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Abstract

Polycystic ovary syndrome (PCOS) is characterised by chronic anovulation, hyperandrogenism, polycystic ovaries, and immunological dysregulation. Immune homeostasis and inflammation management require immune functions. T-regulatory cells (Tregs) regulate PCOS's immune system, inflammation, insulin resistance, ovarian function, and homeostasis. Transforming growth factor βIII (TGF-βIII) and Cytotoxic-T-lymphocyte-associated-protein-4 (CTLA-4) receptors on the surfaces of Treg cells are important for controlling the immune system. The study included 68 PCOS patients and 22 non-PCOS women controls aged 20–45. The waist-hip ratio (WHR) determines obesity. We evaluated serum levels and gene expression of TGF-βIII and CTLA-4 via ELISA and real-time PCR. Women with PCOS had significantly higher TGF-βIII and CTLA-4 levels compared to controls. The study reveals a considerable increase in TGF-βIII and CTLA-4 gene expression in PCOS patients compared to the control. Compared to controls, PCOS aged < and > 25 had higher serum TGF-III and CTLA-4 concentrations. According to the RT-PCR test, the PCOS (> 25) had much higher amounts of TGF-βIII and CTLA-4 gene expression than the control. TGF-βIII and CTLA-4 also increased WHR at < and > 0.8 compared to the control. The RT-PCR results showed that the obese PCOS had much higher levels of TGF-βIII and CTLA-4 gene expression (>0.8) than the control. Based on clinical signs, serum TGF-βIII levels and folding were significantly higher in women with PCOS who had irregular periods compared to women whose cycles were normal. These data indicate a role for TGF-βIII and CTLA-4 in PCOS etiology. This study suggests targeting TGF-βIII and CTLA-4 for PCOS immunotherapy.

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RESEARCH PAPER

The Potential Influence of Immune Modulatory Molecules (TGF-βIII and CTLA-4) in Pathogenicity of PCOS

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Abstract

Polycystic ovary syndrome (PCOS) is characterised by chronic anovulation, hyperandrogenism, polycystic ovaries, and immunological dysregulation. Immune homeostasis and inflammation management require immune functions. T-regulatory cells (Tregs) regulate PCOS's immune system, inflammation, insulin resistance, ovarian function, and homeostasis. Transforming growth factor βIII (TGF-βIII) and Cytotoxic-T-lymphocyte-associated-protein-4 (CTLA-4) receptors on the surfaces of Treg cells are important for controlling the immune system. The study included 68 PCOS patients and 22 non-PCOS women controls aged 20−45. The waist-hip ratio (WHR) determines obesity. We evaluated serum levels and gene expression of TGF-βIII and CTLA-4 via ELISA and real-time PCR. Women with PCOS had significantly higher TGF-βIII and CTLA-4 levels compared to controls. The study reveals a considerable increase in TGF-βIII and CTLA-4 gene expression in PCOS patients compared to the control. Compared to controls, PCOS aged < and >25 had higher serum TGF-III and CTLA-4 concentrations. According to the RT-PCR test, the PCOS (>25) had much higher amounts of TGF-βIII and CTLA-4 gene expression than the control. TGF-βIII and CTLA-4 also increased WHR at < and >0.8 compared to the control. The RT-PCR results showed that the obese PCOS had much higher levels of TGF-βIII and CTLA-4 gene expression (>0.8) than the control. Based on clinical signs, serum TGF-βIII levels and folding were significantly higher in women with PCOS who had irregular periods compared to women whose cycles were normal. These data indicate a role for TGF-βIII and CTLA-4 in PCOS etiology. This study suggests targeting TGF-βIII and CTLA-4 for PCOS immunotherapy.

Keywords: Polycystic ovary syndrome, Inflammation, T regulatory cell, Transforming growth factor β, Cytotoxic T-lymphocyte-associated protein 4

1. Introduction

The intricate interplay of genetic and environmental variables, endocrine hormones, metabolic changes, and immunological systems is believed to have a role in the development of PCOS [1]. Current PCOS treatments focus on treating symptoms due to their unknown aetiology and wide spectrum of clinical manifestations. A definitive cure is still missing. Furthermore, we increasingly understand PCOS as a condition caused by a mismatch between evolution and the modern environment. It presents interconnected metabolic and hormonal symptoms [2,3]. Furthermore, it's critical to remember that the female reproductive tract experiences physiological inflammation during various processes, such as ovulation, menstruation, labor at term, and implantation. However, research suggests that the aetiology of PCOS may potentially involve the development of mild, persistent inflammation [4]. The specific mechanism of inflammation in women with PCOS is unknown; however, chronic low-grade inflammation may contribute to current and future health problems while adopting a Mediterranean diet [5,6]. Studies have revealed that individuals with PCOS show consistently higher levels

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of inflammatory markers in both their serum and ovaries when compared to healthy individuals [7]. Recent studies also suggest that exercise can influence other inflammatory markers in women with PCOS and have a positive effect on reducing C-reactive protein (CRP) levels in these women [8]. The relationship between ovarian dysfunction and inflammatory cytokines suggests that inflammation could be one of the most significant risk factors for PCOS [9]. In PCOS, visceral fat cells affect insulin and glucose processing, similar to how luteinizing hormone (LH) affects theca cells and raises androgens [10,11]. Adipose tissue's immune cells, notably macrophages, release inflammatory substances, intensifying inflammation. PCOS disrupts ovarian function, causing hormone imbalances, elevated androgens, and reduced egg quality. Immune cell imbalances and cytokine disruptions contribute to fertility challenges [12]. Further research on inflammatory mediators in PCOS genesis and development is necessary to understand PCOS biology and establish therapy targets [13]. Previous studies have explained the critical role of immune therapeutic targets in the treatment of PCOS, such as some chemokines, which play significant roles in the promotion, development, and treatment of two reproductive health conditions: PCOS and endometriosis. Chemokine-based therapeutic strategies for PCOS and endometriosis appear promising [14]. PCOS exhibits elevated interleukin-18 (IL-18), complement C3, and insulin levels, indicating inflammation independent of obesity or insulin resistance [15]. During ovulation, menstruation, implantation, and term labour, the female reproductive system experiences physiological inflammation. There is one possible association between this inflammation and the onset of PCOS [16].

