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Aberrant expression of proatherogenic cytokines and growth factors in human umbilical vein endothelial cells from newborns of type 2 diabetic women

Samar Sultan

Abstract

Objectives: This study reports the levels of cytokines, chemokines, and growth factors previously identified as taking part in the pathology of atherosclerosis in human umbilical vein endothelial cells derived from mothers with type 2 diabetes and compares them with those in human umbilical vein endothelial cells derived from healthy mothers under normal glucose conditions.

Methods: Cytokine analysis measures of human umbilical vein endothelial cell lysates were obtained using a multiple analyte profiling (xMAP) assay based on magnetic bead-based technology, using the MAGPIX instrument. The correlation between cytokines, chemokines, and growth factors was examined statistically in human umbilical vein endothelial cells derived from mothers with type 2 diabetes.

Results: This study showed that the expression of proinflammatory cytokine interleukin-1 alpha was significantly greater in human umbilical vein endothelial cells derived from mothers with type 2 diabetes than those derived from healthy mothers. The protein level of granulocyte colony-stimulating factor was higher in human umbilical vein endothelial cells derived from mothers with type 2 diabetes than those derived from healthy mothers. A significant positive correlation was demonstrated between the protein expression of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor in human umbilical vein endothelial cells derived from mothers with type 2 diabetes.

Conclusion: Diabetes evokes a persistent inflammatory phenotype in human umbilical vein endothelial cells, as indicated by the enhanced production of cytokines and growth factors under normal glucose conditions.

Keywords

Proinflammatory cytokines, growth factors, type 2 diabetes, endothelial cells

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Introduction

Diabetes is characterized by subclinical inflammation.[1] Inflammation plays an important role in the pathogenesis of diabetes-associated cardiovascular complications, such as atherosclerosis. Barker[2] hypothesized that adult cardiovascular diseases (CVDs) were programmed during fetal maturation in response to intrauterine or maternal conditions such as insulin resistance or oxidative stress. Subsequent accumulating evidence has suggested a link between being a newborn whose mother has type 2 diabetes (T2D) and the risk of developing CVDs in later life.[3–5] Markers of endothelial dysfunction, vascular abnormalities, and inflammatory irregularities are detectable in the normoglycemic offspring of T2D patients.[6–8] One longitudinal study showed accelerated progression of insulin resistance syndrome from childhood to young adulthood (4–17 years of age) in the offspring of parents with T2D.[9]

Endothelial dysfunction is the first step in the development of atherosclerosis. However, the mechanism by which diabetes leads to vascular inflammation is not clear. Hyperglycemia activates several pathways associated with

Medical Laboratory Technology Department, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

Corresponding author:
Samar Sultan, PhD, Medical Laboratory Technology Department, Faculty of Applied Medical Sciences, King Abdulaziz University, Idara street, Jeddah 21589, Saudi Arabia.
Email: sasultan@kau.edu.sa

the induced oxidative stress that can lead to endothelial dysfunction, including increased levels of advanced glycation end products (AGEs), protein kinase C activation (PKC), polyol pathway, and reduced NO synthesis and expression.[10,11] Prolonged hyperglycemia can lead to persistent glycemic memory of endothelial dysfunction, even after glucose normalization.[12] This observation is supported by our previous study, which showed that fetal human umbilical vein endothelial cells (HUVECs), cultured under normal glucose conditions, obtained from mothers with T2D (T2D.HUVECs) exhibited increased apoptosis, enhanced mitochondrial superoxide generation, altered cell cycle, and differential global expression of genes involved in the inflammatory response and CVDs compared to HUVECs from healthy mothers (C.HUVECs).[13]

Endothelial dysfunction is an early event in the development of atherosclerosis.[14–16] Hyperglycemia-induced oxidative stress can lead to lipid oxidation and subsequent activation of endothelial cells (ECs). Activated ECs secrete cytokines and chemokines, contributing to vascular inflammation through the enhanced recruitment of leukocytes (mainly monocyte-macrophages) into the intima of the inner layer of blood vessels.[17,18] In a previous study, elevated gene and protein expression of interleukin (IL), IL-8, was observed upon exposure to high-glucose (HG) treatment (25 mM) for 24 h.[19] Furthermore, the circulating levels of proinflammatory cytokines, including IL-1 and IL-6, have been found to be increased in the serum of patients with T2D[20] and in the retinas of diabetic patients with retinopathy.[21]

