because of the small number of samples analyzed. Additional studies involving large populations of Colombian women with PCOS are needed to verify the role of the *THADA* gene in this disorder.

1. Introduction

Polycystic ovary syndrome (PCOS) is a endocrine disorder with the highest prevalence in women of reproductive age, affecting between 6 and 10% of this population [1]. According to the Rotterdam criteria, PCOS is diagnosed when the woman presents at least two of the following manifestations: anovulation or oligoovulation, clinical or biochemical hyperandrogenism, and polycystic ovarian morphology [2]. PCOS is considered a heterogeneous disorder that affects multiple aspects of women's general health throughout their lives [3]. In addition, reproductive abnormalities [4], insulin resistance and type 2 diabetes [5], coronary heart [6], atherogenic dyslipidemia [7], cerebrovascular morbidity [8], endometrial cancer [9], obesity [10], anxiety, and depression [11] are pathologies associated with the syndrome.

In recent years, genetic and environmental factors have been identified as contributing to the multifactorial etiology of PCOS [12]. In the first genome-wide association study (GWAS) in Chinese population, three PCOS susceptibility loci were identified: 2p16.3 (rs13405728) where the *LHCGR* gene is located ($p_{meta} = 7.55 \times 10–21$, odds ratio (OR) 0.71); 2p21 (rs13429458) where the *THADA* gene is located ($p_{meta} = 1.73 \times 10–23$, OR 0.67); and 9q33.3 (rs2479106) where the *DENND1A* gene is located ($p_{meta} = 8.12 \times 10–19$, OR 1.34) [13]. To date, seven GWAS: two in Han Chinese women [13, 14], two in women of Korean ancestry [15, 16], and three in women of European ancestry [17, 18, 19], have attempted to identify associations in different populations between single nucleotide polymorphisms (SNPs) in candidate genes and PCOS.

Thyroid adenoma-associated gene (*THADA*) is located on chromosome 2p21 between 43,230,836 to 43,596,046 base pairs on chromosome 2 and is expressed in the pancreas, adrenal medulla, thyroid, adrenal gland, adrenal cortex, testis, thymus, small intestine, and stomach [20]. According to the DisGenET database (https://www.disgenet.org/), *THADA* is associated with clinical conditions such as diabetes mellitus (non-insulin-dependent), nasopharyngeal carcinoma, prostate carcinoma, malignant neoplasm of prostate, Crohn's disease, inflammatory bowel diseases, cleft upper lip, and androgenetic alopecia.

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The SNPs rs13429458, rs12478601, rs12468394, rs6544661, and rs11891936 have been associated with PCOS in different studies [21, 22, 23, 24, 25]. Also, the *THADA* gene is associated with dysfunctions in energy metabolism by reducing energy production and increasing the risk of obesity, which increases the susceptibility to PCOS [26]. In turn, the relationship between *THADA* gene and PCOS has been demonstrated by associations of *THADA* SNPs and type 2 diabetes in rs7578597, affecting the function of beta cells in the pancreas [27]; insulin resistance due to energy imbalance in rs13429458 [28,29]; dyslipidemia due to high levels of low-density lipoproteins; risk factor for cardiovascular diseases; and increase in testosterone levels and subtypes that involve hyperandrogenism in rs12468394, rs13429458 and rs12478601 [30, 31].

Therefore, the present pilot study aimed to evaluate the frequency of rs13429458, rs12478601, rs12468394, rs6544661, and rs11891936 polymorphisms in a sample of Colombian women with PCOS, and their association with clinical, endocrine, and metabolic parameters. Taking into account that the SNPs have different levels of genetic variation across different populations worldwide, we selected those variants in the *THADA* gene, due to their high reported frequency in PCOS association studies [13, 20, 23, 30, 32, 33, 34, 35].

2. Materials and methods

2.1. Subjects

A total of 98 unrelated Colombian women with PCOS (n = 49) and without PCOS (n = 49) were included in the study. PCOS and control groups were recruited from the Central East sub-region (Boyacá-Cundinamarca) of the Andean Colombian region, which is characterized by a predominant European contribution [36]. All samples included did not belong to any ethnic minority community. Inclusion of PCOS patients was based on the Rotterdam criteria, whereby diagnosis certification was made when two of the three conditions were met: anovulation, hyperandrogenism, and the presence of polycystic ovaries [37, 38]. In addition to the confirmed diagnosis of PCOS, women who had already started their sexual life and were over 18 years were included. Excluded from the study were women with pelvic inflammatory disease, reproductive failure, or ovarian surgeries.

The inclusion criteria for the control group consisted of healthy women without any endocrine dysfunctions nor any other kind of diseases, normo-ovulatory, and aged between 18-30 years old. Women with chronic pelvic pain during the menstrual cycle, ovarian premature failure, polycystic ovaries, and hormonal disorders (thyroid and prolactin) or surgical history in the reproductive tract were excluded from this group.