Researchers have found that losing adaptive immunity and regulatory T-cell activity can lead to PCOS. This leads to long-term low-grade inflammation, insulin resistance, hyperandrogenism, ovarian dysfunction, and poor immune homeostasis in the ovary [17,18]. Tregs play an important role in immune imbalance in PCOS, affecting inflammation, insulin resistance, ovarian function, and whole-body homeostasis [19]. Due to a problem with Treg function, there are high levels of inflammatory markers that can interact with the ovulatory disorder. This could make immune homeostasis worse. Transforming Growth Factor βIII (TGF-βIII) and Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are two suppressive cytokines that Treg can use to control the immune response [20,21].

Once TGF-β binds to the receptor complex, it can change the strength and length of signals that come after it, which stops an overactive response. TGF-β is a versatile cytokine integral to cell development, differentiation, death, and immune response modulation. It is a member of a large family of growth factors that control many aspects of cellular function in development and homeostasis. TGF-βIII is one of the three isoforms of TGF-β, a multifunctional cytokine. The three isoforms are TGF-βI, TGF-βII, and TGF-βIII. A separate gene encodes each isoform and has distinct but overlapping functions [22]. Like other TGF-β isoforms, TGF-βIII is involved in wound healing and tissue repair. It contributes to scar tissue formation and aids in wound healing. Also, TGF-βIII can stimulate the production of extracellular matrix components, which are essential for maintaining tissue structure and function [23]. TGF-β also contributes to the pathogenesis of osteoarthritis, colorectal cancer, and asthma [24–26]. Like other TGF-β isoforms, TGF-βIII suppresses the immune system and regulates immune cell function. Additionally, TGF-βIII is crucial for Treg formation and activity [21]. Tregs, a subpopulation of T cells, suppress overactive immunological responses and prevent autoimmunity to preserve immune system homeostasis [27]. TGF-III, known for its immunosuppressive properties, aids in Treg induction and maintenance by inducing Treg differentiation and converting naive CD4+ T cells into Tregs. This process occurs during immune responses, and TGF-βIII is a key factor in promoting the differentiation of these Tregs. TGF-βIII is crucial for the maintenance of the suppressive function of Tregs. Additionally, TGF-βIII helps to establish and maintain immune tolerance by supporting the growth and function of Tregs. This makes sure that Tregs keep their ability to suppress immune responses, stopping the activation and proliferation of effector T cells. This is crucial for preventing autoimmune reactions and maintaining self-tolerance [28].

CTLA-4 is a protein receptor that downregulates and checks the immune system. A cancer study shows that Treg cells generate CTLA-4 constitutively but typical T cells only increase it after activation [29]. It acts as an inhibitory mechanism when it links to CD80 or CD86 on the surface of antigen-presenting cells. Previous research findings from mouse models and human studies indicate that decreased Treg production may contribute to PCOS. Also, CTLA-4, which is unique to Treg cells, can bind to CD80 or CD86 on antigen-presenting cells like macrophages better than CD28. CTLA4 stops T-cells from activating by stopping CD28 from interacting, which lowers levels of IL-1b, IL-6, and interferon-g [30]. Conversely, only active T cells express CTLA-4, which transmits an inhibitory

signal [31]. CTLA-4, a Treg surface protein, is required for immune tolerance. It is associated with several autoimmune conditions, including rheumatoid arthritis, type 1 diabetes mellitus, systemic lupus erythematosus, thyroid disease, and breast cancer, among others [32–34]. CTLA-4 controls T-cell immunity by competing with CD80 and CDCD86 to stop immune responses, which is necessary to keep tolerance [12]. Therefore, any defect in this mechanism can result in an overflow of pro-inflammatory cytokine release that may affect ovary function.

Several pathways, such as inflammation, immune tolerance, and immune function, implicate TGF-βIII and CTLA-4 in the pathogenesis of PCOS. These pathways include ovarian dysfunction, immune dysregulation, and metabolic disturbances. Evidence suggests that elevated levels of inflammatory markers interact with ovulatory disorders, leading to infertility in PCOS patients. This study aims to gain a deeper understanding of PCOS pathogenicity by exploring the roles of immune response regulatory molecules like TGF-βIII and CTLA-4. The goal is to identify new therapeutic targets that could potentially halt the disease's progression and severity, or even cure it.

2. Ethics approval and consent to participate

This study adhered to the Helsinki Declaration. The study received approval from the University of Baghdad Ethics Committee and the Baghdad Health Directorate (Date, November 23, 2022, No. 18363). All study participants gave informed consent.

3. Materials and methods

3.1. Subject collection

This study enrolled a total of 90 subjects from August 2022 to June 2023. The University of Baghdad and the Baghdad Health Directorate verified the study's ethical approval. Additionally, we obtained consent from the administration of Al-Kadhimiya Teaching Hospital, several private clinics, laboratories, and each patient after providing a comprehensive explanation of the study's nature and objectives. Sixty-eight PCOS patients from Al-Kadhimiya Teaching Hospital or some private clinics and laboratories was collected, whose ages ranged from 20 to 45 years, and used 22 age-matched healthy women as controls. Using the 2003 Rotterdam criteria, detailed in the 2003 Rotterdam ESHRE/ASRM Consensus [35], a consulting physician verified a PCOS diagnosis. We excluded patients with thyroid, cardiovascular, autoimmune, diabetes mellitus, hypertension, chronic renal failure, and malignant disorders. Furthermore, we excluded participants who had taken any additional drugs within six months after the sample collection, including lipid-lowering, ovulation-stimulating, corticosteroids, antidiabetic, and antihypertensive drugs.

