Study of MicroRNA-122 as a Diagnostic Biomarker of Sepsis

Noha T. Abou El-Khier[1], Maysaa E. Zaki[2], Nashwa M. Alkasaby [1]

Departments of [1]Medical Microbiology & Immunology and [2]Clinical Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

Sepsis in intensive care units (ICUs) represent a threat with need for rapid and accurate diagnosis. We aimed to assess miR-122 as an early biomarker for diagnosis and outcome prediction in patients with hospital acquired sepsis in ICU. This case control study included 25 adults' patients with sepsis and 25 patients with local wound infections as a control group. C-reactive protein (CRP), total leucocytes count (TLC), liver function, and molecular determination of miR-122 levels were assessed. miR-122 had significant higher area under curve (AUC) when compared with CRP and TLC for differentiation of sepsis from wound infections. The cut off value for miR-122 was 0.16 folds expression with sensitivity, specificity and accuracy 100%. The TLC cut off value was 14.00 x10^3/cmm with 100% sensitivity, 84% specificity and 92% accuracy. While CRP cut off value was 41 mg/l with 76.0% sensitivity, 100% specificity, and 88.0% accuracy. Multivariate logistic analysis revealed non statistically significant difference between survivors and non survivors regarding sepsis biomarkers. Receiver operation curve (ROC) for different biomarkers, CRP, TLC and miR-122 to differentiate patients with poor outcome of sepsis compared to patients with recovery, revealed that AUC was 0.61, 0.6, and 0.45 respectively. miR-122 as a prognostic biomarker for sepsis had 66.6% sensitivity, 50% specificity, and 56.0% accuracy. The present study highlights important points in the use of biomarkers in diagnosis of sepsis in adults' patients above 50 years old. miR-122 is an accurate and specific biomarker for diagnosis of sepsis. miR-122 has limited predictive value for determination of the outcome of patients with sepsis even when used in combination with another biomarker such as CRP and TLC.

Sepsis is considered a major health problem in intensive care units (ICU) with marked morbidity and mortality rates [1]. Moreover, after recovery from this infection, there are several physical, psychological and cognitive sequels with great burden on the health system [2]. The early diagnosis of this infection appears to play a vital role in improving the patients' outcome. The definition of sepsis depends mainly upon the clinical diagnosis [3]. However, appropriate management of sepsis identifies the use of the laboratory diagnosis as supportive method for clinical diagnosis [4]. The combined use of routine laboratory methods of diagnosis such as blood culture and the measurement of C-reactive protein along with the use of biomarker tests such as procalcitonin (PCT) and interleukin 6 (IL-6) appears to influence the proper management of sepsis [4]. Biomarkers used in the diagnosis usually depend upon the innate immunologic processes relating to the body's identification of infection [5, 6]. The use of biomarkers in diagnosis of sepsis not only gives a clue about the presence of infection, but also gives a data upon the response of the patients to the treatment [6]. The molecular identification of sepsis facilitates early laboratory diagnosis with rapid and accurate management [4, 7].

Regulatory microRNA (miR) is a recent recognized biomarker for the diagnosis of many diseases such as cancer and autoimmune diseases. miRs family is short regulatory RNA composed of 21–24 nucleotides in length that acts by regulating gene expression [8]. Recent studies have established the presence of a relatively high

number of miRNAs whose expression can be correlated with sepsis [6].

In sepsis, miRs act as a critical regulator for both the innate and adaptive immunity toward bacterial infections leading to the modulation of the immunity through the pathway of the tumor necrosis factor and the pathway of signals involving toll-like-receptor/NF-κB [9, 10]. This interaction between the immune response and sepsis can be used as a molecular biomarker for the outgoing infection process [10] and even as a biomarker for predicting the survival outcome of sepsis [8].

