Original article

Diagnostic value of $CD14^+$ $CD16^+$ monocytes in neonatal sepsis

Background: *The majority of monocytes (MO) are strongly positive for CD14 and negative for CD16. The phenotype and function of peripheral blood monocytes change after trauma and during sepsis. $CD14^+CD16^+$ monocytes, identified as a minor population of monocytes which constitute a potent phagocytosing and antigen-presenting monocyte subpopulation that expands during acute and chronic infections.*

Objective: *To evaluate monocyte expression of CD14 and CD16 in preterm neonates and to assess it as a possible marker for early diagnosis of neonatal sepsis as the early clinical signs are often insidious and non-specific.*

Methods: *This study was carried out on 45 preterm neonates (1-3 days old) with a mean gestational age of 34.5 ± 1.03 weeks . They were classified into three groups. Group I included 15 neonates with proven sepsis. Group II included 15 neonates with possible or suspected infection. Group III (control group) included 15 healthy age and sex matched neonates. The neonates with possible infection were followed up. Nine of them developed sepsis later on (proved clinically and by laboratory) and they were considered as patients with early sepsis at the time of admission. History taking and clinical examination were performed as well as laboratory investigations including, complete blood count, blood culture and sensitivity (for patients only), measurement of C-reactive protein (CRP) and CD14 and CD16 expression on monocytes by flow cytometry.*

Results: *The proportion of $CD14^+$ $CD16^+$ MO within all circulating monocytes was significantly higher in patients with proven (75.2±13.1%), early (63.9±17.9%) or possible sepsis (55.1±26.8%) than controls (3.86±2.53%) ($p<0.0001$, $p<0.0001$, $p<0.001$, respecctively). It was higher in neonates with proven than possible sepsis ($p<0.05$), whereas it was comparable in the groups of proven and early sepsis ($p>0.05$). There was a significant positive correlation between mean fluorescence intensity (MFI) of $CD16^+$ MO and CRP ($p<0.01$) and a significant negative correlation between it and the platelet count ($p<0.05$) among patients. When neonates with early sepsis were followed up after 48 hours a significant increase in CRP levels and MFI of CD16 expression on monocytes was noted ($p<0.01$ for both). The sensitivity and negative predictive value of $CD14^+$ $CD16^+$ MO% and MFI of $CD16^+$ MO were higher than that of CRP. Specificity and positive predictive value of $CD14^+CD16^+$ MO% were similar to those of CRP. The cut off point (obtained from the ROC curve) for $CD14^+$ $CD16^+$ MO% was 8.6% and that for MFI of $CD16^+$ MO was 9.*

Conclusion*: The measurement of the percentage of $CD14^+$ $CD16^+$ MO among circulating MO is a promising rapid and sensitive test for early diagnosis of neonatal sepsis and exclusion of infection in neonates with high risk to develop sepsis. NICU costs as well as unnecessary antibiotic use can be thus reduced.*

Key words: CD14, CD16, monocyte, neonate, sepsis.

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INTRODUCTION

Neonatal sepsis is a major and frequent cause of morbidity and mortality in the neonatal period. Early diagnosis and treatment are crucial to an improvement in prognosis. Clinical findings are usually nonspecific and indistinguishable from those caused by a variety of neonatal noninfective disorders. Therefore, markers are needed that reliably identify truly infected neonates[1,2].

Peripheral blood monocytes (MO) are members of the mononuclear phagocytic system, which play a central role in immunoregulation and host defense against immunopathogenic organisms. They are heterogeneous in phenotype and function[3]. Many monocyte antigens serve as receptors for recognition and processing of bacterial antigens. The CD14 membrane antigen functions as a receptor for lipopolysaccharides (LPS) from gram-negative bacteria and triggers LPS-induced monocyte activation[4-6]. Moreover, CD14 seems to be involved in the recognition of structures from gram-positive bacteria such as lipoteichoic acid and peptidoglycan[7,8]. In addition, receptors for the fragment crystallisable (Fc) region of IgG are important in the intracellular phagocytosis of IgG-opsonized microorganisms[9]. The Fc gamma receptor III (Fcγ R III) (CD16) exists in two polymorphic forms: the transmembrane from (Fcγ RIIIa) expressed in monocytes/ macrophages and the glycosyl phosphatidylinositol-anchored form (Fcγ RIII b) expressed in granulocytes[9]. The Fcγ RIIIa form on monocytes is reported to mediate important immunophysiological functions such as superoxide generation[10] and antibody-dependent cell mediated cytotoxicity[11], and signal transduction by Fcγ receptor type III is mediated through the γ chain of the Fcγ receptor type III complex[12].