This study was conducted following the Declaration of Helsinki, and the protocol was approved by the Ethics Review Committee of the Universidad Pedagógica y Tecnológica de Colombia (Reference number: VIE 06 2019, SGI 2677), and by the Ethics Review Committee of the Universidad de Boyacá (Reference number: 011-2019 CB, 29/03/2019). All participants signed the informed consent to participate in the study.

2.2. Clinical measurements

The clinical evaluation was carried out using an interview and a physical examination. Data from sociodemographic factors, menstrual and obstetric history, presence of PCOS signs and symptoms, family history of polycystic ovaries, endometriosis, family history of breast-ovarian cancer, or other pathologies were obtained from the questionnaire. In the physical examination, data on height and weight were taken to find body mass index (BMI) with the formula BMI = weight (kg)/height (m2). In addition, for both groups (control and PCOS) a transvaginal pelvic ultrasound was performed using a PHILIPS EnVisor M2540 Ultrasound. Data of antral follicle count (AFC) between 2 and 10 mm in size were recorded. The AFC was measured using the internal diameters, where the final value represents the average of the two perpendicular measurements, and ovarian volume. All transvaginal ultrasounds were performed by a single operator.

2.3. Endocrine and metabolic evaluation

Blood samples were obtained between 7:00–9:00 am after a minimum of 12 h of overnight fasting. Blood sampling was performed during the early follicular phase (between 2 and 5 days of the menstrual cycle) for the detection of levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), antimüllerian hormone (AMH), thyroid-stimulating hormone (TSH), estradiol, dehydroepiandrosterone sulfate (DHEAS), androstenedione and free testosterone. Glycosylated hemoglobin, pre, and post-meal insulin, and glucose levels were also measured.

Pre and post insulin levels, estradiol, and TSH were measured using the amplified enzymatic chemiluminescence technique (SIEMENS-IMMULITE, Germany). Using the chemiluminescence technique, the levels of FSH, LH, and DHEAS were measured. AMH and androstenedione levels were measured using the ELISA immunoassay (MyByosource, San Diego CA.USA, MBS2023458, and DiaMetra, Italy, DKO008, respectively). Free testosterone concentrations were measured using the radioimmunoassay (RIA) technique. Plasma glucose levels were measured using the hexokinase method (GLUC3 GLUCOSE HK GEN.3 04404483190, Roche Diagnostics), and glycosylated hemoglobin levels were measured using the HbA1C monoclonal antibody technique (MyByosource, San Diego CA.USA, MBS2031845) according to the manufacturer's instructions.

The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as follows [fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5], and the homeostatic model assessment for insulin sensitivity (HOMA-IS) was calculated as follows 1/[fasting insulin (mIU/L) × fasting glucose (mmol/L)] [39]. The reference values are detailed in Supplementary able 1.

2.4. DNA isolation and genotyping

Total genomic DNA was extracted from peripheral blood samples using Invisorb R Spin Universal Kit (Stratec Molecular) according to the manufacturer's instructions and kept frozen at −20 °C until use. DNA quantification was performed using an EPOCHTM2 Microplate Spectrophotometer (Biotek).

Five polymorphisms of the *THADA* gene (rs13429458, rs12478601, rs12468394, rs6544661, and rs11891936) were studied. The characterization of the SNPs is shown in Supplementary able 2. Allelic discrimination was performed using the iPLEX Assay and the MassARRAY system from Agena Bioscience. The sequences of the primers designed in the Assay Design Suite (ADS) software for each variant were well established, design data (termination chemistry, first forward sequence, first reverse sequence, length of the amplicon, uniplex amplification score, multiplex amplification score, melting temperature for the extension primer, percentage of GC contained in the first extension, address of the extension first, mass of the first extension, sequence of extension first, first allelic variant, mass of the sequence of the first extension + genotype of the first allelic variant, sequence of the first extension + first allelic variant, second allelic variant, mass of the sequence of the first extension + genotype of the second allelic variant, and sequence of the first extension + second allelic variant) is shown in Supplementary able 3. The procedures of genotyping were detailed previously [40, 41, 42]. After the iPLEX reaction, genotypes for each PCOS patient and control were obtained using the Typer software.

2.5. Statistical analysis

Data obtained from the questionnaire, physical, hormonal and genetic examination, were systematized in Microsoft Excel v15.0 and analyzed in IBM SPSS Statistics v21.0 (https://www.ibm.com/support/pages/downl

oading-ibm-spss-statistics-21). The distribution of the variables was tested with the Kolmogorov-Smirnov and Shapiro-Wilk test. Data were expressed as mean ± SD for parametric variables, median (interquartile range) for nonparametric variables. Data were summarized in absolute frequencies and percentages. Quantitative data were compared with the Student's t or non-parametric Mann-Whitney U test, as appropriate. Pearson's chi-square test or Fisher's exact test were used to determine if there was a difference between two or more groups of categorical variables. The P value for a 2-sided analysis was recorded.