The growth factors granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), are produced by various types of cells, including ECs, under basal conditions. They exert synergistic effects on ECs by enhancing their proliferation,[22] migration,[23] and angiogenesis.[24] The levels of G-CSF and GM-CSF have been shown to be higher under inflammatory conditions such as atherosclerosis[25,26] and T2D.[27,28] Levels were higher under these conditions, possibly due to glucotoxicity,[29–31] lipotoxicity,[32] or oxidative stress.[33] G-CSF is a proinflammatory factor that promotes ECs activation, platelet activation and aggregation, and the progression of atherosclerosis.[34,35] Whether HUVECs from T2D mothers produce these factors when cultured under normal glucose conditions remain unclear. The aim of this study was to evaluate the protein levels of the proatherogenic cytokines, chemokines, and growth factors IL-6, IL-8, IL-1A, G-CSF, and GM-CSF in T2D. HUVECs and compare them with those in C.HUVECs. The correlation between cytokines, chemokines, and growth factors was also investigated in the T2D.HUVECs.

Materials and methods

All materials were obtained from UFC biotechnology (Riyadh, Saudi Arabia) unless otherwise stated.

Study subjects

This was a case–control study. Fresh umbilical cords were collected from four mothers with T2D and three healthy mothers after full-term delivery at King Abdulaziz University Hospital, Jeddah, Saudi Arabia during the period between September 2017 and February 2018. This study was approved by the Ethics Committee of King Abdulaziz University. Informed consent was obtained from all subjects before sample collection. The inclusion criteria for the control subjects were non-diabetic, non-smokers, and did not have hypertension. The inclusion criteria for the diabetic subjects were the previous diagnosis of T2D and plasma glucose concentration (fasting or random) $>$ 7 mmol/L or hemoglobin A1c (HbA1c) of $>$ 7% according to the American Diabetes Association.[36] Glucose concentration and HbA1c were collected at the end of pregnancy. All subjects in this study had no obstetric complications, and all patients with T2D were on insulin.

HUVECs isolation and culture

ECs were harvested from umbilical cords derived from patients with T2D (experimental group) and healthy subjects (control group). The cords were stored at 4°C in phosphate buffered saline (PBS) containing heparin (10 U/mL), Fungizone (2.5 μg/mL), and penicillin/streptomycin (100 U/100 mg/mL) until processing within 24 h according to protocols in a previous study.[37] ECs were isolated from the human umbilical vein by the action of collagenase enzyme as described by Eccles et al.[38] Then, the cells were cultured in complete M199 medium containing 20% fetal bovine serum (FBS) penicillin/streptomycin (100 U/100 mg/mL), Fungizone (2.5 μg/mL) and L-glutamine (2 mM) at 37°C in a humidified 5% CO_2–air atmosphere until confluence was reached. HUVECs were used for up to four passages.

Characterization of isolated HUVECs

Isolated HUVECs were characterized for endothelial-specific CD31 surface marker using flow cytometry. Harvested ECs were suspended in Dulbecco's phosphate buffered saline (DPBS) containing 0.1% (v/v) human serum albumin and incubated for 10 min at 4°C with either mouse monoclonal antibody FITC (fluorescein isothiocyanate)-tag against CD31 (1:20) or PBS as negative control. Then, the ECs were washed with PBS, and fluorescence intensities were assessed using a FACS Aria III flow cytometer (BD Biosciences, San Jose, USA).

Preparation of cell lysate

Confluent cells after seeding on six-well plate at density (1×10^5 cells/well) for 72 h were washed twice with PBS and lysed directly with NP40 cell lysis buffer (50 mM Tris, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4,

Table 1. Mean characteristics of subjects participating in the study.

