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Original article

Role of miRNA-23b as a biomarker of pediatric sepsis "A single-center study"

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ABSTRACT

Background: Pediatric sepsis is one of the most prevalent causes of pediatric mortalities despite all the efforts done to early predict and manage it. This study was designed to assess diagnostic and prognostic role of micro RNA-23b (miRNA-23b) in pediatric sepsis cases by assessing relation between plasma miRNA-23b expression level and results of blood culture as well as patients' outcome. **Method:** This case-control study included 37 clinically diagnosed sepsis cases and 18 control subjects. Assay of miRNA-23b expression was conducted using quantitative real-time PCR. **Results:** The expression levels of miRNA-23 were statistically significantly higher among the case group than the control group (p=0.027). Cases with negative culture witnessed statistically significant higher levels of miRNA-23b (p=0.042). For outcome, discharged cases possessed higher levels of miRNA with a statistically significant difference (p=0.002). Regarding the validity of miRNA-23b expression as a diagnostic marker for sepsis compared to blood culture results as the gold standard for diagnosis of sepsis at a cut-off value 4.08, sensitivity was (91.7%), specificity was (62%), predictive value for positive (PVP) was (73%), predictive value for negative (PVN) was (80%) with 81% accuracy. Regarding the validity of miRNA-23b expression level in prediction of outcome at a cut-off value 1.43 , sensitivity was (85.7%), specificity was (60.9%), PVP was (57.1%), PVN was (87.5%), with 70.3% accuracy. **Conclusion:** MiRNA-23b is a valid diagnostic and prognostic marker for pediatric sepsis. This promising marker would be used for early diagnosis and management of children with sepsis to improve their outcome.

Introduction

A systematic inflammatory reaction caused by an infectious agent is defined as sepsis, which is every pediatric intensivist nightmare despite all the efforts done to early predict and manage pediatric sepsis, it is still the most abundant cause of pediatric mortalities in pediatric intensive care units [1]. It is yet not fully understood the mechanism of sepsis and systemic inflammatory response syndrome (SIRS) development, with a constellation of immune responses, increasing circulating cytokines other inflammatory markers like interleukins (ILs), and tumor necrosis factor (TNF). Understanding this mechanism is important to develop new modalities

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of diagnosis and treatment [2]. Delineating sepsis in the pediatric patient is challenging due to age-related vital signs, and their phantastic physiologic preserve which frequently hides the urgency of their illness [3, 4].

Sepsis is generally identified by bacteriologic blood culture, where the results yields are very late, it may expose to contamination. Its results are highly reliant on preceded antibiotic administration and hardly ever propose any prognostic importance [4]. An empirical antibiotic treatment protocol in sepsis is a universal clinical procedure. In the existence of assumed bacterial infection, the use of random antibiotics is pointless and raises the possibility for the emergence of multi-resistant strains, nevertheless, given the rapid course of the disease, postponing or ceasing antibiotic use in septicemic patient can also be disastrous [5, 6]. Thus, there is an urgent need for biomarkers that can reliably identify sepsis at an early stage and provide prognostic information [7].

MicroRNAs (miRNAs) have gained attention recently as a potential marker for the diagnosis and prognosis of inflammatory diseases, including sepsis [3]. MicroRNAs are a class of short, 19–22 nucleotide, single-stranded, non-coding regulatory RNAs that are involved in a variety of biological activities. They have created new opportunities for the diagnosis and treatment of various illnesses. By attaching to particular messenger RNA (mRNA) molecules, miRNAs function as post-transcription regulators, inhibiting the expression of target genes or breaking down the mRNA, which subsequently supports cell division, development, metabolism, apoptosis, as well as other physiological processes. As a result, miRNAs are evolving as novel regulators of immune and inflammatory responses and subsequently spotlighted as markers for early disease diagnosis and prognosis [8, 9].

Among the microRNAs, miRNA-23b is recognized. Comprehensive review of existing literature indicates that the expression of miRNA-23b is not only regulated by a wide range of stimuli in cells originating from distinct lineages, but also takes part in several gene regulatory feedback loops [10, 11]. Furthermore, research indicates that miRNA-23b may have the ability to avert autoimmune disorders [12]. To some extent, tumor cell growth can be inhibited by suppressing the expression of miRNA-23b in tumor tissue [13].

