Assessment of Neutrophil-to-Lymphocyte Ratio, Platelet-to-Lymphocyte Ratio,Oxidative Stress and Anti Oxidants levels in Polycystic Ovary Syndrome Patients with Low-Grade Chronic Inflammation

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Abstract

Background: The ratio of neutrophils to lymphocytes (NLR) and the ratio of platelets to lymphocytes (PLR)are prognostic factors in many diseases such as inflammatory diseases, cardiovascular diseases and cancer. However, racing NLR and PLR are important, it is important to establish reference values for NLR and PLR in polycystic ovary syndrome (PCOS) patients with mild chronic inflammation.

Objective: To determine the neutrophil-lymphocyte *ratio* (NLR)and platelet-lymphocyte *ratio* (PLR), and the relationship between *oxidative stress* markers *and anti oxidants* markers in PCOS patients *with low-grade chronic Inflammation*. Methodology. The study included 92 patients with PCOS and 46 control. Serum PON1, XO, SOD activities, 8-OHdG and NO levels were measured using enzyme linked immunosorbent assay (ELISA) kits.

Results: Concentration of TNF-α, IL-6, IL-8, IL-10 and CRP were significantly higher in PCOS patients compared with control (P<0.001). Significant increases were recorded in WBC, ANC, PLT, NLR, PLR, and ESR values. A significant decrease in ALC and MPV values in PCOS patients compared with control (P<0.01). Serum XO, SOD activities and NOlevels were higher in PCOS patients than in the control (p<0.001). Serum 8-OHdG levels and PON1 activities were lower in PCOS patients than in the control (p<0.001). Serum CRP values is positively correlated with XO, NO, 8-OHdG and PON1 activity (r=0.377, p<0.008; r=0.560, p<0.007; r=0.387, p<0.006; r=0.481, p<0.009), respectively. Serum CRP values is negatively correlated with SOD activity in PCOS patients (r=−0.444, p<0.004).Serum XO activities were negatively correlated with serum PON1 and SOD. Serum XO activities were Positively correlated with serum NO and 8-OHdG levels.

Conclusion: Inflammation stimulates the oxidation process and reduces the ability of cellular antioxidants in PCOS patients. NLR and PLR are good markers of inflammation, their highest values indicate the severity of the disease and an imbalance between neutrophils and lymphocytes can be linked to cancer and its development.

Keywords: *Polycystic ovary syndrome, Inflammation, Oxidative Stress, Anti Oxidants.*

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Introduction

Polycystic ovary syndrome (PCOS) is a common disorder in women of childbearing years characterized by increased androgen and ovulation and is associated with many metabolic disorders, especially insulin

resistance and obesity [1] . Oxidativestress (OS) indicates an imbalance between oxidizing substances and antioxidants that lead to abnormal cell state [2] . OS occurs when destructive reactive oxygen species (ROS) overcome antioxidants,and stress leads to the accumulation of peroxides and free radicals causing damage to DNA and/orprogrammed cell death and causing damage to proteins,lipids,carbohydrates and other molecules [3] . Chronic inflammation and ROS contribute to the DNA damage of many organs, and ROS causes oxidation of biomolecules such as lipids,proteins,and other molecules which leads to the formation of toxic and mutagenic intermediates [4] .Cells produce ROS and reactive nitrogen species (RNS) as normal products of the biological reduction of molecular oxygen. ROS include superoxide anion ($O2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H2O2) and organic peroxides [5,6] . Superoxide ($O2^{\bullet-}$) is mainly generated at the mitochondrial electron transport chain level and canbe converted to hydrogen peroxide (H2O2) by SODorundergospontaneous disassociation [6] . RNS includes nitric oxide (NO) and its metabolites, which are also highly reactive and toxic [1,5]. Inflammation is one of the main pathophysiological factors in PCOS diseases. Nowadays diagnostic and monitoring markers are used, such as C-reactiveprotein (CRP), erythrocyte sedimentation rate, ferritin, serumalbumin, apolipoprotein A-1, tumor necrosis factor, interleukin-1, interleukin-6 and others [7,8] .Chronic inflammation and infection are an important and major cause of cancer,include NF-κB,reactive oxygen species and nitrogen, inflammatory cytokines,prostaglandins,andR NAs,through proliferation changes,cell death,cellular aging,mutation and methylation in DNA [7] .

