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Evaluation diagnostic performances of novel index based on ascitic fluid MicroRNA-155 and Micro RNA-122, platelet count and end-stage liver disease score in Patients with Spontaneous Bacterial Peritonitis

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Spontaneous bacterial peritonitis (SBP) are a serious complication of liver cirrhosis and a diagnostic model to predict it is needed. This study was designed to test the ability of micro-RNAs (155 and 122) and simple blood tests to predict it.One hundred eighty-seven patients admitted to with liver cirrhosis and ascites were included in our study. Stepwise linear discriminant analysis and area under receiver-operating characteristic curves (AUCs) were used to create a predictive score comprising platelet count, end-stage liver disease (MELD) score and two microRNA (155 and 122).Micro RNA-155 had AUC of 0.64 with sensitivity 60% and specificity 62 % while Micro RNA-122 gave AUC of 0.69 with sensitivity 70% and specificity 64 %. The diagnostic power did not enhance than single markers. The multiple logistic regression analysis indicated that the increase in levels of Micro RNA-155 and MELD score and decrease in levels of platelet count and Micro RNA-122 were significantly associated with the detection of SBP.The diagnostic power of four makes were AUC of 0.93 with 83% sensitivity and 88% specificity. The AUCs of twelve common liver fibrosis scores for detection SBP were evaluated. Our index was the most efficient index with an odds ratio for detection of SBP of 7.2 (2.4 -9.7; $p < 0.0001$), exceeding that of the AAR (0.96), Fibro- Q (0.99), FIB4 (1.0), king (0.86), fibrosis index (3.6) and MELD (6.1). The combination of four markers could improve the diagnosis of SBP in cirrhotic patients.

Keywords: Spontaneous Bacterial Peritonitis, Diagnosis; Ascitic Fluid; Micro RNA-155; Micro RNA-122; Index

INTRODUCTION

spontaneous bacterial peritonitis (SBP), a life-threatening complication of cirrhosis and ascites, is associated with even higher mortality and can be

difficult to diagnosis as the clinical presentation varies significantly, and ascites cultures are rarely positive. The current clinical standard to diagnose SBP is, the ascites neutrophil count (ANC), with 250 ≧ cells/mm^3 indicating peritonitis(Agrawal et al. 2019). The mortality rate exceeds 80% in patients with cirrhosis who develop septic shock secondary to spontaneous bacterial peritonitis. In addition, each hour of delay in appropriate antimicrobial therapy increases the in-hospital mortality rate by 1.86 times (Karvellas et al. 2015)

The existence of microRNA (miR) was first reported in 1993 (Lee et al. 1993). They were described as small, non-coding RNA's of about 21–25 nucleotides (Wagner et al. 2013). They regulate gene expression by binding to messenger RNA (mRNA), and are thereby implicated in essential biological processes, among them liver disease and inflammation(Sagnelli et al. 2018). In addition, due to their size and ability to circulate they are regarded as potential biomarkers for a variety of diseases including liver disease (Lee et al. 1993). While serum levels of miR's are accepted biomarkers for liver disease, their levels and the potential role as biomarkers in other bio fluids such as ascites are unknown in cirrhosis. MiR-155 is involved in macrophage activation, which are next to lymphocytes the predominant cell population in ascites (Fagan et al. 2015). MiR-155 expression increased in peritoneal immune cells in a murine peritonitis model after bacterial challenge(Kanaan et al. 2013) and after intraperitoneal Lipopolysaccarid injection (Billeter et al. 2014). In addition, miR-155 has been shown to be important for antibacterial defense(Zeng et al. 2015). In addition miR-155 was elevated in patients with spontaneous bacterial peritonitis (Lutz et al. 2017). The miR-122 is liver specific and accounts for approximately 70% of all miRs in the liver (Chang et al. 2004). Elevated levels of the liver-specific miR-122 have been found in sera or plasma of patients with chronic hepatitis B infection(Xu et al. 2011, Zhang et al. 2010). Moreover, circulating miR-122 has been proposed as a marker of inflammation in patients with chronic hepatitis C viral infection (Bihrer et al. 2011). Knock out of the miR-122 gene is associated with the loss of the hepatic phenotype and progression to cancer (Coulouarn et al. 2008). So the aim of the work to investigate the level of ascititc fluid Micro RNA-155 and Micro RNA – 122 as a potential biomarkers in spontaneous bacterial peritonitis. Common serum fibrosis scores include fibrosis routine test (FRT)(Attallah et al. 2012), Biotechnology Research Center (BRC) score(Attallah et al. 2013), Goteborg University Cirrhosis Index (GUCI)(Islam et al. 2005), APRI (Wai et al. 2003), fibrosis cirrhosis score (FC)(Ahmad et al. 2011), Lock(Lok et al. 2005), AAR(Sheth et al. 1998), Fibro-Q(Hsieh et al. 2009), FIB-4,(Sterling et al. 2006) King (Cross et al. 2009), fibrosis index (FI)(Ohta et al. 2006) and MELD (Peng et al. 2016).

