

# Neutrophils and IL-12 Levels in Female Patients with Unexplained Infertility

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## ABSTRACT

**Objective:** To evaluate the levels of interleukin-12 (IL-12), a critical cytokine for NK cells, and hemogram parameters in infertile women with unknown causes.

**Study Design:** Descriptive study.

**Place and Duration of the Study:** Department of Gynaecology and Obstetrics, Medical Faculty of Pamukkale University, Denizli, Turkiye, between January and December 2022.

**Methodology:** The study included 43 female patients aged 22-49 years with unexplained infertility who met the inclusion and exclusion criteria, along with 40 healthy control females from the Department of Gynaecology and Obstetrics. The sandwich ELISA method, which depends on antigen-antibody reactivity, was utilised to quantify serum IL-12 levels, and data were analysed using the Mann-Whitney U test. Complete blood cell parameters were also investigated.

**Results:** There was no significant difference in serum IL-12 levels between the Control and Unexplained infertile (UI) groups (median: 16.51, IQR: 14.58 - 21.43 vs. median: 15.92, IQR: 14.4 - 25.47;  $p = 0.623$ , respectively). Sedimentation and CRP levels did not show significant differences between the groups ( $p = 0.327$  and  $0.320$ , respectively). There was also no statistically significant difference in total leucocyte count ( $p = 0.114$ ). The UI Group had a substantially higher neutrophil percentage (median: 62.45, IQR: 56.93 - 68.28 vs. median: 58.75, IQR: 52.08 - 64.48);  $p = 0.017$ ) and absolute neutrophil count ( $5164.4 \pm 1483.29/\mu\text{l}$  vs.  $4408.55 \pm 1153.3/\mu\text{l}$ ;  $p = 0.012$ ) compared to the Control group.

**Conclusion:** While IL-12 levels did not significantly differ among ladies with unexplained infertility, there was a notable increase in the percentage and number of neutrophils.

**Key Words:** Infertility, Interleukin-12 (IL-12), Cytokine, Neutrophil.

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## INTRODUCTION

Infertility is a disorder that affects millions of people throughout the world. Worldwide, 17% of couples are infertile. There are numerous aetiological explanations for infertility. Some of these are related to women, others to men, and some are unexplained.<sup>1,2</sup> Unexplained infertility is reported to affect about 30% of infertile couples.<sup>3</sup> It is invariably diagnosed by exclusion following standard investigative methods.

Nevertheless, a consensus on the standardisation of diagnostic assessment remains lacking. Unexplained infertility is defined as infertility in couples with apparently normal ovarian function, fallopian tubes, uterus, cervix, and pelvis, and with adequate coital frequency, as well as apparently normal testicular function, genitourinary anatomy, and a normal ejaculate, according to the International Committee for Monitoring Assisted Reproductive Technologies (ICMART).<sup>2-4</sup>

Unexplained infertility can be caused by both genetic and immunological problems. The idea of immunological infertility, or infertility brought on by immune factors, has gained attention in recent years. Studies are advancing rapidly in this field, as many questions remain unsolved. Growing evidence suggests that distinct T helper cells and their cytokines are involved in various phases of infertility.<sup>5</sup> Indeed, cellular treatment approaches have recently been tested, including the use of different types of stem cells, lymphocytes, platelet-rich plasma, and peripheral blood mononuclear cells as therapeutic agents.<sup>6</sup>

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Recent studies have also investigated the potential of CD8+ HLA-G+ and CD4+HLA-G+ regulatory T cells to induce maternal immunological tolerance to semi-allogeneic embryos. Additionally, it has been demonstrated that infertile patients have significantly reduced CD8+HLA-G+ regulatory T cells.<sup>5</sup>

Successful implantation depends on specific proinflammatory cytokines. The type 1 helper T-cell (Th1) response and proinflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukins (IL-12, IL-1, IL-15, IL-2, and IL-18) are predominant during trophoblast invasion and delivery. However, cellular immunity triggered by Th1-mediated cytokines may be linked to pregnancy failure.<sup>5</sup>