MiRNA-23b expression in the peripheral blood of sepsis patients is associated with the inflammatory state's development and can be utilized to assess the severity and prognosis of these patients [14]. Additionally, miRNA-23b may be a useful indicator of sepsis in hemocultures from peripheral blood of newborn [15].

This study was designed to assess diagnostic and prognostic role of miRNA-23b in pediatric sepsis cases by assessing relation between plasma miRNA-23b expression level and results of blood culture as well as patients' outcome.

Patients and methods

Study population

This is a case-control study where 37 cases clinically diagnosed sepsis and SIRS were enrolled in the study against 18 controls, all in pediatric age group range from 28 days to 12 years. All cases were recruited from the pediatric intensive care unit at Children's Hospital of Zagazig University from October 2022 to March 2023. Laboratory work was carried out at the Medical Microbiology and Immunology Department and Scientific and Medical Research Center, Faculty of Medicine, Zagazig University. The authors considered the strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines in reporting this research and followed STROBE statement checklist for case-control study.

Inclusion criteria: pediatric patients with clinical features of sepsis and SIRS (e.g., fever, respiratory distress, bradycardia, tachycardia, convulsions, cyanosis). Exclusion criteria: babies less than 28 days, pediatric patients who don’t show signs of sepsis, those who had received antibiotics before sampling, and refusal of parents or legal guardians to participate in the study. In addition, normal healthy children were included with similar age and sex to serve as controls.

All cases were subjected to full history taking and clinical examination, blood culture, C-reactive protein (CRP), complete blood count (CBC), kidney and liver function, procalcitonin, SOFA score [16], hemodynamic support, mechanical ventilation when needed, and Covid-19 testing. Pediatric sepsis and SIRS were diagnosed by the international guidelines for the management of severe sepsis and septic shock in 2012 (Surviving Sepsis Campaign).

Ethical consideration

Approval numbered ZU-IRB #9907/5-10-2022 was granted by the Institutional Review Board (IRB) Committee of Zagazig, Faculty of Medicine to the current study. In accordance with the Helsinki Declaration, samples from all the participating children were utilized after receiving written informed consent from their parents or legal guardians.

Assay of miRNA-23b by quantitative real-time PCR

RNA extraction

From each participant, 5 ml of venous blood was obtained in EDTA-containing tube, and plasma was separated by centrifugation for 15 min at 2000 rpm after incubation of the blood sample at room temperature for 10 minutes then stored at -20°C till extraction. Using the miRNAs' Mini kit (Qiagen, Valencia, Spain), and adhering to the manufacturer's supplementary protocol for serum/plasma, total RNA extraction including miRNA was performed. A spectrophotometric assay was used to determine RNA yield at 260 nm and purity was confirmed by measuring absorbance at 260/280 nm ratio.

Reverse transcription

In this step, complementary DNA (cDNA) is reverse transcribed from total RNA using (TaqMan® MicroRNA Reverse Transcription, Applied Biosystems. Inc., CA, USA) kit. From each sample 5 µl of total RNA was added to the reaction mixture containing 3 µl random hexamer primer, 1.5 µl RT buffer, 0.15 µl of the dNTP's mixture, 0.19 µl RNase inhibitor,1 µl of reverse transcriptase, and the volume was completed to 15 µl with RNase-free water. The reaction tubes were incubated at 16°C for 30 minutes, 42°C for 30 minutes followed by 5 minutes of incubation at 85°C to denature reverse transcriptase. The synthesized cDNA was stored at -20°C until real-time PCR assay.

Assay of miRNA-23b

An assay of miRNA-23b expression by real-time PCR was performed using (TaqMan® Universal PCR Master Mix II, Applied Biosystems. Inc., CA, USA) kit. The PCR reaction mixture consisted of 10 µl of master mix, 1 µl TaqMan assay, 7 µl of cDNA, and 2 µl of nuclease-free water. Reaction conditions were an initial denaturation step at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min using a thermocycler (Agilent technologies Stratagene Mx3005p, Germany). The primers used were designed for miRNA-23b assay, hsa-miRNA-23b (Assay ID 002126, Thermo Fisher, CA, USA). U6 snRNA (Assay ID 001973, Thermo Fisher, CA, USA) was utilized for standardization. Expression level was calculated by the delta-delta CT method [16].