MATERIALS AND METHODS

Patients:

The study will be conducted on 187 patients with liver cirrhosis and ascites attending Department of hepatology, gastroenterology and infectious disease at Benha university hospital and Ahmed Maher teaching hospital. Patients were divided into 2 groups according to presence or absence of SBP. Group I: Includes 97 patients with cirrhosis and ascites and SBP. Group II: Includes 90 patients with cirrhosis and ascites and without SBP. The protocol of this study will be approved by the committee of ethics of scientific research in Benha faculty of medicine in Benha University and Ahmed Maher Teaching Hospital. All patients will give informed written consents for participation in this study. The diagnosis of liver cirrhosis was based on a combination of physical examination, laboratory and ultrasound findings. The Child-Pugh and the widely used MELD scores were used to assess the severity of every patient's liver disease. Exclusion criteria included: Patients with ascites due to causes other than cirrhosis with portal hypertension, recent abdominal surgery, solid organ transplant recipients, patients with documented colitis or enteritis, evidence of gastrointestinal bleeding or bacterial infection in preceding 6 weeks, treatment with non-absorbable antibiotics in preceding 6 weeks, other non-peritoneal infections (skin infections, chest infections, urinary tract infections, meningitis, dental infections gastroenteritis, biliary tract infections) and patients with renal impairment.

All cases will be subjected to full history taking and clinical examination, abdominal ultrasonography and laboratory tests. Complete blood count, liver profile (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), serum bilirubin, albumin, prothrombin time). Hepatitis C virus antibody and hepatitis B virus surface antigen were done. Diagnostic paracentesis stressing on WBC type, count (count of polymorph nuclear leucocytes equal or more than 250 mm^3 considered as spontaneous bacterial peritonitis), malignant cells, total protein, albumin and glucose.

Total RNA extraction and purification was done using a miRNeasy Mini Kit;cat no: 217004 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription: reverse transcription reaction using TaqMan MicroRNA Reverse Transcription (Applied Biosystems, Foster city, USA). Gene expression analysis: The quantification of miR-155 and miR-122 levels analyzed using MX 3000 Applied biosystem machine. All samples concentrations were done by using the equation $2^{\Delta\Delta Ct}$ where the threshold cycle (CT), the cycle number at which the fluorescent signal of the reaction crosses the threshold, was detected and incorporated in quantifying the relative changes in miRNA expression (Rq). ΔCT values are calculated by subtracting the CT value of the endogenous control for a given sample from the CT value of the miRNA for the given sample. Control Assays U6 as an endogenous reference control for normalization purposes.

Data was analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 24*(SPSS Inc., Chicago, IL).* Numerical data was described as median and range or mean and standard deviation as appropriate, while qualitative data were described as number and percentage. Chi-square (Fisher's exact) test was used to examine the relation between qualitative variables as appropriate. Correlation analysis was done between variables using Pearson correlation. Multivariate analysis will be done for variables statistically significant on univariate level to indicate independent predictive factors and to obviate the effect of confounders using logistic regression model. Receiver Operating Characteristics (ROC) curve was done to estimate the best cut off point then calculation of sensitivity, specificity with their 95% confidence interval was done. The multivariate logistic regression was analyzed to estimate the odds ratios (ORs) of the relations between common liver fibrosis scores and spontaneous bacterial peritonitis.