Human pregnancy endocrinology encompasses endocrine and metabolic changes resulting from physiological changes in mother and foetus. Progesterone and estrogen, among other hormones, play an important role. Progesterone enhances Type 2 helper T-cell (Th2) secretion while decreasing Th1 cytokine release, hence preserving pregnancy.<sup>7</sup> Studies have indicated that oestrogen suppresses natural killer (NK) cell activation.<sup>8</sup>

A 2021 study found that successful implantation was associated with a transitory increase in serum proinflammatory cytokine profiles (IFN- $\gamma$  and IL-17), followed by a change to anti-inflammatory cytokine profiles (IL-10 and TGF- $\beta$ 1) before confirmation of pregnancy.<sup>1</sup> IL-12 is among the cytokines responsible for pro-inflammatory and tumour-suppressive reactions, as well as pregnancy. It is a heterodimeric cytokine made by dendritic cells, macrophages, and B cells in response to infections.<sup>9,10</sup> Both NK and NKT cells express the IL-12R $\beta$ 1 and IL-12R $\beta$ 2 receptors, which facilitate their transformation into pro-inflammatory cells upon exposure to IL-12.<sup>9</sup>

This study aimed to compare the levels of IL-12, a crucial cytokine for NK cells, in women who are infertile for unknown reasons to those in the Control group. Additionally, this study investigated these individuals' peripheral blood cells; immunotherapy for infertility may be beneficial from the study's findings.

## METHODOLOGY

The study included 43 female patients aged 22-49 years with unexplained infertility who met the inclusion criteria, as well as 40 healthy control female patients who presented to the Department of Gynaecology and Obstetrics, Medical Faculty of Pamukkale University, Denizli, Türkiye, between January and December 2022.

The reference study's effect magnitude was substantial ( $d = 1.982$ ). Given that a smaller effect size ( $d = 0.8$ ) was obtained because of the power analysis, it was calculated that 80% power could be attained at a 95% confidence level if at least 52 participants (at least 26 persons for each group) participated in the study.

The diagnosis of infertility was based on the inability to conceive after 12 months of regular sexual intercourse without using contraception in women under 35 years of age; and after six

months of regular sexual intercourse without using contraception in women aged 35 and over.<sup>11-13</sup>

Female infertile patients without fallopian tube blockage disorders or ovulation issues met the inclusion criteria. Male partners of the female patients taking part in the study were healthy. Individuals with malignancies were excluded from the study.

On the third day of menstruation, fasting blood samples were drawn from the Unexplained infertile and Control groups, placed in the proper tubes (gel biochemistry tubes), and transported them on the same day to the Medical Biochemistry Laboratory of Pamukkale University. In the same laboratory, sera acquired by centrifuging at 3500 rpm for 10 minutes were stored at -80 degrees until the project was completed. Sufficient samples were collected to meet the power analysis requirements. Commercial kits from BT-Lab (Shanghai, China) were used to examine IL-12 levels.

Based on antigen-antibody reactivity, the sandwich ELISA method was used to measure serum IL-12 levels in patient samples. Before analysis, all kits and samples were collected and brought to room temperature. After preparing the chemicals and standards from the kits, standards and samples were pipetted into the microplate wells. Following that, the samples were coloured using the procedures outlined in the kit protocol. Following the observation of colour creation, a BioTek Elx800 microplate reader (BioTek Instruments Inc., USA) was used to measure absorbance values of the wells at 450 nm. The Gen5 data analysis program was utilised to determine serum concentrations based on absorbance measurements. The IL-12 levels were expressed in ng/ml.

Complete blood count (CBC) results, sedimentation (normal range: 0-30 mm/h), and CRP (normal range: 0-0.5 ug/dl) levels were also measured when the samples were obtained. Coagulation parameters such as activated partial thromboplastin time (aPTT; normal range: 20-38 s), prothrombin time (PT; normal range: 10.2-14.4), and international normalised ratio (INR; normal range: 0.85-1.2) were also used.

Prior to the study, approval was received from the Local Ethics Committee of the Medical Faculty of Pamukkale University, Denizli, Türkiye (25.01.2022/number 02), and it was conducted in compliance with the Helsinki Declaration's legal and regulatory requirements.