Several biomarkers such as CRP, neutrophil-to-lymphocyteratio (NLR) and platelet-to lymphocyte ratio (PLR) were used as markers of inflammation and endothelial damage [8,9] .Complete blood count (CBC) test is widely used,and mainly includes white blood cell (WBC) count, red blood cell (RBC) count and platelet count.WBCs most abundant in healthy individuals are neutrophils,which play important roles during acute and chronic inflammation and may be potential therapeutic targets in several diseases [10,11].

8-Hydroxydeoxyguanosine (8-OHdG) can be a defense mechanism against oxidative stress and preventing inflammatory disease [12]. ROS easily attacks guanine bases in DNA and forms 8-OHdG, which can bind to thymidine instead of cytosine, so the 8-OHdG level is a biological biomarker of mutations resulting from oxidative stress. The oxidative form of DNA damage can be seen by assessing the concentration of 8-OHdG (13) .8-OHdG can reduce ROS production, reduce the pathway of nuclear factor-κB,and reduce expression of inflammatorymediators such as interleukin IL-1,IL-6,cyclo-oxygenase-2,and inducible nitric oxide synthase in addition to the expression of nicotinamide adenine dinucleotide phosphate oxidase (NOX) -1,NOX organizer-1and NOX activator-1 in different cases of PCOS inflammation [2] .To determine the role of OS in PCOS, a set of vital biomarkers were examined including Xanthine oxidase (XO),NO,8-OHdG, Paraoxonase1 (PON1) and Superoxide disututase (SOD) . The aim of present study was to verify individual and group diagnostic accuracy of NLR,PLR,and MPVin PCOS patients diagnosed with CRC. And assess whether there was any relationship between oxidative stress markers and antioxidant markers with inflammation in the development of PCOS.We have investigated oxidative stress by measuring serum XO activity (as a generator of reactive oxygen species) and NO levels.

Material and Method

Study Population: One hundred and thirty-eight women within the (21-38) age group were involved in this study from 12 October 2018 to 16 November 2019. They were divided into two groups:92 patients with PCOS (mean age:34.4±5.8years)and 46 healthy individuals were recruited as a controls (mean age:35.7±5.8years). Patients selected clinically diagnosed with polycystic ovary syndrome fromAl-Saddr Teaching Hospital in Najaf city,Iraq. The diagnosis was based on the presence ofoligomenorrhea or chronic menopause (less than six menstrual periods in the preceding year), hirsutism (Ferriman-Gallweyscore of7),LH/FSH ratio (3.0),hyperandrogenemia, plasma total testosterone concentrations of more than (0.6)ng/ml,and free androgen index (FAI)of less than 5.Patients were excluded if they had evidence of the presence history of malignancy,hype rprolactinemia,diabetes mellitus,hypertension,pregnanc y,thyroid and adrenal dysfunction,any history of cardiac symptoms, myocardial infarction,angina,coronary arterydisease,vascular disease.Womenwho are taking contraceptives because they contain hormones,acetyl salicylic acid,non-steroidal anti-inflammatory drugs or other pharmacological factors that could affect On the results of our study.

Sample Collection: Blood samples were collected

after an overnight fasting of ≥12 hours on the second or third day of the menstrual cycle. Venous blood samples (5ml)were collected into EDTA tubes and centrifuged at 3000 rpm for 5 minutes, and, were separated and stored at -20ºC for estimate some criteria.

Method:On the second or third day of the menstrual cycle, the BMI was calculated according toWHOcriteria based on weight divided by height squared (kg/m^2).Using commercially available diagnostic kits (Bayer Corporation, Tarrytown, NY) in an Advia Centaur Immunoassay System, Follicle stimulating hormone (FSH), lutenizing hormone (LH), and prolactin (PRL)levels were measured. SerumCRP was turbidimetricallydetermined by aclinical chemistrysystem (SPACE, Schiapparelli Biosystems, Woerden, The Netherlands), which gives a quantitative result.SerumC-reactive protein (CRP), tumor necrosisfactor-a (TNF-a), interleukin-6 (IL-6), interleukin-8 (IL-8), andinterleukin-10 (IL-10)were determined using ELISAkits (SunLong Biotech Co, China).Serum PON1, XO, SODactivities, 8-OHdG and NO levels were measured using enzyme linked immuno sorbent assay (ELISA)kits (SunLongBiotechCo, China). Complete blood counts (CBC)tests were performed in anticoagulant samples with EDTA within 4hours after collection, using a Coulter®LH750Hematology Analyzer (Beckman Coulter, USA). Neutrophil-to-lymphocyteratio (NLR) was calculated by dividing the absolute neutrophil count (ANC) by the absolute lymphocyte count (ALC); likewise, platelet to lymphocyte ratio (PLR) was calculated by dividing the absolute platelet count by Absolute lymphocyte count (ALC). Three levels of commercial surveillance material (Beckman Coulter, USA) were run twice daily. Samples were excluded with white blood count (WBC)less than 3.5×10^9/Lor more than 9.5×10^9/Land platelet less than 125×10^9/Lor more than 350×10^9/L.

