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Original article

Association study of uncoupling protein 2-866G/A (rs659366) gene polymorphism and serum myeloperoxidase with cardiovascular disease in type 2 diabetic patients

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Abstract

Diabetic cardiovascular diseases are leading causes of death. In diabetic patients, oxidative stress is key event in cardiovascular disease (CVD) development. Uncoupling protein 2 (UCP2) and myeloperoxidase (MPO) were identified as molecules regulating oxidative stress effects of oxygen free radicals. This work aimed to evaluate association of UCP2-866G/A (rs659366) gene polymorphism and serum MPO with CVD among type 2 diabetic patients. The present work was conducted on 80

Keywords:

Cardiovascular disease,

oxidative stress,

myeloperoxidase,

uncoupling protein 2,

UCP2-866G/A (rs659366) gene polymorphism.

type 2 diabetic Egyptian patients including 40 patients with CVD compared to 40 age- and sex-matched non-CVD patients; recruited from Outpatient Clinics in Beni-suef University Hospital and Kasr Eleiny Hospital. Lipid profile was assayed. Serum MPO was analyzed using enzyme-linked immunosorbent assay. UCP2-866G/A (rs659366) gene polymorphism was determined using polymerase chain reaction-restriction fragment length polymorphism. Statistically significant increase in concentration of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) was found among CVD patients with MPO > 44.3 ng/ml (P=0.01 and 0.019, respectively). Serum MPO showed significant positive correlation with TC and LDL-C (P=0.004 and 0.002, respectively). The UCP2-866 (rs659366) (GA and AA) genotype variants did not demonstrate statistically significant higher risk to develop cardiovascular events, yet were observed to have higher frequency among CVD patients and among patients with MPO > 44.3ng/ml. In conclusion, our results revealed significant correlation between serum myeloperoxidase and CVD lipid risk factors in diabetic patients. This study suggests UCP2 -866G/A (rs659366) gene polymorphism and MPO as target assay studies on CVD etiology and oxidative stress parameters evaluation among type 2 diabetic patients.

1. Introduction:

Diabetes can cause various acute and chronic diseases, the most dangerous of which is cardiovascular disease (CVD) [1]. Diabetic cardiovascular diseases are the leading causes of death among diabetic patients [2]; and include coronary heart disease, cerebrovascular disease, congestive heart failure, and diabetic cardiomyopathy [3, 4].

Sequence events involved in CVD development include endothelial dysfunction, inflammatory atherosclerotic plaque formation and oxidative stress [5].

Oxidative stress (OS) refers to the imbalance between accumulation and scavenging of reactive oxygen species (ROS); consequently oxidation and antioxidants [6]. Oxidative stress is considered one of the most important mechanisms leading to tissue injury [7]. Overproduction of superoxide by mitochondrial electron transport chain through various molecular mechanisms leads to cardiovascular damage [8].

Myeloperoxidase (MPO) is key enzyme catalyzing hydrogen peroxide conversion leading to generation of various ROS which modify lipids, lipoproteins and proteins [9]. Myeloperoxidase plays a central role in lipid peroxidation and conversion of LDL to an atherogenic form taken up by macrophages which is crucial step in formation of foam cells; furthermore MPO extensively oxidize apolipoprotein A1 (apoA1), the major structural protein of HDL, thus impairing the function of promoting cholesterol efflux; where oxidation of apoA1 is much greater in human atheroma than in the plasma [10]. Myeloperoxidase produces its effect on the arterial wall through oxidative products leading to endothelial dysfunction [9]. Circulating MPO has potential implication as a clinical prognostic indicator for patients with cardiovascular disease [11].

Uncoupling proteins (UCPs) are members of the mitochondrial anion transporter family present in inner mitochondrial membrane with six homologs (UCP1-6) which are pivotal physiological regulators of cellular metabolism, mitochondrial membrane potential, metabolic efficiency and energy dispersal [12].