CD 14 is a specific marker for the cells of the monocyte/macrophage lineage refered to as mononuclear phagocytes (MNPs)[13]. According to the extent of expression of the CD14 antigen on the monocytes, the monocytes are separated into two subpopulations: CD14^{high} monocytes (monocytes expressed CD14 at a high density) and CD14^{dim} monocytes (monocytes expressed CD14 antigen at a lower level)[14].

The majority of peripheral blood monocytes strongly positive for CD14 are negative for CD16. However, a subset of monocytes has coexisting CD14 and CD16 known as CD14$^+$CD16$^+$ monocytes. The CD14$^+$ CD16$^+$ MO are considered to represent an activated, more mature, "macrophage-like" subset[15,16]. This subpopulation greatly expands in various infectious and inflammatory diseases, including acquired immunodeficiency syndrome (AIDS), asthma, chronic infections in haemodialysed patients and sepsis[17-19]. The increased proportion and absolute number of CD14$^+$ CD16$^+$ MO correlate with the presence of bacteria in the blood and are preceded by cytokine production[20]. Owing to this, the determination of the proportion of CD14$^+$ CD16$^+$ MO level was suggested as useful for monitoring of patients with high risk of sepsis[21].

To our knowledge, the changes of CD16 expression on MO of preterm newborns during infection or sepsis have not been studied before. So, this study was undertaken to evaluate monocyte expression of CD14 and CD 16 in neonates and to assess it as a marker of early-onset neonatal infection. This may be helpful in early and accurate diagnosis as well as the initiation of appropriate therapy.

METHODS

This study was carried out in the neonatal intensive care unit (NICU) of Benha Children Hospital together with Pediatrics and Clinical Pathology Departments in Ain Shams University Hospitals. It was conducted in the period from April 2003 till February 2004.

The study comprised 30 neonates with possible or proven sepsis and 15 age and sex matched neonates with no clinical or laboratory findings suggestive of sepsis as a control group. All were preterms with gestational age ranging between 33-36 weeks and a mean of 34.53 ± 1.14 weeks. Their ages ranged between 1-3 days with a mean of 1.23 ± 0.5 days. They were divided into 2 groups (I & II) as follows.

Group I (group of neonates with proven sepsis):

It comprised 15 neonates admitted to NICU with proven neonatal sepsis. All had clinical picture suggestive of sepsis and a positive blood culture. They were 8 (53%) males and 7(47%) females. Their ages ranged between 1-3 days with a mean of 1.47 ± 0.64 days.

Group II (group of possible or suspected infection):

It comprised 15 neonates with possible or suspected sepsis i.e. with two or fewer of the following categories of clinical signs of sepsis:

-Respiratory dysfunction (apnea, cyanosis, grunting and intercostal retraction).

-Circulatory dysfunction (tachycardia, poor perfusion and shock).

-Neurological dysfunction (hypotonia, irritability and lethargy).

-Gastrointestinal dysfunction (feeding intolerance, abdominal distension, hepatomegaly and jaundice).

All had initially negative blood culture ± abnormal leukocytic count or high CRP.

There was a high risk factor for infection (as premature rupture of membranes (PROM) > 18 hours, chorioamnionitis, intrapartum maternal fever > 38 ºC, maternal history of urinary tract infection, vaginal bleeding and/or complicated traumatic delivery).

They were 8 (53%) males and 7 (47%) females, in their first day of life.

Group III (control group)

It included 15 healthy age and sex-matched newborns with no clinical manifestations or laboratory findings denoting neonatal sepsis. They were 6 males (40%) and 9 females (60%). Their ages ranged between 1-2 days with a mean of 1.33 ± 0.49 days.

Study design:

Patients and controls were subjected to the following:

I- History taking: Laying stress on antenatal, natal and postnatal periods to detect risk factors for neonatal infection as premature rupture of membranes > 18 hours, maternal fever >38 ºC, vaginal bleeding or presence of foreign body as endo-tracheal or chest tubes and mode of delivery (vaginal, cesarean section or complicated traumatic).

II- Clinical examination:

a- To detect clinical signs of sepsis that have been previously mentioned.

b- General examination (laying stress on) :

- Weight, length, skull circumference.
- Vital signs (pulse, temperature, blood pressure, respiratory rate).
- Neonatal reflexes (Moro's, grasping and suckling).

III- Laboratory investigations which included:

* Complete blood count and blood smears for differential count.
* Blood culture and sensitivity.
* C-reactive protein.
* Assessment of CD 14 and CD16 expression on monocytes by flow cytometry and measuring the percentage of $CD14^+$ $CD16^+$ monocytes within all circulating monocytes.