All statistical analyses were performed using the SPSS software version 25.0 (IBM SPSS Statistics 25; Armonk, NY: IBM Corp.). Continuous data were expressed as mean  $\pm$  standard deviation (SD) and median with interquartile range (IQR: 25<sup>th</sup>-75<sup>th</sup> percentiles), while categorical data were presented as frequencies and percentages. The Shapiro-Wilk test was used to evaluate the normality of data distribution. For normally distributed data, group comparisons were conducted using an independent sample t-test. In cases where the data did not meet the normality assumption, the Mann-Whitney U test was utilised. To assess the relationships between continuous variables, the Spearman's correlation coefficient was computed. A p-value of less than 0.05 was considered statistically significant.

**Table I: Sedimentation and CRP values of the UI and Control groups.**

| Variables     | Control Group   | UI Group         | p-values           |
|---------------|-----------------|------------------|--------------------|
| Sedimentation | 12.76 ± 6.84    | 15.63 ± 6.21     | 0.327 (t = -1.002) |
| CRP           | 1.43 (0.6-3.03) | 1.05 (0.45-2.98) | 0.32 (z = -0.995)  |

\*p < 0.05 statistically significant; All descriptive statistics are summarised with mean ± standard deviation and median (25<sup>th</sup>-75<sup>th</sup> percentiles (IQR)); t: Independent samples t-test; z: Mann-Whitney U test; CRP: C-reactive protein; UI: Unexplained infertile.

**Table II: CBC and serum IL-12 levels of the UI and the Control groups.**

| Variables | Control Group            | UI Group                 | p-values            |
|-----------|--------------------------|--------------------------|---------------------|
| Hb        | 12.89 ± 1.04             | 13.35 ± 1.21             | 0.071 (t = -1.828)  |
| Hct       | 38.94 ± 2.89             | 39.95 ± 3.3              | 0.145 (t = -1.471)  |
| MCV       | 84.3 ± 6.64              | 86.58 ± 4.19             | 0.069 (t = -1.847)  |
| MCH       | 28 (26.55 - 30.08)       | 29 (27.85 - 30.08)       | 0.115 (z = -1.578)  |
| MCHC      | 33 (32.33 - 33.5)        | 33.6 (32.98 - 33.9)      | 0.015* (z = -2.437) |
| RDW       | 14.3 (13.4 - 15.23)      | 13.35 (12.8 - 14.33)     | 0.029* (z = -2.177) |
| WBC       | 7485 ± 1515.27           | 8072.38 ± 1792.41        | 0.114 (t = -1.599)  |
| Neu %     | 58.75 (52.08 - 64.48)    | 62.45 (56.93 - 68.28)    | 0.017* (z = -2.38)  |
| Neu #     | 4408.55 ± 1153.3         | 5164.4 ± 1483.29         | 0.012* (t = -2.583) |
| Lym %     | 32.77 ± 7.21             | 28.95 ± 7.58             | 0.022* (t = 2.331)  |
| Lym #     | 2275 (1905 - 2675)       | 2230 (1782.5 - 2790)     | 0.534 (z = -0.622)  |
| Mon %     | 5.2 (4.9 - 6.6)          | 5.7 (4.98 - 6.2)         | 0.893 (z = -0.135)  |
| Mon #     | 445 (380 - 490)          | 440 (350 - 545)          | 0.93 (z = -0.088)   |
| Baso %    | 0.46 ± 0.25              | 0.42 ± 0.23              | 0.621 (z = -0.495)  |
| Baso #    | 30 (20 - 40)             | 30 (20 - 40)             | 0.624 (z = -0.49)   |
| Eo %      | 1.85 (1.33 - 3.35)       | 1.5 (1.08 - 1.83)        | 0.004* (z = -2.856) |
| Eo #      | 158 (92.5 - 217.5)       | 115 (70 - 160)           | 0.03* (z = -2.174)  |
| NLR       | 1.78 (1.35 - 2.29)       | 2.11 (1.72 - 2.73)       | 0.024* (z = -2.25)  |
| PLT       | 283000 (240250 - 308250) | 276500 (227000 - 311750) | 0.813 (z = -0.237)  |
| MPV       | 9.5 (9.2 - 10.28)        | 9.4 (8.78 - 10.33)       | 0.597 (z = -0.529)  |
| IL-12     | 16.51 (14.58-21.43)      | 15.92 (14.4-25.47)       | 0.623 (z = -0.492)  |

\*p < 0.05 statistically significant; All descriptive statistics are summarised with mean ± standard deviation or median (25<sup>th</sup>-75<sup>th</sup> percentiles (IQR)) according to the normality results; t: Independent samples t-test; z: Mann-Whitney U test. Hb: Haemoglobin; Hct: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; RDW: Red blood cell distribution width; WBC: White blood cell; Neu: Neutrophil; Lym: Lymphocyte; Mon: Monocyte; Baso: Basophil; Eo: Eosinophil; NLR: Neutrophil-to-lymphocyte ratio; PLT: Platelet; MPV: Mean platelet volume.