RESULTS

This study was designed to include 187 patients with liver cirrhosis and ascites divided into two groups according to presence or absence of spontaneous bacterial peritonitis: Group (I): include 90 cirrhotic patients without spontaneous bacterial peritonitis and Group (II): include 97 cirrhotic patients with spontaneous bacterial peritonitis. The mean age (55.4±6.2) was statistically significant higher (< 0.0001) in patients with SBP than in patients without SBP (44.6±11.4). There is no statistically difference between two groups, as regard to gender however SBP tends to be common in males. Cirrhotic patients with SBP complained mostly from abdominal pain (68.4% vs 33.3|%; p= 0.001) and disturbance in conscious (34 % vs 21.1|%; p= 0.04) level with statistically significant difference. No statistically significant difference between group I& II as regard GIT bleeding. Fever was predominant in cases with SBP (54.6 % vs 5.6 %; p= 0.001) with statistically significant difference. No statistically significant difference between group I & II as regards jaundice. No statistically difference between two groups as regard ALT, PT and INR. There is no statistically difference as regards to Child Pough classification between two groups; however, Child C is more common in patients with spontaneous bacterial peritonitis. Splenomegaly (74.2 % vs 56.7 %; p= 0.01) and HCC (8.2 % vs 0 %; p= 0.001) were more common in patient with SBP with a high statistically significantly difference. Marked ascites (54.6 % vs 40 %; p= 0.04) is higher in group II with statistically significantly difference. Table 1 present the laboratory data of complete blood count, liver function tests and creatinine. The mean value of creatinine, total bilirubin, AST and ALP was significantly higher in cirrhotic patients with SBP group II than in the cirrhotic patient without SBP, while the mean value of albumin was significantly lower in SBP group than in the non SBP group. The mean value of TLC and polymorphs was significantly higher in SBP group than non SBP group, while the mean value of ascetic albumin, glucose and protein was significantly lower in SBP group than in non SBP group, no statistically significant difference between the two groups as regard SAAG.

The mean rank of miRNA-122 was significantly down regulated (77.1 vs 112.3; *P* value < 0.0001) while the mean rank miRNA-155 (106 vs 80.6; *P* value < 0.001) was significantly up-regulated.

Table 1: Clinical data of studied groups

Variables	Cases without SBP	Cases with SBP	P value
Age (mean± SD)	44.6±11.4	55.4±6.0	<0.0001
Blood analysis			
Hemoglobin (g/dl)	10.5±1.6	11.1±1.7	0.015

White blood cells (10^9 /L)	6.7±1.0	9.7±4.1	< 0.0001
Red blood cells (10^{12}/L)	4.0±0.5	3.7±0.9	0.006
Platelets count (10^9/ L)	194.6 ±91.5	124.3±25.5	< 0.0001
Creatinine (µmol/L)	91.1± 32.7	135.2 ± 47.7	< 0.0001
Total Bilirubin (µmol/L)	25.9 ± 9.7	39.2 ± 8.4	0.003
ALT (U/L)	68.2±15.2	67.0±18.1	0.62
AST (U/L)	64.5±12.5	73.8±18.9	< 0.0001
ALP (U/L)	85.2±30.6	95.3±30.3	0.025
Albumin (g/dL)	2.7±0.4	2.4±0.8	0.05
AFP (U/l)	9 (7-23)	21 (6.9-45.0)	< 0.0001
INR	1.6±0.25	1.5±0.4	0.288
MELD Score	15.0±3.2	20.7±3.6	< 0.0001
Child Pugh B (no, %) **Child Pugh C (no, %)**	62 (68.9%) 28 (31.1)	57 (58.8%) 40 (41.2)	0.150
Ascitic fluid analysis ([median (IQR)]			
MiRNA 155	0.022 (0.02-0.023)	0.023 (0.02-0.025)	0.001
MiRNA 122	0.23 (0.205-0.24)	0.21 (0.202-0.220)	< 0.0001

Table 2: Diagnostic performances and area under receiver-operating characteristic curve (AUC) for identifying patients with SBP

Variables	AUC (95% CI) [a]	Cutoff	Sn	Sp	Ac
MiRNA -155	0.64 (0.55-0.72)	0.02	60	62	60
MiRNA -122	0.69 (0.61-0.77)	0.22	70	64	69
Platelet count	0.77 (0.69-0.84)	150	70	79	74
MELD score	0.88 (0.84-0.93)	17.5	78	78	78
[*]Score	0.93(0.89-0.97)	1.5	83	88	85

[*]Score: based on platelet count, MELD score, miRNA -155 and miRNA -122
[a] p value was < 0.0001

Table 3: Level and area under ROC of score and twelve common liver fibrosis scores for detection cirrhosis with SBP.