Status mothers	Controls (n = 3)	T2D (n = 4)	*p* (T2D vs controls)
Age (years)	28 ± 0.4	36 ± 0.5	0.15
BMI	26.7 ± 0.6	30.7 ± 0.7	0.62
HbA1c % (mmol/mol)	–	8.1 (65) ± 0.4	–
RPG (mM)	4.9 ± 0.3	10.8 ± 0.6	0.008
FPG (mM)	–	7.85 ± 0.5 (n = 2)	–

RPG: random plasma glucose; FPG: fasting plasma glucose; BMI: body mass index; HbA1c: glycosylated hemoglobin; T2D: type 2 diabetes mellitus, mean ± SEM.
Number of donors donated by (n).

1% Nonidet P40 (NP40), 0.02% NaN_3, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1× protease inhibitor cocktail) on ice for 30 min with vortexing conducted at 10 min intervals. The extract was transferred to microcentrifuge tubes and centrifuged at 13,000 g for 10 min at 4°C. The clear lysate was aliquoted into clean tubes and stored at −80°C until use.

Multiplex immunoassay for protein detection

Multiplex immunoassay is an advanced technique that allows the simultaneous measurement of multiple proteins in one sample. It uses the immunoassay principle with magnetic beads, and it can detect up to five proteins in a sample according to the platform used; in this study, IL-1A, IL-6, IL-8, G-CSF, and GM-CSF were detected. The test was performed using the ProcartaPlex Multiplex immune-bead assay kit provided by Invitrogen. Briefly, the cell culture lysate was transferred to a 96-well plate according to the kit protocol. The antibody was vortexed for 30 s. Then, sonication was carried out for 5 min. A total of 25 µL of beads was then placed into each well. The beads were washed one time with the buffer included with the kit. The serial dilution for standards was preformed according to the manufacturer's instructions. The plate was incubated on a shaker at 500 r/min for 2 h at room temperature. The beads were washed two times with the included buffer, and 50 µL of streptavidin-Phycoerythrin (PE) was added to each well. The plate was again placed on a shaker for 30 min at room temperature. The beads were washed two times with the included buffer, resuspended in the buffer, and shaken for 5 min. Results were obtained by an MAGPIX instrument (Luminex Corporation, Austin, USA) and analyzed using Luminex xPONENT® multiplex assay analysis software (v.4.2.1324.0, Luminex Corporation).

Statistical analysis

The results are presented as the mean ± SEM of three samples from the control group and four samples from the experimental group. Statistical analyses were performed using Student's *t*-test. Correlation analyses were carried out by Pearson correlation coefficient (*r*). A *p*-value of less than 0.05 was considered statistically significant.

Results

Clinical characteristics of the T2D and healthy mothers who participated in this study are summarized in Table 1. There was a significant difference in random plasma glucose between T2D patients and controls ($p = 0.008$) but no significant differences in age and body mass index (BMI).

HUVEC characterization

Isolated HUVECs were identified by the presence of CD31 (platelet EC adhesion molecule-1, PECAM-1) on their surfaces. As depicted in Figure 1(b), all of the isolated cells from the early passages showed high expression of CD31 (95%), confirming that all of the isolated cells were indeed HUVECs. In contrast, negative control cells (cells incubated without anti-CD31 and only with PBS) showed no fluorescence (Figure 1(a)).

Effect of T2D on the production of proinflammatory cytokines and chemokines in HUVECs

To identify the effect of T2D on EC activation, the protein expression of proinflammatory cytokines/chemokines (IL-1A, IL-6, and IL-8) was measured by Multiplex Luminex xMAP Assay. T2D.HUVECs exhibited significantly greater expression of IL-1A than C.HUVECs ($p = 0.04$, Figure 2(a)). The mean IL-1A expression of T2D.HUVECs was 9.6 pg/mL, whereas that of control cells was 2.7 pg/mL. The expression levels of IL-6 did not differ between T2D.HUVECs and control cells (Figure 2(b)). Of note, the mean IL-8 expression of T2D. HUVECs was 55.8 pg/mL, which was greater than that of control cells (40.9 pg/mL), however, it did not reach the statistical significance ($p > 0.05$, Figure 2(c)).