Uncoupling protein 2 (UCP2) negatively modulates energetic membrane potential and ATP synthesis decreasing mitochondrial superoxide formation and ROS production in oxidative stress [13]; by promoting leakage of protons across inner mitochondrial membrane leading to mitochondrial oxidative phosphorylation uncoupling [14]. Consequently, UCP2 have physiological antioxidative effect through maintaining normal mitochondrial dynamics and normal endothelial function, providing optimal mitochondrial membrane potential level sufficient to push ATP synthesis but low enough to limit ROS formation; hence down regulating ROS in endothelial cells [15]. The UCP2 gene is located on human chromosome 11q13; UCP2-866G/A (rs659366) polymorphism is considered a common promoter gene variant [16]. The uncoupling proteins and in consequence the genes that encode them are regarded among cardiovascular diseases as candidate genes [14]. In this study the association of UCP2-866G/A (rs659366)

gene polymorphism and serum MPO with CVD were hypothesized and evaluated as risk parameters for CVD among type 2 diabetic patients.

2. Subjects and Methods:

2.1 Subjects characteristics:

The present work included 80 type 2 diabetic Egyptian patients divided into group I: cardio vascular disease (CVD) group of 40 diabetic patients with history of cardiovascular events (myocardial infarction or angina pectoris), cerebrovascular events (stroke or transient ischemic attacks) or evidence of arterial disease (intermittent claudication); group II: non-cardiovascular disease (non-CVD) group of 40 diabetic patients without any history of cardiovascular events or cardiovascular related diseases such as retinopathy , nephropathy , cerebrovascular events or arterial occlusive diseases. This study excluded patients with co morbidities. All participants were recruited from those attending Outpatient Clinics in Beni-suef University Hospital and Kasr Eleiny Hospital. Approval of this study was conducted by Research Ethical Committee, Faculty of Medicine, Beni-Suef University. Data confidentiality conforming to the Code of Ethics of the Declaration of Helsinki (World Medical Association, 2008) was preserved [17]. Informed consent from all participants in this study was obtained.

2.2 Data collection:

Patients demographic information, full history taking, personal history of CVD and adverse cardiovascular events were collected. Physical examination was performed including weight, height and body mass index (BMI) was calculated = weight/Height.

2.3 Routine laboratory investigations:

Peripheral blood samples were drawn and centrifuged for serum collection. Lipid profile; including triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C); was assayed using an automated chemistry analyzer (Bechman CX5 automated chemistry analyzer, Ireland) by its own commercial kits, according to manufacturer's instructions.

2.4 Serum myeloperoxidase analysis:

Quantitative detection of serum myeloperoxidase (MPO) was assayed by enzyme-linked immunosorbent assay (ELISA) based on solid phase enzyme immunoassay sandwich principle using Human MPO ELISA kit (Boster Bio,

USA) according to manufacturer's instructions.

2.5 Uncoupling protein 2 -866G/A (rs659366) gene polymorphism analysis:

Three ml of the peripheral blood samples anticoagulated with EDTA were obtained for DNA extraction and isolation. The genomic DNA was extracted from each blood sample using TIANamp/Genomic DNA kit (cat No # Dp304-02, Bioscientifics Life Science, USA) according to manufacturer's instructions. Samples were stored at -80°C till analysis. Uncoupling protein 2 -866G/A (rs659366) gene polymorphism was assayed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The PCR primers were: 5′-CACGCTGCTTCTGCCAGGAC-3′ (Forward) and 5′-AGGCGTCAGGAGATGGACCG-3′ (Reverse) [18]. The PCR assay was performed in a final reaction volume of 25 µL for each sample, using12.5 µL of universal master mix, 5 µL of DNA, 1 µL of UCP2 -866G/A forward primer, 1 µL of UCP2 -866G/A reversed primer, and 5.5 µL distilled water. The PCR conditions were performed using thermal cycler Applied Biosystems (Perkin-Elmer 9600, USA) starting by initial denaturation step at 95 °C for 5 min then 35 cycles of: denaturation at 95 °C for 30 s followed by annealing at 51 °C for 30 s, and extension at 72 °C for 45 s. Final extension step was at 72 °C for 10 min. After amplification, the PCR products were treated with 2.5U of MluI restriction enzyme (New England Biolabs, UK) overnight at 37°C [18]. Resolving of the products was then done on 2% agarose gel electrophoresis containing ethidium bromide and then visualized using UV transilluminator. DNA molecular weight marker was used as a reference standard (QIAGEN GelPilot 50-500 bp Ladder {cat no. 239025}) to assess the size of PCR-RFLP products. Single 360 bp band represented homozygous AA genotype, two bands 290 and 70 bp represented homozygous GG genotype, while heterozygous GA genotype was represented by three bands 360, 290, and 70 bp **(Figure 1).**