Variables	Cases without SBP n= 90	Cases with SBP n=97	AUC	
			(95% CI)	P value
FRT	16.2±6.3	20.5±12.8	0.47 (0.9-0.56)	0.55
BRC	16.3±0.4	17.3±13.4	0.53 (0.44-0.62)	0.42
GUCI	1.1±0.59	1.4±1.2	0.61 (0.54-0.70)	0.005
APRI	0.71±0.25	0.75±0.1	0.61 (0.53-0.70)	0.005
FC	0.4±0.2	1.5± 0.96	0.62 (0.53-0.71)	0.004
Lock	1.1±0.11	1.3±0.1	0.63 (0.55-0.72)	0.002
AAR	1.0±0.3	1.3±0.6	0.66 (0.57-0.74)	< 0.0001
Fibro-Q	81.3±40.0	163±109	0.66 (0.57-0.75)	< 0.0001
FIB-4	94.5±41.6	84.5±8.7	0.66 (0.57-0.74)	< 0.0001
King	23.9±13	25.6±23.7	0.67 (0.59-0.75)	< 0.0001
FI	4.1±0.47	2.8±1.7	0.71 (0.62-0.79)	< 0.0001
MELD	15.8±3.2	20.8±3.6	0.88 (0.84-0.93)	< 0.0001

Table 4:Non-invasive scores independently associated with presence of SBP.

Factor	Beta	Standard error	OR	95% CI	*P* Value
AAR	1.8	1.1	0.96	0.61-0.85	0.12
Fibro-Q	- 0.04	0.015	0.99	0.63-0.99	0.007
FIB4	0.006	0.013	1.0	0.97-1.2	0.63

King	0.03	0.05	0.86	0.94-1.1	0.47
FI	0.15	0.58	3.6	0.27-4.1	0.79
MELD	1.2	0.39	6.1	1.7-7.8	0.001
Score	3.1	0.7	7.2	2.4-9.7	< 0.0001

