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The Impact of Polycystic Ovary Syndrome Treatment on Inflammatory Markers and Homocysteine Levels: A Prospective Cohort Study

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ABBREVIATIONS

ALT: alanine transaminase

AMH: anti-Müllerian hormone

AST: aspartate transaminase

BMI: body mass index

COC: combined oral contraceptive

CRP: c-reactive protein

CV: cardiovascular

CVD: cardiovascular disease

DHEAS: dehydroepiandrosterone sulfate

FSH: follicle-stimulating hormone

FT4: free thyroxine

g: gram

HOMA-IR**:** homeostatic model assessment of insulin resistance

IL-1β: interleukin 1 beta

IL-6: interleukin 6

IL-18: interleukin 18

IR: insulin resistance

LH: luteinizing hormone

mg/dL: milligrams per deciliter

PCOS: polycystic ovary syndrome

pg/mL: picograms per milliliter

SD: standard deviation

SNP: single nucleotide polymorphism

TNF-α: tumor necrosis factor alpha

TSH: thyroid-stimulating hormone

μUI/mL: micro-international units per milliliter

17-OH Pg: 17-hydroxyprogesterone

ABSTRACT

Introduction: Polycystic ovary syndrome (PCOS) is a common endocrine disorder among women of reproductive age, characterized by hyperandrogenism, ovulatory dysfunction, and metabolic issues. Chronic low-grade inflammation is a key feature of the syndrome and may contribute to its pathogenesis and the wide range of clinical manifestations. Although numerous studies have evaluated the effect of PCOS treatment on inflammatory markers and homocysteine levels, the findings have often been inconsistent and inconclusive.

Objective: This study aimed to evaluate the impact of treatment on inflammatory markers (IL-6, IL-1β, TNF-α) and homocysteine levels in women with PCOS over a six-month period. Additionally, it sought to assess the effects of different PCOS treatment regimens on these markers, as well as their influence on androgen levels, homeostatic model assessment of insulin resistance (HOMA-IR), and body mass index (BMI).

Methods: This is a multicentric observational prospective cohort study conducted across three outpatient centers in Tirana, Albania, enrolling women aged 18-40 diagnosed with PCOS based on Rotterdam criteria, from February 2022 to March 2024. Patients were categorized into three subgroups based on their clinical and biochemical features (metabolic, hyperandrogenic, and combined phenotype) and received tailored treatment protocols. Inflammatory markers and homocysteine levels were measured at baseline and after six months.

Results: Of the 156 participants, 135 completed follow-up (mean age 26.21 ± 5.33 years). Significant reductions in inflammatory markers and homocysteine levels were observed across the entire cohort: IL-6 decreased from 8.44 ± 11.08 to 4.36 ± 3.55 pg/mL ($p < 0.001$), IL-1β from 15.34 ± 15.37 to 10.19 ± 28.21 pg/mL ($p = 0.012$), TNF-α from 5.88 ± 2.55 to 3.48 ± 1.59 pg/mL ($p < 0.001$), and homocysteine from 10.89 ± 6.01 to 6.77 ± 3.03 μmol/L ($p < 0.001$). Consistent reductions following treatment were noted across all subgroups, with the greatest declines observed in subgroup 3 for IL-6 and TNF-α (mean difference of 4.8 pg/mL for IL-6 and 2.66 pg/mL for TNF-α) and in subgroup 2 for IL-1β (mean difference of 8.38

pg/mL). Statistically significant differences in median levels of inflammatory cytokines and homocysteine were observed across the three subgroups. Additionally, significant reductions in BMI, HOMA-IR, and androgen levels were observed throughout the cohort.

Conclusions: Tailored treatment significantly reduces inflammatory markers and homocysteine levels in women with PCOS. These findings support the effectiveness of phenotype-based treatment approaches for managing inflammation and clinical disturbances in PCOS. However, further research should focus on determining the persistence of these improvements over time and their long-term clinical implications.

1 INTRODUCTION

Polycystic ovary syndrome (PCOS)—a lifelong and heterogeneous endocrine and metabolic disorder—represents the most common endocrinopathy among women of reproductive age, with a prevalence of 10-13% (Teede et al., 2023). Since its initial description in 1935 (Stein & Leventhal, 1935), the characterization of the syndrome has significantly evolved over the years. Initially defined primarily through case reports, subsequent more solid evidence over time have refined the understanding of this complex syndrome and enhanced its clinical recognition.

1.1 Evolving diagnostic criteria for polycystic ovary syndrome

The diagnostic criteria for PCOS have significantly evolved over time as advances in research and clinical understanding have elucidated the syndrome's heterogeneous and intricate nature. Through the years, various international scientific associations have proposed definitions that were initially based on expert opinion, with subsequent revisions aimed at integrating emerging clinical insights and research developments.

The first international conference on PCOS, organized by the National Institute of Health (NIH) in 1990, led to the establishment of what is still referred to as *NIH 1990 criteria* for PCOS diagnosis (Dunaif et al., 1992). These criteria were primarily based on expert clinical consensus rather than extensive analytic studies. Considered the most stringent PCOS diagnostic framework to date, the criteria required the presence of both chronic anovulation and clinical and/or biochemical hyperandrogenism, while excluding other potential diagnoses, such as Cushing's syndrome, non-classic congenital adrenal hyperplasia (NCCAH), and androgen-secreting tumors, as well as thyroid dysfunction and hyperprolactinemia.

The initial effort to define PCOS was later expanded as subsequent analytic studies provided further insights (Azziz et al., 2001; Nestler et al., 1998). These insights were integrated into the revised diagnostic criteria proposed by the Rotterdam European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM)-Sponsored PCOS Consensus Workshop, resulting in the *Rotterdam criteria* for PCOS diagnosis (group, 2004).

The Rotterdam consensus criteria—which largely replaced the NIH criteria in clinical practice—include three diagnostic features, with any two of the three required for PCOS diagnosis (group, 2004). These criteria include: (1) oligo- or anovulation, (2) clinical and/or biochemical hyperandrogenism, and (3) polycystic ovarian morphology (PCOM) on imaging—after excluding other mimicking disorders, as previously described. The inclusion of PCOM as a diagnostic criterion was facilitated by improvements in ultrasound technology, and led to the identification of two additional phenotypes not previously recognized by the NIH criteria: (1) women with ovulatory dysfunctions and PCOM but without hyperandrogenism, and (2) ovulatory women with hyperandrogenism and PCOM. These newly identified phenotypes (group, 2004) represent distinct hormonal and metabolic profiles compared to cases of PCOS diagnosed using NIH criteria. Their inclusion was intended to encompass the whole spectrum of this heterogeneous disorder, with the goal of improving quality of life, reproductive outcomes, and preventing long-term health complications associated with the condition.

Subsequently, ESHRE/ASRM sponsored the 2nd and 3rd PCOS Consensus Workshops, which focused on infertility management (Group, 2008) and provided an update on the available data concerning the syndrome (Fauser et al., 2012).

Meanwhile, in 2009, the Androgen Excess and Polycystic Ovary Syndrome Society (AE-PCOS), following a comprehensive review of the literature and expert consensus, proposed a slightly modified version of the existing diagnostic criteria: (1) clinical and/or biochemical hyperandrogenism, (2) ovarian dysfunction, including oligo/anovulation and/or PCOM, and (3) exclusion of other androgen excess disorders (Azziz et al., 2009). Therefore, in *AE-PCOS criteria*, similar to NIH criteria, androgen excess is a necessary component of the

diagnosis. Unlike the Rotterdam criteria, the AE-PCOS criteria exclude the phenotype of ovulatory dysfunction and PCOM alone, which has been shown to exhibit anthropometric, hormonal, and metabolic profiles similar to control subjects (Azziz et al., 2009).

Over the years, various scientific societies have developed guidelines for the assessment and management of PCOS (Conway et al., 2014; Fauser et al., 2012; Goodman et al., 2015), largely based on expert opinion and consensus. In 2014, AE-PCOS Society based on improvements in ultrasound technology, recommended to increase the follicles threshold for defining PCOM from 12 to around 25 follicles per ovary (Conway et al., 2014), aiming to improve diagnostic accuracy. However, subsequent guidelines lowered this threshold to 20 follicles per ovary (Teede et al., 2018; Teede et al., 2023).

In 2018, the International Evidence-based Guidelines for the Assessment and Management of PCOS were introduced (Teede et al., 2018), marking a transition from consensus-based to evidence-based diagnostic criteria, utilizing the best available evidence at the time, although much of the available evidence was of low to very low quality.

The most recent international guidelines (Teede et al., 2023) integrate newer evidence and methodologies, reflecting advancements over the five-year period, though the quality of evidence remains moderate to low. These guidelines also address gaps identified since 2018, with a particular focus on improving education and awareness among multidisciplinary healthcare providers and promoting personalized treatment approaches to better meet patient needs and improve health outcomes. Another key objective of the guidelines was to standardize the assessment tools and management of PCOS worldwide. While recommending the Rotterdam criteria for PCOS diagnosis, the guidelines suggest that anti-Müllerian hormone (AMH) may be used as an alternative to ultrasound, given that AMH levels independently correlate with PCO morphology (Rosenfield et al., 2012). Therefore, AMH measurement can substitute ultrasound, particularly in settings where it is not readily available. Moreover, combining ultrasound with AMH levels can help identifying more PCOS cases (Fraissinet et al., 2017). The guidelines also emphasize the importance of a

lifelong health plan, promoting a healthy lifestyle, adherence to treatment, optimization of fertility, and the prevention and management of comorbidities such as diabetes, cardiovascular disease, sleep and mood disorders.

Furthermore, the guidelines place increased emphasis on cardiovascular risk factors monitoring, recommending that *all adult and adolescent women* with PCOS, *regardless of age and BMI*, undergo lipid profile and glycemic status assessments, along with blood pressure measurement at diagnosis and at regular intervals thereafter (Teede et al., 2023). Additionally, they strongly advocate recognizing PCOS as an independent risk factor for diabetes and recommend performing a 75-gram oral glucose tolerance test (OGTT) at baseline, during follow-up, in the preconception period, or at the first prenatal visit if it has not been previously conducted.

Notably, a higher prevalence of obstructive sleep apnea has been observed in PCOS patients compared to non-PCOS females, independent of BMI (Fernandez et al., 2018).

Moreover, *all adults and adolescents with PCOS* exhibit higher prevalence of moderate to severe anxiety and depressive symptoms, prompting healthcare professionals to screen for these disorders using regionally validated tools (Chaudhari et al., 2018; Teede et al., 2023).

Finally, the guidelines underscore the importance of providing high-quality, inclusive educational resources to all PCOS patients, highlighting the critical role of education in managing this chronic condition and its long-term health consequences.

To date, despite ongoing efforts, the diagnosis of PCOS remains controversial. This is partly due to the heterogeneity of the syndrome, which encompasses diverse phenotypes, and the clinician's preference for adopting categorical criteria to simplify diagnosis. The evolving nature of diagnostic criteria reflects the complexity of PCOS and underscores the need for greater understanding. Future studies should focus on integrating emerging biomarkers to refine diagnostic guidelines and developing a standardized approach that addresses the variability in clinical presentation.

1.2 PCOS as a chronic low-grade inflammatory disorder: Pathophysiological and clinical insights

In the last two decades, research into inflammatory markers in PCOS has gathered significant attention, particularly following the seminal work by Kelly et al. in 2001 (Kelly et al., 2001), which first identified elevated concentrations of c-reactive protein (CRP) in women with PCOS. This study paved the way for a deeper exploration of chronic low-grade inflammation in PCOS—a mild and persistent inflammatory response characterized by subtle increases in inflammatory markers that may contribute to metabolic dysregulation and tissue damage over time (Deligeoroglou et al., 2012; Duleba & Dokras, 2012).

Chronic low-grade inflammation is now increasingly recognized as a central factor in the pathogenesis of PCOS, contributing significantly to the metabolic (Cooney & Dokras, 2021; Diamanti-Kandarakis & Dunaif, 2012; E. Diamanti-Kandarakis, T. Paterakis, et al., 2006) and reproductive abnormalities associated with the condition (Velez et al., 2021). Additionally, PCOS is characterized by increased oxidative stress, reduced antioxidant capacity, and endothelial dysfunction (E. Diamanti-Kandarakis, K. Alexandraki, et al., 2006; Rudnicka et al., 2021), which along with the inflammatory state contribute to the initiation and progression of atherosclerotic process.

Beyond systemic inflammation, the ovaries in PCOS also exhibit persistent chronic inflammation, as demonstrated by macrophage infiltration of the ovaries and by increased inflammatory cytokines such as IL-6, TNF-α, IL-1β, and IL-18 (Liu et al., 2021). This inflammatory process is closely linked to insulin resistance (IR) and hyperandrogenism, both of which promote and sustain inflammation. Additionally, obesity exacerbates this inflammatory state, creating a vicious cycle (Diamanti-Kandarakis & Dunaif, 2012).

The precise trigger of this inflammatory process remains unclear. However, understanding the complex interplay between inflammation, IR, hyperandrogenism, and obesity is

essential for unraveling the pathophysiological complexities of the disease and recognizing its significant impact on clinical outcomes.