Statistical analysis

All data were collected, tabulated, and statistically analyzed using SPSS 26.0 for Windows (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as the mean ± SD & median (range), and qualitative data were expressed as absolute frequencies (number) & and relative frequencies (percentage). Independent samples Student's t-test was used to compare two groups of normally distributed variables while Mann Whitney U test was used for non-normally distributed variables. The percentage of categorical variables was compared using the Chi-square test. Spearman's rank correlation coefficient was calculated to assess the relationship between various study variables, (+) sign indicates direct correlation & (-) sign indicates inverse correlation, also values near to 1 indicate strong correlation & and values near 0 indicate weak correlation. Validity was tested by a Roc curve. All tests were two sided. p-value < 0.05 was considered statistically significant (S), p-value ≥ 0.05 was considered statistically insignificant (NS).

Results

This study included 37 cases and 18 controls. A statistically insignificant difference existed between the 2 groups under study regarding age and sex, verifying the matching of both groups as shown in **table (1).** There was a statistically significant increase in miRNA-23b expression levels in the case group compared to the control group (p=0.027) as shown in **table (2).**

Nearly half of cases (45.9%) were diagnosed with pneumonia, and multisystem diseases were found in (29.7%) of cases. Lower percentages are diagnosed as acute fulminant hepatitis, CNS infection, and renal injury. More than half of cases (64.9%) were mechanically ventilated, (48.6%) received inotropes and (27%) was COVID-19, positive, **(Table 3).**

By studying the levels and different parameters and its correlations between miRNA-23b expression, weak positive significant correlations were detected between miRNA-23b expression level and each of platelet count and pressure of oxygen in arterial blood (PO2), while weak negative significant ones were found between

miRNA-23b expression level and both procalcitonin and bilirubin levels, **(Table 4).**

The blood culture was positive for more than half of cases, 24 (64.9%), *Klebsiella pneumoniae* (*K. pneumoniae*) was found in 8 (21.6%), each of *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) was found in 4 (10.8%), 3 (8.1%) of cultures obtain Coagulase negative *Staph* (CoNS), 1(2.7%) of cultures had *Acinetobacter baumannii* (*A. baumannii*), and blood culture was negative in 13 (35.1%) cases.

Regarding relation between miRNA-23b expression level and blood culture results, cases with negative culture witnessed statistically significant higher levels of miRNA-23b (p=0.042) than cases with positive culture, table(5). Regarding outcomes of cases, the majority of cases were discharged 23 (62.2%) while 14 (37.8%) of cases died. Regarding the relation between outcomes and miRNA-23b expression level, discharged cases possessed higher levels of miRNA-23b with a statistically significant difference (*p*= 0.002), **(Table 5).**

Regarding the validity of miRNA-23b expression as a diagnostic marker for pediatric sepsis compared to blood culture results as the gold standard for diagnosis of sepsis AUC was (0.788), at a cut-off value 4.08, the value of sensitivity was (91.7%), specificity was (62%), predictive value for positive (PVP) was (73%), predictive value for negative (PVN) was (80%), and (81%) accuracy, achieving a state of validity, **(Table 6)** and **(Figure 1a).**

Regarding the validity of miRNA-23b expression level as a prognostic marker for pediatric sepsis cases outcome AUC was (0.801), at a cut-off value 1.43, sensitivity was (85.7%), specificity was (60.9%), PVP was (57.1%), PVN was (87.5%), with a 70.3% accuracy. Previous data confirmed the validity of miRNA-23b as a prognostic marker for pediatric sepsis outcome, **(Table 6)** and **(Figure 1b).**

Table 1. Patients basic characteristics of the studied groups.

