

## An Analysis of *ESR2* and *CYP19A1* Gene Expression Levels in Women With Endometriosis

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**Abstract.** *Aim: The analysis of oestrogen receptor (ESR2) and cytochrome P450 family 19 subfamily A member (CYP19A1) gene expression in the context of the risk for endometriosis development. Materials and Methods: Tissue specimens, collected from patients with endometriosis (n=100) and from control patients (n=100) embedded into paraffin blocks, provided the material for genetic studies, oriented towards the expression of ESR2 and CYP19A1 genes. The gene expression was assessed by the reverse transcription-polymerase chain reaction technique. Results: Higher expression levels of ESR2 gene were demonstrated in the patients with endometriosis in comparison with the healthy controls. The expression intensity of CYP19A1 gene was associated with endometriosis, manifested as abdominal wall nodules. A relationship was observed between CYP19A1 gene expression and the Revised American Society for Reproductive Medicine classification in the group with ovarian endometrioid cysts, as well as in the group with peritoneal endometriosis. Conclusion: This study suggests the significant role of ESR2 and CYP19A1 gene expression in the pathogenesis of endometriosis.*

Endometriosis is a frequent gynaecological condition, both in Poland and worldwide (1) and is defined as the occurrence of

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uterine mucosa beyond the uterine cavity with a concomitant and chronic inflammatory condition. This disease affects 10-15% of women of reproductive age and 35-50% of women with pain in the pelvis minor or with infertility. One should keep in mind, however, that sporadic cases of endometriosis are also diagnosed in patients after menopause, as well as in young women. No specific marker for endometriosis has yet been identified. Groups of compounds are now searched for which, in a specific combination, would ensure maximum sensitivity and specificity of non-invasive diagnostics for this disease, especially in its early stages (3, 4).

Endometriosis is an oestrogen-dependent disease. It presents a specific ability to receive oestrogen stimulation of its development. An enhanced local production of oestrogens takes place within endometrial tissue as a result of increased expression of the P450 cytochrome aromatase. The product of the *CYP19A1* gene, aromatase converts testosterone and androstenedione into oestradiol and oestrone, respectively (5). Endometriosis lesions, unlike eutopic endometrium, demonstrate some activity of the aromatase (6).

The action of oestrogens on target cells is possible via oestrogen receptors, which act as transcriptive factors, and plays a significant role in the growth and differentiation of endometrial cells, as well as in numerous biological functions, in both, eutopic and ectopic endometrium. The two types of oestrogen receptors thus far identified are ER $\alpha$  and ER $\beta$ , encoded by two different genes (*ESR1* and *ESR2*, respectively). ER $\beta$  is the main oestrogen receptor in patients with endometriosis (7, 8).

A higher expression of P450 cytochrome and of hydroxysteroid 17-dehydrogenase in ectopic endometrium increases the oestrogen level, which activates endothelial cells of the stroma and brings about the development of endometriosis (9, 10). This study aimed to determine whether there were any correlations between the gene

Table I. Characteristic features of patients with endometriosis and of the controls.

	Study group (n=100)	Control group (n=100)
Age, years		
Mean±SD (range)	34.89±7.49 (21-53)	47.93±6.01 (26-67)
BMI, n (%)		
<25 kg/m <sup>2</sup>	68 (68%)	29 (29%)
25≤BMI<30 kg/m <sup>2</sup>	22 (22%)	43 (43%)
≥30 kg/m <sup>2</sup>	10 (10%)	28 (28%)
Deliveries, n (%)		
0	53 (53%)	7 (7%)
1	21 (21%)	36 (36%)
≥2	26 (26%)	57 (57%)
Spontaneous abortion, n (%)		
Yes	5 (5%)	9 (9%)
No	95 (95%)	91 (91%)

BMI: Body mass index; SD: standard deviation.

expression of *ESR2* and *CYP19A1* and the incidence of endometriosis. *ESR2* and *CYP19A1* genes were selected for the study due to their considerable role in hormonal control, in patients with endometriosis, the development of which is largely determined by steroid hormones (10-13).

The goals of the study included: a) An analysis of *ESR2* and *CYP19A1* gene expression levels in patients with endometriosis and in a control group; b) analysis of correlation of the levels of *ESR2* and *CYP19A1* gene expression with clinicopathological data; and c) the assessment of the significance of obtained results in the context of the risk for endometriosis.