Hardy-Weinberg equilibrium and calculations of genotype and allelic associations were carried out using the online tool SNPStats (https://www.snpstats.net/start.htm). The OR and their respective 95% confidence intervals (95% CI) were calculated by contingency tables. Logistic regression analysis was used to test the associations of the *THADA* gene SNPs with PCOS under the five basic inheritance models: codominant, dominant, recessive, overdominant, and additive. The best inheritance model was assessed using the Akaike Information Criteria (AIC) [43]. The model with the lowest value was considered the best fit, and the analysis of genotype-phenotype in the PCOS group was performed using the best model. In cases where there were equal values of AIC, the model that presented the lowest p-value was chosen. The Kruskal-Wallis test and variance analysis (ANOVA) were used to test the hypotheses in more than two independent groups on non-parametric and parametric data, respectively. A value of $p < 0.05$ was considered statistically significant.

Calculation of linkage disequilibrium (D′) and correlation (r2) values between the SNPs was carried out using Haploview software version 4.2 (https://www.broadinstitute.org/haploview), and expressed as D′ and r2 [44]. The relative LD between specific pairs of SNPs is indicated by the color scheme, which represents the LD relationships. Values approaching zero indicate the absence of LD and are shown as shades of pink/red or white, and those approaching 1 indicate complete LD are shown as bright red. LD level was defined like strong LD (D' > 0.8), moderate LD (0.4 < D' ≤ 0.8), and weak LD (D ≤ 0.4) [45]. Haploview was also used to define haplotype blocks and estimate haplotype frequencies (frequency ≥1 % and r2 threshold were 0.8). Haplotype frequencies were compared between cases and controls using chi-square testing.

3. Results

3.1. Study subjects

The clinical and endocrine characteristics of the women included in the analysis are available in Table 1. No significant inter-group differences were recorded for age, height, BMI, menarche, period length, TSH, family history of breast and ovarian cancer, and spontaneous abortion. Compared to controls, women with PCOS had higher weight, menstrual cycle length, AMH, LH, E_2, total ovarian volume, total number of follicles, family history of polycystic ovaries, family history of endometriosis, and early pregnancy loss. The control group had a higher FSH levels and a higher number of pregnancies compared to women with PCOS.

Endocrine-metabolic parameters such as androstenedione, DHEAS, free testosterone, fasting insulin, post-meal insulin, fasting blood glucose, post-meal glucose, HOMA-IR, HOMA-IS, and glycosylated hemoglobin were measured only in women with PCOS and are shown in Table 2. Table 2 also shows the clinical parameters such as acne, hair loss, facial hair, abdominal hair, fatty discharge from scalp and face, acanthosis nigricans, cystic lesion resection, post-coital bleeding, dysmenorrhea, amenorrhea for more than 3 months, and multiple menstrual bleeds in one month, at some point in life of the patients. Contraceptive treatment

Table 1. Clinical and endocrine characteristics of women with polycystic ovary syndrome (PCOS) and control group.

	PCOS (n = 49)	Controls (n = 49)	P-value
Age (years)	28 (24–33)	27 (24–30)	0.448†
Weight (kg)	60.8 (55–74)	60 (52–64)	**0.037**†
Height (m)	1.62 (1.59–1.66)	1.6 (1.56–1.64)	0.064†
BMI (kg/m^2)	23.16 (21.48–25.6)	22.6 (20–24.98)	0.22†
Menarche (years)	13 (12–14)	12 (11.5–14)	0.185†
Menstrual cycle length (days)	31 (29.5–45)	28 (28–30)	**<0.0001**†
Period length (days)	5 (4–8)	5 (4–5)	0.129†
FSH (mUl/ml)	5.95 ± 3.47	9.5 ± 5	**<0.0001**††
AMH (ng/ml)	8.02 (5.07–12.55)	4.87 (3.05–6.77)	**<0.0001**†
LH (mUl/ml)	6.8 (4.55–10.3)	3.2 (2.12–5.17)	**<0.0001**†
LH/FSH ratio	1.27 (0.83–1.74)	0.38 (0.18–0.64)	**<0.0001**†
TSH (mUl/ml)	1.67 (1.29–2.69)	1.65 (1.05–2.47)	0.284†
E_2 (pg/ml)	53.3 (32.72–72.87)	29.7 (15–40.6)	**<0.0001**†
Total ovarian volume (cm^3)	12.25 (9.62–18.75)	7.61 (6.63–9.47)	**<0.0001**†
Total AFC (number of follicles)	27 (23-34,75)	16 (13–20)	**<0.0001**†
FAMILY BACKGROUND			
Family history of polycystic ovaries	22 (44.8%)	6 (12.24%)	**<0.0001**†††
Family history of endometriosis	10 (20.4%)	4 (8.16%)	**0.013**†††
Family history of breast and ovarian cancer	10 (20.4%)	6 (12.24%)	0.196†††
REPRODUCTIVE FEATURES			
Pregnancies	12 (24.48%)	33 (67.34%)	**<0.0001**†††
Early pregnancy loss	8 (16.32%)	2 (4.08%)	**0.045**†††
Spontaneous abortion	7 (14.28%)	2 (4.08%)	0.091†††