Variables		**Cases group (n=37)**		**Control group (n=18)**		**Test**	***p*-value**
		No.	**(%)**	**No.**	**(%)**		
Sex	Female	15	40.5	10	55.6	1.101 **(x^2)**	0.294
	Male	22	59.5	8	44.4		
Age (year)	Mean ±SD Median (IQR)	4.26±4.18 2.5 (0.7-8)		4.44±1.91 5 (3-5)		-1.03 (MW)	0.190

SD: standard deviation, IQR: interquartile range, (MW): Mann Whitney test, (x^2): Chi-Square Tests

Table 2. Expression levels of miRNA-23b in the two studied groups

miRNA-23b	**Cases group (n=37)**	**Control group (n=18)**	**Z**	***p*-value**
Mean ±SD Median (IQR)	2.98±4.98 0.55 (0.21-4.57)	0.28±0.64 0.28 (0.23-0.32)	-2.207	0.027*

SD: standard deviation, IQR: interquartile range, (z): Mann-Whitney Test, ***:** Statistically significant

Table 3. Clinical characteristics of the cases group

Variables		Study group (n=37)	
		No.	(%)
Diagnosis	Acute fulminant hepatitis	2	5.4
	CNS infection	3	8.1
	Multisystem	11	29.7
	Pneumonia	17	45.9
	Renal injury	4	10.8
Mechanical ventilation	No	13	35.1
	Yes	24	64.9
Inotropes	No	19	51.4
	Yes	18	48.6
COVID-19	No	27	73
	Yes	10	27

Table 4. Correlation between miRNA-23b expression levels and different parameters.

Variables	Values		miRNA-23b expression level
CRP (mg/L) Mean±SD Median (IQR)	 117.78±94.56 110 (24-153)	**r** ***p***	-0.120 0.478
Platelets count Mean ±SD Median (IQR)	 253.14±168.57 220 (107.5-387)	**r** ***p***	0.415* 0.011*
PO_2 (mm Hg) Mean ±SD Range	 63.78±20.7 (25-90)	**r** ***p***	0.396* 0.015*
FIO_2 (mm Hg) Mean ±SD Range	66.49±17.98 (30-90)	**r** ***p***	-0.255 0.128
Mean arterial blood pressure (mm Hg) Mean ±SD Range	 69.19±16.39 (50-120)	**r** ***p***	0.253 0.130
Procalcitonin (ng/ml) Mean ±SD Median (IQR)	 14.79±21.66 8 (5-10)	**r** ***p***	-0.342* 0.038*
Urea (mg/dl) Mean ±SD Median (IQR)	 24.93±24.74 13(8.7-29)	**r** **p**	0.245 0.144
Creatinine (mg/dl) Mean ±SD Median (IQR)	 1.12±2.03 0.4(0.2-0.65)	**r** ***p***	0.106 0.532
Albumin (g/L) Mean ±SD Median (IQR)	 3.17±0.65 (1.9-5)	**r** ***p***	0.145 0.393
AST (U/L) Mean ±SD Median (IQR)	 354.11±1847.92 35 (27-56)	**r** ***p***	-0.239 0.153
ALT (U/L) Mean ±SD Median (IQR)	 253.7±1337.39 23 (19-46)	**r** ***p***	-0.058 0.733
Bilirubin (mg/dl) Mean ±SD Median (IQR)	 0.64±0.73 0.5(0.25-0.6)	**r** **p**	**-0.337*** **0.041***
SOFA score Mean ±SD Median (IQR)	 9.35±4.17 9 (5-12)	**r** **p**	-0.078 0.645

CRP: C-reactive protein, **Po2:** pressure of oxygen in arterial blood, **Fio2:** fraction of inspired oxygen given, **AST:** aspartate aminotransferase enzyme, **ALT:** alanine transaminase enzyme, **SOFA score:** the sequential organ failure assessment score, **r:** Spearman's rank correlation coefficient **p:** p-value.

Table 5. Expression levels of miRNA-23b in relation to blood culture results and outcome results within cases group.