## Materials and Methods

**Patients.** Tissue specimens, collected from patients with endometriosis (n=100) and from control patients (n=100), embedded in paraffin blocks, provided the material for studies. The paraffin blocks were provided by the Archive of the Department of Clinical Pathomorphology, Institute-Polish Mother's Memorial Hospital in Lodz, Poland.

The study group comprised 100 patients who underwent surgery for endometriosis at the Department of Operative Gynaecology and Oncological Gynaecology of the Institute - Polish Mother's Memorial Hospital in Lodz, during the years 2015-2016. Endometriosis was confirmed by intraoperative diagnostics and, finally, by histopathological evaluation. See Table I for characteristic features of the patients.

The study material included the following specimens: 63 cases of endometrial ovarian cyst, 10 cases of endometriosis nodules from abdominal walls, 17 cases of peritoneal endometriosis, 10 cases of deeply infiltrating endometriosis. The clinical staging was carried out in the patients using the 1996 Revised American Society for Reproductive Medicine classification of endometriosis (rASRM; Table II) (14).

The control group consisted of 100 patients from the Department of Operative Gynaecology and Oncological Gynaecology of the Institute-Polish Mother's Memorial Hospital in Lodz, Poland.

Table II. The clinical staging of patients with endometriosis (n=100) by the Revised American Society for Reproductive Medicine classification of endometriosis (14).

Clinical stage	Frequency (%)
I	26 (26%)
II	25 (25%)
III	17 (17%)
IV	32 (32%)

Histopathological assessments confirmed normal endometrium in all the controls. The control patients had not suffered from endometriosis. The specimens collected during surgery were immediately cooled to -20°C and then stored for further analysis at a temperature of -70°C. All the specimens were histologically characterised. Formal consent (No. 8/2016) for this study was obtained from the Bioethical Committee of the Institute-Polish Mother's Memorial Hospital in Lodz.

**RNA isolation.** Total RNA was isolated with the Trizol reagent (Ambion, Austin, TX, USA). RNA samples were stored in the temperature of -20°C. Isolated RNA was usually stored for no more than 24-48 hours. This time did not affect the quality or integrity of the RNA.

**Spectrophotometric analysis of RNA purity and concentration.** The purity of obtained RNA specimens was evaluated by the spectrophotometric method, employing a two-fold absorbance measurement of each sample at 260 nm and 280 nm wavelength. A260/A280 values between 1.8 and 2.0 were accepted as criteria for RNA purity. RNA concentrations were measured by spectrophotometric method, based on the absorbance value (OD), measured at 260 nm. That value corresponded to the following relationship: 1 unit OD=40 µg DNA/ml. The mean RNA concentrations and purity degree obtained for the study material met the necessary criteria.

**Reverse transcription polymerase chain reaction (RT-PCR).** A reverse transcription reaction was carried out using a TaKaRa RNA PCR kit (AMV) ver 3.0 (Takara Bio Inc., Tokyo, Japan). The obtained cDNA samples were stored at -20°C. According to the manufacturer's instructions, the amount of RNA was about 500 ng.

**PCR with an analysis of product volume growth in real time.** The reaction mixture included 0.5 µl of cDNA, 0.5 µl of 20×TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA, USA), 5 µl of TaqMan® Universal PCR MasterMix (Applied Biosystems), containing TaqMan® DNA polymerase, dNTP, a reaction buffer and 4 µl of water. Real-Time PCR was carried out in a Mastercycler® ep realplex device (Eppendorf, Germany). The thermal profile of the reaction included a preliminary denaturation in the temperature of 95°C for 10 minutes, followed by 50 cycles of 15-second incubation at 95°C, combined with 1 minute at 60°C. The following, commercially available kits of probes and starters were applied in the real-time PCR: Hs01100353\_m1 for *ESR2* gene, Hs00903411\_m1 for *CYP19A1* gene and Hs02800695\_m1 for hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) gene, being a reference gene. The obtained Ct values were recalculated to the number of mRNA copies of the studied gene per 1,000 mRNA copies of *HPRT1*, according to the following correlations:

