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Expression Of Lipocalin-2 (LNC2) Gene In Polycystic Ovarian Syndrome (PCOS) Patients As Diagnostic Biomarkers

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Abstract:

Background: Recently, research has been conducted on proteins such as lipocalin-2 (LNC2) and peptides that play roles in regulating metabolism, and also research has been conducted on the level of lipids and lipoproteins such as [cholesterol and triglycerides, LDL ,VLDL] which can be considered as potential markers of insulin resistance in some patients. Medical conditions, such as diabetes, obesity, and polycystic ovary syndrome (PCOS). gained in interest. Polycystic ovary syndrome (PCOS) is a common endocrine disorder associated with hyperandrogenemia and anovulatory failure, which is often accompanied by metabolic disorders, including obesity, dyslipidemia, hyperinsulinemia, and insulin resistance. Methods: Venous blood samples (5 ml) were collected from 200 women representing two groups. The first group consisted of 150 blood samples from women with polycystic ovary syndrome, the second group, 50 samples from healthy women. Lipocalin-2 protein concentration was measured using ELISA, lipids and lipoprotein levels were measured by spectrophotometer, and its gene expression was measured by quantitative real-time PCR (qRT-PCR). Objective: This study aims to find out whether the level of LNC2 increases or decreases in patients with polycystic ovary syndrome. Results: Gene expressions and serum LNC2 levels were significantly lower in patients than in controls. Conclusion: The results indicated that the level of LNC2 is increased in patients with PCOS.

Keywords: lipocalin-2, PCOS, ELISA technology, qRT-PCR, insulin resistance markers

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Introduction

PCOS refers to several endocrine and metabolic disorders that occur in adult women of reproductive age (Kakoly e*t al*., 2019). Although the exact cause of polycystic ovary disease remains unknown, genetic and hormonal factors are believed to play an important role in its appearance (Coyle & Campbell, 2019). Polycystic ovary syndrome contributes to disruption of women's health and vitality, as it can cause the risk of menstrual problems, type 2 diabetes, other hormonal disorders, and blood pressure, in addition to visible symptoms such as scalp hair loss, body hair growth, and acne(Deswal *et al*., 2020). Polycystic ovary syndrome is associated with weight loss problems and insulin resistance. Accurate diagnosis of PCOS depends on radio ultrasound and the characteristic clinical symptoms of menstrual irregularity, hypometabolism, or hyperandrogenism, and what is diagnosed in the reproductive stages of life in women. Patients with PCOS suffer from infertility or on murine models. However, in the architecture domain (Fig. 1) and their structure, the human and mouse homologue are highly similar. Table 1 (Lippi *et al*., 2014).Lipocalin-2 expression has been detected in several types of cells, including neutrophils, adipocytes, macrophages, endothelial cells, endometrial cells, splenic cells and hepatocytes (Jaberi e*t al.,* 2021).

symptoms of excess androgens such as testosterone, follicle-stimulating hormone (FSH), and white blood cells(Rao & Bhide, 2020) (LCN2) 24p3, siderocalin, or neutrophil gelatinase-associated lipocalin (NGAL), is the representative of the lipocalins family (Moschen *et al*., 2017). This 178-amino acid protein occurs in three different forms: as a 25-kDa monomer, a 45-kDa homodimer and a 135-kDa heterodimer, forming a complex structure with matrix metalloproteinase 9 (MMP-9) LCN-2 occurs in various molecular forms as a mono-, di- or hetero-dimer with disulfide bonds with neutrophil gelatinase B (Kjeldsen *et al*., 1993).Analyzing the sequence of amino acids among NGAL homologues between various species shows that human NGAL share lower sequence identity with the homologues of mouse and rat (62% and 63% similarities, respectively). This is very important to note because most of the studies on the function of LCN-2 are done

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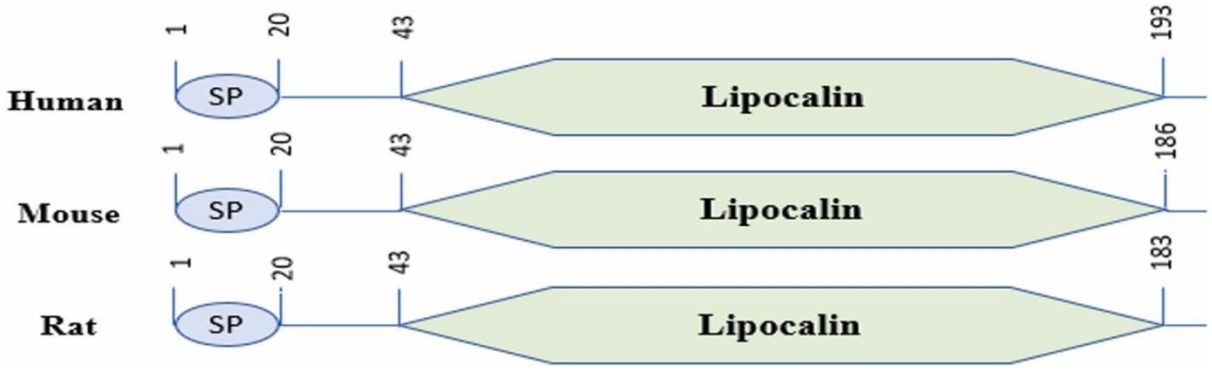