3.2. The waist-to-hip ratio measurement

The waist-to-hip ratio (WHR) assesses the body's fat distribution, specifically around the waist and hip regions. It is a straightforward yet informative indicator of fat distribution. The computation divides the hips, which make up the largest portion of the buttocks, by the waist circumference. We express the formula as WHR = waist circumference/hip circumference. In females, a desirable waist-to-hip ratio is considered to be 0.8. It is noteworthy that women with PCOS had an elevated waist-to-hip ratio of 0.87, indicating the presence of PCOS [36].

3.3. Blood collection

Each patient and control had five millilitres of peripheral blood drawn from them using a disposable syringe. We then divided the blood samples into two parts:

To avoid repeated freeze-thaw cycles, we placed 4 ml in a gel tube for the ELISA study and centrifuged to separate serum into several Eppendorf tubes.

For the molecular study, we added 0.25 mL of blood to 0.5 mL of TRIzol Reagent mixed in a 1.5 mL Eppendorf tube and stored the Eppendorf tube at −20° C for further analysis.

3.4. Enzyme-linked immunosorbent assay (ELISA)

This test uses the "Double Antibody Sandwich" technique (ELISA Kits, MyBioSource, USA). It involves quantifying the concentrations of TGF-βIII and CTLA-4 in the samples. This is achieved by comparing the samples' optical density (O.D.) to a standard curve that has been calculated. To ensure accuracy and consistency, all standards, samples, and reagents were meticulously prepared per the test preparation guidelines provided in the kit leaflet.

3.5. The primers of real-time polymerase chain reaction (RT-PCR)

Table 1 demonstrates the dissolution of primers in nuclease-free water to 100 pmol/l and their storage at −20C. A 10 pmol/μl primer solution was created

Table 1. The primers used in RT-PCR.

Primer Name	Sequence 5′-3′	Ta (°C)	Product size	Ref
TGF-βIII - F	CTGGATTGTGGTTCCATGCA	60	122 bp	[37]
TGF-βIII - R	TCCCCGAATGCCTCACAT			
CTLA-4 - F	CTTCAGTCACCTGGCTGTCA	60	219 bp	[38]
CTLA-4 - R	CTCAGCTGAACCTGGCTACC			

by adding 10 μl of primer stock solution to 90 μl of nuclease-free water. Macrogen supplied all the lyophilized primers.

3.6. Extraction and determination of RNA

The protocol for the TRIzolTM Reagent (Thermo Scientific, USA) guided the isolation of RNA. Quantum fluorometers are used to determine the quality of extracted RNA for downstream applications.

3.7. RT-PCR and gene expression

The specific components of the one-step RT-PCR kit used in this study are as follows: "5 μl of qPCR Master Mix, 0.25 μl of R.T. mix, 0.25 μl of MgCl2, 0.5 μl of forward primer, 0.5 μl of reverse primer, 2.5 μl of nuclease-free water, and 1 μl of RNA, for a total volume of 10 μl. For a single reaction, combine 1 l of the template with 9 l of the Master Mix and add it to an aliquot. We then tested it using real-time PCR. The first stage was a preliminary denaturation at 95 °C for 5 min and a 15-min cycle at 37 °C for cDNA synthesis." In Step 2, the template was denatured at 95 °C for 20 s (A), the primers were annealed to the template at 60 °C for 20 s for TGF-βIII, 65 °C for CTLA-4, and 72 °C for β-globin, and finally the primers were stretched at 72 °C for 20 s. Step three involved heating the green material from 72 to 95 °C. This study used the Livak Method to determine the levels of gene expression.

$$\text{Folding} = 2\text{-}\Delta\Delta CT$$

$$\Delta CT = \text{C.T. gene - C.T. House Keeping gene.}$$

$$\Delta\Delta CT = \Delta CT \text{ Treated or Control - } \Delta CT \text{ Control.}$$

3.8. Statistical analysis

This study analysed the collected data results using the GraphPad Prism (10) programme, applying an independent T-test and one-way ANOVA as appropriate. We performed a chi-square (correlation coefficient) test to determine the presence of a correlation between the studied parameters. The data were presented as mean ± S.E., while significant differences were considered at $p \leq 0.05$. To find the "cut-off value" of optimal sensitivity and specificity for disease diagnosis, we utilised the Receiver Operating Characteristic (ROC) curve technique, which allows us to evaluate the utility of any parameter as a screening or diagnostic tool.

4. Results

Table 2 and Fig. 1 present the mean levels of serum concentration and gene expression parameters in the PCOS and control groups. The study found that the levels of TGF-βIII and CTLA-4 were significantly higher in people with PCOS (466.06 ± 25.09 pg/mL vs. 821.72 ± 33.50 pg/mL) compared to people who did not have PCOS (159.18 ± 6.52 pg/mL vs. 320.41 ± 22.10), with a difference that was statistically significant ($P < 0.0001$). In RT-PCR findings, the TGF-βIII gene expression level (fold change) indicated a notable upregulation in the PCOS group (3.61 ± 0.60 folds) as opposed to the control group (1.16 ± 0.30) with a significant difference at a P value of 0.001. Similarly, the CTLA-4 gene expression level (fold change) results showed a significant increase in PCOS (3.27 ± 0.66 folds) compared to the control group (1.11 ± 0.15), with a P value of 0.003.

In Table 3 and Fig. 2, the serum levels of TGF-βIII and CTLA-4 in PCOS were examined concerning age, revealing a substantial increase at both < and >25 years of age (426.50 ± 35.64 & 508.22 ± 35.70) for

Table 2. Serum level of TGF-βIII and CTLA-4 in PCOS and control.