Patients with possible sepsis were followed up to detect the development of other clinical manifestations of sepsis. CRP was repeated after 24 and 48 hours and blood culture was repeated after 48 hours. The patients who developed sepsis later as evidenced by clinical manifestations of sepsis listed above and positive blood culture were considered as patients with early sepsis at the time of admission (n=9).

Methods:

Sample collection:

Three ml of venous blood were collected from each preterm newborn under aseptic conditions. Two ml were dispensed into tubes containing K- EDTA as an anticoagulant, one ml was immediately used for flow cytometric analysis and one ml was taken for complete blood count. The remaining one ml was left to clot, then centrifuged and the separated serum was used for CRP assay.

Complete blood count:

Blood samples were assayed by coulter counter on the same day (Coulter Microdiff 18, USA), it included (total leukocytic count, absolute neutrophilic count, immature / total neutrophilic ratio (I/T ratio), red blood cell count, hemoglobin level and platelet count. Leishman stained peripheral blood films were done for all samples and examined for differential blood count laying stress on neutrophil number and band cell number.

C-reactive protein (CRP):

CRP was measured semi-quantitatively using latex agglutination test kit (Avitex-CRP Omega Diagnostics Ltd. , UK). The cut-of point of CRP is 6 mg/L[22].

Flow cytometric analysis :

Fluorescein isothiocyanate (FITC) labeled anti CD14 monoclonal antibodies (CD14 - FITC) and phycoerythrin (PE) labeled anti CD16 monoclonal antibodies (CD16-PE) were used in the present study (Cymbus Biotechnology LTD, Hamsphere, U.K), together with FITC and PE negative isotype (Cymbus Biotechnology LTD, Hamsphere, U.K) controls (in order to determine the non specific binding of monoclonal antibodies under study).

Total leukocyte count (TLC) in each blood sample was adjusted at $5\text{-}10x10^3$ cell/ ul and 100 ul of each sample were added to a tube containing 10 ul of each of CD14-FITC and CD16-PE, then tubes were gently vortexed and were incubated for 15 minutes in the dark. One ml of working lysing solution (ammonium chloride 1.5 mmol/l, potassium biphosphate 100 mmol/l and tetrasodium EDTA 10 mmol/L) was added to each tube which was vortexed and incubated again for 5-10 minutes, followed by centrifugation at 3000 rpm for 5 minutes. The supernatant was then discarded and tubes were washed twice with phosphate buffered saline (PBS) and cells were then resuspended in 300 ul of PBS and analysed using Coulter Epics XL flow cytometer (Profile Instrument, Coulter Electronics, Hieloch, FL. USA).

Monocytes were gated by forward and side scatter and CD14, CD16 expression was then assessed on the gated monocytes. $CD14^{high}$ ($CD14^{bright}$) and $CD14^{dim}$, CD16 expressing cells were then electronically determined. Results were expressed by percent of cells co-expressing both markers CD14 and CD16 within all circulating monocytes in comparison to isotype matched control and by mean fluorescence intensity (MFI) of CD16 expression on the CD14 gated monocytes.

Statistical methods:

The results were analyzed by commercially available software package (Stat View, Abacus Concepts, Inc, Berkley, CA, USA). The data are presented as mean, standard deviation (SD), median and interquartile range (IQR). IQR is the range between 25th percentile till 75th percentile. Student's "t" test was used for comparing parametric data between each two groups, whereas Mann-Whitney U test was used for non-parametric data. Wilcoxon signed rank test (for non parametric data) was used to compare between the same group in two repeated measurements such as the initial results of the group of early sepsis versus the results after 48 hours. Correlation between variables was determined by Spearman's rank correlation coefficient. Chi square test was used to compare between categorial variables. After analysis, a probability p value <0.05 was considered significant.

The diagnostic performance of a variable (ability to discriminate between 2 conditions) was evaluated using Receiver Operating Characteristic (ROC) curve analysis. The ROC curve is a graph of the true positive rate (Sensitivity) against the false positive rate (1- specificity) at different cut-off points. The optimal cut-off point is the point that gives the highest sensitivity and specificity.

RESULTS

CD14 and CD16 expression on monocytes in the different groups:

Range, mean, SD, median and IQR of the following: proportion of CD14$^+$ CD16$^+$ monocytes within all circulating monocytes (CD14$^+$ CD16$^+$ MO %), Mean fluorescence intensity of CD16 expression on the CD14 gated monocytes (MFI), proportion of CD14^{dim} CD16$^+$ MO and CD14^{high} CD16$^+$ MO within all circulating monocytes are shown in table (1).