Statistical Analysis: SPSS software 22 was used for statistically analysis. The data are expressed as mean±SD. Significance of the difference between the mean value of the measured parameters between groups were evaluated by Student's t-test. and chi-square. Correlation was indicated by Pearson correlation tests and P<0.05 is consider significant.

Results

The demographic characteristics and hormones levels in PCOS patients and control are shown in Table 1. Concentration of pro-inflammatory cytokines (TNF-a, IL-6and IL-8), anti-inflammatory cytokines (IL-10), and CRP were significantly higher in PCOS patients compared with control (P<0.001) Table 2. A significant increases in WBC, ANC, PLT, NLR, PLR and ESR values in PCOS patients compared to controls.A significant decrease in ALC and MPV values in PCOS patients compared with control (P<0.01) Table 3 and Fig.1. Serum XO, SOD activities and NOlevels were higher in the PCOS patients compared with control (p<0.001). Serum 8-OHdG levels and PON1 activities were lower in women with PCOS than in the control (p<0.001)Table 4. Serum CRP values is positively correlated with XO, NO, 8-OHdG and PON1 activity (r=0.377, p<0.008; r=0.560, p<0.007; r=0.387, p<0.006; r=0.481, p<0.009), respectively. Serum CRP values is negatively correlated with SOD activity in PCOS patients (r=−0.444, p<0.004) Table 5. SerumXO activities were negatively correlated with serum PON1 and SOD activities (r=−0.465, p<0.008; r=−0.375, p<0.007), respectively. Serum XO activities were Positively correlated with serum NO and 8-OhdG levels (r=0.483, p<0.009; r=0.472, p<0.006), respectively Table 6.

Table 1. Characteristics in PCOS patients and control.

Parameter	PCOS (n=92) Mean ± SD	Controls (n=46) Mean ± SD	*p*-Value
Age (Years)	23.40±2.80	24.72±1.68	NR
BMI (kg/m^2)	29.33±3.56	22.48±2.87	<0.01*
FSH (mIU/ml)	5.22±2.20	6.10±2.11	<0.001
LH (mIU/ml)	10.33±2.11	5.01±2.77	<0.001
PRL (mIU/ml)	40.42±1.28	18.88±1.18	<0.001

*p<0.05 was considered significant; NR,Not significant.

Table 2. Concentration of pro-inflammatory cytokines, anti-inflammatory cytokinesand CRP in PCOS patients and control.

p-Value	Control (n=46) Mean ± SD	PCOS (n=92) Mean ± SD	Parameter
< 0.001	8.11±2.21	43.30±3.42	CRP (mg/l)
< 0.001	5.42 ± 1.66	97.33 ± 1.74	TNF-a(pg/ml)
< 0.001	38.32±6.32	142.31±5.24	IL-6 (pg/ml)
< 0.001	31.34±5.95	127.44±7.10	IL-8 (pg/ml)
< 0.001	40.14±2.27	77.62±2.22	IL-10 (pg/ml)

CRP, C-reactive protein; TNF-α, tumor necrosis factor-α; IL-6,interleukin-6; IL-8, interleukin-8.p<0.05 was considered significant

Table 3. Laboratory values in PCOS patients and control.

p-Value	Controls (n=46) Mean ± SD	PCOS (n=92) Mean ± SD	Parameter
<0.01	4.55±7.33	12.02±4.31	WBC(10^9/L)
<0.01	4.33 ± 1.62	7.55 ± 2.66	ANC (%)
<0.01	5.13 ± 0.85	1.60 ± 0.75	ALC (%)
<0.01	220.38 ± 51.88	263.04 ± 80.12	PLT (10^9/L)
<0.01	1.81±0.22	6.82±0.97	NLR
<0.01	93.78±18.33	182.04±41.11	PLR
<0.01	9.77 ± 1.65	5.53 ± 1.24	MPV (fL)
<0.01	15.86±4.76	31.11±8.21	ESR(mm/hour)

p<0.05 was considered significant; WBC, white blood count. Tumor necrosis factor-alphaCBC,Complete blood counts;ANC,Absolute neutrophil count; ALC,Absolute lymphocyte count.