Fig. 1. Human, mouse and rat LCN-2 domain architecture showing high degree of similarity, consisting of a 20 amino-acid signaling peptide (SP) placed at its N-terminal.

Species	Gene	Alternative names	Location of chromosome	Number of amino acids	Percentage resemblance (%) to human LCN-2
Human	LCN2	24p3, MSF1, NGAL	9q34	198 a.a	-
Mouse	LCN2	RP23–161B9, 11–003, 24p3, AW212229, Sip24, OTTMUSP00000013 951, Ngal, SV40- I24p3P	2A3, 2 27.0 cM (Chr. 2 – NC_000068.6)	200 a.a	**62**
Rat	LCN2	Ngal, p25, lipocalin- 2, oncogene 24p3, α-2 U-GRP, α-2-MRP	3p11	198 a.a.	**63**

Table 1 Genetic structure of lipocalin-2 homologues in mammalian species

Materials and Methods

This current study was conducted on women of reproductive age with polycystic ovary syndrome, at Samarra General Hospital in the city of Samarra, Salah al-Din

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Governorate, and they were diagnosed according to clinical criteria. In this study, samples were collected from 1/12/2023 to 4/5/2024, when 200 blood samples were collected from women, representing two groups. The first group consisted of 150 blood samples from infected women, in addition to the information obtained according to the questionnaire. As for polycystic ovary syndrome and the second group, 50 samples from healthy women (control samples) were measured, and the level of protein lipocalin-2, which is associated with insulin resistance and metabolism, and its gene expression were measured.

Collect blood samples

A 5 ml venous blood sample was drawn from each study patient. Medical syringes with a capacity of 5 ml were used, then blood was drawn and the procedure was performed under the necessary sterile conditions.Then, about 250 microliters of blood is placed in a 2 ml Eppendorf tube, with about 750 microliters of Trizol added to it, with a simple mixing of the sample, and the sample number, patient's name, and group number are written on it using an adhesive tape to be stuck on it. Store in the refrigerator at -20 degrees until testing

lipocalin-2 concentration

The concentration of LNC2was measured using a kit produced by the Chinese company Sun Long using ELISA technology and according to the company's steps and method of work.

Lipid profile concentration

The Lipid profile concentration was measured using a kit produced by the French company Biolabo using a spectrophotometer and according to the company's steps and method.

Measuring gene expression

mRNA was isolated from the samples as a basic step of the real-time polymerase chain reaction real-time PCR). In order to estimate the level of gene expression, an RNA extraction kit was used Transzol Up plus (RNA Kit) Supplied by the company TRANS was used, and the primers shown in Table (2) were used.

No.	Primer Name	Foreword Sequence	Reverse Sequence

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1	LNC2	ACGCTGGGCAACATTAAGAG	CGAAGTCAGCTCCTTGGTTC
2	(Housekeeping)	ATCGCCAAGAGATCAAAGATAA	TCTGAAGACATCCTTATTGACG

Table (2) shows the primers used in the reaction real-time polymerase chain reaction real-time PCR

Quantitative Real-Time PCR (qRT-PCR) Run

Quantitative Real-Time PCR (qRT–PCR) was carried out using the Real-time PCR method . The levels of Gene expression and fold change were identified by measuring the threshold cycle (Ct) using the TransStart® Top qPCR SuperMix Kits components. The Ct threshold cycle was calculated using real-time cycler software for each sample. All samples were run in duplicate, and mean values were calculated.- Expression data of selected genes were normalized against housekeeping. The ΔΔCt method was used as was recommended for data analysis, and results were expressed as folding change in gene expression as follow:

For each sample, the difference between the Ct values (ΔCt) for each gene of target and the housekeeping gene was calculate

Statistical Analysis

A statistical software (SPSS V20) was used for the analysis of data. Measured data were expressed as mean ± Standard Error (SE). Differences between means were assessed by independent samples T test. (Cleophas & Zwinderman, 2016) Differences with a probability value that equals or less than 0.05 were considered significant. The fold change was calculated by the following equations:

1. ΔCt (control) = Ct (gene) – Ct (HKG)

2. ΔCt (patient) = Ct (gene) – Ct (HKG)

4 ΔΔCT=ΔCT of each sample – average control ΔCT.