Effect of T2D on the production of growth factors in HUVECs

The effect of T2D on growth factors (G-CSF and GM-CSF) in HUVECs was evaluated using Multiplex Luminex xMAP Assay. Interestingly, the level of G-CSF protein production in T2D.HUVECs was significantly increased compared to

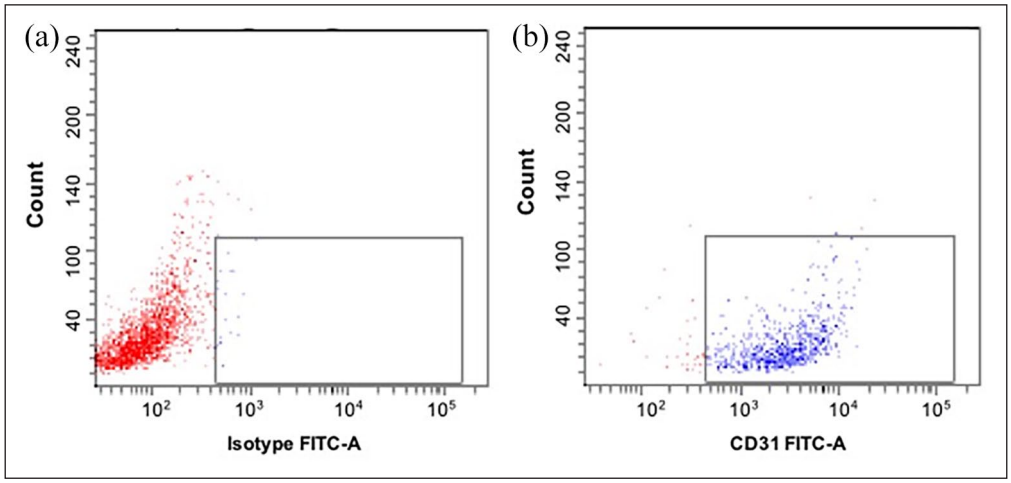

Figure 1. Flow cytometric analysis of the endothelial cell-specific marker CD31: (a) isotype negative control and (b) positive CD31 marker.

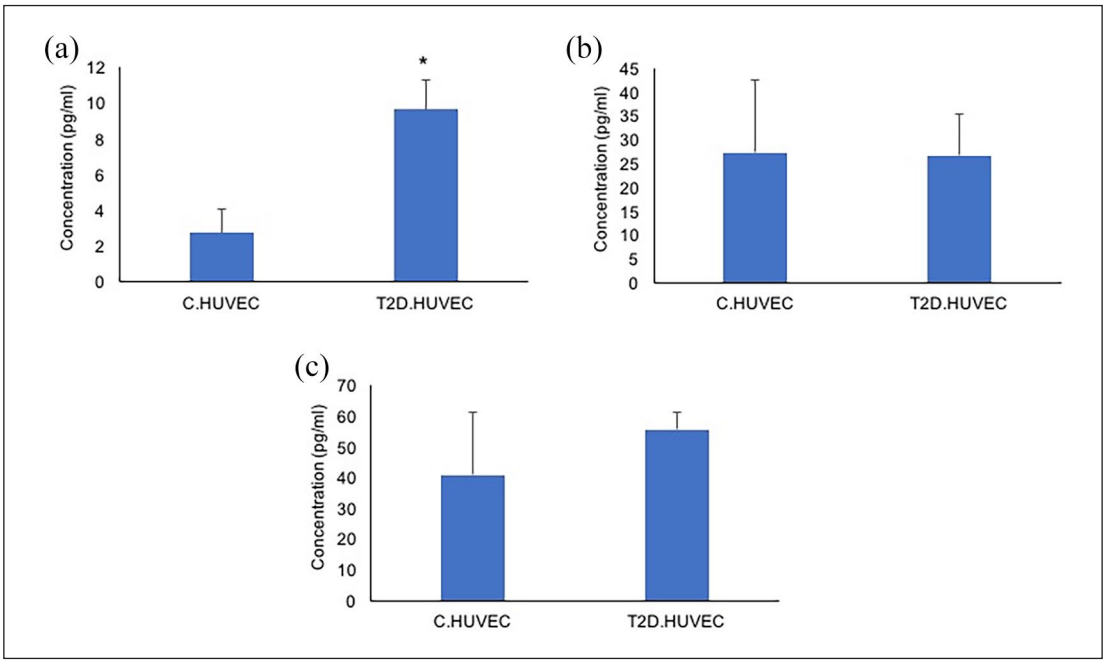

Figure 2. Protein levels of cytokines and a chemokine in HUVECs. The protein levels of (a) IL-1A, (b) IL-6, and (c) IL-8 in cell lysates of T2D.HUVECs and C.HUVECs were analyzed using ProcartaPlex Multiplex cytokine assay.
The values are mean ± SEM. *$p < 0.05$ compared with control.