Table 4. Serum oxidative stress markers (XO, NO and 8-OHdG) and antioxidative stress markers (SOD and PON1) in PCOS patients and control.

p-Value	Controls (n=46) Mean ± SD	PCOS (n=92) Mean ± SD	Parameter
< 0.001	0.73±0.30	4.07±0.22	XO (U/mL)
< 0.001	6.82±3.11	10.06±2.52	NO (µmol/L)
< 0.001	220.32 ± 64.81	136.72 ± 45.34	8-OHdG (pg/mL)
< 0.001	748.66±20.77	855.44±41.44	SOD (U/gm of Hb)
< 0.001	196.10±81.83	127.50±77.26	PON1 (U/L)

p<0.05 was considered significant

Table 5. Correlation between Oxidative stress markers, Antioxidative stress markers and CRP in PCOS patients.

Parameter	PCOS (n=92)		Control (n=46)	
	r	p-value	r	p-value
XO (U/mL)	0.377	<0.008	0.228	<0.005
NO (µmol/L)	0.560	<0.007	0.217	<0.008
8-OHdG (pg/mL)	0.387	<0.006	0.636	<0.005
SOD (U/gm of Hb)	−0.444	<0.006	0.338	<0.002
PON-1 (U/L)	0.481	<0.009	0.568	<0.007

Table 6. Correlation between XO activity and PON1, SOD activity, and NO, 8-OHdG levels in PCOS patients.

Parameter				p-value
8-OHdG (pg/mL)	NO (μmol/L)	SOD (U/gm of Hb)	PON1 (U/L)	
0.472	0.483	−0.375	−0.465	r
<0.006	<0.009	<0.007	<0.008	