## RESULTS

The study comprised 43 female patients in the UI group and 40 individuals in the Control group. The study included female patients with healthy male partners. All volunteers who participated in the study had sedimentation and C-reactive protein (CRP) values that were within the normal range when compared to the reference points (Table I).

Haemoglobin levels did not differ significantly between the Control group and the UI group (12.89 ± 1.04 g/dL vs. 13.35 ± 1.21 g/dL; p = 0.071). Likewise, no statistically significant difference was found between the two groups in terms of platelet counts (median: 283000, IQR: 240250-308250 mm<sup>3</sup> vs. median: 276500, IQR: 227000 - 311750 mm<sup>3</sup>; p = 0.813; Table II).

No statistically significant difference was observed between the Control group and the UI group in terms of total leucocyte count (7485 ± 1515.27 /mm<sup>3</sup> vs. 8072.38 ± 1792.41/mm<sup>3</sup>; p = 0.114; Table II).

The neutrophil percentage was found to be significantly higher in the UI group than in the Control group (median: 62.45, IQR: 56.93 - 68.28 vs. median: 58.75, IQR: 52.08-64.48; p = 0.017). The absolute neutrophil count was also found to be significantly higher in the UI group than in the Control group (5164.4 ± 1483.29/μl vs. 4408.55 ± 1153.3/μl; p = 0.012; Table II).

The percentage of lymphocytes was significantly lower in the UI group than in the Control group (28.95 ± 7.58 vs. 32.77 ± 7.21; p = 0.022). However, the absolute lymphocyte count did not differ significantly between the two groups (p = 0.534; Table II).

**Table III: The correlation analysis of IL-12.**

| IL-12         |   | Control Group | UI Group |
|---------------|---|---------------|----------|
| Age           | r | -0.067        | -0.248   |
|               | p | 0.682         | 0.109    |
| Hb            | r | -0.115        | -0.332*  |
|               | p | 0.481         | 0.032    |
| Hct           | r | -0.123        | -0.237   |
|               | p | 0.450         | 0.131    |
| WBC           | r | -0.012        | -0.187   |
|               | p | 0.943         | 0.235    |
| Neu %         | r | 0.124         | 0.014    |
|               | p | 0.447         | 0.932    |
| Neu #         | r | 0.090         | -0.180   |
|               | p | 0.579         | 0.254    |
| Lym %         | r | -0.191        | 0.027    |
|               | p | 0.238         | 0.865    |
| Lym #         | r | -0.168        | -0.081   |
|               | p | 0.301         | 0.609    |
| Mon %         | r | -0.373*       | 0.061    |
|               | p | 0.018         | 0.702    |
| Mon #         | r | -0.066        | -0.090   |
|               | p | 0.687         | 0.571    |
| Baso %        | r | -0.059        | -0.004   |
|               | p | 0.717         | 0.979    |
| Baso #        | r | 0.028         | -0.114   |
|               | p | 0.866         | 0.473    |
| Eo %          | r | 0.114         | -0.040   |
|               | p | 0.486         | 0.799    |
| Eo #          | r | 0.114         | -0.091   |
|               | p | 0.485         | 0.565    |
| NLR           | r | 0.174         | -0.006   |
|               | p | 0.284         | 0.969    |
| PLT           | r | -0.056        | -0.267   |
|               | p | 0.731         | 0.088    |
| Sedimentation | r | 0.185         | 0.619    |
|               | p | 0.478         | 0.102    |
|               | p | 0.755         | 0.699    |
| CRP           | r | 0.324         | -0.065   |
|               | p | 0.142         | 0.688    |
| INR           | r | -0.145        | 0.026    |
|               | p | 0.444         | 0.870    |
| PT            | r | -0.121        | 0.047    |
|               | p | 0.524         | 0.770    |
| APTT          | r | -0.237        | -0.334*  |
|               | p | 0.207         | 0.033    |