that in control cells ($p = 0.02$, Figure 3(a)). The mean value of G-CSF expression in T2D.HUVECs was 26.7 pg/mL, whereas that in control cells was 12.2 pg/mL. Similarly, the level of GM-CSF protein in T2D.HUVECs was nonsignificantly increased relative to the level in control cells ($p > 0.05$, Figure 3(b)).

Correlation between proinflammatory cytokines, chemokines, and growth factors in T2D.HUVECs

The results showed a significant and positive correlation ($r = 0.96$, $p = 0.03$) between protein expression of G-CSF and GM-CSF in T2D.HUVECs (Table 2). However, no other significant correlation was noted.

Discussion

Our study showed for the first time that, under normal glucose conditions, T2D.HUVECs exhibited significantly higher levels of the proinflammatory cytokine IL-1A and the growth factor G-CSF than did the control cells. This indicated that the damaging effect of elevated glucose during gestation persisted in ECs even after they returned to normoglycemic conditions. In this study, we analyzed the protein expression of

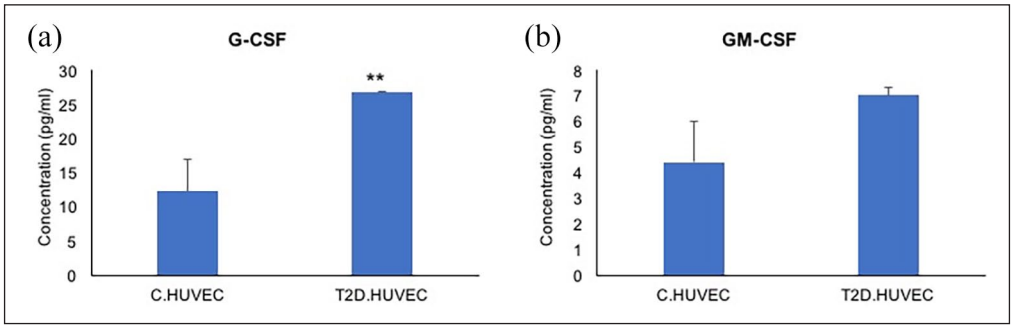

Figure 3. Protein levels of growth factors in HUVECs. The protein levels of (a) G-CSF and (b) GM-CSF in cell lysates of T2D.HUVECs and C.HUVECs were analyzed using ProcartaPlex Multiplex cytokine assay.
The values are mean ± SEM. $^{**}p < 0.01$ compared with control.

Table 2. Pearson correlation coefficient (*r*) between cytokines, chemokines, and growth factors in T2D.HUVECs.

	IL-1A	IL-6	IL-8	G-CSF	GM-CSF
IL-1A	1.00	–	–	–	–
IL-6	**0.48**	1.00	–	–	–
IL-8	−0.13	**0.56**	1.00	–	–
G-CSF	**0.32**	**0.59**	0.09	1.00	–
GM-CSF	**−0.49**	**0.50**	−0.23	**0.96***	1.00

IL-1A: interleukin-1 alpha; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor.
*Significant correlation ($p < 0.05$), values in bold indicate correlation coefficient ⩾ 0.25 or ⩽ −0.25.

proinflammatory cytokines, chemokines, and growth factors related to the pathogenesis of atherosclerosis: IL-1A, IL-6, IL-8, GM-CSF, and G-CSF. The levels of cytokines and growth factors were measured in cell lysates because some of the cytokine precursors stored in the cells such as IL-1.[39] In a previous study, we showed that HUVECs from T2D patients exhibited dysfunction due to increased oxidative stress, decreased proliferation, increased apoptosis, disrupted cell cycle distribution, and alterations in the global expression of genes involved in CVD in these cells relative to those from healthy subjects.[13] Endothelial dysfunction is the initial event of atherogenesis and contributes to its progression and the formation of atherosclerotic plaque.[40]