A set of histograms that represent flow cytometric analysis of CD14 and CD16 expression on monocytes is shown in figure (1).

The percentage of CD14$^+$CD16$^+$MO was significantly higher in patients with proven, early and possible sepsis than controls (z = 4.67, p<0.0001; z = 4.02, p<0.0001; z = 3.57, p<0.001, respectively). It was higher in neonates with proven than possible sepsis (z = 2.3, p<0.05), whereas there was no significant difference between the groups of proven and early sepsis (z = 1.64, p>0.05) (Fig. 2A).

Mean fluorescence intensity of CD16 expression on the CD14 gated monocytes (MFI of CD16$^+$MO) was higher in neonates with proven sepsis and early sepsis than controls (z = 3.84, p<0.0001; z = 4.02, p<0.0001, respectively), whereas it was comparable in the group of possible infection and controls (z = 1.87, p>0.05). It was significantly higher in neonates with proven sepsis than those with early and possible infection (z = 2.3, p<0.05; z = 3.38, p<0.001 respectively) (Fig. 2B).

The prevailing subset of monocytes in patients and controls

There was no significant difference in the prevalence of CD14^{high} CD16$^+$ MO and CD14^{dim} CD16$^+$ MO among patients and controls. Percentage of CD14^{high} CD16$^+$ MO was higher than that of CD14^{dim} CD16 MO in both patients and controls (Table 2, Fig. 3).

Correlation between CD14 & CD16 expression on monocytes and clinical characteristics of patients and controls:

There was no significant correlation between CD14$^+$ CD16$^+$ MO% and gestational age, age or weight neither in the studied patients (r = -0.09, p>0.05; r = 0.04, p>0.05; r = -0.18, p>0.05, respectively) nor in the control group (r = 0.2, p>0.05; r = -0.08, p>0.05; r = 0.13, p>0.05, respectively).

There was no significant difference between males and females as regards CD14$^+$ CD16$^+$ MO% neither in the studied patients nor in the control group (z = 0.5, p>0.05; z = 0.71, p>0.05, respectively).

Correlation between CD14 & CD16 expression on MO and indices of neonatal sepsis.

Table (3) shows that there was a significant positive correlation between MFI of CD16$^+$ MO and CRP (Fig. 5); and significant negative correlation with RBCs count and platelets count. While there was a significant positive correlation between CD14^{dim} CD16$^+$ MO% and CRP level in the studied patients, whereas there was no significant correlation between CD14$^+$ CD16$^+$ MO% or CD14^{high} CD16$^+$ MO% with any of the laboratory indices of neonatal sepsis.

Table 1: CD14 and CD16 expression on monocytes in the studied groups.

Variable \ Group		Proven Sepsis	Possible Infection	Early Sepsis	Control Group
$CD14^+$ $CD16^+$ MO% *	Range	52.5-95.4	2.01-94.2	42-94.2	0.6-8.6
	Mean ± SD	75.24±13.12	55.19±26.8	63.92±17.9	3.86±2.53
	Median	74	59.7	63.2	2.3
	IQR	64.95-85.6	44.15-74.8	49.6-75.1	2.15-6.55
MFI of $CD16^+$ MO	Range	11.8-58.7	1.7-21.7	10.2-21.7	1-19.1
	Mean ± SD	28.53±14.62	12.58±5.61	15.89±3.7	8.28±6.78
	Median	24.7	12.3	16.4	6.7
	IQR	16.5-36.65	9.5-17	14.9-17.8	2.35-14.85
$CD14^{dim}$ $CD16^+$ MO% *	Range	7.8-56.5	0.6-20	6.6-13.5	0.08-2.1
	Mean ± SD	19.53±14.48	10.45 ± 4.39	10.82 ± 2.44	0.98 ±0.75
	Median	14.4	11.8	12	0.6
	IQR	10.35-20.6	8.3-12.2	8.9-12.1	0.55-1.7
CD14high CD16+ MO%*	Range	6.4-78.8	1.1-60	33-60	0.4-6.2
	Mean ± SD	44.39±23.71	39.67 ±16.27	46.61±10.81	2.36 ±1.58
	Median	48.5	41.2	42	1.6
	IQR	26.75-63.7	34.5-48.55	39-59	1.35-3.5

*: % of all circulating monocytes. SD: Standard deviation. IQR: Interquartile range