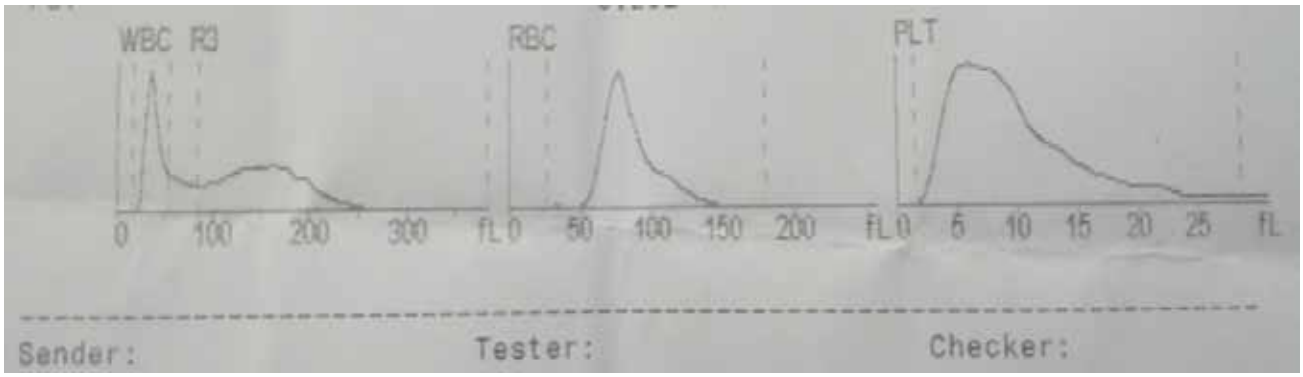

Fig. 1. Values of WBC, RBC and PLT in PCOS patients

Discussion

Enlarged adipose tissue leads to infiltration of macrophages and imbalance are pro-inflammatory and anti-inflammatory factors secreted by adipose tissue, which leads to enhanced inflammation, impairment of insulin sensitivity and dysregulation of lipid metabolism. Obesity-related inflammation is caused by extra nutrients that initially activate many pathways of metabolic signals, such as JNK, NF-kB, and protein kinase R pathways [2, 14].In obese PCOS patients, BMI, free fatty acids, IL-6, and CRPlevels increased, while androgen levels decreases with gonadotropin-releasing hormone (Gn RH) agonist for a long term [15]. These results are consistent with Our current study. Activation of these pathways leads to the production of many inflammatory cytokines and then leads to chronic low grade inflammation [6]. Increased production of ROSleads to disruption of the organism, therefore, the enhancement of OS in tissues and organisms causes related damage;harms mitochondrial components (mtDNA, proteins, lipids, etc.), and induces apoptosis [1]. Our results indicate the concentration of pro-inflammatory cytokines (TNF-α, IL-6 and IL-8), anti-inflammatory cytokines (IL-10), and CRP were significantly higher in PCOS patients compared with control (P< 0.001, foreach) Table 2. Several recent studies have indicated that chronic inflammation have a role in causing PCOS because many markers of inflammation are elevated in those women with this disease. On the other hand, a relationship was found between the proinflammatory condition and PCOS, associated with polymorphism of TNF-α, IL-6, and its receptor[16,17]. Inflammation are involved in the pathogenesis of numerous chronic diseases. Where there is a relationship between chronic low-grade Inflammation and the development of PCOS [4].

Our results indicate a significant increases in WBC, ANC, PLT, NLR, PLR and ESR values, and a significant decrease in ALC and MPV values in PCOS patients compared with control (P<0.01, for each) Table 3 and Fig. 1. These results are consistent with other studies [9, 18]. NLR (neutrophil-to-lymphocyte ratio)and PLR (platelet to lymphocyte ratio) were good inflammatory markers and can be used to assess disease activity and in some diseases they are used to differentiate infections [16]. On the other hand, these parameters are related to the early diagnosis of diseases[11]. MPV is used as a marker for platelet size and activity [10]. MPV has been identified as an inflammatory marker for cerebrovascular, cardiovascular, digestive and rheumatic diseases [18]. Also, as a marker of early diagnosis in detecting types of cancer [9, 10]. Ahigh BMI can be explained by considering that obesity can also affect MPV [19].

Oxidants and antioxidants are involved in regulating gene expression in physiological and pathological

conditions, and therefore ROS modulate proliferation (1). The results indicated a significantly increased Serum XO activity in women with PCOS compared with control (p<0.001)Table 4.XO plays an important role in the catabolism of purines and generating ROS in PCOS patients [20], Thus it can be said that the increase is due to the increase in oxidative stress in patients. These results are consistent with previous studies [20, 21].PCOS can be considered a purely oxidative state in which the body's antioxidants cannot exceed the overproduction of free radicals [3]. This indicates that the increase in XO activity is closely related to the changes and increase in markers of inflammation during the development of PCOS.XO is a key enzyme and source of ROS and can generate superoxide anion radicals, capable of lipid peroxide and protein degradation [21]. This enzyme catalyzes oxidation of hypoxanthine to xanthine and oxidation of xanthine to uric acid [20].Free radicals seek stability by taking electrons from other stable molecules, which creates a chain reaction of free radical formation that can cause damage to body cells, proteins, andDNA.In addition, aging and/or environmental stressmay enhance this oxidation and may cause chronic inflammation, which may exacerbate damage and increase the risk of cancer. NO plays a major role in the pathophysiology of PCOS. The results showed that NO levels were statistically higher in patients with PCOS compared to control (p<0.001) Table 4. RNS, uch as NOwith the unpaired electron are also highly reactive and toxic.OS occurs when destructive ROSovercome antioxidants, causing DNA damage and/or programmed cell death. Our current results are consistent with similar studies [6, 22].Increased NO levels may be toxic to host cells, as they are also produced in immune responses by monocytes, macrophages, and neutrophils [6, 23], indicated that nitrite/ nitrate concentration is an indicator of NOlining.On the other hand, an analytical study showed that the mean NO level had no statistically significant difference in patients with PCOS compared to controls [24].Moreover, several data indicated a significant negative correlation between NO and fasting insulin levels and HOMAin PCOS patients [2].