Then the fold change was calculated using the Livak method (Livak & Schmittgen, 2001 ; Al-Doury,et al.,2019)

5. Fold change = $2^{-\Delta\Delta Ct}$

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Results

LNC2 levels were measured in serum samples of PCOS patients and control with significant differences ($p < 0.05$), The results of the current study showed a significant increase at the $P \leq 0.05$ level for some biochemical indicators, such as (FBS, TG, TC, VLDL-C), while (HDL-C, LDL-C) did not show significant differences at the ($P>0.05$) in patients with polycystic ovary syndrome compared to the control group Table (3).

Parameters	**Mean ±SD**	
	control	Patients
lipocalin-2 (pg/mL)	142.9± 60.7	64.6± 14.2*
cholesterol	134.6±36.8	155.5±51.9*
triglycerides	94.7±19.0	129.7±45.9*
HDL	48.0±11.6	50.0±12.4 **n.s**
LDL	73.2±20.8	81.1±26.8**n.s**
VLDL	18.95±3.80	25.94±9.18*

Table:3 Serum levels of LNC2 in PCOS patients and control.

- **Where (*) represents a significant difference**
- **n.s (non-significant) represents no significant difference**

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LNC2 gene expression

The gene expression level of the LNC2 gene was measured in all samples of patients and the control group. The LNC2 melting curve was sharply defined with a weak peak, indicating that pure and homogeneous PCR results were obtained.

ΔRh is the size of the fluorescence signal resulting from the dye (Reporter Dye) for each amplification cycle in the real time PCR technique (ΔRh=Rh-baseline), which shows us the function of the cycle number, and irregular amplification can be verified, and also from it the threshold limit for each cycle can be known.

(Ct) cycle threshold, which represents the cycle number in PCR at which fluorescence begins against the threshold limit and represents a function of the good location and to identify abnormal values that must be avoided in calculations Fig. 3. Rt-PCR results of related mRNA expression tested by threshold cycle (Ct) The results revealed that LNC2 gene expression was significantly lower in PCOS patients compared to controls (p = 0.001), as shown in Table 4.

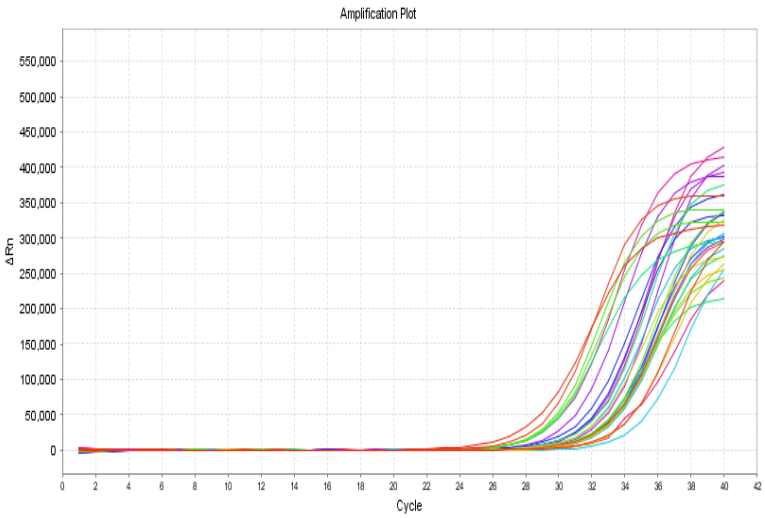

Fig. 3. Rt-PCR results of related mRNA expression tested by threshold cycle (Ct)

mRNA	Mean ΔCT± ES for control	Mean ΔCT± ES for Patients	p-value	ΔΔCt	Folding expression
lipocalin-2 mRNA	6.57±1.40	7.59±1.59*	0.05	0.311	0.806

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Table:4 LNC2 gene expression in PCOS patients and control.