Insulin resistance and inflammation—IR plays a critical role in mediating increased cardiometabolic risk in women with PCOS (Randeva et al., 2012; Zhao et al., 2023). IR and compensatory hyperinsulinemia can be observed in 65–95% of women with PCOS, affecting the majority of overweight and obese women, as well as more than half of women with normal weight. Importantly, IR is independent of, but exacerbated by obesity (Cassar et al., 2016; Tosi et al., 2017).

The etiology behind increased IR and compensatory hyperinsulinemia in PCOS remains unclear. Although the number, structure, and function of insulin receptors are largely intact, a post-binding defect in insulin signaling has been identified, primarily due to increased receptor and insulin receptor substrate-1 (IRS-1) serine phosphorylation, which selectively impairs metabolic, but not mitogenic pathways, in classical insulin-target tissues and possibly in the ovaries (Diamanti-Kandarakis & Dunaif, 2012; Gonzalez, 2012). Notably, a tissue-specific difference in insulin resistance has been identified, with the ovaries exhibiting preserved or even exaggerated insulin action, particularly in the mitogenic pathway, which consequently increases androgen synthesis. Thus, in the context of systemic insulin resistance, the ovaries remain sensitive to insulin (Diamanti-Kandarakis et al., 2012).

The pro-inflammatory cytokine TNF-α is a known mediator of insulin resistance, primarily by promoting serine phosphorylation of IRS-1 (Ebejer & Calleja-Agius, 2013; Gonzalez, 2012). In insulin-resistant states, sources of cytokines include the insulin-target tissues themselves—particularly adipose tissue—and, to a larger extent, activated tissue macrophages. In PCOS, the activation of macrophages derived from mononuclear cells (MNC) in response to inflammation skews toward the M1 pro-inflammatory phenotype, shifting away from the M2 anti-inflammatory phenotype (Feng et al., 2023). These macrophages, which largely infiltrate adipose tissue, release pro-inflammatory cytokines that act in a paracrine manner, activating nuclear factor kappa-B (NF-κB), which enhances

the transcription of genes encoding inflammatory cytokines, such as IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1), among others (Deligeoroglou et al., 2012; Ebejer & Calleja-Agius, 2013; Gonzalez, 2012). These cytokines, in turn, further perpetuate macrophage recruitment and activation, resulting in a self-sustaining inflammatory cycle. Additionally, in women with PCOS, dietary triggers such as glucose can provoke oxidative stress and an inflammatory response from MNC-derived macrophages, a phenomenon independent of obesity (Gonzalez, 2012).

In summary, insulin resistance likely plays a pivotal role in initiating and sustaining inflammation in PCOS, promoting a vicious cycle that involves adipose tissue dysfunction, macrophage activation, and the release of pro-inflammatory cytokines.

Androgens and inflammation—Although the relationship between androgen excess and inflammation in PCOS is not fully understood, evidence suggests a bidirectional interaction (Ebejer & Calleja-Agius, 2013). Androgens contribute to adipocyte hypertrophy by activating enzymes and proteins involved in lipid and protein synthesis and insulin signaling. They also promote differentiation of pre-adipocytes in mature adipocytes especially in the abdominal area, facilitating the development of visceral-type obesity, which is present in 38 to 88 % of patients with PCOS (Deligeoroglou et al., 2012).

Hyperandrogenism has also been shown to activate MNC in the fasting state—further increasing their sensitivity to glucose intake—leading to another important component: diet-induced inflammation (Ebejer & Calleja-Agius, 2013; Gonzalez, 2012). Thus, glucose either in vivo, whether in vitro stimulates the MNC-derived macrophages to release TNF-α and IL-6, thereby promoting an inflammatory response (Gonzalez et al., 2005; Gonzalez et al., 2006). In turn, these cytokines, particularly TNF-α, stimulate androgen production in ovarian thecal cells (Gonzalez, 2012), creating a vicious cycle. Moreover, TNF-α has been shown to induce in vitro proliferation of ovarian thecal cells in rats (Spaczynski et al., 1999). This suggests that inflammation directly stimulates the polycystic ovaries to produce androgens, thus causing hyperandrogenemia.

A strong link exists between insulin resistance and androgens in PCOS (Yanes Cardozo et al., 2017). Thus, insulin resistance and the compensatory hyperinsulinemia increase androgen production both directly in the ovaries and indirectly by stimulating luteinizing hormone (LH) release. It also decreases the synthesis of sex hormone-binding globulin (SHBG), thereby increasing free androgen levels.

Androgen levels positively correlate with hyperinsulinemia in women with PCOS.

Additionally, a positive correlation between elevated plasma androgen levels and several cardiometabolic risk factors, including endothelial dysfunction, increased blood pressure, and obesity has been reported (Barber et al., 2015; Moulana, 2023; Paradisi et al., 2001). Notably, these parameters tend to improve with androgen reduction (Amiri et al., 2014). Interestingly, recent systemic reviews of population-based studies suggest that aging women with PCOS—due to natural decline in circulating androgen levels with age—may not have an increased risk of CVD compared to healthy controls (Kazemi Jaliseh et al., 2017; Ramezani Tehrani et al., 2020).

Despite the strong evidence linking plasma androgen levels and cardiometabolic risk factors, the exact mechanisms by which androgens drive these disturbances remain unclear.

Obesity and inflammation—The onset of the inflammatory process in PCOS is intricately linked to increased adiposity, particularly with the abdominal adiposity (Dumesic et al., 2016; Gonzalez, 2012). Obesity is a crucial contributor to inflammation, as adipose tissue is a major site of inflammatory cytokine production. Thus, in obesity, the associated adipocyte hypertrophy cause hypoperfusion due to stromal vessel compression. The resulting adipocyte hypoxia triggers the activation of NF-κB, which induces the production and secretion of several pro-inflammatory cytokines, including TNF-α, IL-6, IL-18, IL-1β and CRP. These cytokines, in turn, promote the macrophage recruitment in the adipose tissue, which maintain the inflammatory state, impair adipocyte cell function, and promote cellular necrosis, thus further aggravating inflammation and setting up a self-perpetuating vicious cycle (Deligeoroglou et al., 2012; Ebejer & Calleja-Agius, 2013; Spritzer et al., 2015).

In summary, insulin resistance and hyperandrogenemia represent key drivers in initiating and sustaining the inflammatory state in PCOS, with obesity further exacerbating this complex interplay (Diamanti-Kandarakis & Dunaif, 2012; Ding et al., 2021; Yanes Cardozo et al., 2017).

1.2.1 C-reactive protein in PCOS: Inflammation and metabolic implications

The majority of studies investigating chronic low-grade inflammation in PCOS have been focused on the measurement of CRP, an acute phase protein produced by the liver in response to stimulation by IL-6 and TNF-α. CRP is also directly produced by adipose tissue. It represents a key marker of systemic inflammation and one of the most reliable predictors of cardiovascular disease risk (Ridker, 2009). The studies, which consistently corroborated earlier findings, further demonstrated a correlation between elevated CRP levels and various metabolic factors, such as a positive correlation with the degree of obesity and an inverse correlation with insulin sensitivity (E. Diamanti-Kandarakis, K. Alexandraki, et al., 2006; Evanthia Diamanti-Kandarakis et al., 2006; Rudnicka et al., 2020).

Importantly, even normal-weight women with PCOS exhibit significantly higher CRP levels compared to weight-matched healthy controls, indicating that the inflammatory state in PCOS is not solely attributable to obesity (Aboeldalyl et al., 2021; Escobar-Morreale et al., 2011; Tarkun et al., 2004; Tola et al., 2017). However, obesity and hyperinsulinemia may exacerbate the underlying chronic low-grade inflammation associated with the syndrome (Rudnicka et al., 2020). Moreover, the inflammatory background appears to be persistent over time (Talbott et al., 2004).

Currently, no consistent association has been found between androgen levels and CRP concentrations in women with PCOS (Papalou et al., 2015); however, other inflammatory markers, such as white blood cells (WBC), have shown a positive correlation (Rudnicka et al., 2020).

1.2.2 Pro-Inflammatory cytokines

Cytokines are critical signaling molecules involved in intercellular communication and immune regulation. They are produced by a variety of cells, including immune cells, adipocytes, and endothelial cells, and play an important role in coordinating both physiological and pathological inflammatory responses.

Over the past three decades, a wide range of pro-inflammatory cytokines has been extensively studied considering their involvement in numerous biological processes, such as promoting macrophage recruitment, vascular smooth muscle cell proliferation, and the migration of these cells from tunica media to the tunica intima. These mechanisms are central to the pathogenesis of atherosclerosis and metabolic disorders (Escobar-Morreale et al., 2011; Rudnicka et al., 2020). In the context of metabolic disorders, such as polycystic ovary syndrome, dysregulation of pro-inflammatory cytokines contributes to the chronic low-grade inflammation that underpins many of the syndrome's clinical features, such as insulin resistance, hyperandrogenism, and obesity (Rudnicka et al., 2021).

Pro-inflammatory cytokines interact in a cascade, where one promotes the synthesis of the next. Thus, IL-18 induces the production of TNF-α, which in turn promotes IL-6 synthesis, and the latter regulates the hepatic production of CRP. IL-18, which belongs to IL-1 superfamily, is closely associated with insulin resistance and metabolic syndrome, and is an important predictor of long-term cardiovascular mortality (Rudnicka et al., 2021). In PCOS, increased IL-18 concentrations are observed regardless of the presence of insulin resistance and obesity, although obesity and hyperinsulinemia further increase IL-18 levels in these women (Escobar-Morreale et al., 2004; Kaya et al., 2010).

Furthermore, key pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β have been extensively studied in relation to their roles in disease pathogenesis and their correlation with various PCOS phenotypes. This has spurred research into the genetic regulation of

pro-inflammatory pathways, with studies focusing on polymorphisms in interleukin genes and their promoter regions, investigating their potential role in shaping the inflammatory and metabolic features of PCOS (Chen et al., 2018; Escobar-Morreale et al., 2001; Wu et al., 2015; Yun et al., 2011).

TNF-α—A multifunctional pro-inflammatory cytokine primarily produced by macrophages and monocytes, but also by adipocytes, endothelial cells, and fibroblasts, with a crucial role in mediating inflammatory responses (Deligeoroglou et al., 2012). It is also produced by granulosa and thecal cells of the ovarian follicles, influencing the follicular development and ovulation in response to the LH surge (Duffy et al., 2019).
TNF-α is a well-known mediator of inflammation-induced insulin resistance, primarily by promoting serine phosphorylation of IRS-1, which disrupts insulin signaling (Ebejer & Calleja-Agius, 2013).

Conflicting results have been reported regarding the association between TNF-α levels and PCOS. While two meta-analyses found no clear association (Escobar-Morreale et al., 2011; Toulis et al., 2011), others reported higher levels of TNF-α in PCOS compared to controls (Gao et al., 2016). A recent systematic review and meta-analysis found no significant differences in TNF-α levels between PCOS patients and controls, while observing increased CRP levels in PCOS women, independent of obesity (Aboeldalyl et al., 2021).

Several studies have explored the association between polymorphisms in the promoter region of TNF-α gene and PCOS, yielding controversial results, particularly when across different ethnic groups (Escobar-Morreale et al., 2001; Wu et al., 2015; Yun et al., 2011). A recent meta-analysis addressing ethnic differences in single nucleotide polymorphisms (SNPs) demonstrated that while -238 G/A, -857 C/T polymorphisms might influence susceptibility to PCOS in the overall population, the -308 G/A variant is specifically associated with PCOS risk in the Asian population (Zhang et al., 2020). In addition, the -308G/A polymorphism has been linked to higher androgen levels, suggesting a role in modulating the clinical expression of the syndrome (Escobar-Morreale et al., 2001).

However, given the heterogeneity and varying quality of the studies included in meta-analyses, the findings should be interpreted with caution. Future studies involving larger, multi-ethnic cohorts of well-matched PCOS patients and controls are needed to clarify the role of SNPs of TNF-α gene in PCOS susceptibility and its phenotypes.

IL-6—A multifunctional pro-inflammatory cytokine produced by various cells, including macrophages, adipocytes, endothelial cells, and granulosa and theca cells in ovarian follicles (Rostamtabar et al., 2021). It plays a crucial role in immune regulation and metabolic processes, particularly in the context of obesity and insulin resistance, both of which are commonly associated with PCOS (Deligeoroglou et al., 2012; Escobar-Morreale et al., 2003).

IL-6 has garnered particular attention in studies exploring the association between serum interleukin levels and the phenotype of PCOS. This is due to various factors: its direct role in stimulating hepatic synthesis of CRP—a major marker of systemic inflammation and of cardiovascular risk (Ebejer & Calleja-Agius, 2013); its close association with obesity, insulin resistance, and cardiovascular diseases (Deligeoroglou et al., 2012; Vgontzas et al., 2006); and its influence on ovulation, fertilization, and implantation—processes that are often impaired in women with PCOS (Kicinska et al., 2023).

Serum levels of IL-6 are reported to be elevated in women with PCOS compared to healthy controls, with even higher concentrations observed in obese PCOS women. Higher BMI, insulin resistance, and hyperandrogenemia contribute to increased IL-6 concentrations (Lin et al., 2011). Moreover, its secretion from adipocytes is correlated with adipocyte size, leading to higher IL-6 release in response to weight gain (Hotamisligil, 2006; Rostamtabar et al., 2021). However, despite these findings, the association between elevated serum IL-6 levels with PCOS remain inconsistent.