Among the studied miRs in the diagnosis of sepsis is miR-122. The miR-122 circulating in the blood is tissue specific RNA produced mainly by the liver and it represents around 70% of all liver miRs [11]. The miR-122 acts on the liver cells mainly affecting their differentiation, proliferation, and apoptosis through many genes and had been shown to be a specific biomarker for hepatocyte affections in different liver diseases [12]. The disturbance of the serum levels of miR-122 has been associated with acute hepatic injuries in the various inflammations and sepsis [13, 14]. miR-122 is considered a useful biomarker for early mortality prediction according to Sepsis-1 definition [15]. There is great interindividual variability and the reduced predictive value of miR-122expression in human studies [15].

Thus, the aim of the present study was to assess miR-122 as an early biomarker for diagnosis and outcome prediction in patients with hospital acquired sepsis in ICU.

Subjects and Methods

This case control study included 25 adults' patients admitted to ICUs in Mansoura University hospital during the period from March 2018 to October 2018 with clinical and laboratory confirmed sepsis as previously defined [16] and with positive blood cultures. All patients were followed up for 30-days for outcome calculated from day 1 of the diagnosis of sepsis. Patients with chronic liver diseases were excluded from the study. In addition, 25 patients with hospital acquired wound infections without obvious sepsis and with negative blood cultures were enrolled as a control group.

Written informed consent was obtained from each participant ahead of enrollment in this study. The study protocol was agreed upon by the Ethical Committee of Faculty of Medicine, Mansoura University, code number R/ 18.09.268.

Each patient was subjected to full clinical history and clinical examination. Blood samples were obtained from each patient after meeting the clinical criteria of the diagnosis. Samples were analyzed for blood culture, complete blood counts, liver functions tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and Real-Time PCR for miR-122

Blood sample collection, preparation, and storage

Ten ml. of blood was obtained from each subject. Blood culture bottles Bact/ Alert were first inoculated for culture and the remaining blood samples were divided into three aliquots; one with EDTA for complete blood counts and two aliquots without anticoagulant for liver function tests and for miR-122 study. Blood counts were performed by automated hematology analyzers (Sysmex) and liver function by Dialab autoanalyzer system.

For miR-122 Testing: serum was separated within 30 minutes and stored at -80°C until analysis. Sample aliquots were thawed for miR-122 analysis one time only for extraction to avoid degradation from multiple freezing and thawing procedures.

Serum MicroRNA extraction

Extraction of miR-122 was performed by the use of 400-µl serum with miRVana Kit (Ambion, Life Technologies Corporation, USA) according to the manufacturer´s instructions. Total RNA was eluted in 100 µl of RNase-free water and stored at -70°C until further use.

Preparation of cDNA for miR-122

The extracted RNA was subjected to reverse transcription using ready to use TaqMan MicroRNA Reverse Transcription Kit with specific primers for MicroRNA (Applied Biosystems, USA). The reaction

mixtures preparation included 0.075 ml of 100 mM dNTP, 0.5 ml RT enzyme (50 U/ml), 0.75 ml 10 RT buffer, 0.094 ml RNAse inhibitor (20 U/ml), 2.5 ml eluted RNA, and 2.081 ml nuclease-free water. The procedures were 16°C for 30 min, 42° C for 30 min, 85° C for 5 min, and 4° C until the end of the reaction. cDNA was produced at the end of the reaction [17].

Real-Time PCR for miR-122

Transcribed cDNA was used for quantitative measurement of miR-122 by the use of TaqMan MicroRNA assays (Applied Biosystems, USA). The reaction mixture contains 10.0 µ universal master mix, 1.0 µ of 20x Real Time probes, 1.0 µ of cDNA, and 8.0 µ of nuclease-free water. The amplification conditions were 95° C for 10 min, followed by 40 cycles at 95° C for 15 seconds, and 60° C for 1 min [17].