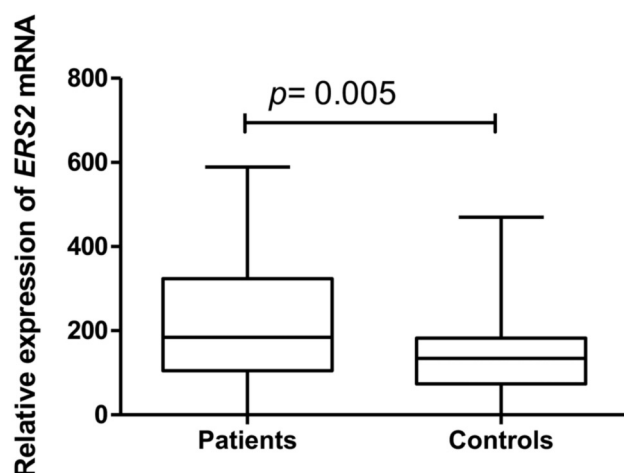


Figure 1. Relative oestrogen receptor  $\beta$  (*ESR2*) gene expression in study groups.

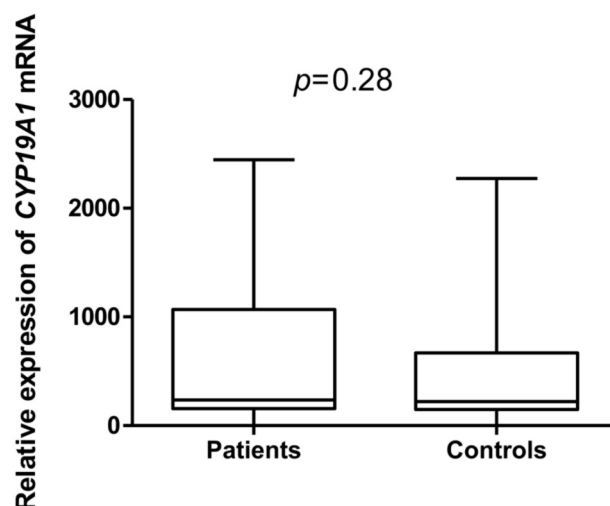


Figure 2. Relative gene expression of cytochrome P450 family 19 subfamily A member (*CYP19A1*) in study groups.

$$\Delta C_t = C_{t \text{ study gene}} - C_{t \text{ reference gene}}$$

$$L = 1,000 \times 2^{-\Delta C_t}$$

where L was the number of mRNA copies of the study gene per 1,000 copies of the reference gene mRNA. The mean Ct values, obtained for both neoplastic and normal material, met the necessary criteria.

**Statistical analysis.** The obtained results were statistically processed by means of the STATISTICA 11 software (StatSoft, Poznan, Poland). The significance of differences was analysed at the level of gene expression and mRNA, using non-parametric tests (the *U* Mann-Whitney test and the Kruskal-Wallis test) for a lack of distribution normality of the obtained results, as confirmed by the Shapiro-Wilk test. The Spearman rank-order correlation coefficient test was used to assess correlations between variables. A statistical significance was confirmed at  $p < 0.05$ .

## Results

The study demonstrated higher expression of *ESR2* gene in 100 examined patients with endometriosis in comparison with the healthy controls (Figure 1). The *ESR2* gene expression in the study group did not correlate with the age of the patients nor with their body mass index, the level of cancer antigen-125 (CA125) marker nor with the number of previous pregnancies. No relationship was revealed between *ESR2* gene expression and rASRM classification scores in the study group. Neither were any relationships demonstrated between the *ESR2* gene expression level and the clinicopathological factors when the patients were classified to the particular types of endometriosis, such as: peritoneal, deeply infiltrating or endometrial ovarian cysts. Only in the case of endometrial nodules in abdominal walls, was *ESR2* gene expression significantly higher compared with the control group.

Regarding *CYP19A1* gene in the study, no correlation with endometriosis was identified in the study group ( $n=100$ ) (Figure 2).

However, negative correlation was confirmed between *CYP19A1* gene expression level and body mass index (Figure 3). The expression of the *CYP19A1* gene showed a statistically significant relationship with the level of CA125 marker in the patient group (Table III); expression was higher in the group with a concentration of CA125  $< 35$  U/ml (Figure 4).