Some meta-analyses have reported no significant association between IL-6 levels and PCOS, suggesting that the increased IL-6 levels previously observed may be primarily related to obesity rather than to PCOS itself (Escobar-Morreale et al., 2011; Toulis et al., 2011). In contrast, a later meta-analysis found significantly higher IL-6 levels in PCOS patients and

linked these elevations to insulin resistance and testosterone levels, proposing that IL-6 could serve as a useful biomarker for monitoring the treatment of PCOS (Peng et al., 2016). However, a more recent meta-analysis corroborated earlier findings, showing no association between IL-6 levels and PCOS (Aboeldalyl et al., 2021).

Similar to TNF-α, several studies have explored the association between polymorphisms in IL-6 gene and its promoter region and PCOS susceptibility. Previous studies have reported a link between specific SNPs and hyperandrogenemia—particularly with increased levels of testosterone and 17-hydroxyprogesterone (17-OH Pg) (Villuendas et al., 2002; Walch et al., 2004). The most commonly studied SNP in the promoter region of the IL-6 gene is -174 G/C, which has been proven to alter the gene's transcription rate. However, meta-analyses have shown conflicting results, with some reporting no association (Guo et al., 2015; Wu et al., 2015), while others showing this SNP to be associated with decreased susceptibility to PCOS (Chen et al., 2018).
Further studies with larger, well-matched populations are needed to clarify these findings.

IL-1β—A pleiotropic cytokine playing a significant role in the inflammatory response, as well as in cell proliferation, differentiation, and apoptosis. It is primarily secreted by monocytes, macrophages, and epithelial cells, but also by granulosa and theca cells in ovarian follicles. IL-1β is produced by the cleavage of its inactive precursor, pro-IL-1β, through the activation of caspase-1. This activation is triggered by the assembly of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome—a multi-protein complex located within the cytoplasm of immune cells that is primarily responsible for activating inflammatory processes in response to several harmful cellular signals. IL-1β activates NF-κB signaling, which in turn promotes the production of IL-1β, creating a positive feedback loop that sustains and amplifies the inflammatory process (Rostamtabar et al., 2021). Alongside other pro-inflammatory cytokines, IL-1β plays an important role in ovulation by inducing the expression of proteases responsible for the degradation of the extracellular matrix surrounding the follicle and by modulating prostaglandin production.

Additionally, it influences the process of fertilization, and its increased levels can disrupt endometrial receptivity and embryo implantation (Duffy et al., 2019).

A significant positive association has been observed between PCOS and elevated serum IL-1β levels (Zafari Zangeneh et al., 2017). Moreover, increased IL-1β levels are observed in the follicular fluid of women with PCOS, further suggesting its influence in altering the follicular microenvironment and possibly affecting ovarian function (Liu et al., 2021).

Genetic studies investigating SNPs of IL-1β gene and their association with susceptibility to PCOS has yielded mixed results, particularly across different ethnic groups. Thus, no significant association was found between the IL-1β -511 C/T polymorphism and PCOS risk, as well as several clinical and biochemical parameters of PCOS (Guo et al., 2015). In a more recent meta-analysis, IL-1β -51 C/T polymorphism was associated with increased PCOS susceptibility in the Asian populations (Zhang et al., 2020).

In summary, pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β play significant roles in the pathophysiology of PCOS. Increased levels of these cytokines have been observed in PCOS—particularly correlated with obesity and insulin resistance—suggesting a link between chronic inflammation and metabolic features. Emerging genetic studies aim to identify specific SNPs in the interleukin genes and their promoter regions associated with the risk of PCOS and its phenotypes.

1.2.3 Impact of pharmacological approaches on pro-inflammatory cytokines in PCOS

1.2.3.1 Metformin and Inositols

Metformin, a well-known insulin-sensitizing agent, has shown potential in addressing the chronic low-grade inflammation often associated with PCOS (Evanthia Diamanti-Kandarakis et al., 2006). However, research shows conflicting results regarding its impact on pro-inflammatory cytokines, such as IL-6 and TNF-α. Some studies report no significant changes in plasma IL-6 levels with metformin treatment, while TNF-α levels have been

found to decrease significantly (Jakubowska et al., 2008). Other studies suggest that reductions in IL-6 levels correlate with improvements in insulin sensitivity following metformin therapy (Luque-Ramirez & Escobar-Morreale, 2010). Combined treatment with metformin and other insulin-sensitizing agents, such as pioglitazone, has been associated with greater reductions in IL-6 levels compared to metformin alone, further suggesting a link between insulin resistance improvement and inflammatory markers reduction (Ali et al., 2019; Ciaraldi et al., 2013). Conversely, a meta-analysis reported no significant decrease in IL-6 levels with metformin treatment, highlighting the need for further investigation (Wang et al., 2017).

Inositols, particularly myo-inositol and D-chiro-inositol, influence PCOS pathogenesis by improving insulin signaling and reducing insulin resistance and hyperinsulinemia (Dinicola et al., 2021; Moretti et al., 2024). However, there is limited direct evidence evaluating their effects on the inflammatory status associated with PCOS. Therefore, comprehensive studies are warranted to explore these associations and assess the potential direct effects of inositols on inflammatory markers in PCOS.

Collectively, existing studies on the impact of metformin and inositols on inflammation in PCOS patients are limited by small sample sizes, short follow-up durations, and heterogeneity in treatment protocols. Future research should prioritize larger, homogeneous cohorts with extended follow-up periods to elucidate the role of these pharmacological approaches in modulating inflammatory status in PCOS.

1.2.3.2 Combined oral contraceptives (COC)

COC use has been associated with the induction of an inflammatory profile in healthy individuals, raising concerns that their use may exacerbate the low-grade inflammation typically observed in PCOS (Sorensen et al., 2014). On the other hand, COCs contain progesterone—an important immune-modulator, which exerts anti-inflammatory effects by modulating the activity of NF-κB, thereby decreasing the expression of TNF-α and IL-1β

(Tait et al., 2008). In addition, studies assessing the impact of COCs on inflammatory markers in PCOS have produced mixed results.

In a 1-year randomized controlled trial comparing metformin alone versus metformin in combination with COC, neither treatment model was associated with changes in inflammatory markers (Glintborg et al., 2014). Similarly, a recent meta-analysis concluded that COCs do not consistently and significantly increase IL-6 and TNF-α levels in PCOS patients (de Medeiros et al., 2018). In contrast, this meta-analysis, as well as earlier research (Dardzińska et al., 2014), indicates that COC use is associated with elevated CRP levels, regardless of the COC formulation, suggesting a potential increased risk of atherogenesis. Moreover, IL-1β levels were increased with COCs use (Manzoor et al., 2019).

Overall, the studies examining changes in inflammatory cytokines with COCs treatment in PCOS are hindered by small sizes, variation in COC compositions, and short follow-up periods. Larger studies with more homogeneous treatment protocols are necessary to clarify the influence of COCs—a mainstay of PCOS treatment—on the inflammatory status of these patients.

1.2.3.3 Statins and anti-inflammatory therapies

Several studies have explored the role of statins in the management of PCOS (Duleba et al., 2006; Raval et al., 2011). However, findings regarding their anti-inflammatory effects have been inconsistent.

Atorvastatin was shown to significantly decrease CRP levels in PCOS (Sathyapalan et al., 2009). Moreover, a prospective randomized trial comparing the role of metformin and simvastatin reported that both drugs produced comparable reductions in inflammatory markers after 3 months of therapy, with combined therapy offering no significant advantage over simvastatin alone (Banaszewska et al., 2009). A subsequent review highlighted the lack of sufficient evidence to recommend statins specifically for their anti-inflammatory effects in PCOS, except in cases where dyslipidemia coexist (Sirmans et al., 2012).

Other therapies with potential anti-inflammatory effects in PCOS include omega-3 fatty acids (Tosatti et al., 2021), vitamin D (Akbari et al., 2018), and resveratrol (Karimi et al., 2022).

Despite emerging research points to the promise of these interventions, more robust data from larger, homogenous studies with extended follow-up periods are required to define their role in modulating inflammation in PCOS.

1.3 Clinical implications of homocysteine in PCOS

Homocysteine is a sulfur-containing amino acid that plays a crucial role in cellular metabolism and the methylation cycle. Increased levels of homocysteine are associated with cardiovascular risk, oxidative stress, and endothelial dysfunction independently of other traditional CV risk factors (Coppola et al., 2000; Ganguly & Alam, 2015; Nehler et al., 1997). Women with PCOS exhibit higher homocysteine levels compared to healthy controls, regardless of the degree of obesity, IR, or androgen levels (Meng et al., 2016; Murri et al., 2013). Some studies, however, indicate a strong association between increased serum homocysteine and insulin resistance in PCOS (Badawy et al., 2007; M. Schachter et al., 2003). In addition, a positive correlation has been observed between increased androgen levels and homocysteine (Gul et al., 2008). The increased levels of this CV risk marker in PCOS raises significant concerns due to the cardiovascular vulnerability to which these women are already predisposed.

1.3.1 Impact of different therapeutic interventions on homocysteine levels in PCOS

Metformin— Numerous studies have shown an increase of homocysteine levels with metformin treatment in PCOS, seemingly in a dose-dependent manner, and suggested the use of doses below 1700 mg/day in these patients to mitigate this effect (Li et al., 2021; Zhang et al., 2016). Conversely, other studies report a decrease in homocysteine levels following metformin treatment (Kilic et al., 2011), while a meta-analysis reported no significant effect of metformin in homocysteine levels in PCOS patients (Meng et al., 2016). These conflicting

results emphasize the need for further investigation to elucidate metformin influence on homocysteine levels in PCOS and its clinical significance.

Combined oral contraceptives—The impact of COCs on homocysteine levels in PCOS remains inconclusive. While the majority of studies considering this topic are randomized, they typically involve small sample sizes and short follow-up durations, limiting the generalizability of findings (de Medeiros et al., 2018). Different COC formulations seem to yield varying results. For example, COCs containing drospirenone have been associated with both increases and decreases in homocysteine levels among women with PCOS (Harmanci et al., 2013; Mancini et al., 2010). In contrast, COCs containing ethinyl estradiol (EE) and cyproterone acetate decreased the levels, while EE and Desogestrel showed no significant impact (Cagnacci et al., 2006; Gul et al., 2008). This variability in findings may suggest that, among other factors, the influence of COCs on homocysteine levels may be formulation-dependent, highlighting the need for head-to-head trials with larger, more homogeneous cohorts.

Statins—Limited research has indicated that statins can reduce serum homocysteine levels in PCOS women (Kaya et al., 2009). Despite this, the role of statins as modulators of homocysteine in PCOS is not yet fully established, warranting further exploration.

2 RATIONALE FOR THE STUDY AND STUDY OBJECTIVES

The primary aim of this observational prospective cohort study was to evaluate the impact of phenotypic treatment approaches for PCOS on inflammatory markers and homocysteine levels. The study hypothesized that these treatments may modulate the inflammatory background and homocysteine levels in PCOS patients by enhancing insulin sensitivity and decreasing androgen levels.

To address the heterogeneity within the PCOS population, patients were categorized into three distinct subgroups based predominantly on their clinical and biochemical profiles:

- **Metabolic phenotype (subgroup 1)**: This subgroup comprises patients predominantly exhibiting indicators of insulin resistance (BMI levels indicative of overweight/obesity, acanthosis nigricans), and/or increased HOMA-IR;
- **Hyperandrogenic phenotype (subgroup 2)**: This subgroup includes patients predominantly exhibiting clinical signs of hyperandrogenism (hirsutism, acne, and male-pattern hair loss) and/or elevated serum androgen levels (*i.e.* hyperandrogenemia);
- **Combined phenotype (subgroup 3)**: This subgroup comprises patients with an equivalent/similar expression of the features of subgroup 1 and 2.

STUDY OBJECTIVES

Primary objectives:

- To evaluate the impact of PCOS treatment on inflammatory markers (IL-6, IL-1β, and TNF-α) and homocysteine levels by assessing changes in these markers following 6 months of treatment in women with PCOS.
- To assess the impact of different PCOS treatment regimens on the levels of IL-6, TNF-α, IL-1β, and homocysteine by evaluating changes in each subgroup.

Secondary objectives:

- To evaluate the effects of treatment on androgen levels, HOMA-IR and BMI in the overall cohort and each subgroup.
- To assess the differences in inflammatory cytokine levels among the three subgroups at baseline and post-treatment.

3 MATERIALS AND METHODS

3.1 Study design and population

This multicentric observational prospective cohort study was conducted across three outpatient centers in Tirana, Albania. Patients diagnosed with polycystic ovary syndrome were recruited from February 2022 to March 2024 and followed for 6 months.

The study included females aged 18-40 years who had been diagnosed with PCOS according to the Rotterdam criteria and the latest guidelines for the diagnosis of PCOS (Teede et al., 2023). The exclusion criteria comprised patients with PCOS who were pursuing pregnancy; those with recognized chronic infectious illnesses or acute infectious illnesses (in the last month); diabetes mellitus; arterial hypertension; patients with a family history of deep venous thrombosis; current cigarette smoking (ex-smokers required a cessation period of at least 6 months); females on pharmacologic treatment for PCOS within 1-3 months prior to the diagnostic assessments, as well as females on treatment with antilipidemic, antihypertensive medications, folate antagonists, and anti-epileptic drugs.