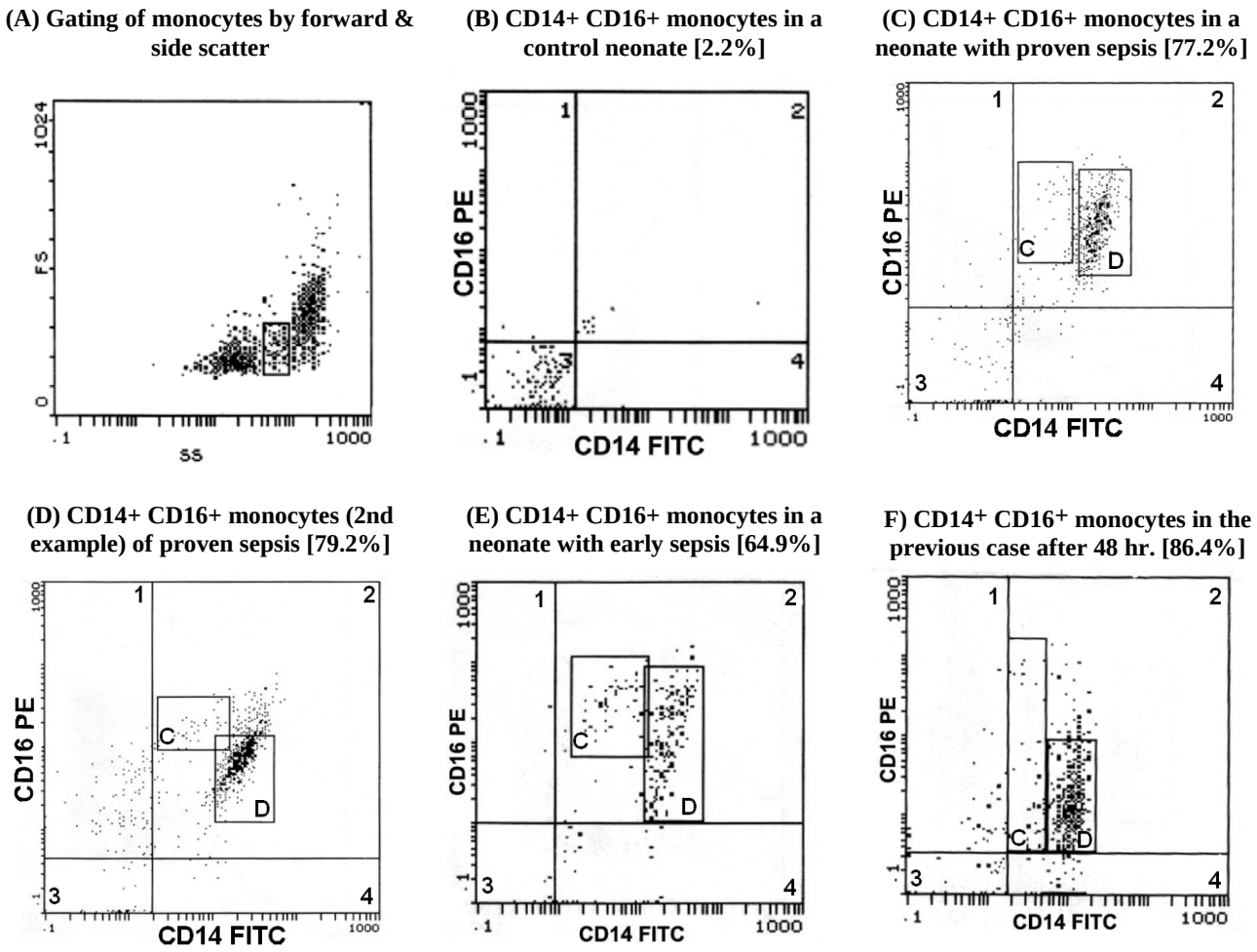

Figure 1: A set of histograms that represent flow cytometric analysis of CD14 and CD16 expression on monocytes. Upper and lower right quadrants (2 & 4) represent all $CD14^+$ cells, upper right and left quadrants (1 & 2) represent all $CD16^+$ cells, upper right quadrant (2) represents cells having coexpression of both CD14 & CD16. Square in (A) represents the forward scatter (FS) and side scatter (SS) gated monocytes. Squares C & D in (C), (D), (E) and (F) represent $CD14^{dim}$ $CD16^+$MO, $CD14^{high}CD16^+$ monocytes respectively. The percentage of $CD14^+CD16^+$ monocytes is given in parentheses.