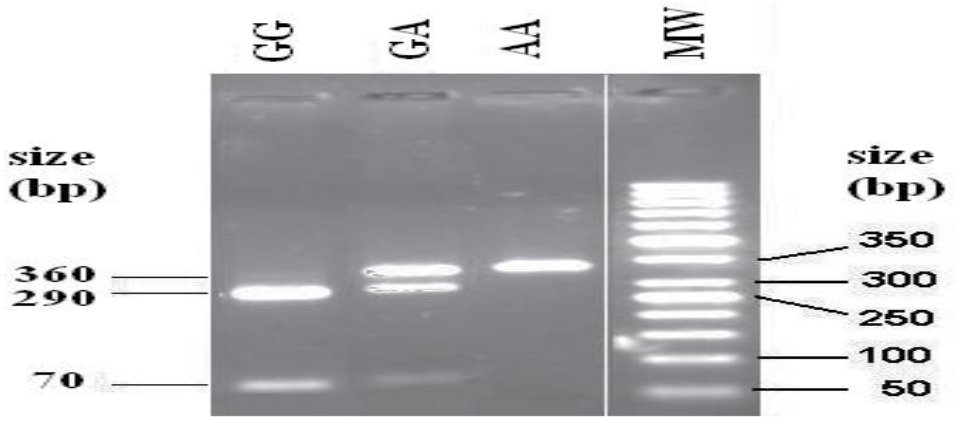

Figure (1): Gel patterns of UCP2-866G/A (rs659366) genotypes using MluI restriction enzyme on agarose gel electrophoresis

Lane 1 GG genotype represented by 2 bands (290, 70)

Lane 2 GA genotype represented by 3bands (360, 290, 70)

Lane 3 AA genotype represented by 1 band (360)

Lane 4 MW is a 50 bp ladder

2.6 Statistical analysis:

Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test and Fisher's exact test were used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using either Student's t-test or Mann-Whitney test (non-parametric t-test). Correlation between numerical variables was done using Pearson product-moment. Receiver operating characteristic (ROC) curve was used for prediction of cut off values. Odds ratio (OR) and its 95% confidence interval (95%CI) of mutation was calculated between groups. A P-value < 0.05 was considered significant [19].

3. Results:

Mean age of CVD group was 53.9 ± 11 years versus 54.1 ± 8.5 years among non-CVD group. This study included 28 males (70%) and 12 females (30%) as CVD group versus 21 males (52.5%) & 19 females (47.5%) among non-CVD group. Both groups were age- and sex- matched (P=0.929 and 0.108, respectively). Comparison between CVD and non-CVD groups regarding different studied variables is shown in **Table 1.**

Table (1): Statistical comparison between CVD and non-CVD groups as regard different studied variables

Variable	CVD group (n=40) mean± SD	Non-CVD group (n=40) mean± SD	P-value
BMI (kg/m2)	29.1±4.4	31±5.0	0.561
TG (mmol/L)	2.15±1.12	1.9±0.85	0.522
TC (mmol/L)	4.95±0.98	5.12±1.1	0.491
HDL-C (mmol/L)	0.98±0.26	0.91±0.2	0.181
LDL-C (mmol/L)	2.97±0.91	3.41±1.06	0.061
MPO (ng/ml)	44.3 (12.1-216.8)*	48.9 (23.2-212.8)*	0.153

BMI: Body mass index; TG: Triglycerides; TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; MPO: Myeloperoxidase

*MPO is expressed by median (25^{th}- 75^{th} percentiles)

Intergroup comparison was done using Independent t-test

P-value>0.05 is not significant

Using ROC curve, no reasonable cutoff for MPO could be chosen to differentiate between CVD and non-CVD groups since area under the curve was very small (Figure 2).