Informed consent was obtained from all participants prior to their inclusion in the study. Patients were informed that their treatment would adhere to the latest clinical guidelines for the management of PCOS (Teede et al., 2023) and that biochemical assessments of inflammatory cytokines (IL-6, TNF-α, IL-1β) and homocysteine would be conducted before and after six months of treatment solely for research purposes. The study was performed in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki and its later amendments.

3.2 Baseline assessments

To ensure a relevant study population, patients with either a suspected or confirmed diagnosis of PCOS were carefully examined. The patients underwent clinical, biochemical, and ultrasonographic assessments.

The **clinical assessment** included a detailed medical history, anthropometric measurements and physical examination. The medical history involved a detailed menstrual history (including age at menarche, onset of menstrual irregularities, current frequency of menstrual cycles, duration, and flow), the presence of additional symptoms (such as weight gain, hair loss, mood changes, etc.), the pathologic history (known chronic diseases, past medical history), family history of metabolic and cardiovascular diseases (e.g., diabetes, cardiovascular disease, PCOS), and lifestyle factors (such as diet, smoking status, and physical activity). Anthropometric measurements included assessments of weight (in kilograms) and BMI (in kg/m^2). The physical examination included evaluation of the presence of abdominal violaceous striae, acanthosis nigricans, hirsutism (using the modified Ferriman-Gallwey score), acne, male-pattern hair loss and blood pressure measurement.

Biochemical assessments were conducted by collecting blood samples between 8 and 10 a.m. after an overnight fasting. Serum levels of follicle-stimulating hormone (FSH), LH, estradiol (E2), prolactin (PRL), thyroid-stimulating hormone (TSH), free thyroxine (FT4), cortisol, total testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEAS), and 17-OH Pg, which were assessed during the early follicular phase of the menstrual cycle (days 2-5), or on a random day in females with amenorrhea. Fasting glucose and insulin, alanine transaminase (ALT), aspartate transaminase (AST), azotemia, and creatinine were also measured. Insulin resistance was evaluated using HOMA-IR, calculated using the following formula:

HOMA-IR = (fasting insulin (μUI/mL) x fasting glucose (mg/dL)/405 (Matthews et al., 1985).

Ultrasonographic assessment was performed using a Voluson E6 ultrasound system (2015), equipped with a transvaginal transducer (RIC5-9-D probe) and abdominal transducer (RAB6-D). A transvaginal approach was utilized, whenever possible and permitted by the patients, during the early follicular phase of the menstrual cycle or on a random day for patients presenting with amenorrhea. The assessment began with ruling out the presence of dominant follicles, cysts, or corpora lutea. PCOM was then evaluated by measuring ovarian volume and follicle count. Ovarian volume was determined both automatically by

the ultrasound machine and calculated using the three-dimensional diameters of each ovary. The number of follicles measuring 2-9 mm in diameter was counted per cross-sectional image.

Following the clinical, biochemical, and ultrasonographic assessments, the diagnosis of PCOS was further considered according to the Rotterdam criteria. Additional appropriate diagnostic tests were performed in some patients to exclude NCCAH, using a high-dose ACTH stimulation test for basal 17-OH Pg > 2 ng/ml; Cushing's syndrome, through repeated serum cortisol measurements and low-dose dexamethasone suppression test (LDDT); and androgen-secreting tumors, through abdominal ultrasound and/ or computed tomography.

Inflammatory markers and homocysteine assessment: Venous samples were taken in a fasting state at a single central laboratory. After proper centrifugation, 3 mL of serum was divided in three Eppendorf tubes. Samples for homocysteine and IL-6 analysis were stored at -20°C and assessed within 1-2 months, while samples for IL-1β and TNF-α were stored at -80°C and evaluated within 3-6 months. The following laboratory methods were employed:

- IL-1β: Serum levels of IL-1β were measured with enzyme-linked immunosorbent assay (ELISA), using the DEMEDITEC IL-1 Beta ELISA kit (Catalog No. DE4437, DEMEDITEC Diagnostic GmbH, Germany).
- TNF-α: Serum levels of TNF-α were assessed with ELISA, using the DEMEDITEC TNF-α ELISA kit (Catalog No. DE4641, DEMEDITEC Diagnostic GmbH, Germany).
- IL-6: Serum levels of IL-6 were assessed with chemiluminescent immunoassay (CLIA), using the Maglum IL-6 CLIA kit (Catalog No. 130616004M, Maglum, Germany).
- Homocysteine**:** Serum levels of homocysteine were assessed using the enzymatic assay method with the Cobas Integra 400 plus analyzer (REF No. 05385415 190, Roche Diagnostic).

The manufacturer's instruction for use of every kit were followed for optimal performance (Diagnostic, 2022; Diagnostic, 2021; GmbH, 2021a, 2021b).

3.3 Subgroup categorization and specific treatment protocols

Patients were divided into three subgroups based predominantly on their clinical and biochemical features, as described in the section titled "Rationale for the study and study objectives".

Specific treatment protocols

In accordance with the guidelines for the treatment of PCOS, lifestyle intervention (diet and physical activity) and optimal weight management were emphasized to all patients (Teede et al., 2023). However, no specific structured dietary or physical activity regimes were prescribed and adherence to these recommendations was not formally measured.

Treatment protocols were tailored for each subgroup as follows:

Subgroup 1—Patients in this subgroup received a combined treatment with Metformin and Inositols. Metformin was administered at a dose of 1-2 g/day, with dosage escalation every 1-2 weeks. In cases of adverse gastrointestinal effects, the dosage was reduced or switched to extended-release formulations. Inositols were administered at a daily dosage of 2000 mg Myo-inositol and 400 mg D-chiro-inositol.

Subgroup 2—A combined oral contraceptive was administered in this subgroup, with a regimen consisting of 24 active tablets, each containing 20 mcg Ethinyl estradiol and 3 mg of Drospirenone, followed by 4 placebo tablets.

Subgroup 3—Patients in this subgroup received a combination of the therapies from subgroup 1 and 2. This included the same COC regimen as outlined is subgroup 2, along with the combined Metformin and Inositols treatment as specified in subgroup 1.

Before initiating treatment, relative and absolute contraindications to COC use were assessed in accordance with the Centers for Disease Control and Prevention (CDC) eligibility criteria for contraceptive use (Nguyen et al., 2024). Additionally, patients who experienced side effects from COC use during the follow-up period were switched to alternative formulations or discontinued COC use and were subsequently excluded from the study to maintain cohort homogeneity and ensure consistency in treatment outcomes.

3.4 Follow-up assessments and monitoring

Follow-up visits were conducted at 1, 3, and 6 months after the initiation of treatment. Each visit involved clinical assessments, with the final visit also including biochemical evaluations.

During the first follow-up visit at one month, the focus was on monitoring treatment adherence and identifying potential side effects. The physical examination included body weight assessment and blood pressure measurement.

The second follow-up visit at three months involved repeating the clinical assessment and physical examination as in the first visit, along with an additional evaluation of hirsutism using modified Ferriman-Gallwey scale.

At the six-month follow-up visit, the clinical assessment and physical examination were repeated and biochemical evaluations were performed. Blood samples were collected for fasting glucose and insulin to assess HOMA-IR, as well as total testosterone, DHEAS, androstenedione, 17OH Pg, IL-6, IL-1β, TNF-α, and homocysteine, utilizing the laboratory assays described in the section “Baseline assessments”.

4 STATISTICAL ANALYSIS

The collected data were exported from Microsoft Excel to the *Statistical Package for Social Sciences (SPSS) version 25.0*, where all statistical analyses were performed.

Categorical variables (*nominal*, including *binary/dichotomous*, and *ordinal*) were described using frequencies (absolute counts) and corresponding percentages (%). For numerical (*continuous*) variables, measures of central tendency (mean, median) and dispersion (standard deviation [SD] and interquartile range [IQR]) were calculated. The distribution of the variables was assessed using the Shapiro-Wilk test, which indicated a normal distribution of the variables in the entire cohort, while within subgroups the variables exhibited non-parametric distributions. Accordingly, *arithmetic means ± standard deviations* were reported for normally distributed data, whereas *medians and interquartile ranges* were provided for non-parametric data.

To analyze differences between two groups for continuous quantitative variables, the paired Student's t-test for normally distributed data, while the related-samples Wilcoxon signed rank test was applied for non-normally distributed data. For comparisons involving more than two groups, a one-way analysis of variance (ANOVA) was performed.

The results were presented in both simple and composite tables and supported by graphical representations. A p-value of ≤ 0.05 was considered statistically significant.

5 RESULTS

Between February 2022 and March 2024, a total of 156 patients diagnosed with PCOS were enrolled in the study according to the established inclusion criteria. The mean age of participants at baseline was 26.44 ± 5.37 years. Of these, 135 patients (mean age 26.21 years, SD = 5.33) completed the six-month follow-up. The remaining 21 patients were excluded during the study period due to pregnancy (n = 3), adverse effects necessitating treatment changes (n = 2), and discontinuation of treatment and follow-up (n = 16). **Figure 1** illustrates the study flowchart, showing patient enrollment, follow-up completion and subgroup classification.

5.1 Baseline characteristics of overall patients completing the study and patients in subgroups

The baseline characteristics of the 135 patients who completed the six-month follow-up were assessed to provide an overview of the study population and establish reference points for evaluating changes following the treatment period. Among these patients, 25 belonged to subgroup 1 (metabolic phenotype), 47 to subgroup 2 (hyperandrogenic phenotype), and 63 to subgroup 3 (combined phenotype). The baseline assessments included BMI, HOMA-IR, LH/FSH ratio, total testosterone, 17-OH Pg, androstenedione, DHEAS, inflammatory markers (IL-6, IL-1β, TNF-α), and homocysteine.

In the entire cohort, the mean BMI was 27.54 ± 5.9 kg/m^2. As expected, in the context of subgroups, subgroup 1 exhibited the highest BMI levels (34.13 ± 5.74 kg/m^2). Similarly, HOMA-IR levels were higher in subgroup 1 (5.78 ± 4.06) and 3 (4.07 ± 2.54), while averaging 3.48 ± 2.94 in the overall cohort.

The overall mean values of total testosterone, DHEAS, androstenedione, 17-OH Pg were 0.55 ± 0.24 ng/mL, 336.4 ± 128.9 μg/dL, 3.30 ± 1.24 ng/mL, and 1.3 ± 0.74 ng/mL, respectively. As expected, androgen levels were comparable in subgroups 2 and 3, and in both groups higher than in subgroup 1. Specifically, the baseline testosterone levels were 0.56 ± 0.19 ng/ml in subgroup 2, 0.59 ± 0.28 ng/ml in subgroup 3, while averaging 0.41 ± 0.17 ng/ml in subgroup 1. Other androgens showed similar trends among the subgroups.

Inflammatory markers exhibited considerable variability across participants. Thus, baseline IL-6, IL-1β, and TNF-α levels were 8.44 ± 11.08, 15.34 ± 15.37, and 5.88 ± 2.55 pg/mL, respectively, in the overall cohort. In the context of subgroups, slightly higher levels and greater variability were observed in IL-6 and IL-1β in subgroup 3, averaging 10.4 ± 15.59 pg/mL and 15.45 ± 17.38 pg/mL, respectively.

Baseline homocysteine levels were 10.89 ± 6.01 μmol/L across the cohort. When examining subgroups, subgroup 3 again displayed slightly higher homocysteine levels (11.26 ± 6.95 μmol/L) compared to subgroup 1 and 2 (11.09 ± 5.58 and 10.3 ± 4.82 μmol/L, respectively).

Table 1 summarizes the main clinical and biochemical evaluations of the entire cohort at baseline, while **Table 2** presents the clinical and biochemical evaluations within subgroups 1, 2, and 3.

5.2 Study outcomes

5.2.1 Overall and subgroup-specific changes in inflammatory markers and homocysteine levels

Significant reductions in inflammatory cytokines and homocysteine levels were observed across the entire cohort and each subgroup when comparing baseline values to post-treatment assessments.

Overall, IL-6 levels significantly decreased from 8.44 ± 11.08 to 4.36 ± 3.55 pg/mL ($p < 0.001$) after 6 months of treatment. This decline was consistent across all subgroups: subgroup 1 (from 6.58 ± 3.43 to 3.99 ± 2.09 pg/mL), subgroup 2 (from 6.8 ± 3.78 to 2.89 ± 2.18 pg/mL), and subgroup 3 (10.4 ± 15.59 to 5.60 ± 4.33 pg/mL), with the greatest reduction observed in subgroup 3 (mean difference: 4.8 pg/mL). A statistically significant reduction following treatment was found in the median IL-6 levels across three subgroups, 6.65 (4.44) vs 3.4 (3.8); 7.9 (6.04) vs 2.53 (2.47); 6.89 (6.63) vs 4.57 (4.0) (all $p < 0.001$), respectively for subgroup 1, 2, and 3.