Synthetic hsa-microRNA-122 and cel-miR-39 (Shanghai GeneChem, Shanghai, China) were serially diluted to final concentrations of 200 nM, 20 nM, 2 nM, 0.2 nM, 0.02 nM, 2 pM, 0.2 pM, 0.02 pM, 2 fM, and 0.2 fM. hsa-microRNA-122 and cel-miR-39 serial dilutions were reverse-transcribed and assayed using real-time PCR analysis concurrently with RNA extracted from serum samples. Standard curves for hsa-microRNA-122 and cel-miR-39 were included on each plate of the miRNA TaqMan assays to convert the cycle threshold (Ct) values of each sample into the corresponding number of microRNA copies. Thus, the microRNA extraction efficiencies for each sample could be calculated by the yield ratios of added cel-miR-39, to obtain the absolute serum microRNA-122 quantification results [17].

Statistical Analysis

Data was analyzed using Statistical Package for Social Science software computer program version 22 (SPSS, Inc., Chicago, IL, USA). Quantitative parametric data were presented as mean and standard deviation (SD), while qualitative data were presented as frequency. Student's t-test (unpaired) was used for comparing quantitative parametric data while chi-square "$\chi 2$", exact tests or Monte-Carlo, as indicated, were used to compare the qualitative data. Pearson correlation coefficient test was used correlating different parameters. The sensitivity and specificity of miR-122, CRP and TLC to differentiate between Sepsis & wound groups and also between survival & non-survival of sepsis groups were examined at different cutoff points using Receiver operation curve (ROC) analysis to determine the best cutoff point. *P* value less than 0.05 was considered statistically significant.

Results

The study included 25 patients with sepsis their mean age was 52.3 ± 14.6 years, they were mainly females (68%). There was statistically insignificant difference between patients with sepsis and patients with wound infections as regard age and gender distribution (*P*=0.5, *P*=0.2 respectively). The markers of infections had significantly higher values in patients with sepsis as regards CRP (55.7± 27.5 mg/l), TLC 18.0± 2.6 x10^3/cmm), and miR-122 (0.22± 0.03 folds expression), P=0.001. The microbiological culture from blood had significant high rates of isolation of *Klebsiella pneumoniae*, *E. coli* and *Enterobacter* spp. Compared to culture of wounds, *P*=0.01. On the other hand, microbiological culture of the wounds had significantly higher rates of *S. aureus* isolation compared to blood culture isolates (*P*=0.01), table 1.

Table 1. Comparison of Demographic and laboratory findings between patients with wound infection versus sepsis

		Wound (n=25)	Sepsis (n=25)	*P* value
Age (years) (mean±SD)		52.32±14.62	54.68±10.87	NS
ALT (IU/l) (mean±SD)		30.48±10.95	50.32±12.85	<0.001*
AST (IU/l) (mean±SD)		31.88±9.21	50.56±15.19	<0.001*
Bilirubin (mg/dl) (mean±SD)		0.92±.16	0.83±.26	NS
Mir-122 (copies/μl)		0.12±.01	0.22±.03	<0.001*
CRP (mg/dl) (mean±SD)		26.28±5.18	55.68±27.47	<0.001*
WBCS (10^3/cmm) (mean±SD)		12.06±2.81	17.95±2.62	<0.001*
Sex [No (%)]	Male	13 (52.0%)	8 (32.0%)	NS
	Female	12 (48.0%)	17 (68.0%)	
Culture [No (%)]	No growth	6 (24.0%)	0 (0%)	0.02*
	Growth	19 (76.0%)	25 (100%)	
Bacteria [No (%)]	No	5 (20.0%)	0 (0.0%)	0.01*
	E. coli	1 (4.0%)	6 (24.0%)	
	Enterobacter	0 (0.0%)	1 (4.0%)	
	Klebsiella pneumoniae	3 (12.0%)	7 (28.0%)	
	S. aureus	16 (64.0%)	11 (44.0%)	

P>0.05 is not significant (NS). Test used: Student's t-test for data expressed as mean±SD and Chi-square or fisher exact for data expressed as frequency

The use of miR-122 had significant higher area under curve (AUC) when compared with CRP and TLC for differentiation of sepsis from wound infections. The cut off value for miR-122 was 0.16 folds expression with sensitivity, specificity and accuracy 100%. TLC cut off value 14.00 x10^3/cmm had sensitivity 100% and 84% specificity with 92% accuracy. CRP cut off value 41 mg/l had sensitivity 76.0%, specificity 100% and 88.0% accuracy, figure 1, tables 2, 3.