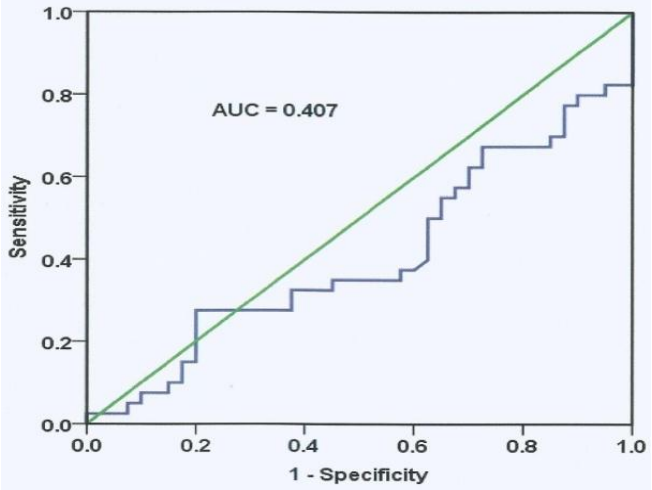

Figure (2): Receiver operating characteristic curve of serum myeloperoxidase

Lipid profile parameters where compared among CVD group at MPO median level **(Table 2).** Statistically significant increase in TC and LDL-C levels was found among patients with serum MPO > 44.3 ng/ml versus those with serum MPO ≤44.3 ng/ml (P=0.01 and 0.019, respectively).

Table (2): Lipid profile variables at median MPO level among CVD patients

Variable	MPO ≤ 44.3 ng/ml mean± SD	MPO > 44.3 ng/ml mean± SD	P-value
TC (mmol/L)	4.8±1.02	5.44±1.11	0.01*
HDL-C (mmol/L)	0.96±0.22	0.91±0.25	0.148
LDL-C (mmol/L)	2.96±0.91	3.6±1.05	0.019*

TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; MPO: Myeloperoxidase

Intergroup comparison was done using Independent t-test

* P-value <0.05 is significant

Using the Pearson correlation, a statistically significant positive correlation of MPO was observed with TC and LDL-C (p=0.004 and 0.002, respectively) (Figures 3 and 4, respectively); while negative correlation was found with HDL-C (P=0.545) **(Table 3).**

Table (3): Correlation between MPO and lipid profile variables

Variable	MPO (ng/ml)	
	R	**P-value**
TC (mg/dl)	0.321	0.004*
HDL (mg/dl)	-0.069	0.545
LDL (mg/dl)	0.334	0.002*

TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; MPO: Myeloperoxidase

Pearson correlation was done between numerical variables

* P-value <0.05 is significant

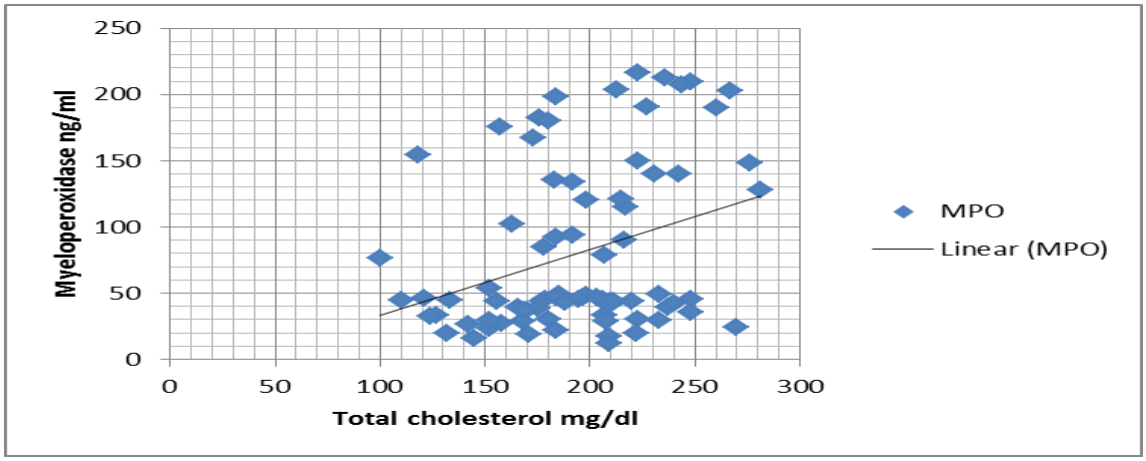

Figure 3: Correlation between myeloperoxidase and total cholesterol

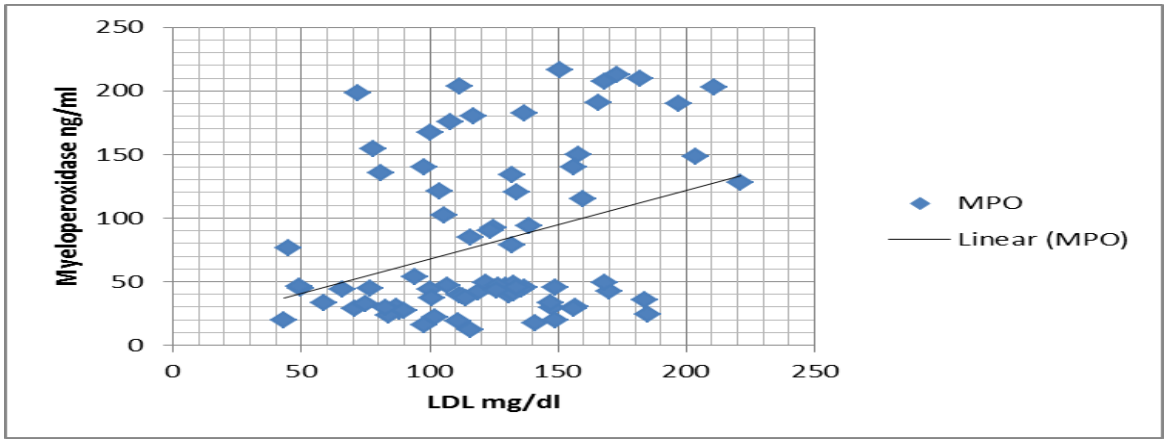

Figure 4: Correlation between myeloperoxidase and LDL-cholesterol

Genotype frequency of UCP2-866G/A (rs659366) gene polymorphism between CVD and non-CVD groups is demonstrated in **Table 4.**

Table (4): Genotype frequency of UCP2-866G/A (rs659366) gene polymorphism between CVD and non-CVD groups

Genotype	**CVD group (n=40) Frequency n (%)**	**Non-CVD group (n=40) Frequency n (%)**
AA	8 (20%)	13(32.5%)
GA	20 (50%)	12(30%)
GG	12 (30%)	15(37.5%)

The (GA and AA) genotype variants were grouped together to enhance statistical power and were observed to have higher frequency among CVD group. Yet, the calculated odds ratio between the studied groups did not demonstrate a higher risk to develop cardiovascular events if they harbor the variant allele **(Table 5).**

Table (5): Genotype frequency of UCP2-866G/A (rs659366) gene polymorphism between CVD and non-CVD groups

Genotype	**CVD group (n=40) Frequency n (%)**	**Non-CVD group (n=40) Frequency n (%)**	**OR (95% CI)**	**P-value**
GA&AA	28(70%)	25(62.5%)	1.400 (0.552- 3.552)	0.478
GG	12(30%)	15(37.5%)		

Intergroup genotype distribution was done using Binary logistic regression

P-value>0.05 is not significant

Lipid profile was compared between UCP2-866G/A (rs659366) genotype variants **(Table 6).**

Table (6): Genotype variants frequency of UCP2-866G/A (rs659366) and lipid profile

Variable	**GA+AA mean± SD**	**GG mean± SD**	**P-value**
TC (mmol/L)	5.02±1.1	5.07±0.95	0.992
HDL-C (mmol/L)	0.97±0.25	0.89±0.18	0.157
LDL-C (mmol/L)	3.15±1.06	3.27±0.9	0.749

TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol

Intergroup comparison was done using Independent t-test

P-value>0.05 is not significant

Genotype frequency was compared at MPO median level among CVD group where higher frequency of (GA and AA) variants was observed at serum MPO > 44.3ng/ml versus serum MPO ≤ 44.3ng/ml with no statistical significance **(Table 7).**