Similarly, both IL-1β and TNF-α decreased significantly in the overall cohort, with IL-1β decreasing from 15.34 ± 15.37 to 10.19 ± 28.21 pg/mL ($p = 0.012$), and TNF-α decreasing from 5.88 ± 2.55 to 3.48 ± 1.59 pg/mL ($p < 0.001$). The greatest reduction of IL-1β was observed in subgroup 2 (mean difference: 8.38 pg/mL), while subgroup 3 had the greatest reduction in

TNF-α (mean difference 2.66 pg/mL). Additionally, statistically significant reductions in median IL-1β and TNF-α levels were observed across all subgroups following treatment.

Homocysteine levels were significantly reduced from 10.89 ± 6.01 μmol/L to 6.77 ± 3.03 μmol/L in the entire cohort ($p < 0.001$). Statistically significant reduction of median homocysteine levels was observed in subgroup 1 [from 10.2 (4.11) to 6.9 (5.0) μmol/L, $p < 0.001$], subgroup 2 [from 9.1 (4.43) to 5.9 (4.2) μmol/L, $p < 0.001$], and subgroup 3 [from 10.8 (6.2) to 6.6 (4.5) μmol/L, $p < 0.001$].

Tables 3 and 4 provide detailed summaries of the changes in inflammatory markers and homocysteine levels across the entire cohort and individual subgroups, respectively. **Figure 2** presents boxplots illustrating the overall cohort changes, while **Figures 3, 4,** and **5** present boxplots of the results in subgroup 1, 2, and 3.

5.2.2 Secondary outcomes

BMI showed a significant reduction across the entire cohort, decreasing from 27.54 ± 5.9 to 26.76 ± 5.54 kg/m^2 ($p < 0.001$). The greatest reduction in BMI was found in subgroup 3 (mean difference: 1.14 kg/ m^2). A statistically significant decrease was noted in median BMI levels in subgroup 2 [22.1 (3.7) to 21.9 (3.7) kg/m^2, $p = 0.001$] and 3 [27.1 (5.7) to 26.7 (4.7) kg/m^2, $p < 0.001$] following treatment.

Moreover, HOMA-IR levels decreased significantly in the overall cohort, from 3.48 ± 2.95 to 3.06 ± 2.68, $p < 0.001$, with subgroup 1 exhibiting the highest reduction (mean difference: 0.59). All subgroups showed consistent statistically significant reductions in median HOMA-IR levels.

Androgen levels followed similar patterns: total testosterone decreased from 0.55 ± 0.24 to 0.36 ± 0.15 ng/mL, DHEAS from 336.4 ± 128.9 to 231.2 ± 89.6 μg/dL, 17-OH Pg from 1.3 ± 0.74 to 0.72 ± 0.35 ng/mL, and androstenedione from 3.30 ± 1.24 to 2.19 ± 0.81 ng/mL (all $p <$

0.001). The most pronounced decreases in androgen levels were seen in subgroup 2 (mean difference: 0.24 ng/mL for total testosterone; 142.89 for DHEAS μg/dL; 0.89 ng/mL for 17-OH Pg; 1.55 ng/mL for androstenedione).

Tables 5 and **6** presents the differences in BMI, HOMA-IR, testosterone, DHEAS, 17-OH Pg, androstenedione in all cohort and subgroups, respectively. **Appendix Figure 1** illustrates boxplots of changes in these parameters in the overall cohort.

Lastly, when comparing baseline levels of inflammatory cytokines and homocysteine among the three subgroups, no significant differences were observed. In contrast, post-treatment analysis revealed statistically significant differences in IL-6 levels ($p < 0.001$) and homocysteine levels ($p = 0.013$) across the three subgroups. In addition, the pairwise comparison between subgroup 1 and 2 revealed a significant difference in post-treatment TNF-α levels ($p = 0.023$).

Table 7 illustrates the differences in baseline levels of inflammatory markers and homocysteine levels across subgroups, while **Table 8** presents the differences in post-treatment values for these parameters.

6 DISCUSSION

In our study the inflammatory markers, particularly IL-6 and IL-1β, displayed considerable baseline variability across the cohort and subgroups. Similar variability has been reported by meta-analyses and systematic reviews considering inflammatory markers in PCOS (Peng et al., 2016), emphasizing the syndrome's inflammatory heterogeneity. Notably, subgroup 3 exhibited higher variability in cytokine levels, probably because the larger sample of this subgroup likely captures a wider range of individual inflammatory patterns. Moreover, it may reflect the complex interactions between metabolic dysfunction and hyperandrogenism that characterizes the subgroup (Deligeoroglou et al., 2012; Gonzalez,

2012; Rosenfield & Ehrmann, 2016). However, the mean levels of inflammatory markers did not demonstrate significant differences between subgroups at baseline, suggesting comparable inflammatory profiles, despite the varying clinical presentations and patient characteristics across subgroups. Such uniformity at baseline may indicate a shared underlying pathophysiological mechanism influencing inflammatory marker levels across different phenotypes of PCOS, as suggested by previous studies (Duleba & Dokras, 2012; Escobar-Morreale et al., 2011; Rostamtabar et al., 2021).

Similarly, comparable homocysteine levels were observed in all subgroups at baseline, with higher variability in subgroup 3, potentially reflecting diverse clinical characteristics within this larger subgroup. These observations are consistent with meta-analyses of case-control studies that report no correlation between homocysteine and the degree of obesity, IR, or androgen levels in women with PCOS (Meng et al., 2016; Murri et al., 2013). Conversely, other studies observed a correlation between homocysteine, insulin resistance and increased androgen levels in PCOS (Esmaeilzadeh et al., 2017; Lin et al., 2013; Morey Schachter et al., 2003).

The study demonstrated significant reductions in inflammatory markers and homocysteine levels across the entire cohort and in each subgroup following treatment. These findings suggest that, despite the inherent heterogeneity of PCOS, pharmacologic intervention can effectively reduce low-grade inflammation associated with the condition. The observed reductions, potentially driven by enhancements in insulin sensitivity (significant reduction in HOMA-IR levels following treatment) and reductions in androgen levels, contribute to further reinforcing clinical improvements. Furthermore, targeting the predominant clinical features appears to contribute to the overall improvement in the inflammatory profile, underscoring the therapeutic potential of tailored treatment strategies in managing the inflammatory component of PCOS.

However, previous research on this topic has yielded inconsistent results. While some studies, in line with our findings, have shown that reductions in inflammatory cytokines are linked to improvements in insulin sensitivity and androgen levels (Ali et al., 2019; Amiri

et al., 2014; Ciaraldi et al., 2013; Luque-Ramirez & Escobar-Morreale, 2010), other studies have found no significant impact of treatment on the inflammatory status of women with PCOS (de Medeiros et al., 2018; Wang et al., 2017).

Interestingly, the most pronounced reductions in inflammatory markers were observed in subgroup 2 and 3 (IL-6 and TNF-α in subgroup 3 and IL-1β in subgroup 2), likely due to the greater decline in androgen levels observed in these subgroups. This finding supports the hypothesis that hyperandrogenism plays a central role in driving the inflammatory response in PCOS (Deligeoroglou et al., 2012; Rudnicka et al., 2020). Slightly less pronounced reductions were noted in subgroup 1, which, on the other hand, were associated with substantial reductions in BMI and HOMA-IR, aligning with observations from other research (Ali et al., 2019; Luque-Ramirez & Escobar-Morreale, 2010).

Regarding treatment regimens, most patients (subgroup 1 and 3) had received daily metformin dosage of 1-1.7 g, consistent with dosages used in other studies (Jakubowska et al., 2008; Kilic et al., 2011). The metformin and inositols combination were associated with significant improvements in HOMA-IR and modest, but significant improvements in androgen levels, consistent with the results of a recent meta-analysis of six randomized controlled trials (Kelly et al., 2024). Additionally, Metformin may operate in ameliorating inflammatory status independently of weight loss and insulin resistance, due to its known anti-inflammatory properties, such as reducing cytokine production and inhibiting NF-κB activation (Bai & Chen, 2021).

In our study, a standardized approach was taken by using a single type of combined oral contraceptive containing ethinyl estradiol 20 mcg and Drospirenone 3 mg to enhance treatment homogeneity. Previous studies on the impact of this COC on inflammatory cytokines in PCOS patients has yielded inconsistent findings (de Medeiros et al., 2018). However, progesterone in the content of COCs has been shown to have an overall significant immune-modulating effect, and exert anti-inflammatory properties by

modulating the activity of NF-κB, thereby decreasing the expression of TNF-α and IL-1β (Tait et al., 2008).

On the other hand, while inconsistent results have been reported from studies investigating the effects of drospirenone-containing COCs on insulin resistance (Kriplani et al., 2010; Orio et al., 2016), in our study significant reductions were observed in HOMA-IR levels in both subgroups 2 and 3.

Homocysteine levels showed similar reductions across all three subgroups, suggesting that the treatment regimens had a comparable effect on lowering homocysteine, despite the baseline heterogeneity among subgroups. Consistent with our findings, previous studies have reported that metformin treatment can reduce homocysteine levels, independently of obesity (Kilic et al., 2011). Conversely, a meta-analysis by Liu et al. (Li et al., 2021) indicated that metformin therapy could increase homocysteine levels, recommending a dosage of less than 1700 mg/day in PCOS patients. Another meta-analysis found no significant change in homocysteine levels with metformin treatment (Meng et al., 2016).

The observed reduction in homocysteine levels in subgroup 2 with COC therapy suggests that the decrease in androgen levels may play a role, supporting a potential correlation between homocysteine and androgen levels in PCOS, as reported in previous studies (Lin et al., 2013). Regarding the specific COC used in this study, which contained drospirenone, prior research has shown that drospirenone does not significantly affect homocysteine levels (Mancini et al., 2010). However, when combined with other anti-androgen therapies, it has been associated with increased homocysteine levels (Harmanci et al., 2013).

The findings from this study have several clinical implications. First, the results emphasize the importance of pharmacologic interventions targeting androgen excess and insulin resistance, which effectively improve low-grade inflammation associated with the syndrome. Furthermore, the long-term management strategies, including lifestyle modifications and pharmacotherapy, are essential for maintaining improvements in terms of inflammation, insulin resistance, and hyperandrogenism, as well as mitigating the long-

term metabolic and cardiovascular risk factors in PCOS—although further research is necessary to confirm the effectiveness of long-term treatment protocols. Lastly, incorporating inflammatory biomarkers into routine monitoring of PCOS patients—along with other clinical and biochemical parameters—could provide valuable insights into disease activity and treatment response, thus helping healthcare providers optimize treatment for maximal benefits.

While the study provides important insights, it also has several limitations. First, the sample size, though sufficient for detecting significant differences in the primary outcomes, may limit the generalizability of the findings to diverse populations with PCOS. Additionally, the relatively short duration of follow-up may not adequately capture long-term changes in inflammatory markers and homocysteine levels, nor the persistence of the observed improvements.

The reliance on BMI as a marker of weight status also presents a limitation, as it does not account for differences in body composition that may vary significantly among individuals with PCOS. Furthermore, while HOMA-IR is a commonly used marker of insulin resistance, it may not completely reflect the complex nature of insulin resistance in these population.

Another limitation of this study is the methods used for quantifying inflammatory cytokines (CLIA for IL-6, ELISA for IL-1β and TNF-α) and homocysteine levels (enzymatic assay). Although these methods are widely used in clinical research, they may lack the precision and sensitivity of more advanced methods, such as mass spectrometry liquid chromatography (Metzemaekers et al., 2021), potentially leading to less accurate quantifications of these biomarkers. The laboratory methods used for measuring androgen levels also constitute a limitation of the study; although standard immunoassay techniques are commonly utilized, they may not provide the sensitivity and specificity necessary for accurate assessment in females (Legro et al., 2010).

Moreover, the potential subjectivity involved in categorizing patients into subgroups represents another limitation. This categorization is partly influenced by the heterogenous clinical presentation of patients, particularly in conditions like hirsutism. Although the hirsutism score was evaluated by a single investigator, local intervention in some patients may have impacted the accuracy of scoring and led to inconsistencies in subgroup classification.

Lastly, the study did not account for potential lifestyle factors that could have influenced the results (Alenezi et al., 2024).

Future research should aim to explore the long-term effects of various therapeutic interventions in different PCOS phenotypes, as well as the role of lifestyle modifications in enhancing treatment outcomes. Studies with larger cohorts and longer follow-up periods are needed to validate the observed effects and assess the sustainability of the improvements. Additionally, exploring the role of adipose tissue function, gut microbiota, and other metabolic regulators may provide deeper insights into the complex interplay between inflammation, insulin resistance, and androgen excess in PCOS.

7 CONCLUSIONS

This study demonstrates that a six-month pharmacologic intervention in women with PCOS can significantly reduce systemic inflammation and homocysteine levels, with consistent effectiveness across different clinical phenotypes. These findings suggest that addressing insulin resistance and hyperandrogenism may play a crucial role in mitigating inflammatory responses and potentially reducing long-term metabolic and cardiovascular risks associated with the syndrome. However, further research with larger sample sizes and extended follow-up periods is warranted to corroborate our findings, confirm the persistence of these improvements over time, and better understand their long-term clinical implications.