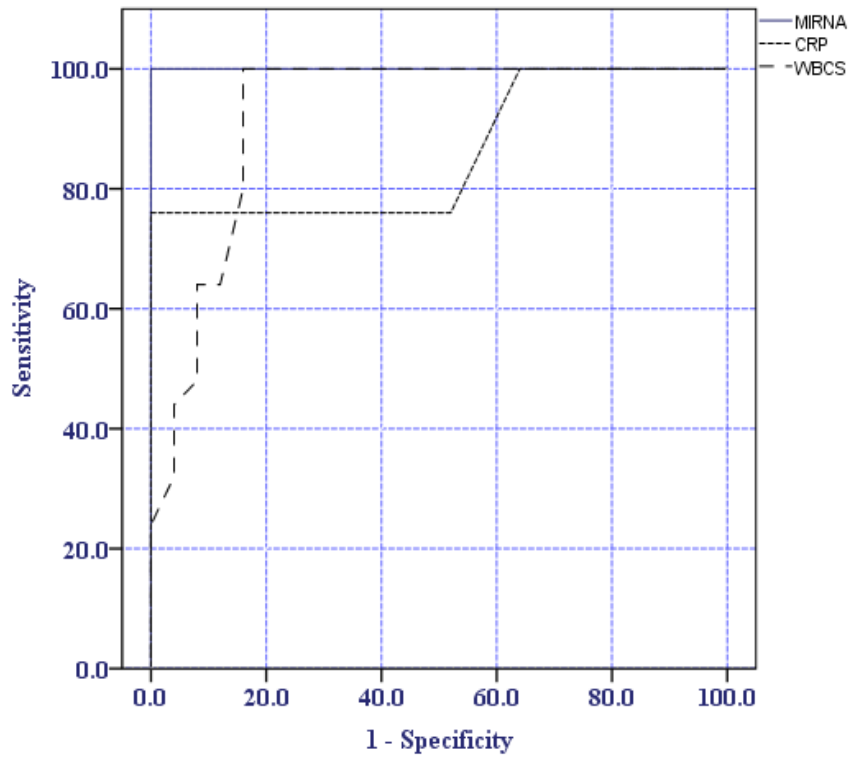

Figure 1. ROC curve for miR-122, CRP and TLC for differentiation between patients with sepsis and patients with wound infections.

Table 2. The Roc curve results and area under curve for miR-122, CRP and TLC for differentiation of patients with sepsis from patients with wound infection.

	AUC (CI 95%)	*P* value
miR-122	1.00 (1.00-1.00)	<0.001*
CRP	0.86 (0.75-0.97)	<0.001*
TLC	0.92 (0.85-1.00)	<0.001*

AUC: area under curve CI: confidence interval *P*<0.05 is significant.

Table 3. Diagnostic values of miR-122, CRP and TLC for detection of sepsis

	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR-122	0.165	100	100	100	100	100
CRP	41.50	76.0	100.0	100.0	80.6	88.0
TLC	14.00	100.0	84.0	86.2	100.0	92.0

PPV: Positive predictive value; NPV: negative predictive value.

The multivariate logistic analysis for different demographic, clinical and laboratory data for risk factors associated with mortality in patients with sepsis, the statistical significant marker was elevated ALT level (59.00 15.6 IU/l, P=0.008), while elevated sepsis biomarkers, CRP and miR-122 had no statistical significant difference between survivors and non-survivors (*P*=0.3, *P*= 0.45, respectively), table 4.