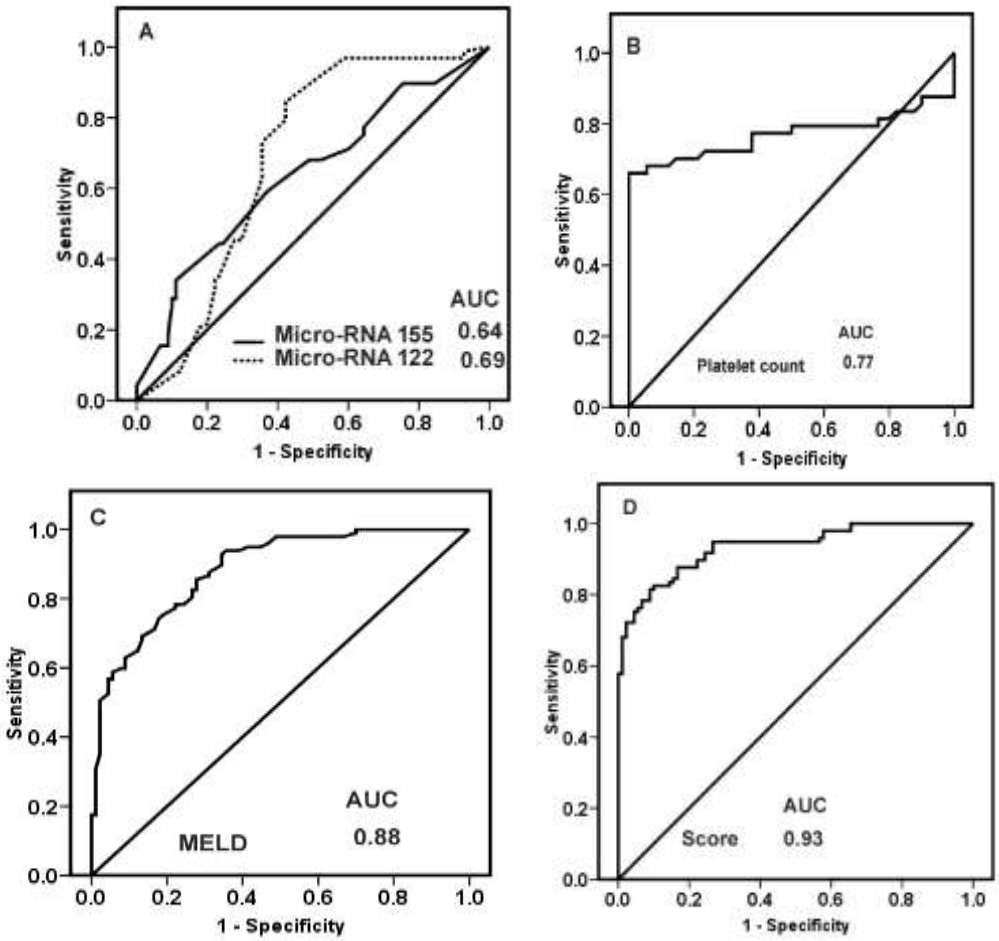

Figure 1: ROC curves of single markers and novel score for discriminated SBP among patients with liver cirrhosis.

There is a no statistically significant correlation between Micro RNA-155 with TLC, Polymorphs, MELD score and Child Pough score values among patients with SBP.

The diagnostic performances of Micro RNA-155, at optimum cutoff 0.022 that gave optimum balance between sensitivity and specificity were AUC of 0.64 with sensitivity 60% and specificity 62 %. At optimum cutoff 0.218, Micro RNA-122 gave AUC of 0.69 with sensitivity 70% and specificity 64%. Micro RNA-122 had a greater AUC than Micro RNA-155 for discriminating cirrhotic patients with SBP. The combination of two micro-RNA (155+122), did not enhance than single markers gave AUC of 0.64. The AUCs of platelets count and MELD score were 0.77 and 0.88 for discriminating cirrhotic patients with SBP; respectively; figure 1. MELD score was the most efficient index for SBP detection in cirrhotic patients. So, we have been taking MELD score as the basic index to combine with other markers to SBP detection.

The multiple logistic regression analysis indicated that the increase in levels of Micro RNA-155 and MELD score and decrease in levels of platelet count and Micro RNA-122 were significantly associated with the detection of SBP. Based on the AUC of ROC analysis, the performances of the four biomarkers were evaluated for detection of SBP and presented in Table 2. Therefore, in an attempt to rise the diagnostic power of our score, we inserted platelet

count and MELD (Micro RNA-155, Micro RNA-155, platelet count and MELD score) for discriminating patients with SBP. The index is illustrated as follows: (Micro122 X 0.414+ Micro155 X 4.66 + MELD X 0.059 + Platelets count (10^3/ul) X 0.002 – 0.04). The diagnostic power of four makes were AUC of 0.93 with 83% sensitivity and 88% specificity; table 2; figure 1.

Level (mean ± SD) and area under ROC of score and twelve common liver fibrosis scores for detection SBP were presented in table 3. The AUC of six indices overlapped with that of our score and so was compared in a multivariate logistic regression to determine independence and rank value. This showed that our index was the most efficient index with an odds ratio (95% CI) for detection of SBP of 7.2 (2.4 -9.7; $p < 0.0001$), exceeding that of the AAR, Fibro- Q, FIB4, king, fibrosis index and MELD; table 4.

DISCUSSION

The incidence of Spontaneous bacterial peritonitis (SBP) occurs in about 20 % of liver cirrhosis patients and its mortality rates is about 40% (Singal et al. 2014). As such, there is a real need to find a noninvasive prognostic scoring system to predict patients more liable to develop SBP, as early treatment could reduce the mortality rate (Lutz et al. 2015). Multiple laboratory tests have been introduced as predictive for SBP, including C-reactive protein (CRP) level (Wehmeyer et al. 2014), platelet count, impaired prothrombin time, creatinine level, bedside liver disease scoring systems like Child-Pugh and the model of end-stage liver disease (MELD) scores. The aim of this study was to identify clinical and laboratory parameters that predict SBP in patients with cirrhotic ascites and to develop a score which allows a rapid diagnosis of SBP or to exclude SBP.