8 TABLES AND FIGURES

Table 1. Baseline clinical and biochemical assessments in 135 patients concluding the follow-up period.[1]

Baseline characteristics	**Mean (SD)**	**Minimum**	**Maximum**
Age, years	26.21 (5.33)	18.00	40.00
BMI, kg/m^2	27.54 (5.90)	18.40	48.60
Modified Ferriman-Gallwey scale	9.41 (3.23)	2.00	21.00
Fasting glucose, mg/dL	92.92 (9.49)	69.90	119.90
Fasting insulinemia, µUI/mL	14.72 (11.45)	1.01	76.59
HOMA-IR	3.48 (2.94)	0.20	19.4
FSH, mUI/mL	5.12 (1.62)	1.70	9.80
LH, mUI/mL	11.56 (8.21)	1.12	56.43
LH/FSH	2.51 (2.02)	0.12	13.00
Total testosterone, ng/mL	0.55 (0.24)	0.14	1.86
17-OH Progesterone, ng/mL	1.30 (0.74)	0.44	5.09
Androstenedione, ng/mL	3.30 (1.24)	0.69	9.70
DHEAS, µg/dL	336.44 (128.92)	76.11	767.00
IL-6, pg/mL	8.44 (11.08)	0.50	91.00
IL-1β, pg/mL	15.34 (15.70)	0.68	93.60
TNF-alfa, pg/mL	5.88 (2.55)	0.65	22.00
Homocysteine, µmol/L	10.89 (6.01)	1.10	39.33

[1] For the full list of abbreviations (tables 1-8), see 'Tables abbreviations' below.

Table 2. Baseline clinical and biochemical evaluations within subgroups 1 (metabolic), 2 (hyperandrogenic), and 3 (combined phenotype). Data are expressed as mean (SD)**.**

Baseline characteristics	**Subgroup 1 (n = 25)**	**Subgroup 2 (n = 47)**	**Subgroup 3 (n = 63)**
BMI, kg/m^2	34.13 (5.74)	22.52 (2.69)	28.66 (4.40)
Modified Ferriman-Gallwey scale	4.92 (1.73)	11.36 (2.32)	8.17 (2.50)
HOMA-IR	5.78 (4.06)	1.45 (0.53)	4.07 (2.54)
Total testosterone, ng/mL	0.41 (0.17)	0.56 (0.19)	0.59 (0.28)
17-OH Progesterone, ng/mL	0.89 (0.42)	1.51 (0.8)	1.31 (0.75)
Androstenedione, ng/mL	2.9 (0.96)	3.44 (1.05)	3.35 (1.43)
DHEAS, µg/dL	299.16 (143.03)	338.36 (133.7)	349.79 (118.26)
IL-6, pg/mL	6.58 (3.43)	6.8 (3.78)	10.4 (15.59)
IL-1β, pg/mL	14.93 (15.3)	15.39 (13.72)	15.45 (17.38)
TNF-alfa, pg/mL	5.86 (1.81)	5.3 (1.9)	6.33 (3.12)
Homocysteine, µmol/L	11.09 (5.58)	10.3 (4.82)	11.26 (6.95)

Table 3. Baseline and post-treatment differences in inflammatory markers and homocysteine levels in the entire cohort. p-values are calculated using paired t-test.

Variable	Baseline mean (SD)	Post-treatment mean (SD)	p-value
IL-6, pg/mL	8.44 (11.08)	4.36 (3.55)	< 0.001
IL-1β, pg/mL	15.34 (15.7)	10.19 (28.21)	0.012
TNF-α, pg/Ml	5.88 (2.55)	3.48 (1.59)	< 0.001
Homocysteine, µmol/L	10.89 (6.01)	6.77 (3.03)	< 0.001

Table 4. Baseline and post-treatment differences in inflammatory markers and homocysteine levels in each subgroup. p-values are calculated using the Related-Samples Wilcoxon Signed Rank Test.

	Subgroup 1 (metabolic phenotype, n = 25)				**Subgroup 2 (hyperandrogenic phenotype, n = 47)**				**Subgroup 3 (combined phenotype, n = 63)**			
Variables	**Mean (SD)**	**Median (IQR)**	**Mean difference**	**p-value**	**Mean (SD)**	**Median (IQR)**	**Mean difference**	**p-value**	**Mean (SD)**	**Median (IQR)**	**Mean difference**	**p-value**
Baseline IL-6	6.58 (3.43)	6.65 (4.44)	2.59	<0.001	6.80 (3.78)	7.90 (6.04)	3.91	<0.001	10.4 (15.59)	6.89 (6.63)	4.8	<0.001
Post-treatment IL-6	3.99 (2.09)	3.40 (3.8)			2.89 (2.18)	2.53 (2.47)			5.60 (4.33)	4.57 (4.0)		
Baseline IL-1β	14.93 (15.3)	12.20 (7.65)	7.66	<0.001	15.39 (13.72)	11.10 (9.68)	8.38	<0.001	15.45 (17.38)	11.80 (8.3)	1.73	<0.001
Post-treatment IL-1β	7.27 (4.49)	6.40 (5.8)			7.01 (13.62)	3.90 (5.0)			13.72 (39.38)	6.2 (6.8)		
Baseline TNF-α	5.86 (1.81)	5.20 (1.92)	1.87	<0.001	5.30 (1.9)	5.16 (2.62)	2.35	<0.001	6.33 (3.12)	5.80 (3.53)	2.66	<0.001
Post-treatment TNF-α	3.99 (1.37)	3.90 (1.7)			2.95 (1.59)	3.00 (1.98)			3.67 (1.59)	3.5 (1.7)		
Baseline homocysteine	11.09 (5.58)	10.20 (4.11)	3.67	<0.001	10.30 (4.82)	9.10 (4.43)	3.81	<0.001	11.26 (6.95)	10.8 (6.2)	4.54	<0.001
Post-treatment homocysteine	7.42 (3.3)	6.90 (5.0)			6.49 (2.72)	5.90 (4.2)			6.72 (3.11)	6.6 (4.5)		

Table 5. Baseline and post-treatment differences in BMI, HOMA-IR, total testosterone, DHEAS, 17-OH Progesterone, androstenedione in the entire cohort. p-values calculated using paired t-test.

Variable	Baseline mean (SD)	Post-treatment mean (SD)	p-value
BMI, kg/m^2	27.54 (5.9)	26.76 (5.54)	< 0.001
HOMA-IR	3.48 (2.94)	3.06 (2.68)	< 0.001
Total testosterone, ng/mL	0.55 (0.24)	0.36 (0.15)	< 0.001
DHEAS, µg/Dl	336.44 (128.92)	231.24 (97.64)	< 0.001
17-OH Progesterone, ng/mL	1.3 (0.74)	0.72 (0.35)	< 0.001
Androstenedione, ng/mL	3.3 (1.24)	2.19 (0.81)	< 0.001

Table 6. Baseline and post-treatment differences in BMI, HOMA-IR, total testosterone, DHEAS, 17-OH Progesterone, androstenedione in subgroups. p-values calculated using the Related-Samples Wilcoxon Signed Rank Test.

	Subgroup 1 (metabolic phenotype, n = 25)				Subgroup 2 (hyperandrogenic phenotype, n = 47)				Subgroup 3 (combined phenotype, n = 63)			
Variables	**Mean (SD)**	**Median (IQR)**	**Mean difference**	**p-value**	**Mean (SD)**	**Median (IQR)**	**Mean difference**	**p-value**	**Mean (SD)**	**Median (IQR)**	**Mean difference**	**p-value**
Baseline BMI	34.13 (5.74)	33.70 (7.6)	0.61	0.146	22.52 (2.69)	22.1 (3.7)	0.39	0.001	28.66 (4.4)	28.66 (5.7)	1.14	<0.001
Post-treatment BMI	33.53 (5.21)	32.80 (7.96)			22.13 (2.47)	21.9 (3.7)			27.52 (3.99)	26.7 (4.7)		
Baseline HOMA-IR	5.78 (4.06)	4.4 (3.65)	0.59	<0.001	1.45 (0.53)	1.40 (0.7)	0.14	0.025	4.07 (2.54)	3.2 (2.8)	0.55	0.005
Post-treatment HOMA-IR	5.19 (3.72)	3.9 (2.9)			1.31 (0.43)	1.20 (0.5)			3.52 (2.38)	2.9 (1.7)		
Baseline total testosterone	0.41 (0.17)	0.39 (0.18)	0.07	<0.001	0.56 (0.19)	0.54 (0.26)	0.24	<0.001	0.59 (0.28)	0.55 (0.03)	0.19	<0.001
Post-treatment total testosterone	0.34 (0.13)	0.34 (0.19)			0.32 (0.13)	0.32 (0.2)			0.4 (0.16)	0.34 (0.16)		
Baseline 17-OH Pg	0.89 (0.42)	0.81 (0.36)	0.20	<0.001	1.51 (0.8)	1.38 (0.78)	0.89	<0.001	1.31 (0.75)	1.12 (0.56)	0.5	<0.001
Post-treatment 17-OH Pg	0.69 (0.27)	0.62 (0.29)			0.62 (0.23)	0.61 (0.28)			0.81 (0.42)	0.71 (0.28)		
Baseline androstenedione	2.9 (0.96)	2.9 (1.34)	0.58	<0.001	3.44 (1.05)	3.40 (1.44)	1.55	<0.001	3.35 (1.43)	3.5 (1.56)	0.99	<0.001
Post-treatment androstenedione	2.32 (0.88)	2.1 (1.2)			1.89 (0.73)	2.10 (1.3)			2.36 (0.8)	2.4 (1.3)		
Baseline DHEAS	299.16 (143.03)	290.8 (125.0)	64.84	<0.001	338.36 (133.76)	346.00 (175.2)	142.89	<0.001	349.79 (118.26)	330.6 (180.85)	93.09	<0.001
Post-treatment DHEAS	234.32 (102.64)	232 (153.05)			195.47 (75.86)	198.00 (75.8)			256.7 (103.07)	251.8 (120.0)		

Table 7. Differences in the baseline inflammatory markers and homocysteine levels across subgroups. Overall p-values were calculated using one-way ANOVA, while p-values for subgroup comparisons were determined using Bonferroni tests.

Variables (baseline)	Subgroup	N	Mean	SD	SE	95% Confidence interval for mean		Min	Max	F	Overall ANOVA p-value	Subgroup comparisons p-values
						Lower bound	Upper bound					
IL-6	1	25	6.58	3.43	0.69	5.16	7.99	1.65	15.19	1.874	0.158	p (1 vs 2) = 1.0
	2	47	6.80	3.78	0.55	5.69	7.91	0.50	14.20			p (1 vs 3) = 0.434
	3	63	10.40	15.59	1.96	6.47	14.32	0.50	91.00			p (2 vs 3) = 0.277
IL-1β	1	25	14.93	15.30	3.06	8.61	21.25	4.20	83.30	0.010	0.990	p (1 vs 2) = 1.0
	2	47	15.39	13.72	2.00	11.37	19.42	1.10	74.90			p (1 vs 3) = 1.0
	3	63	15.45	17.38	2.19	11.07	19.83	0.68	93.60			p (2 vs 3) = 1.0
TNF-α	1	25	5.86	1.81	0.36	5.12	6.61	3.80	10.60	2.192	0.116	p (1 vs 2) = 1.0
	2	47	5.30	1.90	0.28	4.75	5.86	0.65	9.90			p (1 vs 3) = 1.0
	3	63	6.33	3.12	0.39	5.54	7.11	2.30	22.00			p (2 vs 3) = 0.115
Homocysteine	1	25	11.09	5.58	1.12	8.78	13.39	1.50	28.80	0.350	0.705	p (1 vs 2) = 1.0
	2	47	10.30	4.82	0.70	8.89	11.72	3.30	36.80			p (1 vs 3) = 1.0
	3	63	11.26	6.95	0.88	9.51	13.00	1.10	39.33			p (2 vs 3) = 1.0

Table 8. Differences in the post-treatment inflammatory markers and homocysteine levels across subgroups. Overall p-values were calculated using one-way ANOVA, while p-values for subgroup comparisons were determined using Bonferroni tests.

Variables (post-treatment)	Subgroup	N	Mean	SD	SE	95% Confidence interval for mean		Min	Max	F	Overall ANOVA p-value	Subgroup comparisons p-values
						Lower bound	Upper bound					
IL-6	1	25	3.99	2.09	0.42	3.13	4.85	1.00	8.20	8.984	**<0.001**	p (1 vs 2) = 0.561
	2	47	2.89	2.18	0.32	2.24	3.53	0.20	11.56			p (1 vs 3) = 0.133
	3	63	5.60	4.33	0.55	4.51	6.69	0.90	26.00			**p (2 vs 3) < 0.001**
IL-1β	1	25	7.28	4.49	0.90	5.42	9.13	0.10	19.20	0.923	0.40	p (1 vs 2) = 1.0
	2	47	7.01	13.62	1.99	3.01	11.01	0.10	93.20			p (1 vs 3) = 1.0
	3	63	13.72	39.38	4.96	3.80	23.63	0.10	313.00			p (2 vs 3) = 0.659
TNF-α	1	25	3.99	1.37	0.27	3.43	4.55	0.20	6.90	0.782	0.46	**p (1 vs 2) = 0.023**
	2	47	2.95	1.59	0.23	2.49	3.42	0.20	8.20			p (1 vs 3) = 1.0
	3	63	3.67	1.59	0.20	3.27	4.06	0.30	9.80			p (2 vs 3) = 0.055
Homocysteine	1	25	7.42	3.30	0.66	6.06	8.78	1.31	15.70	4.523	**0.013**	p (1 vs 2) = 0.654
	2	47	6.49	2.77	0.40	5.68	7.31	0.40	14.45			p (1 vs 3) = 0.99
	3	63	6.72	3.11	0.39	5.93	7.50	0.20	17.50			p (2 vs 3) = 1.0

Tables Abbreviations (for tables 1-8): BMI = body mass index; DHEAS = dehydroepiandrosterone sulfate; F = F-statistic; HOMA-IR = homeostasis model assessment of insulin resistance; IL = interleukin; IQR = interquartile range; kg/m^2 = kilograms per square meter; mg/dL, ng/dL, μg/dL = milligrams, nanograms, micrograms per deciliter; pg/mL = picograms per milliliter; μmol/L = micromoles per liter; SD = standard deviation TNF = tumor necrosis factor.