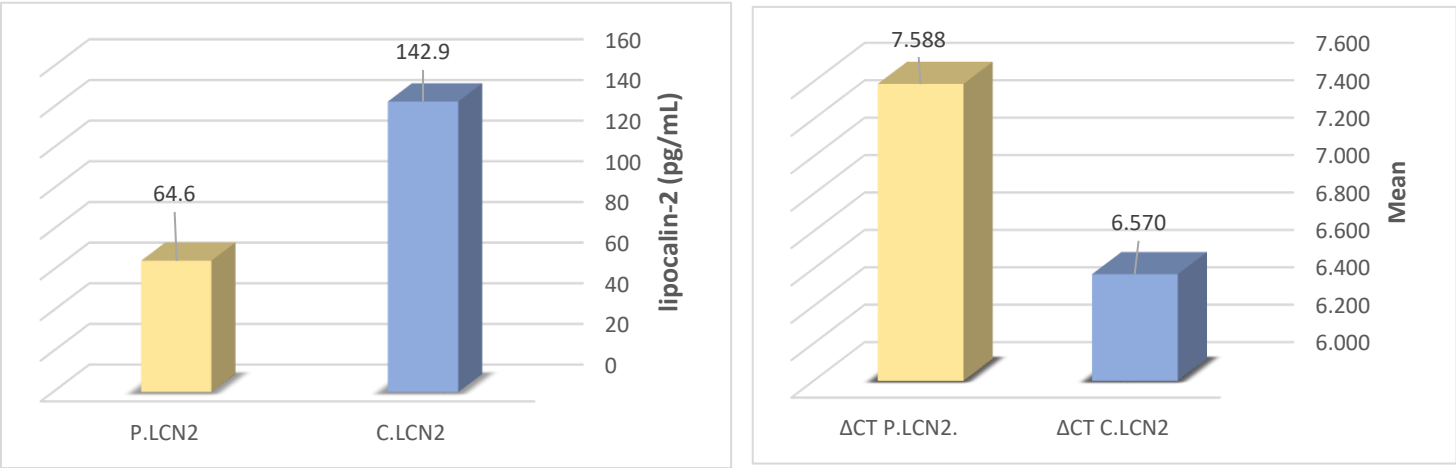

Fig. 4. A and B LNC2 gene expression in PCOS patients and control.

Discussion:

The percentage of fats in the blood is important for assessing the health status of women with polycystic ovary syndrome. High levels of these fats may affect the body, increasing the risk of blood, blood vessel and arterial diseases, and androgenic hormone disorders (male hormones) (Kiranmayee *et al*., 2017).

An increase in the level of cholesterol and triglycerides is affected by several factors, including obesity, insulin resistance, as well as genetic factors. Our results showed a significant increase in women with PCOS. On the other hand, our results showed that there were no significant differences in the levels of high-density lipoprotein and low-density lipoprotein, and also showed an increase in the levels of cholesterol and triglycerides. In the level of very low-density lipoprotein, our results are also consistent with the study of Ibrahim et al., where they indicated an increase in the level of triglycerides and cholesterol in women with PCOS compared to healthy women, while in the level of HDL and LDL, there were no significant and statistically significant differences, while our results agree with Onyegbule. Others indicated that there was a significant increase in the level of very low-density protein

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(VLDL) (Ibrahim et al., 2020; Onyegbule et *al.*, 2022).

LCN-2 is known to regulate inflammatory pathways and cytokine secretion and plays a role in chronic inflammatory diseases, such as obesity, TDM2, or NASH (Ibrahim *et al*., 2020). It has been confirmed, in animal studies, that LCN2 deficiency may protect against the development of insulin resistance by regulating the levels of lipoxygenases and catechins in adipose tissue (Onyegbule *et al*., 2022). Since the 2000s, LCN2 has gained increasing interest, due to its potential role as a biomarker for PCOS; However, the results of studies aimed at confirming this hypothesis remain ambiguous (Law *et al*., 2010). Kakal et al. A cross-sectional study investigating LCN2 as a marker of insulin resistance in PCOS populations was conducted and found significantly higher serum lipocaine-2 levels in PCOS individuals compared to age- and BMI-matched healthy controls (Law et al., 2010) Similar results were presented by (Yilmaz *et al.*, 2017). In the study by Panidis et al., serum LCN2 levels were slightly elevated, but the results were not statistically significant, despite the larger number of participants. However, the study and control groups did not differ in terms of HOMA-IR (Cakal *et al*., 2011). In fact, HOMA-IR values differed significantly between the PCOS and control groups in the previously mentioned studies conducted by Cakal et al. (Law *et al.,* 2010) Yilmaz et al(Yilmaz et al., 2017). Surprisingly, there are also reports of lower LCN2 levels in PCOS patients compared to healthy individuals (Panidis *et al*., 2010) underscoring the need for larger studies. There are also mixed data when it comes to the relationship of LCN level with HOMA-IR, as an association between them has been identified in some studies (Gencer *et al*., 2014) but not found in others (Diamanti-Kandarakis et al., 2008) . In the most recent study, Cheng et al. observed the relationship between serum LCN-2 level and the development of diabetes mellitus in PCOS patients (Cheng *et al*., 2021) . Regarding obesity, Martinez-Garcia et al. investigated LNC2 elevation in non-obese PCOS patients compared with non-obese controls. Conversely, in obese PCOS subjects, the serum lipocalin-2 level was lower than that in obese controls (Martínez-García *et al*., 2013). In fact, some researchers have concluded that elevated LCN2 levels are associated with PCOS-related obesity, not

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PCOS alone (Koiou *et al*., 2012). Therefore, more studies are needed

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