There was significant correlation between ALT, AST, total bilirubin in patients with sepsis and miR-122 (P=0.001, P=0.01, *P*=0.002 respectively), table 5

Table 4. Risk factors analysis for survivors and non-survivors from sepsis as regards demographic, clinical and laboratory data

		Survivors (n=16)	Non-Survivors (n=9)	*P* value
Age (mean±SD)		54.56±12.30	54.89±8.42	NS
ALT (IU/l) (mean±SD)		45.44±8.03	59.00±15.56	0.008*
AST (IU/l) (mean±SD)		46.19±11.78	58.33±18.03	NS
Bilirubin (mg/dl) (mean±SD)		0.78±0.23	0.92±0.29	NS
Mir-122 (copies/μl) (mean±SD)		0.21±0.03	0.22±0.03	NS
CRP (mg/dl) (mean±SD)		52.50±28.01	61.33±27.13	NS
TLC (10^3/cmm) (mean±SD)		18.25±2.98	17.41±1.87	NS
Sex [No (%)]	Male	5 (31.3%)	3 (33.3%)	NS
	Female	11 (68.8%)	6 (66.7%)	
CVC inserted [No (%)]	Yes	9 (56.3%)	3 (33.3%)	NS
	No	7 (43.8%)	6 (66.7%)	
Urinary catheter inserted [No (%)]	Yes	5 (31.3%)	1 (11.1%)	NS
	No	11 (68.8%)	8 (88.9%)	
Infection //fever [No (%)]	Yes	8 (50.0%)	4 (44.4%)	NS
	No	8 (50.0%)	5 (55.6%)	
Device associated [No (%)]	Central	8 (50.0%)	6 (66.7%)	NS
	VAP	1 (6.3%)	0 (0.0%)	
	No device	7 (43.8%)	3 (33.3%)	
BSI Type [No (%)]	Primary	13 (81.3%)	6 (66.7%)	NS
	Secondary	3 (18.8%)	3 (33.3%)	

Test used: Student's t-test for data expressed as mean±SD and Chi-square or fisher exact for data expressed as frequency, BSI (blood stream infection), CVC (central venous catheter). *P*>0.05 is not significant (NS).

Table 5. Correlations between miR-122 and age, liver functions tests, CRP and TLC in patients with wound infections versus sepsis.

Groups			Age	ALT	AST	Bilirubin	CRP	WBCS
Wound	miR-122	r	0.234	0.027	0.368	-0.089	-0.030	0.211
		P value	NS	NS	NS	NS	NS	NS
Sepsis	miR-122	r	-0.249	0.624	0.506	0.600	0.329	-0.226
		P value	NS	0.001*	0.010*	0.002*	NS	NS

P>0.05 is not significant (NS). r:Pearson's correlation coefficient

Table 6. AUC for miR-122, CRP and TLC to differentiate survivors from non survivors.

	AUC (CI 95%)	*P* value
miR-122	0.61 (0.38-0.85)	NS
CRP	0.60 (0.37-0.83)	NS
TLC	0.45 (0.22-0.68)	NS

AUC: area under curve CI: confidence interval *P*>0.05 is not significant (NS).

ROC curve for different biomarkers, CRP, TLC and miR-122 to differentiate patients with poor outcome of sepsis compared to patients with recovery, revealed that AUC was 0.61, 0.6, and 0.45 respectively. The value of miR-122 as a prognostic biomarker for sepsis was 66.6%, 50%, 56.0% for sensitivity, specificity and accuracy, respectively, table (7).

When biomarkers were used in combination, the AUC for miR-122 and CRP, miR-122 and TLCs, TLC and CRP, miR-122 with CRP and TLCs were 0.66, 0.65.0.63, 0.66 respectively, figures 2, 3, tables (8).

Table 7. Diagnostic values for miR-122, CRP and TLC to differentiate survivors from non survivors.