Figure 1. Study flowchart

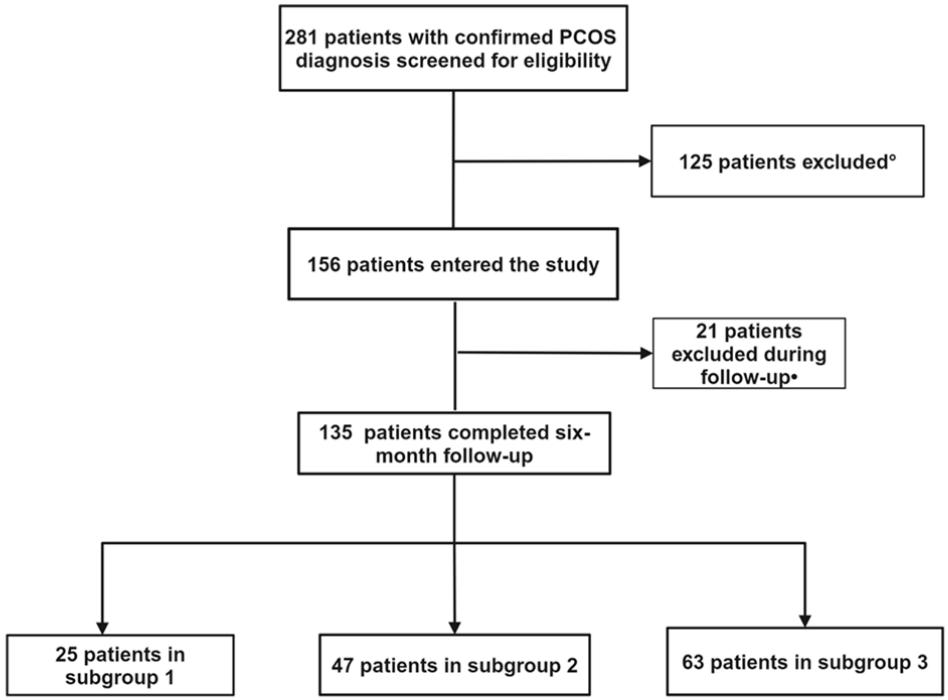


Figure 1. Study flowchart. Created with BioRender.com. °**Reasons for exclusion**: pursuing pregnancy (n = 94), type 2 diabetes (T2DM) (n = 1), hypertension (HTA) (n = 2), and treatment refusal (n = 28). •**Reasons for exclusion during follow-up**: pregnancy (n = 3), adverse effects (n = 2), non-compliance (n = 16). Abbreviations: PCOS = polycystic ovary syndrome.

Figure 2. Boxplots of IL-6, IL-1β, TNF-α, and homocysteine levels at baseline and post-treatment in the overall cohort.

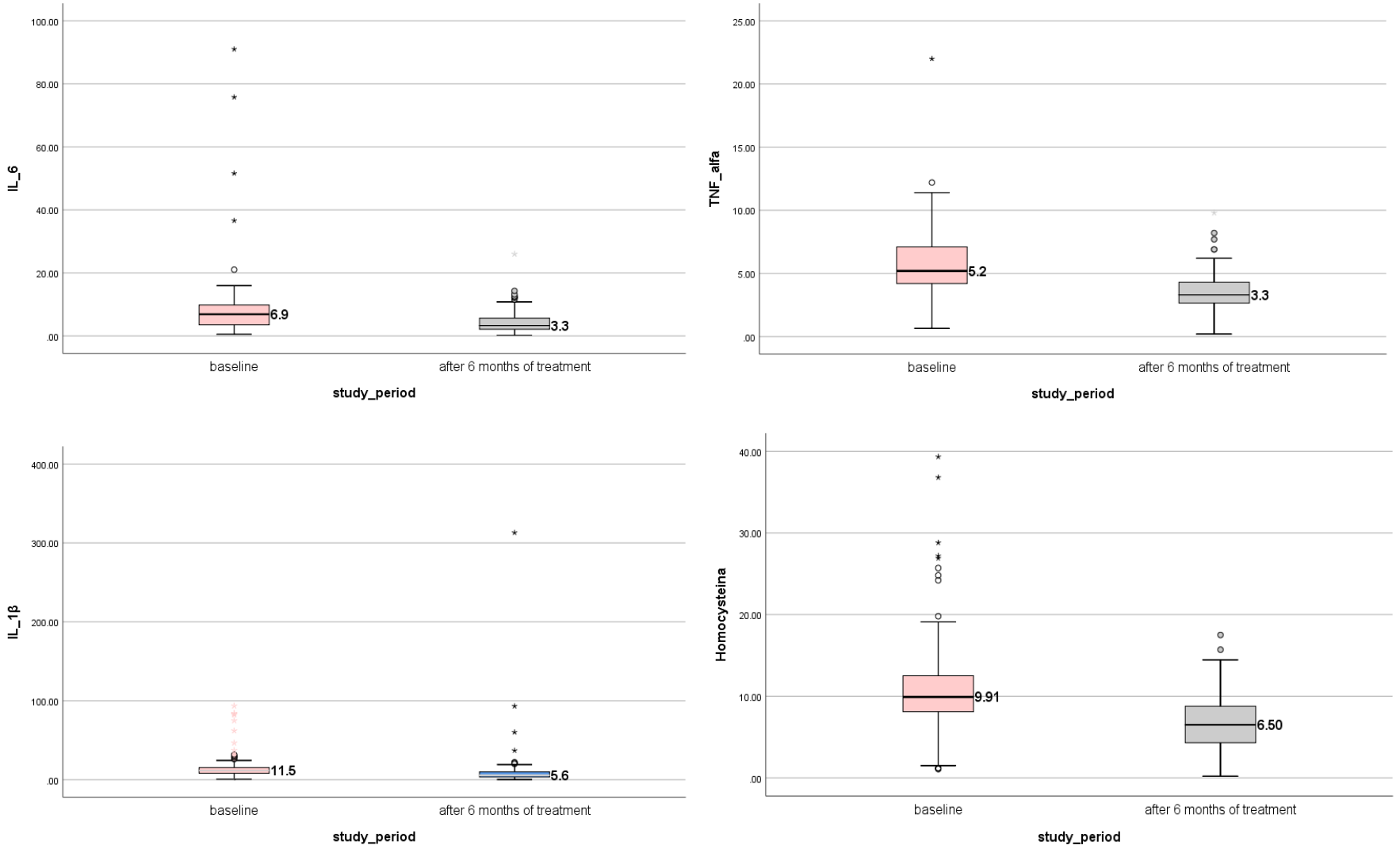


Figure 3. Boxplots of IL-6, IL-1β, TNF-α, and homocysteine levels at baseline and post-treatment in subgroup 1.

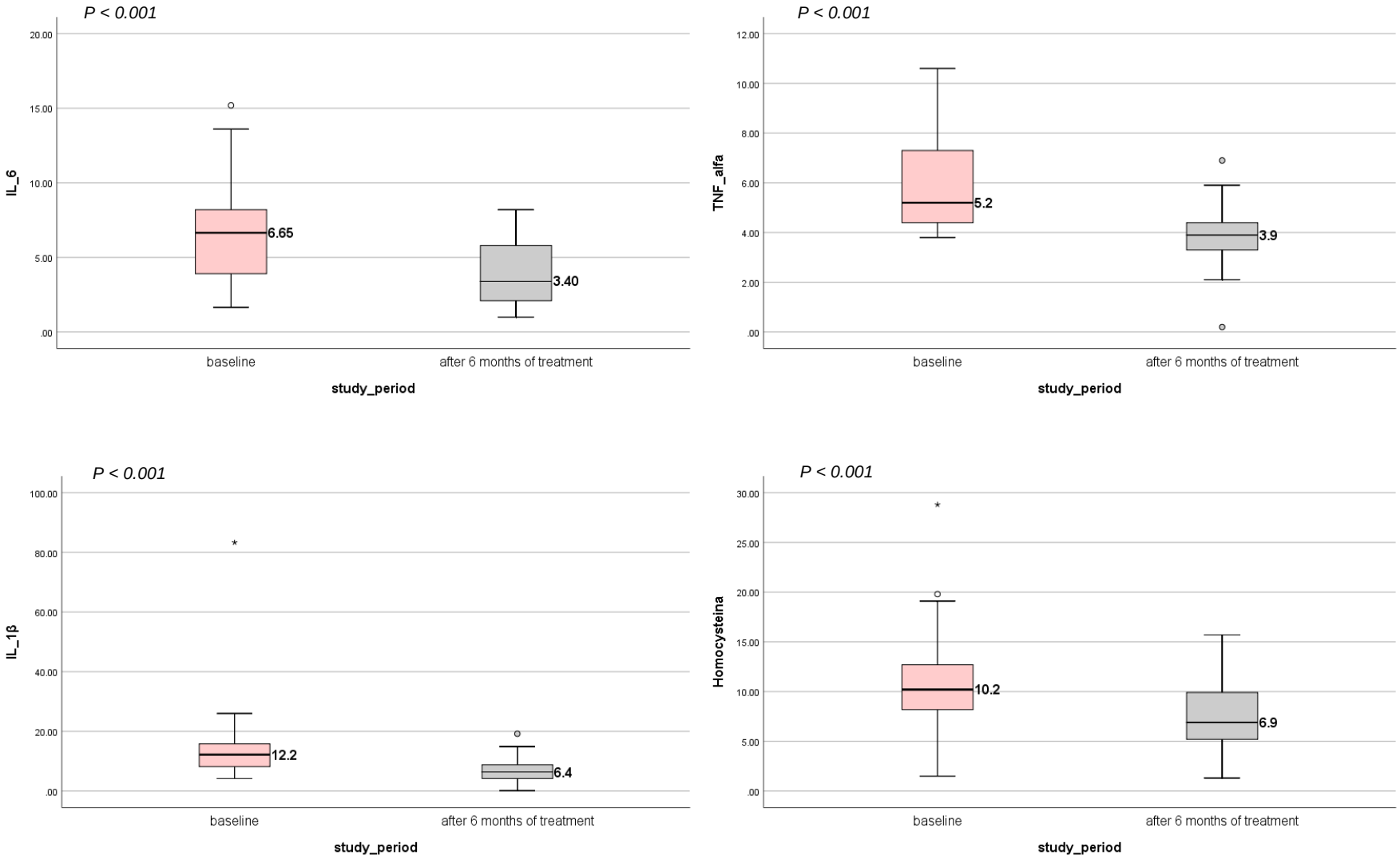


Figure 4. Boxplots of IL-6, IL-1β, TNF-α, and homocysteine levels at baseline and post-treatment in subgroup 2.