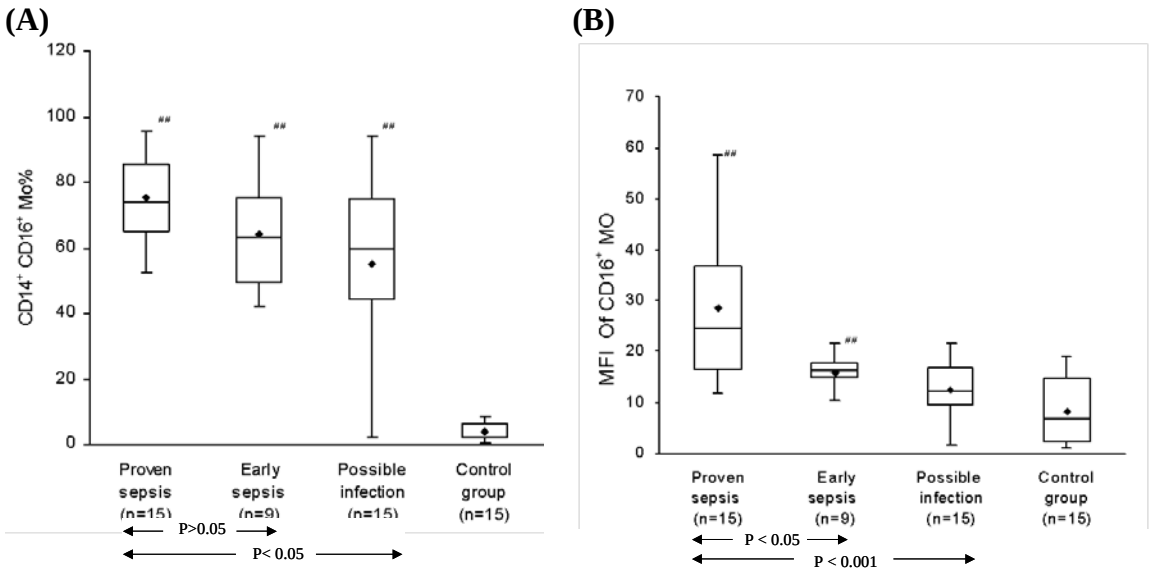

Figure 2: Comparison of CD14^{+} CD16^{+} MO (A) and MFI of CD16^{+} MO (B) among the studied groups. Horizontal lines indicate median values and boxes enclose interquartiles (25th and 75th percentiles). Ranges are marked as maximum and minimum, and dots represent mean values. Mann-Whitney U test is used in comparison. ## Highly significant difference with the control group ($p < 0.01$).

Table 2: Comparison of prevalence of CD14^{high} CD16^{+} MO & CD14^{dim} CD16^{+} MO among all studied patients and controls.

CD14^{dim} CD16^{+}MO /CD14^{high} CD16^{+} MO	All patients (n = 30)		Controls (n = 15)		Total (n = 45)		Chi-square	p
	n	%	n	%	n	%		
CD14^{high}CD16^{+}MO> CD14^{dim} CD16^{+}MO	28	93%	15	100%	43	96%	1.05	>0.05
CD14^{dim}CD16^{+}MO> CD14^{high} CD16^{+}MO	2	7%	0	0%	2	4%		

Table 3: Correlation between CD14 & CD16 expression on monocytes and laboratory indices of neonatal sepsis in all studied patients.

Parameter		***CD14^{+}CD16^{+} MO%***	***MFI of CD16^{+} MO***	***CD14^{dim}CD16^{+} MO%***	***CD14^{high}CD16^{+} MO%***
Hb (gm/dL)	r	-0.17	-0.20	- 0.14	-0.25
	p	> 0.05	> 0.05	> 0.05	> 0.05
RBCs (x10^{6}/mm^{3})	r	-0.16	-0.39	-0.08	-0.13
	p	> 0.05	< 0.05*	> 0.05	> 0.05
TLC (x10^{3}/mm^{3})	r	-0.05	-0.06	-0.34	0.17
	p	> 0.05	> 0.05	> 0.05	> 0.05
ANC (x10^{3}/mm^{3})	r	0.17	0.01	-0.17	0.20
	p	> 0.05	> 0.05	> 0.05	> 0.05
Immature neutrophils (x10^{3}/mm^{3})	r	0	0.02	-0.22	0.16
	p	> 0.05	> 0.05	> 0.05	> 0.05
I/T ratio	r	0.21	0.14	0.26	0.09
	p	> 0.05	> 0.05	> 0.05	> 0.05
Platelets (x10^{3}/mm^{3})	r	-0.32	- 0.36	-0.33	-0.16
	p	> 0.05	< 0.05*	> 0.05	> 0.05
CRP (mg/L)	r	0.17	0.55	0.40	-0.1
	p	>0.05	<0.01**	<0.05*	>0.05

p > 0.05= Not significant, p < 0.05*= Significant, p < 0.01, 0.001, 0.0001**= Highly Significant
Hb=hemoglobin, RBCs=red blood cells, TLC=total leukocytic count, ANC=absolute neutrophilic count, I/T ratio=immature/total neutrophil ratio, CRP=C-reactive protein.