Abbreviations: BMI: Body mass index; FSH: Follicle-stimulating hormone; AMH: Antimüllerian hormone; LH: Luteinizing hormone; TSH: Thyroid-Stimulating hormone; E2: Estradiol; AFC: Antral follicular count.

Data in bold indicate statistically significant results ($p < 0.05$).

† Mann-Whitney U-test (nonparametric variables). Data are expressed as median (interquartile range).

†† Student's t-test (parametric variables). Data are expressed as mean ± standard deviation.

††† Fisher's exact test and chi-square test was used for analyzing the associations between categorical variables. P value for a 2-sided analysis was recorded. Data are expressed as a number of cases (percentage).

Table 2. Endocrine-metabolic and clinical characteristics in women with polycystic ovary syndrome.

Endocrine-Metabolic parameters	Value[†]
Androstenedione (ng/ml)	1.49 ± 0.59
DHEAS (ug/dL)	152.8 ± 64.51
Free testosterone (pg/ml)	1.34 (0.91–2.40)
Fasting insulin (uUl/ml)	4.68 (2.62–9.16)
Post meal insulin (uUl/ml)	28.3 (13.1–43.6)
Fasting blood glucose (mg/dL)	83.91 ± 8.51
Post meal glucose (mg/dL)	80.5 (72.5–95)
HOMA-IR	0.84 (0.48–1.95)
HOMA-IS	0.49 (0.02–0.08)
Glycosylated hemoglobin (%)	5.24 (5.01–5.74)
Clinical parameters	**n (%)**[††]
Acne	30 (60%)
Hair loss	43 (86%)
Facial hair	34 (68%)
Abdominal hair	30 (60%)
Fatty discharge from scalp and facial	33 (66%)
Acanthosis nigricans	10 (20%)
Cystic lesion resection	2 (4%)
Menstrual bleeding stopped for more than 3 months	30 (60%)
Multiple menstrual bleeds in one month	25 (50%)
Postcoital bleeding	5 (10%)
Dysmenorrhea	29 (58%)
Contraceptive treatment	17 (34%)
Smoker	9 (18%)
Exercise regularly	28 (56%)
Daily coffee consumption	32 (64%)

Abbreviations: DHEAS: Dehydroepiandrosterone sulfate; HOMA-IR: Homeostasis model Assessment-Insulin resistance; HOMA-IS: Homeostasis model Assessment-Insulin sensitive.

[†] The parametric variables are expressed as mean ± standard deviation, and nonparametric variables are expressed as median (interquartile range).

[††] Data are expressed as a number of cases (percentage).

(Etinilestradiol 0.02 mg + drospirenone 30 mg, one tablet oral administration at the first day of the menstrual cycle during 21 days), smoking, regular exercise, and daily coffee consumption, were also described in women with PCOS (Table 2). It is worth mentioning that there were no significant differences in the clinical, endocrine, and metabolic characteristics between PCOS women with and without contraceptive treatment (Supplementary able 4).

3.2. Genotype and allele frequency distribution

Table 3 summarizes the association between *THADA* SNPs and PCOS in both groups (cases and controls). The genotypic frequencies of the 5 polymorphisms in *THADA* were consistent with the Hardy-Weinberg equilibrium for both groups; non-significant P values suggest that alleles are in equilibrium [46]. Minor allele frequency (MAF) for each variant coincided with those reported by the 1000 Genomes Project (Supplementary able 2). Although the data shown here correspond to a pilot study, the statistical power for each SNP studied was calculated using the Open Epi tool (https://www.openepi.com) (Supplementary able 5).

Supplementary igure 1 illustrates the distribution of the genotypes obtained, which were clustered for all women included in the study. Although not statistically significant differences were observed in genotypes distribution between cases and controls, variants rs1247860 and rs6544661 presented a greater number of heterozygous individuals (TC and GA respectively) concerning the wild-type homozygous genotype (CC ad AA respectively) in both groups. In the rs12468394 a higher frequency of wild-type homozygous genotype CC was observed, while in controls more heterozygous CA were observed. In turn, in the rs13429458 and rs11891936, no woman in the PCOS group presented the genotypes CC and TT, respectively. For the other SNPs, the polymorphic homozygous genotypes were found in at least one woman. We found no statistically significant differences between the PCOS and the control groups according to the inheritance models (Table 4).