\*p < 0.05 statistically significant correlation; r: Spearman's correlation coefficient. Hb: Haemoglobin; Hct: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; RDW: Red blood cell distribution width; WBC: White blood cell; Neu: Neutrophil; Lym: Lymphocyte; Mon: Monocyte; Baso: Basophil; Eo: Eosinophil; NLR: Neutrophil-to-lymphocyte ratio; PLT: Platelet; MPV: Mean platelet volume.

Neutrophil/lymphocyte ratio (NLR) was significantly higher in the UI group than in the Control group (median: 2.11, IQR: 1.72-2.73 vs. median: 1.78, IQR: 1.35-2.29; p = 0.024; Table II).

The eosinophil percentage was also found to be significantly lower in the UI group (p = 0.004). The absolute eosinophil count was also found to be significantly lower in the UI group than in the Control group (p = 0.03; Table II).

Between the Control group and the UI group, there was no significant difference in IL-12 levels (median: 16.51, IQR: 14.58-21.43 vs. median: 15.92, IQR: 14.4 - 25.47; p = 0.623, Table II).

aPTT was found to be significantly higher in the UI group (p = 0.005). However, the INR and PT values did not differ significantly between the Control group and the UI group (p = 0.632; p = 0.455).

The correlation analysis revealed a statistically significant negative association between IL-12 and Hb in the UI group (r = -0.332, p = 0.032; Table III).

No statistically significant correlation was detected between IL-12 and white blood cell (WBC) in the UI group ( $r = -0.187$ ;  $p = 0.235$ ). Likewise, no statistically significant correlation was detected between IL-12 and monocyte percentage ( $r = 0.061$ ;  $p = 0.702$ ) and absolute monocyte count ( $r = -0.09$ ;  $p = 0.571$ ) in the UI group (Table III).

The correlation analysis showed a statistically significant negative association between IL-12 and monocyte percentage in the Control group ( $r = -0.373$ ;  $p = 0.018$ ; Table III).

A statistically significant negative relationship was found in the correlation study between IL-12 and aPTT in the UI group ( $r = -0.334$ ;  $p = 0.033$ ; Table III).

## DISCUSSION

There was no statistically significant relationship between the Control group and the UI group in terms of IL-12 in this study. The UI group had considerably greater neutrophil percentage and absolute neutrophil count. However, no significant difference was observed between the two groups in terms of inflammatory indicators such as sedimentation rate and CRP. Lymphocyte percentage, eosinophil percentage, and absolute eosinophil count were all considerably lower in the UI group. The UI group had significantly longer aPTT tests, although they were within the usual reference range.

Unexpectedly, there was no difference in IL-12 levels despite a statistically significant increase in neutrophils in the UI group. IL-12 is produced by neutrophils, activated macrophages, and B cells,<sup>14</sup> and it is a key cytokine in neutrophil activation.<sup>15</sup> Neutrophils may not create IL-12 in this case, or the presence of another cytokine, such as IL-10, may prevent the synthesis of IL-12. The UI group had significantly lower percentages and absolute amounts of eosinophils than the Control group. Given that eosinophils are a major source of IL-13, it would be more rational to investigate factors other than IL-13. IL-12 is significant in neutrophil phagocytic and microbicidal activation, as opposed to IFN-gamma-induced migration.<sup>15,16</sup> In that study, neutrophils increased in unexplained infertile patients, but IL-12 levels remained unchanged, indicating that other variables influencing neutrophils may exist.

Neutrophils in the local environment have been shown to remove sperm by trophocytosis.<sup>17</sup> However, as this research showed, a systemic increase in peripheral blood neutrophils may also be a sign that the body is alert for sperm. Neutrophils are often regarded as the first-line of innate immune protection, capable of rapidly killing or trapping pathogens but also inflicting tissue harm in the event of overactivation. The presence and activity of neutrophils in the female reproductive system are strictly regulated. In this situation, the cyclic steroid sex hormones that are present during the menstrual cycle and pregnancy are probably significant.<sup>18</sup> The increase in neutrophils may be linked to growing levels of FSH and estrogen because the blood samples used in this investigation were from the follicular period.