	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR-122	0.2050	66.6	50	42.9	72.7	56.0
CRP	36.00	88.9	31.2	42.1	83.3	52.0
TLC	17.35	55.6	56.2	41.7	69.2	56.0

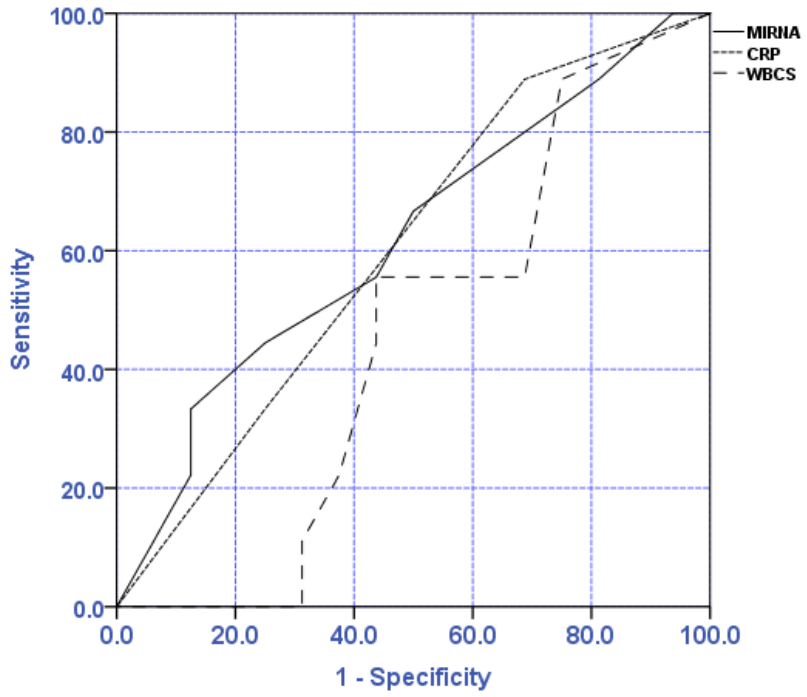

Figure 2. ROC curve for miR-122, CRP and TLC to differentiate survivors from non survivors.

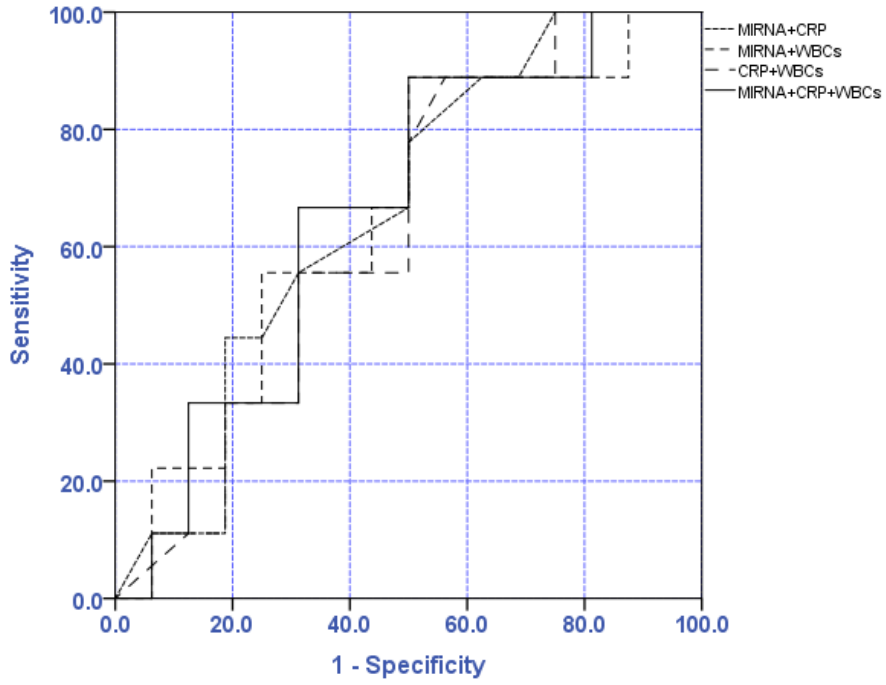

Figure 3. ROC curve for miR-122, CRP and TLC as prognostic biomarkers.