Serum8-OHdGlevels was decrease inPCOSpatients compared with control (p<0.001)Table 4. The reason for the decrease in 8-OHdG level may be due to changes in antioxidant levels as response to increased oxidative stressthat causes DNA damage these results are consistent with previous studies [25, 26], which have indicated that metformin therapy leads to reduced serum 8-OHdG level in obese patients.On the other hand, 8-OHdG levels are associated with body weight and the marker of inflammationCRP [13].Conversely, several studies indicated an increase in the 8-OHdG level in PCOS patients compared to control [27, 28].On the other hand, Several studies have shown increased levels of 8-OHdG in diseases associated with oxidation.ROS easily attacks guanine bases in DNA and forms 8-OHdG, which can bind to thymidine instead of cytosine, so the 8-OHdG level is a biological biomarker of mutations resulting from oxidative stress [12]. The oxidative form of DNA damage can be seen by assessing the concentration of 8-OHdG [13].

SODs are a family of enzymes that catalyze the breakdown of $O2^{-}$ to H2O2, which is a toxic substance converted to water by GPx [29, 30].Serum SOD activity increased in patients with PCOS compared to control (p<0.001)Table4.These results are consistent with other studies [22, 31, 32].Excessive expression of SODmay be an adaptive response against increased ROS levels, and leads to increased dissociation of superoxide from hydrogen peroxide [33].Several studies have suggested that an increase in antioxidant enzymes may represent compensatory swelling in response to an increase in oxidative stress [34,35].Serum SODactivity can be a clinical marke for determining oxidative stress in PCOSpatients. SODenzymes act as pro-oxidant producing H2O2;for this reason, other antioxidant enzymes such as GPXand CAT are urgently needed.On the other hand, the results of our study are not compatible with what previous studies have found [21, 36].

PON1 is an antioxidant responsible for removing oxidized toxins from lipids [37].Serum PON1activity decreased in PCOSpatients compared to control (p<0.001)Table4.Similar results have been reported in other studies [20, 38]. The mechanism of decrease serum PON1 activity is unclear. Consumption of PON1 to prevent oxidation can lead to decreased activity in PCOS patients, or the reason for the decrease is due to the increased disruption of PON1 as a result of increased ROS generation in patients [38].Therefore, it can be speculated that the superoxide anionsproduced by XO are responsible for the decreased PON1 activity due to the change in structure of protein. PON1 associated with lipid peroxidation, therefore an anti-inflammatory indication due to its activity in paroxonase which does not represent its total physiological activity.PON1/HDL activity was a better abnormal parameter in several diseases including PCOS [20].

Serum CRPvalues is positively correlated with XO, NO, 8-OHdG and PON1activity (r=0.377, p<0.008; r=0.560, p<0.007; r=0.387, p<0.006; r=0.481, p<0.009; respectively). Serum CRP values is negatively correlated with SODactivity in PCOSpatients (r=−0.444, p<0.006) Table 5. These results are consistent with other studies [20, 21]. OS and chronic inflammation are closely related mechanisms, the release of many active substances by inflammatory cells into inflammatory sites results in the overgeneration of the OS [39]. This relationship is confirmed by the increase in the levels of associated circulatory markers with inflammation, such as CRP, IL-6, TNF-α, IL-8, monocyte chemoattractant protein-1 (MCP-1), soluble intercellular adhesion molecule-1, and WBC in PCOS patients[16, 39]. XO was used as an indicator of oxidative stress (as agenerator of ROS); Serum XO activities were negatively correlated with serum PON1 and SOD activities (r=−0.465, p<0.008; r=−0.375, p<0.007; respectively).SerumXO activities were positively correlated with serumNO and8-OHdG levels (r=0.483, p<0.009; r=0.472, p<0.007; respectively)Table 6. These results are consistent with other studies[2, 20]. To our knowledge, this is the first study that combines NLR, PLR and MPV and has a high diagnostic accuracy. In addition, this is the first study among the Iraqi population, which is important given the regional prevalence of PCOS. **In conclusion**. An imbalance in oxidative stress and antioxidants system toward increased of ROS generation and cause PCOS and its development. Inflammation stimulates the oxidation process and reduces the ability of cellular antioxidants in PCOS patients. Diagnostic effectiveness of NLR, PLR, and MPV can be used as biomarkers and test markers to detect PCOS even in the early stages of the disease, taking into account that it is part of a routine blood work analysis, and its higher values disease severity and imbalance between neutrophils and lymphocytes then to cancer and its development.

Ethical Clearance: Ethical clearance taken from Al-Saddr Teaching Hospital committee in Najaf city, Iraq.

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