Although several studies have investigated the role of miRs in liver diseases, prospective studies examining the role of ascitic miRNAs in SBP are still rare. In this study, we evaluated if the ascitic miRNAs levels of miR-155 and miR-122 might be a suitable marker for SBP detection. In this study, we evaluated associations of ascites miR levels to common complications of liver cirrhosis. Ascites fluid is body fluid, which might reflect SBP better than peripheral blood and easily accessible in cirrhotic patients. We evaluated levels of miR-155 and miR-122, which have been involved in macrophages activation, regulation of innate immunity responses(Lutz et al. 2017). To our knowledge, we provide the first data on ascites microRNA levels (155, 122) in SBP patients with liver cirrhosis. MiR-155, and MiR-122 levels were significant different in patients with and without SBP as indicator of liver cirrhosis severity. Ascites miR-155 was significantly upregulated during SBP in our patients, similar observation in human and after injection of Klebsiella pneumoniae in mouse model(Barnett et al. 2013). MiR-155 has been several biological functions includes B cell maturation, receptor ligation, antibacterial effect, Therefore, miR-155 has been important role in response to ascites infection and a novel marker of SBP(Lind et al. 2013, Patop et al. 2019).

Many genes in the hepatocytes that control the cell cycle, differentiation, proliferation and apoptosis are regulates by miR-122 (Boutz et al. 2011). miR-122 levels were reduced in patients with hepatic decompensation P = 0.012), ascites (P =0.007), spontaneous bacterial peritonitis(P =0.003) and hepatorenal syndrome (P = 0.037) , variceal bleeding (P = 0.933), hepatic encephalopathy (P = 0.550) or hepatocellular carcinoma (P = 0.132) had significantly lower miR-122 levels than patients without these complications(Waidmann et al. 2012). The model for end-stage liver disease (MELD) score is a simple and accurate score in predicting patients with cirrhosis, and is used worldwide for listing patients for liver transplantation. MELD score is also shown to be higher among SBP patients and to predict mortality in these patients(Bal et al. 2016, Khan et al. 2019). Platelets have many immunologic functions such as activating neutrophil granulocytes in bacterial infections. Thus, thrombopenia might result in reduced activation of neutrophils in cirrhotic patients and might be a risk factor for the development of infections in cirrhotic patients in general (Clark et al. 2007). In this study, for detection SBP, Micro RNA-155 had AUC of 0.64 with sensitivity 60% and specificity 62 % while Micro RNA-122 gave AUC of 0.69 with sensitivity 70% and specificity 64%. The diagnostic power did not enhance than single markers (gave AUC of 0.64). For the diagnosis of SBP, ascetic amyloid A, the sensitivity and specificity were 90% and 60% respectively(Badawi et al. 2019). The AUCs of ascetic fluid miR-155 and calprotectin were 0.95 and 0.90; respectively while blood CD64 was with AUC of 0.93. Combined CD64 and calprotectin had a diagnostic accuracy of 0.99, CD64 and miR-155, had AUC of 0.99, and that for calprotectin and miR-155 was 0.99. (Nabiel et al. 2019). Urinary-NGAL showed 95% sensitivity and 76% specificity for detection of SBPs(Fouad et al. 2019). For the diagnosis of SBP, AUC of ascitic PGE2 was 0.75, and the AUC of Prostaglandin E2

(PGE2), white blood cell count and PGE2 with neutrophils were 0.90 and 0.90, respectively(Luo et al. 2019). A new simple SBP prognostic scoring system consisting of age, platelet count and CRP level was recently developed by Wehmeyer et al (Wehmeyer et al. 2014).

CONCLUSION

This work provides four markers MELD, platelets count, miR-122, and miR-155 are independent predictors and could be a valuable diagnostic score for SBP.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

El-Toukhy N, Mohamed A, Sultan A, designed and performed the experiments. Omran M help in writing the manuscript and data analysis. Ezzat O, Fawazy N, Sakr Y, Abdelhaleem A, Ahmed S, Raafat K and Fathalla L revised manuscript and help in publication. All authors read and approved the final version.

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