3.3. Association between the genotypes and clinical-endocrine-metabolic parameters in PCOS women

Table 5 shows significant associations identified in the genotype-phenotype association analysis. Using a codominant model in rs13429458 no significant differences were found ($p > 0.05$). Employing the additive model in rs12478601, we observed that the homozygous polymorphic genotype TT presented higher levels of free testosterone compared to the TC genotype (2.09 vs 0.96, pg/ml, $p = 0.033$). Similarly, using the overdominant model in rs12468394, we observed differences in free testosterone between the CC + AA and CA genotypes, the CC + AA genotype presented higher levels of free testosterone (1.67 vs 0.96, $p = 0.023$). In this same SNP, the CC + AA genotype presented higher levels of androstenedione compared to the CA genotype (1.66 vs 1.27, $p = 0.03$).

Free testosterone levels also appear differences between the rs6544661 genotypes following the additive model. The AA genotype presented increased levels compared to the AG genotype (1.64 vs 0.94, $p = 0.041$). Similarly, the GG genotype presented increased levels compared to the AG genotype (2.09 vs 0.94, $p = 0.024$). No analysis was performed for rs11891936 because the best inheritance model for this SNP was recessive, and no woman of the PCOS group presented de polymorphic homozygous genotype TT. Supplementary ables 6, 7, 8, and 9 shows all associations between the genotypes of each variant and clinical and endocrine-metabolic parameters in the group of women with PCOS according to the best inheritance model. No associations were observed with insulin-related parameters.

3.4. Linkage disequilibrium and haplotype analysis

Haploview analysis demonstrated strong ($D' > 0.8$) and complete LD ($D = 1$) among the SNPs of the *THADA* gene. One block with all *THADA* SNPs was constructed spanning 189 kb Figure 1 shows the D' and r^2 values.

To assess the combined effects of SNPs in the *THADA* gene, the haplotypic frequency was calculated between cases and controls (Table 6). The distribution of haplotypes was very similar and equivalent in the two groups. Although there were no significant differences, it is worth mentioning that the haplotype CCAAC was the most frequent in both groups. However, the statistical power of this analysis is low because of the small number of samples analyzed.

4. Discussion

This is the first study to explore *THADA* gene variants and their association with PCOS in Colombian women. Although this pilot study includes a low number of samples, which consequently yields a very low statistical power, it represents a first indication of the behavior of these variants in a Colombian sample with PCOS. The findings shown here should be corroborated in later population studies that include sample size and statistical power sufficient to establish associations of these SNPs with clinical, endocrine, and metabolic parameters, as has been established in other populations with PCOS of the world.

Since the identification of the *THADA* gene as a candidate gene in PCOS, several association studies in different populations have been carried out reporting particular results. The first GWAS included 744 PCOS and 780 controls in the first stage and the second stage 2,840 PCOS cases and 5,012 controls from northern Han Chinese (Replication 1). 498

Table 3. Genotypic and allelic frequencies for *THADA* gene variants in polycystic ovary syndrome (PCOS) women and control group.

Variant	Genotype	PCOS frequency (n = 49)	Control frequency (n = 49)	OR (95% CI)	P- value[†]
rs13429458	Genotypes				0.081
	AA	37 (0.76)	41 (0.84)	Reference	
	CA	12 (0.24)	6 (0.12)	2.22 (0.76–6.50)	
	CC	0	2 (0.04)	NC	
	H–W test	1	0.061		
	Alleles				0.651
	A	86 (0.88)	88 (0.9)	Reference	
	C	12 (0.12)	10 (0.1)	1.23 (0.50–2.99)	
rs12478601	Genotypes				0.85
	CC	17 (0.35)	19 (0.39)	Reference	
	TC	22 (0.45)	22 (0.45)	1.12 (0.46–2.70)	
	TT	10 (0.20)	8 (0.16)	1.40 (0.45–4.35)	
	H–W test	0.57	0.76		
	Alleles				0.561
	C	56 (0.57)	60 (0.61)	Reference	
	T	42 (0.43)	38 (0.39)	1.18 (0.67–2.09)	
rs12468394[††]	Genotypes				0.59
	CC	22 (0.47)	21 (0.43)	Reference	
	CA	20 (0.43)	25 (0.51)	0.76 (0.33–1.77)	
	AA	5 (0.1)	3 (0.06)	1.59 (0.34–7.50)	
	H–W test	1	0.32		
	Alleles				0.967
	C	64 (0.68)	67 (0.68)	Reference	
	A	30 (0.32)	31 (0.32)	1.01 (0.55–1.86)	
rs6544661	Genotypes				0.84
	AA	16 (0.33)	18 (0.37)	Reference	
	GA	23 (0.47)	23 (0.47)	1.12 (0.46–2.73)	
	GG	10 (0.20)	8 (0.16)	1.41 (0.45–4.43)	
	H–W test	0.77	1		
	Alleles				0.562
	A	55 (0.56)	59 (0.6)	Reference	
	G	43 (0.44)	39 (0.4)	1.18 (0.67–2.09)	
rs11891936	Genotypes				0.29
	CC	37 (0.76)	38 (0.78)	Reference	
	CT	12 (0.24)	9 (0.18)	1.37 (0.52–3.63)	
	TT	0	2 (0.04)	NC	
	H–W test	1	0.18		
	Alleles				0.83
	C	86 (0.88)	85 (0.87)	Reference	
	T	12 (0.12)	13 (0.13)	0.91 (0.39–2.11)	