Variable		**miRNA-23b expression**	**Test**	
			Z	***p*-value**
Blood culture result Mean±SD Median (IQR)	**Positive Group (n=24)**	1.60±2.51 0.46 (0.21-1.57)	-1.941	0.042*
	Negative Group (n=13)	5.54±7.16 4.49 (0.27-6.9)		
Outcome	**Died Group (n=14)**	1.08±2.16 0.21 (0.03-0.74)	-3.038	0.002*
	Discharged Group (n=23)	4.15±5.83 2.47 (0.45-5.7)		

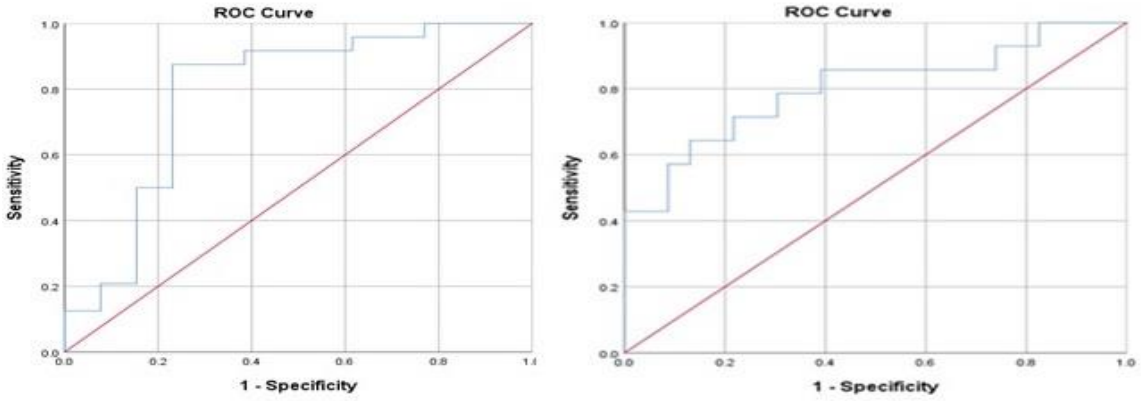
SD: standard deviation, IQR: interquartile range , (z): Mann-Whitney Test,
*: Statistically significant

Table 6. Validity of miRNA-23b expression level as diagnostic and prognostic marker for pediatric sepsis.

miRNA-23b expression level	**AUC**	**95%CI**	**Cut off**	**Sensitivity**	**Specificity**	**PVP**	**PVN**	**Accuracy**
Blood culture Result	0.788	0.613-0.964	4.08	91.7%	62%	73%	80 %	81%
Sepsis outcome	0.801	0.644-0.959	1.43	85.7%	60.9%	57.1 %	87.5 %	70.3%

AUC: area under the curve, CI: confidence interval, PVP: predictive value for positive,
PVN: predictive value for negative.

Figure 1. Roc curve illustrating validity of miRNA-23b expression as diagnostic and prognostic marker for pediatric sepsis.



(a) Validity of miRNA-23b expression level as diagnostic marker for pediatric sepsis cases.
(b) Validity of miRNA-23b expression level as prognostic marker for pediatric sepsis cases.

Discussion

Pediatric sepsis is one of the most common causes of pediatric mortalities, despite all the efforts made to early predict and manage it. MiRNAs have gained attention recently as a potential marker for the diagnosis and prognosis of inflammatory diseases. Our study added to what is known about the role of miRNA-23b in sepsis and other systemic inflammatory syndromes; only sparse studies were done to evaluate its role as a diagnostic and prognostic factor for these conditions.

In our study, samples were taken from the pediatric age group at different stages of the clinical progress of sepsis and other inflammatory processes. We noted that the levels of miRNA-23b were higher in cases than in controls, the difference was statistically significant ($p = 0.027$). This agrees with a study done by **Fatmi et al.** who stated that miRNA-23b was higher in neonates who were

diagnosed with early-onset sepsis than controls and showed that the difference in expression of miRNA-23b was related to the timing of sepsis as it was higher in early onset sepsis and lower in late onset sepsis than controls [15]. Another study observed that miRNA-23b was lower in cases than in controls, but in adult sepsis patients [17]. These different results point to the expression levels of miRNA-23b may be related to age and stage of sepsis itself.

Regarding the clinical characteristics of the case group, we reported that nearly half of cases (45.9%) were diagnosed with pneumonia, more than half of cases (64.9%) were mechanically ventilated, and 48.6% received inotropes. These results are in agreement with **Nour et al.'s** results, who reported that about half of the sepsis cases (45%) were caused by pneumonia, 72.5% of cases needed mechanical ventilation, and 60% of cases received inotropic support [18].