ELISA	Control (n = 22)	PCOS (n = 68)	P value
TGF-βIII (pg/mL) Mean ± S.E	159.18 ± 6.52	466.06 ± 25.09	<0.0001**
CTLA-4 (pg/mL) Mean ± S.E	320.41 ± 22.10	821.72 ± 33.50	<0.0001**
Gene expression	**Control (n = 20)**	**PCOS (n = 35)**	**P value**
TGF-βIII Folding (Mean ± S.E)	1.16 ± 0.30	3.61 ± 0.60	0.001**
CTLA-4 Folding (Mean ± S.E)	1.11 ± 0.15	3.27 ± 0.66	0.003**

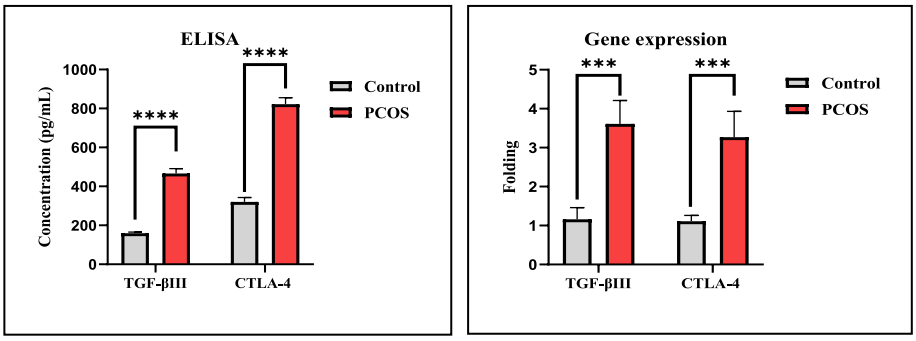

Fig. 1. Serum levels and gene expression of TGF-βIII & CTLA-4 in PCOS and control.

Table 3. The distribution of (TGF-βIII & CTLA-4) serum level and gene expression according to age in understudied groups.

Age					
	Control (Mean ± S.E)		*PCOS* (Mean ± S.E)		
ELISA	≤25	>25	≤25	>25	P value
TGF-βIII concentration	155.88 ± 7.90	170.40 ± 9.50	426.50 ± 35.64[a]	508.22 ± 35.70[a]	<0.0001**
CTLA-4 concentration	328.82 ± 26.92	291.80 ± 34.11	816.53 ± 50.72[a]	839.36 ± 47.42[a]	<0.0001**
Gene Expression	≤25	>25	≤25	>25	P value
TGF-βIII Folding	1.41 ± 0.43	0.79 ± 0.38	3.23 ± 0.48	4.19 ± 1.35[a]	0.07 NS
CTLA-4 Folding	1.31 ± 0.23	0.83 ± 0.11	2.72 ± 0.48	4.10 ± 1.49[a]	0.127 NS

[a] Significant difference vs. control > and <25.

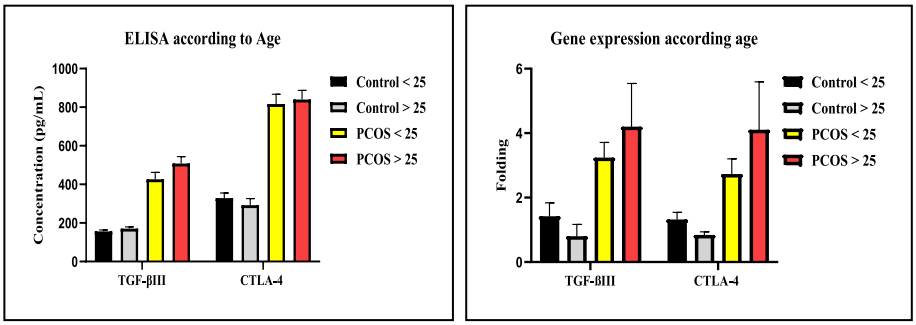

Fig. 2. Serum level gene expression of TGF-βIII & CTLA-4 in PCOS and control according to age.

TGF-βIII and (816.53 ± 50.72 & 839.36 ± 47.42) for CTLA-4, respectively, compared to the control group (155.88 ± 7.90 & 170.40 ± 9.50 for TGF-βIII) and (328.82 ± 26.92 & 291.80 ± 34.11 for CTLA-4). In the RT-PCR results, the TGF-βIII and CTLA-4 gene expression levels (fold change) demonstrated a significant upregulation in the PCOS (>25) group (4.19 ± 1.35 & 4.10 ± 1.49) when compared to the control < and >25 groups (1.41 ± 0.43 & 0.79 ± 0.38 for TGF-βIII, respectively) and (1.31 ± 0.23 & 0.83 ± 0.11 for CTLA-4, respectively).

According to WHR, revealing a substantial increase at both < and >0.8 of WHR (384.67 ± 69.65 & 473.94 ± 26.61 for TGF-βIII) and (940.33 ± 129.75 & 810.24 ± 34.55 for CTLA-4), respectively, compared to the control group (162.20 ± 6.79 & 129.00 ± 6.00 for TGF-βIII) and (323.50 ± 24.16 & 289.50 ± 30.50 for CTLA-4). According to RT-PCR results, the TGF-βIII and CTLA-4 gene expression levels (fold change) recorded a significant upregulation in the PCOS (>0.8) obese group (3.83 ± 0.65 and 3.30 ± 0.72, respectively) when compared with the >0.8 obese control group (1.23 ± 0.53 and 1.43 ± 0.71, respectively). Table 4 and Fig. 3 present this result.

Table 5 and Fig. 4 of the ELISA and RT-PCR study present the demographic characteristics of PCOS, based on data from PCOS patients. It includes the menstrual cycle, disease duration (years), and family history of PCOS. The statistical analysis showed that the TGF-βIII serum level and folding were

Table 4. The distribution of (TGF-βIII & CTLA-4) serum level and gene expression according to WHR in understudied groups.