Abbreviations: OR (CI 95%): Odds ratio and 95% confidence intervals; H–W test: Hardy-Weinberg equilibrium test; NC: Not calculated.

[†] Pearson's chi-square test, was used to evaluate the association between SNP and groups (PCOS and control).

[††] The genotypes in rs12468394 were not obtained in two PCOS women.

cases and 780 controls from southern and central Han Chinese (Replication 2) were also included. These GWAS showed strong evidence of associations between PCOS and rs13429458 of the *THADA* gene (p_{meta} = 1.73 × 10–23, OR 0.67) [13]. Later studies have reported different results regarding the association of *THADA* gene variants with an increased risk of PCOS [22, 24, 25, 28].

We did not identify any significant statistical difference ($p < 0.05$) between the genotypes of the variants evaluated between the PCOS and control groups. Similarly, previous studies have not identified any association between *THADA* gene variants with the susceptibility of PCOS in European population such as rs1342958 [20,53,54], rs12468394, and rs12478601 [31]. However, in other studies, some variants have been associated with PCOS risk in European cohorts such as rs7563201 [18] [19], rs11891936 [20], rs13429458 [19], and rs12468394 [33] [20].

An association between rs13429458 and PCOS has been found in multiple populations such as women of Western Saudi Arabia [24], the Indian population [22], and Asian women using five genetic random effects models including the allelic, recessive, dominant, homozygous, and heterozygous genetic models [28]. Equally, in Xinjiang Uygur women [23], the Han Chinese population [55], and in the Hainan Chinese population using dominant, and additive genetic models In this last population, the rs12478601 also has been associated with PCOS using dominant model analysis [32].

Using genotype-phenotype association analysis in the PCOS group, we found an increased frequency of the TT genotype of rs12478601, CC + AA genotypes under overdominant inheritance model of rs12468394, and the homozygous polymorphic genotype GG of rs6544661, in patients with a higher level of free testosterone. Also, we observed an increased frequency of the CC + AA genotypes under overdominant inheritance model of rs12468394 in patients with a higher level of androstenedione. A study in the European population likewise revealed an association between the minor allele frequency adenine-A of rs12468394 and the

Table 4. Association between *THADA* SNPs and polycystic ovary syndrome (PCOS) risk under multiple models of inheritance adjusted for age and body mass index.