By looking at patient laboratory parameters, we notice a positive correlation between platelet counts as well as Po2 and levels of miRNA-23b; in other words, patients who had favorable laboratory parameters were noticed to have higher levels of miRNA-23b; on the contrary, levels of miRNA-23b were negatively correlated with elevated markers of sepsis and organ failure such as procalcitonin and bilirubin. This points to the suppressive role of miRNA-23b on the inflammatory process. Another observation by **Zhang et al.** proved the same result, demonstrating that levels of miRNA-23b were significantly negative regulators of the inflammatory response in sepsis [19].

In the current study, blood culture was positive for more than half of cases (64.9%) (*K. pneumonia* was 21.6%; each of *P. aeruginosa*, *S. aureus* and *E. coli* was 10.8%; 8.1% obtained CoNS; and 2.7% *A. baumannii*). A similar result was reported by **Nour et al.** as sepsis cases revealed *K. pneumonia* as the causative pathogen in 30% of cases, *S. aureus* in 27.5%, *P. aeruginosa* in 15%, and *Candida* in 12.5% [18]. Moving forward to the relation between miRNA-23b expression level and blood culture results, an important aspect of our study, as we were looking for rapid alternative gold standard marker for pediatric sepsis, we observed that levels of miRNA-23b were much higher in cases with negative culture; that elevation was statistically significant (p = 0.042), which goes in agreement with **Fatmi et al.** who reported significantly lower miRNA-23b expression with positive hemoculture [15].

Also, we observed that cases who were discharged had a higher blood level of miRNA-23b compared to those who died, with a statistically significant difference (p = 0.002). **Ou et al.** agree with our results, who observed that miRNA-23b levels were much lower in cases who died from sepsis in comparison to the survivors; the same observation was found by **Fatmi et al.** [15, 17]. Also, Wu et al.'s study confirmed the role of miRNA-23b in preventing circulatory failure in sepsis cases [20]. All point to the protective role of miRNA-23b and suggest that a high blood level may be considered a favorable marker for sepsis outcome.

Regarding the validity of miRNA-23b plasma level as a diagnostic and prognostic marker for pediatric sepsis, this is the first study to determine it. Meta-analysis studies results indicated circulating miRNAs could have the ability to monitor the progress of sepsis as diagnostic and prognostic markers [21, 22].

We found that miRNA-23b expression level is a valid diagnostic marker for pediatric sepsis compared to blood culture results with AUC 0.788 and a cut-off value of 4.08 (sensitivity, 91.7%; specificity, 62%; and accuracy, 81%). Another study by **Nour et al.** which targeted other miRNAs (miRNA-122, -181b, -223, and -146a), found that the expression levels of all of them are valid for the early detection of pediatric sepsis [18].

Also, our results confirmed that miRNA-23b expression level is a valid prognostic marker for pediatric sepsis with AUC of 0.801 and a cut-off value of 1.43 (sensitivity, 85.7%; specificity, 60.9%; and accuracy, 70.3%). Another study reported that blood cultures from neonates who died from sepsis had significantly lower miR-23b expression levels and had a significant negative correlation with deaths, indicating that it may be able to predict fatal outcomes [23]. These findings predict the future role of miRNA-23b as a valid diagnostic and prognostic factor for pediatric sepsis and other inflammatory states.

This work encountered some limitations as it was carried out in a single-center children's hospital, so the results need to be verified in the future by multicenter studies. On the other hand, further studies are required to measure the expression level of miRNA-23b in sepsis cases in

different age groups to assess its potential use as a comprehensive biomarker for sepsis.

Conclusion

MiRNA-23b is a valid diagnostic and prognostic marker for pediatric sepsis. This promising marker would be used for the early diagnosis and management of children with sepsis to improve their outcome. It offers a rapid, reliable test to early diagnose and predict the outcome of sepsis cases, and it can be correlated with both clinical and laboratory parameters of sepsis.

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Conflict of interest

All authors declare that there is no conflict of interest related to this work that could inappropriately influence (bias) the authors' actions.

Author contributions

H.A. and A.N. designed the study research. A.A. and A.N. enlisted patients and collected their data. H.A. and S.G. operated the laboratory work of this work. All authors contributed in data analysis, implemented the statistics and wrote the paper. H.A. submitted the final manuscript.

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