Table 8. AUC for miR-122, CRP and TLC to differentiate survivors from non survivors

	AUC (CI 95%)	*P* value
miR-122+ CRP	0.66 (0.44-0.87)	NS
miR-122+ TLC	0.65 (0.42-0.87)	NS
CRP+ WBCS	0.63 (0.40-0.85)	NS
miR-122+ CRP+ TLC	0.66 (0.44-0.88)	NS

P>0.05 is not significant (NS).

Discussion

The biomarkers of infections had significantly higher values in patients with sepsis as regards CRP, TLC and miR-122 compared to patients with local infections. The diagnosis of sepsis depends upon clinical signs such as fever or hypothermia, tachycardia, tachypnea and elevated inflammatory markers such total leucocytes counts, CRP and procalcitonin [18, 19]. However, there is a need to include other biomarkers for diagnosis of sepsis in adults, especially older than 50 years [20, 21]. Moreover, new definition for sepsis included the inclusion of acute sepsis diagnosis related to specific organ dysfunction [22]. The important function of such biomarker is distinct differentiation between local infections and sepsis and better prediction of the patients `prognosis [23]. In this context, the results of miR-122 appear to have a value.

The use of miR-122 had significant higher area under curve (AUC) when compared with CRP and TLC for differentiation of sepsis from wound infections. Several studies revealed that alteration in the level of miR-122 could be used for diagnosis of sepsis [15, 24].

There was statistically significant association between elevated ALT level and mortality outcome in patients with sepsis. Previous report demonstrated altered liver functions in sepsis associated with worse outcome [25]. Other report found no specific association between transaminase activity and acute liver dysfunction in sepsis [26]. Thus, there is a need to use other biomarker to support acute liver injury in sepsis.

In the present study, there was significant correlation between ALT, AST, total bilirubin in patients with sepsis and miR-122.miR-122 was introduced as hepatic biomarker for detection of sepsis associated liver dysfunction [27] and to detect mortality outcome in those patients [15]. Organ malperfusion may up-regulate circulating miR-122. This is supported by showing increased miR-122 expression in several ischemia and reperfusion models [14, 28].

The value of miR-122 to predict mortality was limited in the present study with poor specificity and sensitivity, though, the area under curve increase when miR-122 was combined with CRP and with WBCs [29, 30]. In these studies, increased miR-122 serum concentrations were detected in critically ill patients with sepsis compared with healthy controls. Thus, study of miR-

122 as a prognostic biomarker for sepsis remain elusive.

In the present study, there was no significant correlation between miR-122 expression and CRP and TLC as biomarkers of sepsis. This finding is similar to previous reports [13, 31]. It was reported that miR-122 is an independent marker from leucocytes counts and CRP as this marker is not regulated by the common inflammatory process [13, 32].

The other biomarkers used in the present study for diagnosis of sepsis, CRP and TLC had sensitivity 76.0% & 100 respectively, and 100 & 84 respectively. Previous studies reported that CRP and TLC can be used as an accurate marker for diagnosis of sepsis [18, 33]. There is a correlation between CRP and TLC production. In the present study, CRP discriminated between patients with sepsis and positive blood cultures from patients with local infection with negative blood culture. Previous reports supported these findings in pediatric patients [34, 35].

The current study highlights important points in the use of biomarkers in diagnosis of sepsis in adults' patients. miR-122 is accurate and specific biomarkers for diagnosis of sepsis. miR-122 has limited predictive value for determination of the outcome of patients with sepsis even when used in combination with other biomarkers CRP and TLC. Elevated level of Alanine aminotransferase can predict the poor outcome of patients with sepsis. Further studies are recommended to validate these findings.

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