Variant	Model	Genotype	PCOS frequency (n = 49)	Control frequency (n = 49)	OR (95% CI)	P- value	AIC
rs13429458	Codominant	AA	37 (0.76)	41 (0.84)	Reference	0.11	137.8
		CA	12 (0.24)	6 (0.12)	2.23 (0.75–6.65)		
		CC	0	2 (0.04)	NC		
	Dominant	AA	37 (0.76)	41 (0.84)	Reference	0.3	139.2
		CA + CC	12 (0.24)	8 (0.16)	1.70 (0.62–4.72)		
	Recessive	AA + CA	49 (1)	47 (0.96)	Reference	0.13	137.9
		CC	0	2 (0.04)	NC		
	Overdominant	AA + CC	37 (0.76)	43 (0.88)	Reference	0.12	137.9
		CA	12 (0.24)	6 (0.12)	2.33 (0.78–6.95)		
	Additive				1.25 (0.53–3.00)	0.61	140
rs12478601	Codominant	CC	17 (0.35)	19 (0.39)	Reference	0.89	142
		TC	22 (0.45)	22 (0.45)	1.18 (0.48–2.90)		
		TT	10 (0.20)	8 (0.16)	1.30 (0.41–4.18)		
	Dominant	CC	17 (0.35)	19 (0.39)	Reference	0.65	140.1
		TC + TT	32 (0.65)	30 (0.61)	1.22 (0.53–2.81)		
	Recessive	CC + TC	39 (0.80)	41 (0.84)	Reference	0.75	140.2
		TT	10 (0.20)	8 (0.16)	1.19 (0.41–3.42)		
	Overdominant	CC + TT	27 (0.55)	27 (0.55)	Reference	0.84	140.2
		TC	22 (0.50)	22 (0.50)	1.08 (0.48–2.45)		
	Additive				1.15 (0.65–2.02)	0.63	140
rs12468394	Codominant	CC	22 (0.47)	21 (0.43)	Reference	0.71	138.2
		CA	20 (0.43)	25 (0.51)	0.78 (0.33–1.84)		
		AA	5 (0.10)	3 (0.06)	1.41 (0.28–6.96)		
	Dominant	CC	22 (0.47)	21 (0.43)	Reference	0.7	136.8
		CA + AA	25 (0.53)	28 (0.57)	0.85 (0.37–1.94)		
	Recessive	CC + CA	42 (0.90)	46 (0.94)	Reference	0.55	136.5
		AA	5 (0.10)	3 (0.06)	1.59 (0.34–7.44)		
	Overdominant	CC + AA	27 (0.58)	24 (0.49)	Reference	0.48	136.4
		CA	20 (0.42)	25 (0.51)	0.74 (0.33–1.69)		
	Additive				0.98 (0.51–1.89)	0.96	136.9
rs6544661	Codominant	AA	16 (0.33)	18 (0.37)	Reference	0.88	142
		AG	23 (0.47)	23 (0.47)	1.20 (0.49–2.98)		
		GG	10 (0.20)	8 (0.16)	1.32 (0.41–4.29)		
	Dominant	AA	16 (0.33)	18 (0.37)	Reference	0.63	140
		AG + GG	33 (0.67)	31 (0.63)	1.23 (0.53–2.89)		
	Recessive	AA + AG	39 (0.80)	41 (0.84)	Reference	0.75	140.2
		GG	10 (0.20)	8 (0.16)	1.19 (0.41–3.42)		
	Overdominant	AA + GG	26 (0.53)	26 (0.53)	Reference	0.83	140.2
		AG	23 (0.47)	23 (0.47)	1.09 (0.49–2.47)		
	Additive				1.16 (0.65–2.06)	0.62	140
rs11891936	Codominant	CC	37 (0.76)	38 (0.78)	Reference	0.26	139.6
		CT	12 (0.24)	9 (0.18)	1.35 (0.50–3.65)		
		TT	0	2 (0.04)	NC		
	Dominant	CC	37 (0.76)	38 (0.78)	Reference	0.81	140.2
		CT + TT	12 (0.24)	11 (0.22)	1.13 (0.43–2.92)		
	Recessive	CC + CT	49 (1)	47 (0.96)	Reference	0.13	137.9
		TT	0	2 (0.04)	NC		
	Overdominant	CC + TT	37 (0.76)	40 (0.82)	Reference	0.49	139.8
		CT	12 (0.24)	9 (0.18)	1.42 (0.52–3.82)		
	Additive				0.94 (0.40–2.17)	0.88	140.2

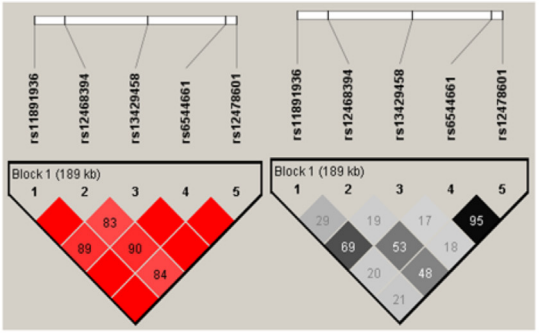
Abbreviations: OR (CI 95%): Odds ratio and 95% confidence intervals; AIC: Akaike Information Criteria.

increase in testosterone levels [31]. Although not in the same SNPs, a similar investigation identified the same association with the AA wild-type genotype of the rs13429458 variant [30].

Haploview analysis demonstrated strong and complete LD among SNPs in the *THADA* gene. Similar results have been found in other studies, where rs7567607, rs13029250, rs13429458, rs7582497, rs7605725, rs6746064, rs12478601 in the *THADA* gene were linked together in all possible combinations with D'>0.6 [32]. It should be noted that although there was a very strong LD, the r^2 values were below 0.8 indicating that possibly the SNPs neither represent each other nor are irreplaceable between them, therefore these variants would add their effects on the *THADA* gene function [56]. Further, it is interesting to mention that the combined rs6544661-rs12478601 variants presented a complete LD (D' = 1) and a strong correlation (r^2 = 0.95), which could

Table 5. Associations between rs12478601, rs12468394, rs6544661, and rs11891936 of *THADA* gene and endocrine-metabolic parameters identified in Colombian women with polycystic ovary syndrome.