Table (7): Genotype frequency of UCP2-866G/A (rs659366) gene polymorphism at MPO median level among CVD group

Genotype	**≤ 44.3 ng/ml Frequency (%)**	**> 44.3 ng/ml Frequency (%)**	**OR (95% CI)**	**P-value**
GA &AA	12(60%)	16(80%)	2.71 (0.64-10.62)	0.168
GG	8(40%)	4(20%)		

Intergroup genotype distribution was done using Binary logistic regression

P-value>0.05 is not significant

4. Discussion:

Myeloperoxidase and uncoupling protein 2 were identified as molecules that participate in regulating the effects of oxygen free radicals [20]. The current study evaluated uncoupling protein 2-866G/A (rs659366) gene polymorphism and serum myeloperoxidase with cardiovascular disease among type 2 diabetic patients.

Myeloperoxidase is a potent mediator for cardiovascular diseases and is linked with cardiovascular disease risk [21]. In our study, statistically significant increase in TC and LDL-C levels was found among CVD patients with serum MPO > 44.3 ng/ml (P=0.01 and 0.019, respectively). Furthermore, MPO showed significant positive correlation with TC and LDL-C (P=0.004 and 0.002, respectively). These results came in agreement with another research which reported that dyslipidemia with increased TC and LDL-C levels induces increase in concentration of myeloperoxidase [22]. Moreover, Peroxidation reaction system of myeloperoxidase correlates with progressive low density lipoprotein modifications leading to increased LDL-C level [9]. Strong interaction of MPO with low density lipoproteins (LDL) induces proatherogenic modification of LDL in the form of MPO-modified LDL (Mox-LDL) pathognomonic atherosclerotic foam cells which accumulates in macrophages; where preventing this modification and accumulation was found as a promising

approach in atherosclerosis prophylactic therapy[23]. Interestingly, it was reported that MPO may potentially be appropriate therapeutic target for preventing coronary disease [24].

Evaluation of uncoupling proteins and their encoding genes are recommended among CVD patients [14]. The (GA and AA) genotype variants were grouped together in this study to enhance statistical power .Yet, the calculated odds ratio between the studied groups did not demonstrate a statistical significance although higher frequency was observed among CVD group; probably due to small sample size as a limitation in the current study. In other studies, UCP2-866 (rs659366) gene polymorphism has been significantly associated with risk of CVD where A allele was reported as risk allele [14, 25]. The UCP2-866A allele was associated with T2DM in studies performed among Russian population [26] and Chinese population [27]. UCP2-866 (rs659366) gene polymorphism is associated with disturbance in lipid metabolism, T2DM, and cardiovascular diseases through influencing UCP2 expression [16]. This association was explained by finding that presence of UCP 2-866A gene variant was associated with decreased UCP2 gene expression [28]. The UCP2 is an important molecule in protecting cardiovascular system against various oxidative stress signals incriminated in pathogenesis of CVD [25]. Up regulation of UCP2 in myocardium in response to hypoxia alleviates ischemia insults [29]. Researches on genes that affect UCP2 expression is of remarkable value as UCP2 could be used as a protective marker for oxidative stress and mitochondrial injury among various cardiac insults [29, 30, 31].

5. Conclusion and Recommendations:

In conclusion, this study revealed an association between serum myeloperoxidase and CVD lipid risk factors among type 2 diabetic Egyptian patients. Our study suggests UCP2 -866G/A (rs659366) gene polymorphism and MPO as target assays for studies on CVD etiology, risk stratification and oxidative stress parameters evaluation. The small sample size is one of the limitations of this study; further studies on a large cohort of patients with diabetes are needed. Increase in the number of patients can potentially enhance the statistical power. It is necessary to perform more studies as well as proper Meta analysis before MPO can be a routinely adopted marker in clinical practice.

Declaration of competing interest

The authors do not have any conflict of interest.

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Ethical approval

The study was conducted in accordance with the Declaration of Helsinki. All participants provided informed consent, and the Research Ethical Committee of Faculty of Medicine, Beni-Suef University approved the study protocol.

Informed consent

A signed consent form was obtained from each study participant.

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