Table 4: Comparison between the initial results of CRP and CD14 & CD16 expression on monocytes and the results after 48 hrs (at the time of positive blood culture) in the group of early sepsis.

Parameters	***Initial***		***After 48 h***		***z***	***p***
	Mean	***± SD***	***Mean***	***± SD***		
CRP	22.67	28.74	72	54.99	2.67	<0.01**
$CD14^{+}$ $CD16^{+}$MO%	63.92	17.9	67.4	15.69	0.65	>0.05
MFI of $CD16^{+}$ MO	15.89	3.7	23.73	4.99	2.67	<0.01**
$CD14^{dim}$ $CD16^{+}$MO%	10.82	2.44	12.97	6.21	1.24	>0.05
$CD14^{high}$ $CD16^{+}$MO%	46.61	10.81	47.98	13.11	0.3	>0.05

z: Wilcoxon signed rank test. p > 0.05 = Not significant, p < 0.05*=Significant, p < 0.01, 0.001, 0.0001**= Highly Significant

Table 5: Sensitivity, specificity and predictive values of CRP, $CD14^{+}$ $CD16^{+}$ MO and MFI of $CD16^{+}$ MO in diagnosis of neonatal sepsis.

Variable	Optimal Cutoff	Sensitivity %	Specificity %	+ve predictive value	-ve predictive value
CRP	> 6	83.3	100	100	78.9
$CD14^{+}$ $CD16^{+}$ MO%	> 8.6	100	100	100	100
MFI of $CD16^{+}$ MO	> 9	100	66.7	82.8	100

+ve = positive, -ve = negative

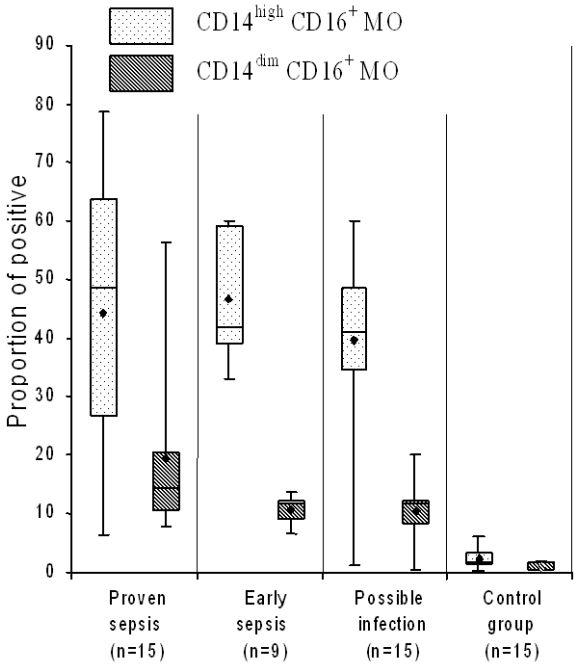

Figure 3: The proportion of $CD16^{+}$ monocytes with dim and high expression of CD14 among the studied groups. Horizontal lines indicate median values and boxes enclose interquartiles (25^{th} and 75^{th} percentiles). Ranges are marked as maximum and minimum, and dots represent mean values. Mann-Whitney U test is used in comparison.

Follow up of neonates with early sepsis

When neonates with early sepsis were followed up for 48 hours, a significant increase in CRP levels and MFI of CD16 expression on monocytes was noticed, whereas no significant difference in $CD14^{+}$ $CD16^{+}$ MO%, $CD14^{dim}$ $CD16^{+}$ MO% and $CD14^{high}$ $CD16^{+}$ MO% was found (table 4).

Sensitivity, specificity and predictive values of $CD14^{+}CD16^{+}$MO

When the patients with proven and early sepsis were compiled as the positive group and the controls were considered as the negative group, the cut off point (obtained from the ROC curve) for $CD14^{+}$ $CD16^{+}$ MO% was 8.6% and that for MFI of $CD16^{+}$ MO was 9. The sensitivity and negative predictive value of $CD14^{+}$ $CD16^{+}$ MO% and MFI of $CD16^{+}$ MO were higher than that of CRP. The specificity and positive predictive value of $CD14^{+}CD16^{+}$ MO% were similar to those of CRP, whereas those for MFI of $CD16^{+}$ MO were less than the corresponding parameters of CRP (table 5).