In most pregnancies, there is no tissue compatibility between mother and father. Therefore, for a successful pregnancy, the mother's immune system must be suppressed. It is believed that NK cells are required for this immunological suppression. These cells, called the uterine NK cells, belong to the CD56 bright NK cell subtype, which is characterised by strong cytokine output and poor cytotoxicity. In early pregnancy, they make up 70% of uterine leucocytes. Regarding uterine NK cell development, there is no consensus. Growing evidence shows that uterine NK cells can originate from a variety of sources, including decidua, endometrium, or peripheral blood. Several studies have shown that uterine NK cells can be generated from CD34(+) cell precursors identified from the decidua or endometrium, or by attracting CD34(-) CD117(+) CD94(-) NK precursor cells from the peripheral blood *via* decidua or endometrium.<sup>19</sup> There was no statistically significant difference in total leucocyte count between the control and UI groups in this study. Although it was not possible to explicitly investigate NK cells in this study, this may be resolved in subsequent research.

The most prevalent causes of female infertility include ovulation problems, endometriosis, fallopian tube diseases, and unexplained infertility.<sup>1</sup> A more comprehensive review of unexplained infertility reveals growing evidence in favour of both autoimmune and alloimmune processes, in which autoantibodies and NK cells might have vital functions. A Th2-type lymphocyte response, as well as elevated levels of IL-18, IL-12, and IL-15, and other unknown soluble factors reliant on NK cells, is likely to contribute to successful pregnancies. Most NK cells in the implantation site are uterine cells, and their activity, features, and quantity suggest that they engage in the decidualisation process, which causes NK activation and recruitment during each menstrual cycle. The pathophysiology of immunological infertility is assumed to be impacted by changes in the quantity of circulating NK cells, which are most likely a primary event rather than the result of current inflammation or medication taken during an inflammatory response. Alterations in NK cells may be linked to poor pregnancy outcomes.<sup>20</sup> There was no discernible difference in IL-12 levels between the UI and Control groups, but a study showed that infertile women with endometriosis had higher blood levels of IL-12p70 than the Control group.<sup>21,22</sup>

Polymorphonuclear neutrophils are the most prevalent form of leucocyte in the peripheral bloodstream. A relatively recent discovery about these cells is their capacity to export their DNA into the extracellular environment, creating termed neutrophil extracellular traps (NETs) that capture and eliminate bacteria. The formation of reactive oxygen species, nicotinamide adenine dinucleotide phosphate oxidase, and the combined activities of neutrophil elastase, myeloperoxidase, and histone deamination by human peptidylarginine deiminase 4 (PAD4) are important for this unusual type of cell death known as NETosis. Neutrophil NETs may be involved in several phases of the reproductive period, starting with fertility and perhaps concluding with foetal death.<sup>18</sup> In this study, neutrophils rose in



the UI group. The issue of NET generation should be investigated further in future studies.

There were a few limitations of this study. To support the IL-12 level, another test, such as flow cytometry displaying NK cell surface markers (CD3-CD56+), might have been employed. Aside from this, even if the power analysis achieved an adequate number of patients for the trial, the number may have been greater.

## CONCLUSION

Infertile women with unclear aetiology had similar IL-12 levels compared with the Control group. However, the neutrophil percentage and absolute neutrophil count were observed to be greater in these patients. More research on cytokines linked to infertility is needed.

## ETHICAL APPROVAL:

Ethical approval was obtained from the Local Ethics Committee of the Medical Faculty of Pamukkale University, Denizli, Türkiye (Approval. 25.01.2022/number 02), and it was conducted in compliance with the Helsinki Declaration's legal and regulatory requirements.

## PATIENTS' CONSENT:

Informed consent was taken from all the patients.

## COMPETING INTEREST:

The authors declared no conflict of interest.

## AUTHORS' CONTRIBUTION:

BUK, EA: Study design, data collection, analysis, data evaluation, drafting, and editing.

SF, VF, AA, HS: Data collection, data analysis, and data interpretation.

All authors approved the final version of the manuscript to be published.

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