WHR					
	Control (Mean ± S.E)		*PCOS* (Mean ± S.E)		
ELISA	≤0.8	>0.8	≤0.8	>0.8	P value
TGF-βIII concentration	162.20 ± 6.79	129.00 ± 6.00	384.67 ± 69.65[a]	473.94 ± 26.61[a]	<0.0001**
CTLA-4 concentration	323.50 ± 24.16	289.50 ± 30.50	940.33 ± 129.75[a]	810.24 ± 34.55[a]	<0.0001**
Gene Expression	≤0.8	>0.8	≤0.8	>0.8	P value
TGF-βIII Folding	1.15 ± 0.34	1.23 ± 0.53	1.35 ± 0.58	3.83 ± 0.65[b]	0.05*
CTLA-4 Folding	1.07 ± 0.16	1.43 ± 0.71	2.91 ± 1.10	3.30 ± 0.72[b]	0.05*

[a] Significant differences vs control > and <0.8.
[b] Significant differences vs control >0.8 (p = 0.05).

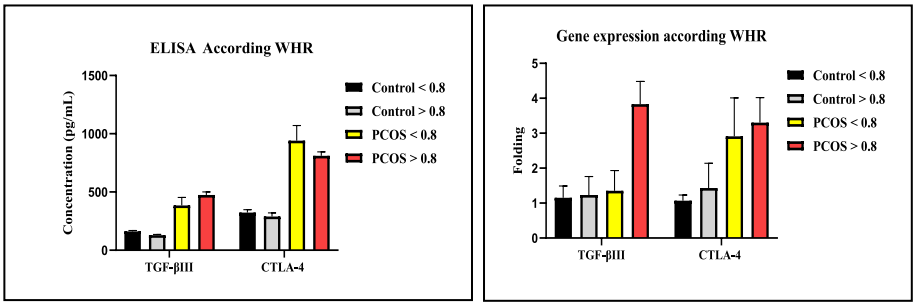

Fig. 3. Serum level gene expression of TGF-βIII & CTLA-4 in PCOS and control according to WHR.

Table 5. Baseline and Clinical Characteristics Parameters in Patients with PCOS of ELISA study.

ELISA	Menstrual cycle (Mean ± S.E)<		Disease duration (Mean ± S.E)		History in family (Mean ± S.E)	
	Regular	Irregular	≤1Yrs	>1Yrs.	No	Yes
TGF-βIII	255.75 ± 76.28	479.20 ± 25.44	431.32 ± 37.0	500.79 ± 33.36	470.96 ± 27.52	447.14 ± 61.74
P value	<0.05		0.114		0.664	
CTLA-4	604.00 ± 124.36	835.33 ± 34.23	778.24 ± 38.70	865.21 ± 54.26	823.07 ± 37.37	816.50 ± 78.25
P value	0.068		0.146		0.93	
Gene expression	Regular	Irregular	≤1Yrs	>1Yrs.	No	Yes
TGF-βIII Folding	0.46 ± 0.43	3.81 ± 0.63	3.89 ± 0.98	3.33 ± 0.72	3.91 ± 0.71	2.18 ± 0.69
P value	<0.05		0.596		0.214	
CTLA-4 Folding	3.68 ± 3.02	3.68 ± 3.02	4.21 ± 1.17	2.27 ± 0.49	1.88 ± 0.99	3.56 ± 0.76
P value	0.857		0.820		0.261	

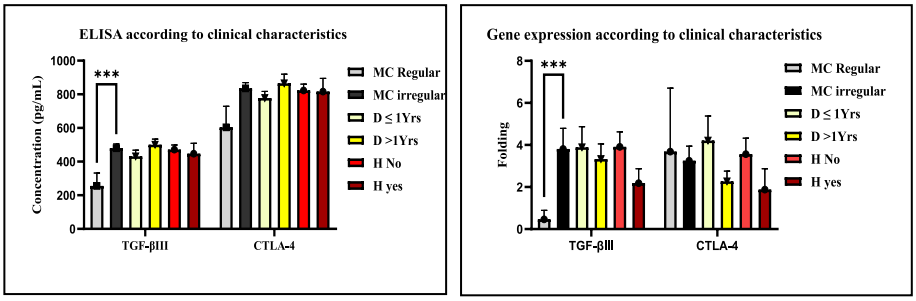

Fig. 4. Serum level gene expression of TGF-βIII & CTLA-4 in PCOS according to clinical characteristics of PCOS (MS: Menstrual cycle, D: Duration and H: History).

significantly higher in women with PCOS who had irregular periods compared to women with regular periods. Other demographic factors did not show any significant differences.

According to the Pearson correlation coefficient, there has been a significant positive correlation between TGF-III and CTLA-4 levels at r = 0.760 in PCOS, and a weak positive correlation between TGF-III and CTLA-4 folding at r = 0.305. Table 6 illustrates the results.

Tis study examined the effectiveness of TGF-III and CTLA-4 levels and gene expression as biomarkers for PCOS prediction using ROC analysis in PCOS patients and healthy controls. The results are in Table 7 and Figs. 5 and 6. The results showed that the area under the curve (AUC) for TGF-βIII levels was 0.96 for people with PCOS and 97% for healthy controls. The specificity and sensitivity were also very high, at 87% and 89%, respectively. These AUC values determined the best cut-off metrics. The TGF-βIII and CTLA-4 genes' area under the curve (AUC) values were 0.746 and 0.691, respectively, indicating a fair level of accuracy. The specificity values were 80% and 99%, and the sensitivity values were 63% and 48%. The best cut-off values were determined by comparing the AUC values in patients with PCOS to the control group, which were 1.8839 and 2.44, respectively.

Table 6. Correlation of studied parameters.