The *CYP19A1* gene expression intensity was associated with endometriosis nodules of the abdominal wall. In the group of patients with endometrial ovarian cysts, a statistically significant negatively relationship was confirmed between the expression levels of *CYP19A1* gene and the number of pregnancies and deliveries in those patients (Table IV). A relationship was observed between *CYP19A1* gene expression and rASRM classification scores in the group with ovarian endometrioid cysts, as well as in the group with peritoneal endometriosis (Figure 5): *CYP19A1* gene expression level was higher in patients with a higher degree (III-IV) of disease.

## Discussion

The expression levels of *ESR2* and *CYP19A1* genes were assayed in patients with endometriosis and compared with a healthy control group, while also studying their association with increased prevalence of endometriosis among Polish women.

Disorders at the level of *ESR2* gene expression associated with endometriosis and related to the types and sites of lesions have been confirmed by world literature reports. The team of Maekawa *et al.* employed RT-PCR to assay the expression levels of *ESR1*, *ESR2* and progesterone receptor (*PGR*) genes in cases of ovarian endometriosis and eutopic

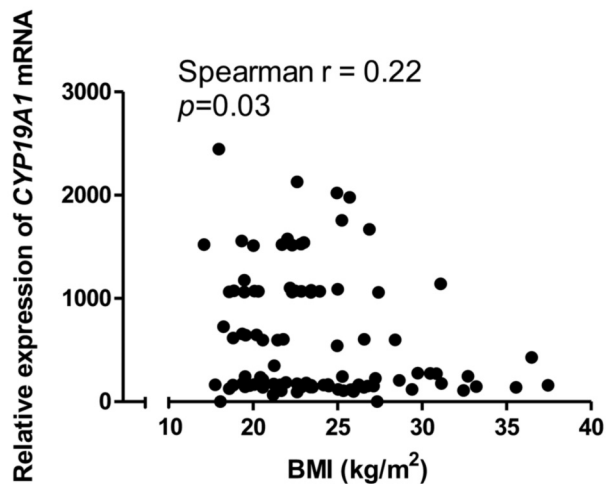


Figure 3. Correlation between body mass index (BMI) and the relative gene expression of cytochrome P450 family 19 subfamily A member (CYP19A1) for the patient group.

endometrium (15). The *ESR1* and *PGR* expression levels were substantially lower, while the level of *ESR2* gene expression was significantly higher in endometriosis than in normal endometrium. DNA methylation levels were also studied. DNA methylation of *ESR1* gene was significantly higher in endometriosis than in eutopic endometrium. No major differences were observed in the degree of DNA methylation for *ESR2* nor for *PGR*. The researchers made a suggestion that the observed, abnormal DNA methylation was associated with a low level of *ESR1* expression. While the degree of methylation did not affect the expression changes of *ESR2* or *PGR* genes in endometriosis (15).

The latest study of the team of Yilmaz and Bulun demonstrated that endometrial stromal cells, which participate in the inflammatory process, are induced by oestrogen, and play a role in the formation of prostaglandins, revealed an extremely low ratio of *ESR1* to *ESR2* expression, resulting from an excessive increase in the expression of the latter gene (12). The cells also demonstrated a deficit of *PGR* protein, which leads to resistance to progesterone and defective retinoid synthesis. The expression of nuclear receptors, including low levels of *ESR1* and *PGR* under a high level of *ESR2*, corresponds to the expression of factors found in the stem cells of uterine fibroid. It is suggested that endometrial stromal cells may present certain features of stem cells present in other uterine tissues.

In the study of Colon-Caraballo *et al.*, the expression of *ESR1*, *ESR2* and *PGR* was assayed in various types of endometrial change and in eutopic endometrium of women with endometriosis and in a control group (11). Changes in ovarian ducts were characterised by high expression levels of

Table III. Relative gene expression of cytochrome P450 family 19 subfamily A member (CYP19A1) with regard to level of the marker cancer antigen CA125.