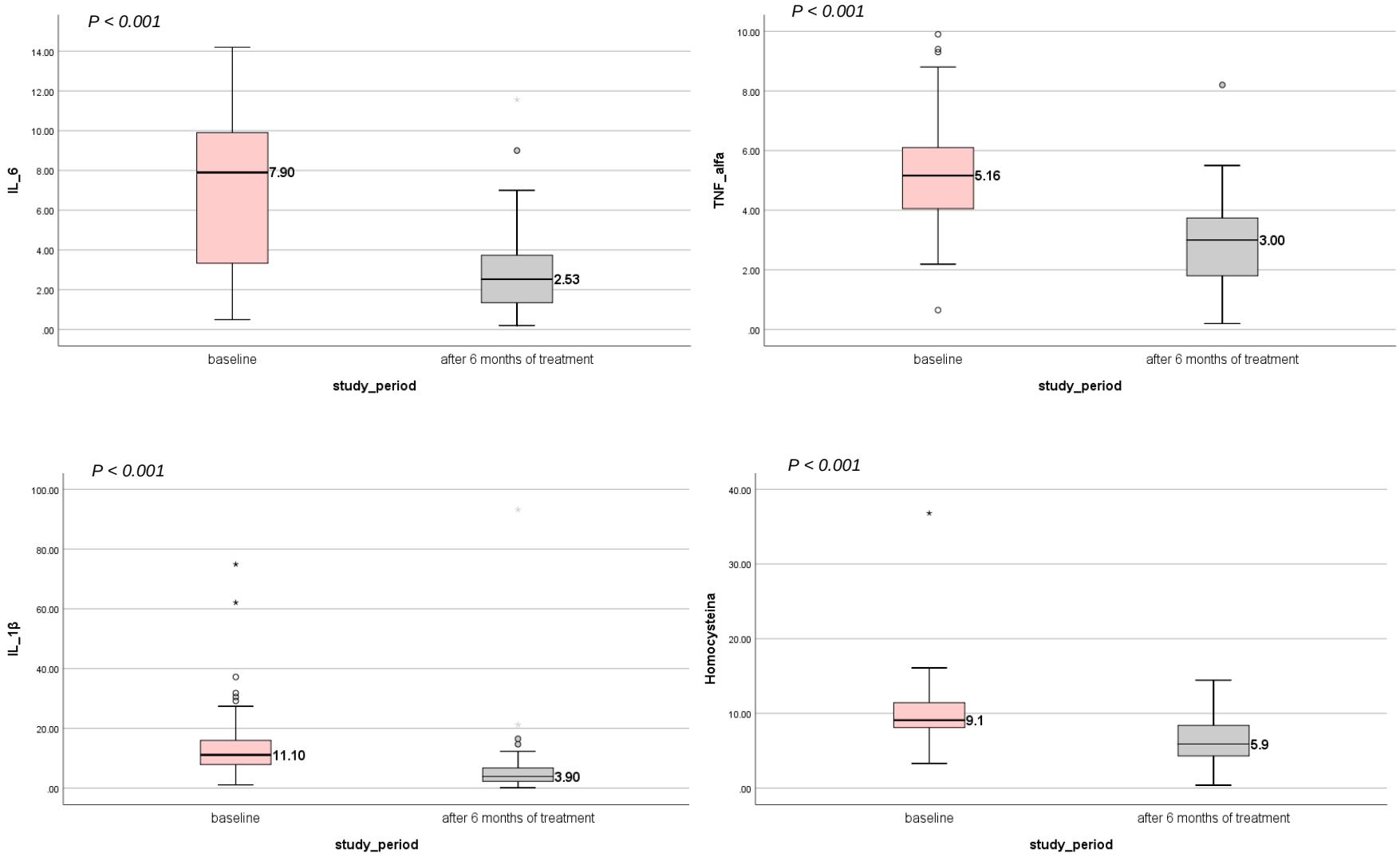


Figure 5. Boxplots of IL-6, IL-1β, TNF-α, and homocysteine levels at baseline and post-treatment in subgroup 3.

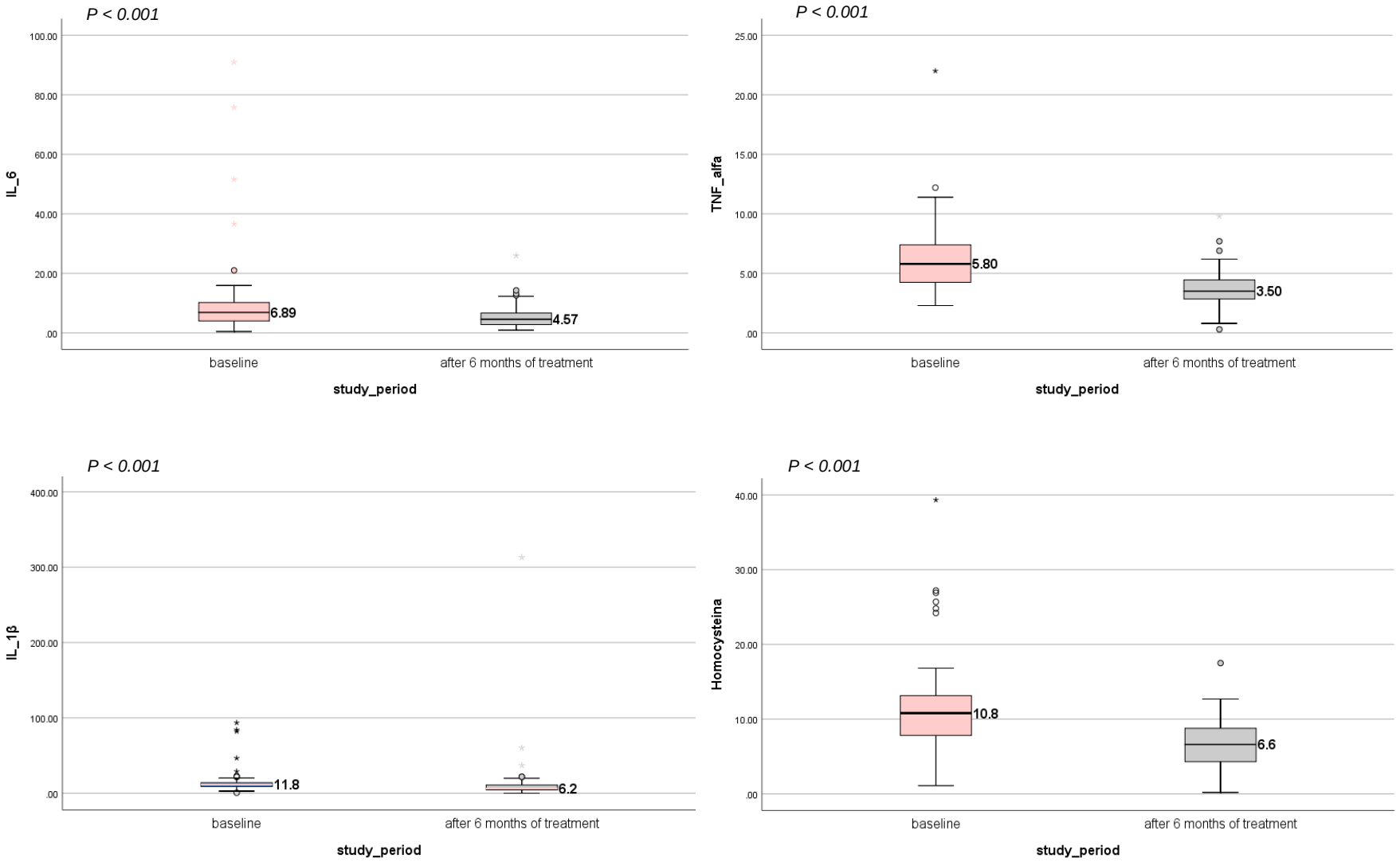
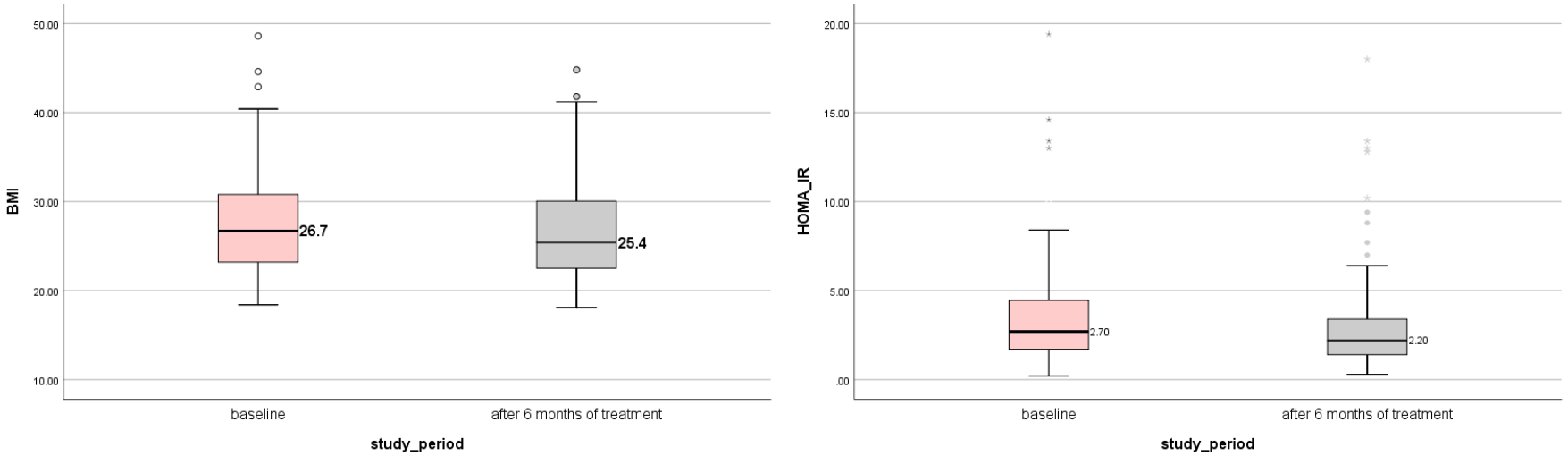


Figure legends (for figures 2-5):

Boxplots of interleukin-6 (IL-6), tumor necrosis alpha (TNF-α), interleukin-1beta (IL-1β), and homocysteine levels in overall cohort and subgroups, comparing baseline and post-treatment values. Statistical analysis revealed significant differences in all variables between baseline and post-treatment levels (all $p < 0.05$). Units of measurement: IL-6, TNF-α, IL-1β—picograms per milliliter (pg/mL); homocysteine—micromoles per liter (μmol/L).

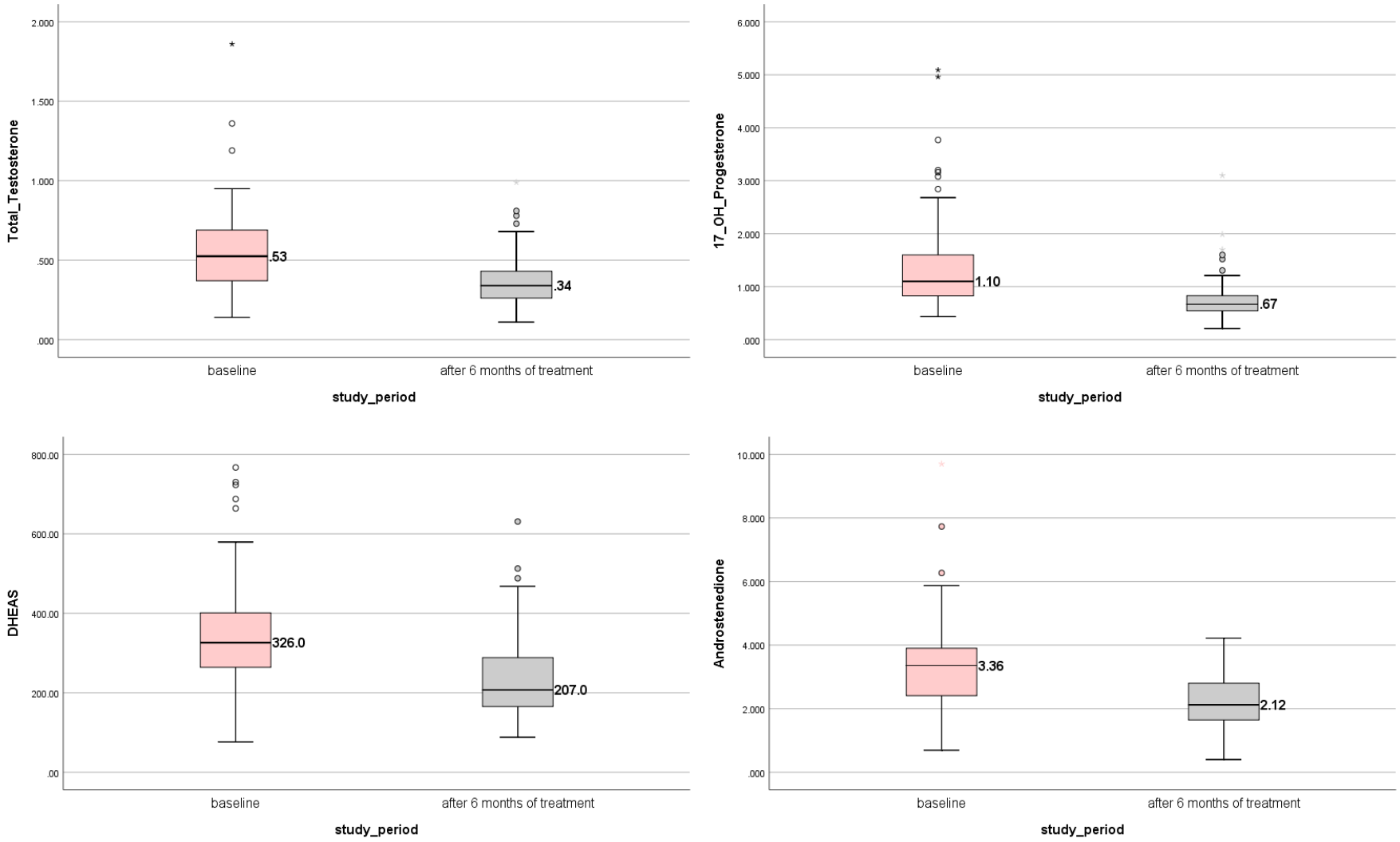
APPENDIX. Boxplots of secondary objectives results.

Appendix Figure 1. Boxplots of BMI (kg/m²) and HOMA-IR values at baseline and post-treatment in overall cohort.[2]



[2] Abbreviations and units of measurement in Appendix Figure 1 and 2 are provided in 'Table Abbreviations' above.

Appendix Figure 2. Boxplots of total testosterone, 17-OH progesterone, DHEAS, and androstenedione levels at baseline and post-treatment in overall cohort.



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