Parameter	Pearson Correlation coefficient	P-value
TGF-βIII and CTLA-4 level	0.760**	0.0001
TGF- βIII and CTLA-4 Folding	0.305*	0.03
TGF- βIII folding and TGF-βIII level	0.116	0.422
TGF- βIII folding and CTLA-4 level	0.067	0.646
CTLA-4 folding and CTLA-4 level	0.093	0.523
CTLA-4 folding and TGF-βIII level	0.149	0.3

5. Discussion

PCOS is often associated with low-grade inflammation and altered immune function [39]. This study revealed, as illustrated in Table 2 and Fig. 1, a significant increase in the serum level of TGF-III in PCOS patients compared to the control, and a significant upregulation of TGF-III in patients. This study is considered the first to explain the role of TGF-βIII in PCOS. In various organs, deregulation of TGF-III signaling is associated with fibrotic diseases, where excessive TGF-III activity can lead to abnormal tissue scarring and fibrosis [40]. Earlier research has demonstrated that fibrillins are crucial in the extracellular matrix (ECM) and PCOS pathogenesis. Mutations in fibrillins lead to TGF-b dysregulation, which in turn leads to TGF-β dysregulation. This dysregulation may play a role in

Table 7. ROC curve results for all studied parameters in PCOS compared with controls.

Parameter	AUC	Cut-off	Sensitivity %	Specificity %	Confidence interval (95%)	P Value
TGF- βIII	0.95	207	87	97	0.90–0.99	<0.0001
CTLA-4	0.96	525.5	89	98	0.93–1.00	<0.0001
TGF- βIII gene	0.746	1.8839	63	80	0.60–0.88	0.006
CTLA-4 gene	0.691	2.44	48	99	0.55–0.83	0.03

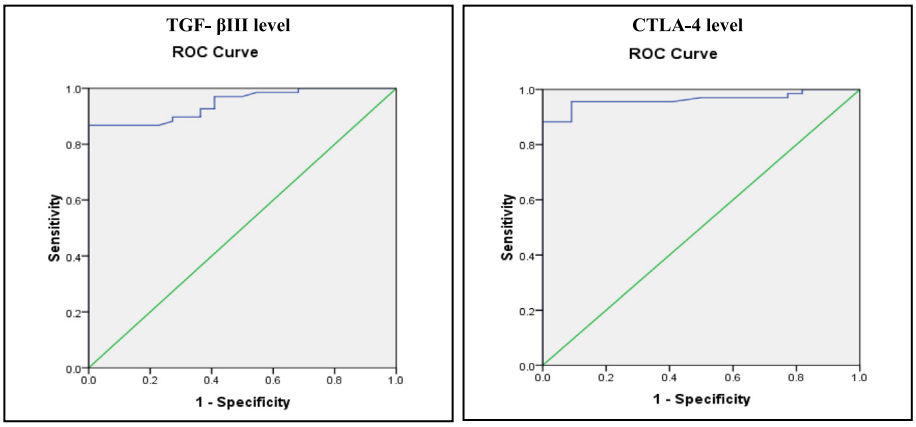

Fig. 5. The (ROC Curve) for TGF- βIII and CTLA-4 levels in patients and control groups, shows cut-off value, sensitivity, specificity, and area under the curve (AUC).

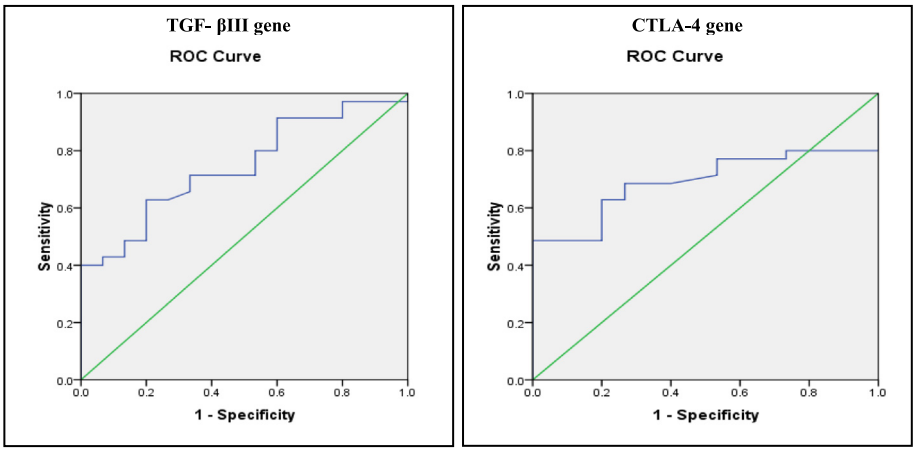

Fig. 6. The (ROC Curve) for TGF- βIII and CTLA-4 genes in patients and control groups, shows cut-off value, sensitivity, specificity, and area under the curve (AUC).