	CA125	
	≤35 U/ml	>35 U/ml
Number of specimens	58	38
Mean±SD (range)	642.1±618.7 (66.67-2,446)	367.3±387.5 (0.0-1,542)

Mann-Whitney test,  $p=0.046$ .

all three receptors. Differences observed among endometrial specimens included lower *ESR1* expression and higher *ESR2* expression in the proliferative endometrium from the study group. A tendency towards a lack of nuclear activity of *PGR* was identified in the proliferative endometrium from patients. The highest *ESR2:ESR1* expression ratio was observed in those with endometrial changes of the ovaries and in the secretory endometrium. The results obtained by Colon-Caraballo *et al.* are valuable as they extend our knowledge regarding the potential for individual reactions to hormonal therapies, providing a basis to develop personalised therapeutic methods for women with endometriosis (11).

*CYP19A1* was another gene whose expression was analysed in the reported study. *CYP19A1* encodes cytochrome P450 aromatase, an enzyme participating in the biosynthesis of oestrogens. In regard to *CYP19A1*, the goals of the study focused on correlations between the level of gene expression and the prevalence of endometriosis, as when the study was planned there was no basis for the changes we subsequently found. The identification of variable expression levels of the aromatase-encoding gene in this study may make a significant contribution to the understanding of the mechanism responsible for the enhanced production of oestrogens observed in endometriosis.

There are literature data suggesting that the expression of *CYP19A1* gene may not change in endometriosis (6), which was partially confirmed by the results of our study.

The reduced *CYP19A1* expression in endometriosis may result from epigenetic modifications in the regulatory regions of the gene, for example, DNA methylation or the modification of histones (16). Changes in the methylation status of CpG islands, contained in *CYP19A1* gene were confirmed in endometriosis by Izawa *et al.* (17). A CpG island is localised approximately 70 kb below exon 1.1 of the *CYP19A1* gene. It was found to be hypomethylated in endometriosis (studies were run on stromal cells, obtained from chocolate ovarian cysts). However, its hypermethylation was found in eutopic endometrium (stromal cells obtained from women, not suffering from endometriosis) (17).

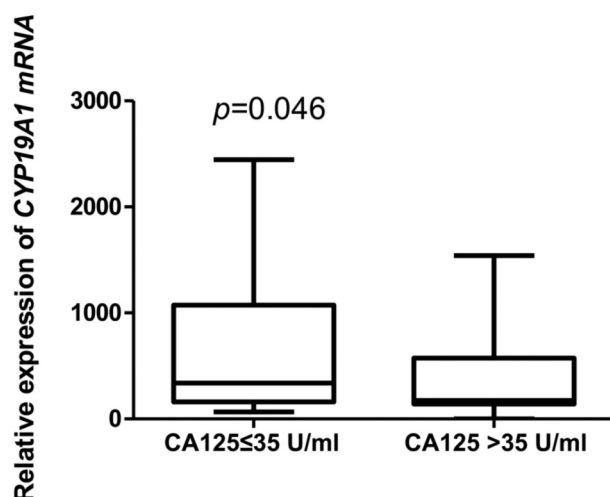


Figure 4. Relative gene expression of cytochrome P450 family 19 subfamily A member (CYP19A1) according to the level of the marker cancer antigen 125 (CA125).

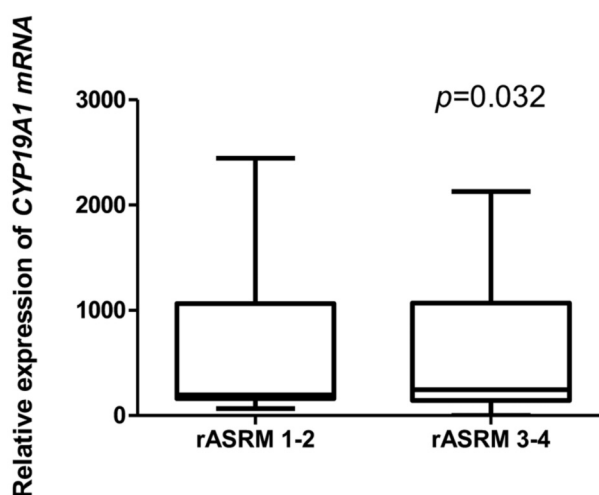


Figure 5. Relative gene expression (in peritoneal endometriosis) of cytochrome P450 family 19 subfamily A member (CYP19A1) according to the Revised American Society for Reproductive Medicine (rASRM) classification of endometriosis.