Variant	Best inheritance model	Endocrine parameter	Genotypes			P-value
rs12478601	Additive	Free testosterone (pg/ml)	CC	TC	TT	$P_{TC\ and\ TT}$: 0.033
			1.61 (1.17–2.69)	0.96 (0.74–1.84)	2.09 (1.10–3.40)	
rs12468394	Overdominant		CC + AA	CA		
		Androstenedione (ng/ml)	1.66 ± 0.53	1.27 ± 0.63		**0.03**
		Free testosterone (pg/ml)	1.67 (1.11–2.7)	0.96 (0.57–1.74)		**0.023**
rs6544661	Additive		AA	AG	GG	
		Free testosterone (pg/ml)	1.64 (1.24–2.82)	0.94 (0.69–1.79)	2.09 (1.1–3.4)	$P_{AA\ and\ AG}$: **0.041**
						$P_{AG\ and\ GG}$: **0.024**



L1	L2	D'	r²
rs11891936	rs12468394	1	0.29
rs11891937	rs13429458	0.89	0.69
rs11891938	rs6544661	1	0.2
rs11891939	rs12478601	1	0.21
rs12468394	rs13429458	0.83	0.19
rs12468395	rs6544661	0.9	0.53
rs12468396	rs12478601	0.84	0.48
rs13429458	rs6544661	1	0.17
rs13429459	rs12478601	1	0.18
rs6544661	rs12478601	1	0.95

Figure 1. Linkage disequilibrium (LD) map and haplotype block map for all the SNPs of the *THADA* gene. The *THADA* SNPs were analyzed by Haploview.

Table 6. Haplotype frequencies across five *THADA* SNPs analyzed.

Haplotype[a]	Global Frequency	PCOS frequency	Control frequency	$\chi 2$[c]	P-value
Block 1[b]					
CCAAC[d]	0.564	0.528	0.6	1.012	0.3144
CAAGT	0.166	0.162	0.171	0.031	0.8594
CCAGT	0.104	0.124	0.083	0.857	0.3546
TACGT	0.102	0.102	0.102	0	0.9952
TAAGT	0.026	0.021	0.031	0.213	0.6447
CAAAC	0.018	0.033	0.002	2.635	0.1045
CCCGT	0.011	0.021	0.001	1.898	0.1683
CAAGC	0.01	0.01	0.01	0	1

[a] Underlined indicate the minor allele frequency.
[b] *THADA* SNPs within Block 1 haplotypes were: rs11891936, rs12468394, rs13429458, rs6544661, rs12478601.
[c] Two-sides $\chi 2$ test/Fisher's exact tests.
[d] Reference haplotype.

show that these variants would be substitutable among themselves, and therefore, they could add joint effects on PCOS. In agreement with previous studies, we have not found significant differences in the frequency of haplotypes between PCOS and controls, additional genetic factors could influence the risk of this multi-factorial disorder [32].

Although this study could provide evidence of the role of genetic variants in a sample of Colombian women with PCOS, the potential limitations of our study should be mentioned. We had a small sample size and limited power to accurately test for association. Therefore, the associations identified do not correspond to definitive results for our Colombian population. We suggest studying the polymorphisms analyzed here as well as others that have been associated with PCOS in other populations [35]. Thus, we propose to extend this research, by using a larger sample, with adequate power to detect associations for polygenic conditions such as PCOS. Therefore, an effective sample size can be defined as the minimum number of samples that achieves adequate statistical power (e.g., 80% power) [57] Likewise, for future studies, we propose to recruit a large-scale homogeneous cohort without therapy for PCOS to avoid a confounding factor for endocrine and metabolic profiling, and use the Ferriman Gallwey score to assess hirsutism.

5. Conclusion

In this pilot study, no association was observed between the rs13429458, rs12478601, rs12468394, rs6544661, and rs11891936 variants of the *THADA* gene, and PCOS. However, we found associations between endocrine parameters such as increased free testosterone and androstenedione levels and variants of the *THADA* gene in a sample of Colombian women with PCOS. A very strong LD among the SNPs of the *THADA* gene was observed. Due to the small size of the sample, these results cannot be considered definitive. Therefore, it is necessary to

replicate this study in a larger cohort with an adequate power to detect associations for polygenic conditions such as PCOS.

Declarations

Author contribution statement

Maria Camila Alarcón-Granados: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Harold Moreno-Ortíz: Performed the experiments; Analyzed and interpreted the data.

Clara Inés Esteban-Pérez, Atilio Ferrebúz-Cardozo: Performed the experiments.

Gloria Eugenia Camargo-Villalba: Conceived and designed the experiments; Performed the experiments.

Maribel Forero-Castro: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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