(1) PCOS's fetal roots, (2) reproductive problems, and (3) cardiovascular and metabolic abnormalities. Furthermore, PCOS is characterised by a dysregulation of TGF-β, as well as changes in Follicle-stimulating hormone (FSH) decrease and LH increase, leading to hyperandrogenism. This condition heightens mechanical stress and contributes to PCOS pathogenesis [41,42]. Previous studies showed that the level of TGF-β1 was increasing in PCOS patients' sera and ovaries compared to non-PCOS women [43,44]. TGF-β family members, such as TGF-βIII, play a role in ovarian function and folliculogenesis [45]. PCOS is marked by problems with follicular development [46]. As a study by Ref. [23] mentioned, TGF-βIII involves wound healing and tissue repair. Since TGF-III aids in the formation of scar tissue and aids in wound resolution, we propose that its increased expression plays a crucial role in tissue healing. This, in turn, could alter the architecture or fibrosis of the ovaries, potentially impacting their function, a topic that warrants further investigation. Based on this study, increasing TGF-βIII may have an effect on ovarian dysfunction in PCOS through immune-related pathways. The current study suggests that increases in TGF-III could potentially modulate inflammation and metabolic dysfunction observed in some PCOS cases [47,48]. Another study showed increased TGF-beta in serum levels with PCOS, suggesting an association between PCOS and chronic inflammation [49]. This study suggests that the higher TGF-βIII gene expression and serum level in PCOS are caused by symptoms that may cause low-level inflammation and changes in how the immune system works [50]. TGF-βIII may play two roles in inflammation: it may help reduce inflammation in the early stages so it goes away, but it may also play a part in making inflammation worse when it lasts for a long time [28]. TGF-βIII has been implicated in regulating immune cell differentiation, influencing the development of T cells, B cells, and other immune cell lineages [51]. This substance, TGF-βIII, is known to have immunomodulatory properties [28]. It is very important for stopping autoimmunity and overactive immune responses by changing naive T cells into Tregs. Tregs are essential in suppressing excessive immune responses and maintaining immune tolerance. Forkhead box protein P3 (Foxp3) is a transcription factor that is important for Treg development and function [28]. TGF-βIII helps to make Foxp3. PCOS is often associated with low-grade inflammation and altered immune function [50], so TGF-βIII could potentially influence the immune response in women with PCOS. Altered TGF-βIII levels or their signalling pathways might contribute to the immune dysregulation observed in some individuals with PCOS. In other words, TGF-βIII can exhibit a pro-inflammatory effect. The effects of TGF-βIII can vary depending on the cellular microenvironment and the presence of other signalling molecules. TGF-βIII signalling can sometimes interact with other signalling pathways or mediators that cause inflammation, which can change the way pro-inflammatory cytokines are made [52]. The current study showed a highly significant elevated serum level of CTLA-4 and upregulation of gene expression in patients versus controls. A study [53] found that genetic variation led to a decrease in CTLA-4 gene expression. This made the researchers think that PCOS is associated with a higher risk of developing abdominal fat tissue and a low baseline of inflammation. These findings provide more evidence that CTLA4 is associated with obesity. These findings show that

CD4+ T cells' metabolism changes in a way that leads to more glycolysis and differentiation into Th2 cells, both of which are very important in PCOS [54]. When CTLA-4 is overexpressed or hyperactive, it can lead to immune suppression and tolerance of self-antigens. So, it can contribute to developing autoimmune diseases, where the immune system mistakenly attacks the body's tissues [55]. A study by Ref. [56] demonstrated that soluble CTLA-4 levels were elevated in patients with untreated rheumatoid arthritis, as a greater concentration of sCTLA-4 was detected in these individuals. Since T cells have the most impact on R.A. pathogenesis, it makes sense that if CTLA-4 is involved in R.A. pathogenesis, it is by blocking B7, which causes T cells to become overactive and react inappropriately. For example, a different study [57] found that soluble CTLA-4 levels were rising in people with Systemic Lupus Erythematosus (SLE). One important part of how SLE affects the immune system is that T-lymphocyte stimulation is out of whack, which leads to the production of soluble T-cell costimulatory molecules that are not working properly. The concentration of CTLA in plasma may serve as a surrogate indicator of the severity of SLE disease [57]. A study conducted by Ref. [58] found that there was an upregulation of CTLA-4 expression in CD4+ T cells (but not CD8+ T cells) and an increase in Th17 cells in individuals with asthma. These findings indicate that there is an activation of regulatory T cells (Tregs) to inhibit inflammation associated with asthma. The present study is the first to report a statistically significant association between PCOS patients, serum CTLA4, and gene expression of CTLA-4. However, the role of CTLA-4 in PCOS development is not clearly defined. CTLA-4 likely plays a vital role in regulating the immune system, and its impact may vary based on the activation status of the cells. sCTLA-4 has the ability to suppress CD80/CD86-CD28 interactions in inactive cells, thereby disrupting T-cell activation. Inhibition of CD80/CD86-CTLA-4 interactions on activated T-cells, specifically when the transmembrane version of CTLA-4 is present, could potentially stop the decrease in T-cell responses, which might play a role in PCOS development [59]. Higher levels of CTLA are believed to maintain T cell activation, perhaps causing autoreactivity [56,60]. Another suggestion suggests that the high expression of the CTLA4 gene may be attributed to the high percentage of T cells resulting from low-grade chronic inflammation in PCOS patients. Furthermore, CTLA molecules lack the ability to regulate the immune response. In the current study, the gene expression results agreed with the ELISA test. This increase may contribute to the polymorphism of CTLA-4 in PCOS patients, and it may produce an inactive form. However, this conclusion needs further studies to confirm it.

Regarding age (TGF-βIII and CTLA-4) gene expression, our results demonstrated a significant upregulation in the PCOS (>25) versus control < and >25 groups. Elevated TGF-βIII indicates inflammation in females in reproductive ages, especially the ovary, impacted before any other organ system; this can cause infertility, miscarriages, congenital defects, and systemic deterioration due to estrogen depletion [61]. As women with PCOS age, hormonal and metabolic changes, such as a decline in ovarian function and changes in sex hormone levels, can influence the presentation of PCOS symptoms [62]. It has been shown that chronic low-grade inflammation is associated with PCOS, and age-related changes may contribute to the inflammatory state in individuals with PCOS [63]. Therefore, upregulation of TGF-βIII and CTLA-4, particularly in patients with PCOS, may indicate an inflammatory response, especially in patients >25 years of age. Age is a major and important factor in PCOS syndrome, so this study suggests that as the disease increases, it may progress due to several factors, including lifestyle, type of nutrition, and the psychological state of patients. It is known that PCOS patients are exposed to psychological stress due to not having children, which increases the level of corticosteroid secretion, which weakens the functioning of the immune system, making them vulnerable to infection with many pathogens, and thus increases the level of inflammatory mediators that may affect ovarian function. Despite the statistical analysis's lack of significant impact on the disease's duration, it remains a crucial factor in the disease's progression mechanism. As patients age, their chances of conceiving decrease, leading to an increase in psychological pressure. This, in turn, triggers the symptoms we previously discussed in relation to the age factor.