Van Kaam *et al.* demonstrated that methyl-CpG-binding proteins, such as MBD1 and MeCP2, which contain the methyl-CpG-binding N-terminal domain and the transcription inhibition domain on the carboxyl terminus, were not bound with the hypermethylated region of a CpG island in eutopic tissues (19). It may explain the 'muting' of aromatase expression in eutopic endometrium. The treatment of endometrial stromal cells with 5-aza-deoxycytidine (an irreversible inhibitor of DNA methyltransferase, which is necessary to maintain the status of genomic DNA methylation) induces the expression of aromatase (18).

Demethylation of CpG islands in CYP19A1 gene may be significant for the control of aromatase levels, although other explanations are also possible, since 5-aza-deoxycytidine changes the expression levels of a broad spectrum of genes which may either indirectly or directly affect aromatase expression (19).

Maia *et al.* obtained results clearly indicating that CYP19A1 expression was not strictly associated with endometriosis (20). The researchers carried out their studies to see whether the expression of aromatase in eutopic endometrium correlated with the presence of endometriosis and its clinical staging in 106 women of reproductive age and with symptoms of dysmenorrhoea and infertility. The patients were submitted to laparoscopy and hysteroscopy. A control group consisted of 16 patients with asymptomatic endometriosis. The results of the study demonstrated that aromatase expression in the endometrium was associated with the occurrence of dysmenorrhoea and infertility, regardless of whether endometriosis was present or not (20).

Table IV. Relative gene expression of cytochrome P450 family 19 subfamily A member (CYP19A1) with regard to the number of pregnancies and deliveries.

	Pregnancies=0; Deliveries=0	Pregnancies>0; Deliveries>0
Number of specimens	38	24
Mean±SD	685.4±627.4 (0-2,446)	504.2±489.9 (94.16-2,128)

Mann-Whitney test,  $p=0.031$ .

The results of this study, concerning the analysis of ESR2 and CYP19A1 genes, unveil certain correlations of their expression with endometriosis. It should, however, be emphasised that this study involved a numerically small population (100 patients) and thus requires continuation on much larger study groups. Taking into account the significance of the obtained results, such studies should be continued. The results, obtained in the study, contribute to better knowledge of and information on the molecular mechanisms which support the development of endometriosis. The study pointed out differences in the expression levels of the studied genes between the studied groups of patients, which may potentially foster the development of the condition. In consideration of the rather small number of studies on the expression of CYP19A1 and ESR2 genes in patients with endometriosis from the Polish population, these findings may bring real added value, broadening the knowledge of the effects of genetic factors on the development of endometriosis.

Determining the relationships between *ESR2* and *CYP19A1* gene expression and endometriosis may help design and develop new therapeutic concepts and strategies for this disease. Studies on gene expression aspects are likely to constitute a *prima facie* target of major concern regarding personalised therapy.

## Conclusion

A significant increase of *ESR2* gene expression was observed in the group of women with endometriosis, which may indicate a role of this factor in the pathogenesis of endometriosis. The lack of statistically significant difference in *CYP19A1* gene expression in the study group *versus* the control indicates no participation of the gene in the pathogenesis of endometriosis. A higher expression of *ESR2* gene was demonstrated in the group of patients with endometrial nodules of abdominal walls. This shows a significant role of *ESR2* gene in the pathogenesis of this form of endometriosis. In the group of patients with endometrial ovarian cysts and peritoneal endometriosis, the levels of *CYP19A1* expression were associated with the rASRM clinical staging of endometriosis. In the case of nulliparous patients with endometrial cysts, a statistically significant positive correlation was demonstrated with *CYP19A* gene expression.

## Declarations

Ethics approval and consent to participate. This work was supported by the Institute of Polish Mother's Memorial Hospital, Lodz, Poland from the Statutory Development Fund. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the study participants gave their written-informed consent.

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## Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

## Authors' Contributions

Conceived and designed the study: TS and BS. Performed the experiments – case group: MB and EF. Case group design and collection: TS, HR, KS, BK, MS and BM. Performed the study – control group: MB and EF. Analysed data: BS. Contributed reagents/materials/analysis tools: MB and EF. Contributed to the writing of article: BS and TS. All Authors approved the final article.

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