Under inflammatory conditions, ECs express both IL-1 alpha (IL-1A) and IL-1 beta (IL-1B).[41] Members of the IL-1 family serve as key inflammatory mediators of atherosclerotic events.[18,42] IL-1 induced by oxidative stress has been shown to impair endothelial function, and an IL-1 receptor antagonist has been shown to improve endothelial dysfunction in streptozotocin-induced diabetic rats.[43] The high levels of IL-1A in T2D.HUVECs in the current study may have been caused by HG-induced oxidative stress. This interpretation is also supported by a study showing the effect of HG on the levels of IL-1 to mimic a diabetes environment, and this work showed similar increases in the levels of IL-1.[44–46] Another study showed that IL-1A increased vascular endothelial permeability,[47] which might contribute to vascular endothelial injury. One possible mechanism underlying increased expression of IL-1A in ECs could be epigenetic methylation, because exposure of monocytes to HG has been found to cause H3K9 demethylation at IL-1A promoter regions.[48] In this study, an increase in the level of IL-8 protein was observed in T2D.HUVECs, but the difference did not reach statistical significance. Also, there was no significant difference in the protein levels of IL-6 between HUVECs from T2D subjects and those from healthy controls. This is consistent with the findings of a previous study showing that the level of IL-6 released from diabetic human saphenous vein ECs was not affected by exposure to HG (30 mM) for 48 h.[49]

GM-CSF (CSF2) and G-CSF (CSF3) are glycoprotein growth factors that stimulate the growth, survival, and differentiation of hematopoietic cells. They are produced by resting or stimulated stromal cells (fibroblasts and ECs) and by immune cells (monocytes and macrophages).[50] In addition to acting on hematopoietic cells, they regulate the migration and proliferation of ECs.[22] The presence of oxidized lipids has been reported in atherosclerotic tissue,[51] and one study showed that treating ECs with oxidized lipid led to strong induction of the expression of G-CSF and GM-CSF,[52] suggesting the involvement of these growth factors in the autocrine regulation of EC function under stress conditions.

Our data provide the first report that HUVECs from T2D subjects produce greater amounts of the growth factors G-CSF and GM-CSF than control HUVECs do. This is supported by a previous study showing that upon exposure to diabetic-mimicking HG conditions, the amount of G-CSF increased in trophoblast.[30]

Our correlation analyses also show a significant and positive correlation between the protein levels of pro-inflammatory growth factors G-CSF and GM-CSF. This association has not been previously reported in T2D.HUVECs. Although we have not found any significant correlation between these growth factors and IL-1A (Table 2), it is possible that their higher protein levels in T2D.HUVECs relative to C.HUVECs may have been partially due to autocrine stimulation by IL-1A. A previous study showed that IL-1A stimulates the release of G-CSF and GM-CSF from vascular ECs.[53] Thus, the increased protein expression of these growth factors by

ECs exposed to hyperglycemia in utero might contribute to vascular endothelial injury.

In our previous microarray study, both IL-1A and G-CSF transcripts appeared in the functional network analysis of T2D.HUVECs associated with the inflammatory response and RNA posttranscriptional modification, respectively.[13] These findings indicated that T2D induces persistent changes in T2D.HUVECs even after being kept in a normal glucose environment for several passages. However, the mechanisms whereby T2D.HUVECs maintain the inflammatory phenotype even after glucose normalization remain undetermined.

Despite the small number of HUVECs analyzed in this study, our results are supported by a strong statistical analysis. Nevertheless, the few studies that have been published in this field all have an equally small number of samples. The sample size/power analysis was not performed in this study. Our data need to be confirmed in a larger cohort study.

Conclusion

This study showed that T2D promotes the production of pro-inflammatory cytokines IL-1A and G-CSF in HUVECs under normal glucose conditions and there was a positive correlation between G-CSF and GM-CSF, which may contribute to the pathology of diabetes-associated complications.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Ethical approval for this study was obtained from the Ethics Committee of King Abdulaziz University (Approval number: HA-02-J-008).

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Informed consent

Written informed consent was obtained from all subjects before the study.

ORCID iD

Samar Sultan https://orcid.org/0000-0003-1194-8545

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