Regarding WHR, the result of CTLA-4 in both ELISA and gene expression recorded a significant increase in patients (<0.8 and >0.8) versus control. Treg expresses CTLA-4 constitutively or after T-cell activation via CD28 and TCR signaling. PCOS is characterised by increasing fat tissue weight and minimal inflammation.; there is an association between increased weight gain and inflammation [64]. In conditions of well-established systemic inflammation, Tregs perform their functions [65], so increases in CTLA-4 are a mean increase in Treg [66]. According to research [67], the Tregs promote insulin resistance and obesity through a Blimp-1/IL-10 axis, suggesting that CTLA4 regulates T-cell

activation, which could have a role in the metabolic pathways that lead to obesity and insulin resistance. Also, the statistical analysis shows an increase in TGF-βIII levels in < and >0.8 in PCOS compared to the control. However, in gene expression, the study found upregulation of TGF-βIII in obese PCOS compared to the control. Obesity is a distinctive feature of women with polycystic ovary syndrome [68]. Previous research has shown that TGF-β plays a similar molecular role in the inflammation of lung diseases linked to obesity, like asthma and chronic obstructive pulmonary disease (COPD) [69]. Also, TGF-β is implicated in inflammatory processes in obese women [70]. TGF-β is involved in the regulation of inflammation and fibrosis [71]. In obesity, there is often a chronic low-grade inflammatory state in adipose tissue [72], and TGF-β signalling may contribute to this inflammation and be associated with tissue fibrosis [71]. Also, TGF-β has been implicated in remodelling adipose tissue [73]. In obesity, there can be changes in the structure and function of adipose tissue, and TGF-β may be involved in these processes. TGF-β signalling has been linked to insulin resistance, a critical metabolic abnormality associated with obesity, and insulin resistance can contribute to developing type 2 diabetes [74]. Furthermore, the Western diet may affect the microbiota, which may release compounds that affect PCOS women's hormonal patterns.

Obesity dysfunctions adipose tissue, causing chronic low-grade inflammation with pro-inflammatory cytokines and immune cell infiltration [64]. Endotrophin promotes immune cell recruitment and activation, which increases adipose tissue inflammation. Higher endotrophin levels in PCOS patients may have predicted PCOS with enhanced insulin resistance index homeostasis model assessment [75]. Endotrophin can also regulate the activity of TGF-beta, a multifunctional cytokine involved in immune regulation and tissue remodelling [76] so this study suggests endotrophin may influence TGF-beta signalling by directly interacting with TGF-beta receptors or indirectly modulating downstream signalling pathways in PCOS. Also, endotrophin-mediated inflammation in adipose tissue [76] could indirectly impact immune responses regulated by CTLA-4. Chronic inflammation associated with obesity and metabolic disorders may dysregulate immune checkpoints such as CTLA-4, leading to altered T cell function and immune tolerance in PCOS.

In Table 5, the study found a significant increase of TGF-βIII in serum and gene expression in PCOS with irregular menstrual cycles compared with PCOS with regular menstrual cycles. Chronic stress, autoimmune disorders, obesity, and hormonal balance—all these signs can lead to menstrual irregularities and inflammation [77,78]. This study suggests that high TGF-βIII levels in serum and genetic levels in PCOS women with irregular menstrual cycles are because these women have a high percentage of inflammatory factors that may lead to irregular menstruation. The interaction between TGF-βIII and other cytokine signalling is essential in establishing the balance of immunity and tolerance. The statistical results indicate that the history of the disease has no significant impact on the incidence of PCOS. This finding supports previous hypotheses that suggest no genetic factors play a role in the occurrence of the disease, and the cause remains unknown. This was one of the reasons that prompted us to conduct this study to find out the role of the disorder in the immune regulation mechanism in increased severity and exacerbation of the disease.

In the last previous study about PCOS, some evidence suggests the role of inositols in oocyte maturation, fertilisation, implantation, and post-implantation development in PCOS [79]. Inositols are a group of naturally occurring sugar alcohols that have been studied for their potential therapeutic effects in various conditions, including PCOS [80]. Therefore, this study suggested that inositols could influence immune responses and inflammation, indirectly impacting CTLA-4-mediated immune regulation. For example, inositols may modulate cytokine production or immune cell activation, which could affect the expression or activity of CTLA-4 on T cells. Also, inositols may influence insulin sensitivity, which could indirectly modulate the TGF-β effects on metabolic processes. Additionally, inositols may have anti-inflammatory effects, which could impact TGF-beta-regulated inflammatory responses.

The study found a positive correlation between TGF-III and CTLA-4 in Table 6, suggesting that these two molecules are distinct and involved in immune regulation. TGF-III may influence the function of regulatory T cells, potentially interacting with CTLA-4 to modulate immune responses due to immune system dysfunction in PCOS. These results hold significance as they have the potential to impact the immune response and interfere with hormone imbalances. Additionally, an increase in TGF-III and CTLA-4 may alter the tissue's composition, potentially serving as a direct indicator of immune system changes within the female reproductive system's environment. Therefore, this study suggests examining these parameters in uterine fluid or vaginal secretions, along with measuring Foxp3 and detecting Treg using flow cytometry, as these findings may

reveal significant immune response factors, particularly at the affected anatomical site.

6. Conclusion

The result of the current study recorded a significantly elevated serum level as well as an upregulation of both genes (TGF-βIII and CTLA-4) in females with PCOS. These two genes play a crucial role in immune suppression; therefore, these results may help in a deeper understanding of PCOS pathogenesis and may explain the occurrence of multiple disorders in those women, such as insulin resistance and cardiovascular disease. Perhaps the most important conclusion is that these two molecules could be used as a potential therapeutic target to stop the severity of PCOS or even cure it.

7. Study limitation

The study encountered a critical limitation, namely, the sample size of PCOS. Although PCOS has been a disease for a long time, its causes and treatment are still unknown.

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