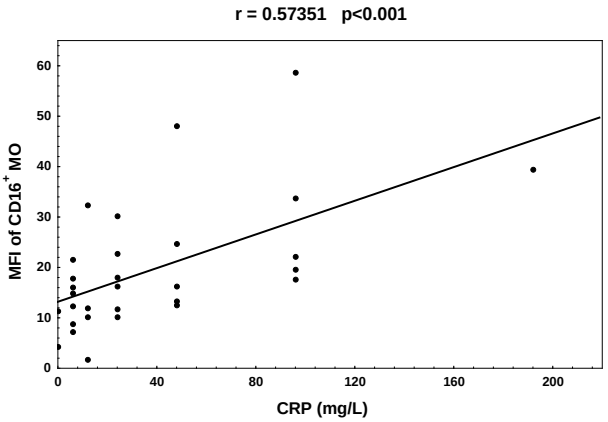

Figure 4: Correlation of MFI of $CD16^{+}$MO with CRP among studied patients.

DISCUSSION

The CD14$^+$ CD16$^+$ monocytes are considered to represent an activated more mature, "macrophage-like subset" as opposed to the prevailing CD14$^+$ CD16$^-$ MO[15,16]. This subpopulation greatly expands in various infections and inflammatory diseases including sepsis[17-19].

Our results revealed that CD14$^+$CD16$^+$ MO% was significantly increased in neonates with proven sepsis and early sepsis than in the control neonates. These CD14$^+$CD16$^+$ cells comprised 3.86 ± 2.53% of all circulating monocytes in the control group, and comprised 75.24 ± 13.12% and 63.92 ± 17.9% in the groups of proven sepsis and early sepsis respectively.

In agreement with our results, Fingerle et al.[23] reported that in adult patients with septicemia, CD14$^+$CD16$^+$ MO became the predominant cell type and accounted for more than 50% of all monocytes while in the control group these cells accounted for 9.3% ± 5.2 % of all monocytes.

Similarly, Nockher and Scherberich[18] reported that CD14$^+$CD16$^+$ monocytes accounted for 8% ± 4% of all monocytes in healthy adult controls of their series. In their hemodialysed patients suffering from chronic infection, these CD14$^+$CD16$^+$ cells constituted 24% of all blood monocytes.

After trauma and during sepsis, significant monocytosis was observed[24,25]. In the present study monocytes % within all leukocytes was significantly higher in the neonates with proven sepsis (11.14 ± 3.87%) than controls (7.42 ± 2.01%). In accordance with our results Skrzeczynska et al.[26] reported that CD14$^+$ cells comprised about 15% of all nongranulocytes (gated PBMCs) in the control group, 21% in children with clinical symptoms suggesting sepsis and 23% in children with symptoms of sepsis and positive blood culture. They postulated that the higher proportion of CD14$^+$ cells within PBMCs in patients with symptoms of sepsis is due to the increased proportion of CD14$^+$CD16$^+$ MO.

In the current study, CD14$^+$CD16$^+$ MO% was significantly elevated in patients with proven sepsis and early sepsis compared with the control group. This agrees with the findings of Skrzeczynska et al.[26]. They found that, CD14$^+$CD16$^+$ MO% was significantly increased both in samples obtained from patients with positive blood culture and in those with clinical symptoms of sepsis as compared to controls. They also found that, the risk group was comparable to controls. Their study included thirty children with median age of 4 months (range 5 days to 2 years) admitted to the intensive care unit. All children in their study were considered to be at high risk of developing sepsis based on their clinical condition such as burns over 30%, trauma or major surgical treatment owing to correction of congenital defects. To our knowledge our study is the first to elucidate the changes of CD14 and CD16 expression on MO in preterm neonates.

To exclude the possibility that underlying disease, drug treatment or intensive care treatment in themselves might induce the CD14$^+$CD16$^+$ cells, Fingerle et al.[23] have screened a panel of 155 patients with various conditions. These included patients with cardiovascular, inflammatory , infectious (viral and bacterial), metabolic and neoplastic diseases. In addition, they have tested 113 ICU patients without sepsis syndrome. None of these patients exhibited increased CD14$^+$CD16$^+$ cells.

We found that the mean CD14$^+$CD16$^+$ MO% was higher in neonates with proven sepsis than in the early sepsis group, however the difference did not reach statistical significance. Meanwhile, MFI of CD16$^+$ MO was significantly higher in neonates with proven sepsis than the early sepsis group. Fingerle et al.[23] reported that the increase of CD14$^+$CD16$^+$ cells in sepsis was not a persistent phenomenon, but usually lasted for only 1 to 3 days within a two weeks time span of screening. Low CD14 on monocytes was not a constant phenomenon either, but specific fluorescence intensity for CD14 was frequently still low when CD14$^+$CD16$^+$ cells had returned to the control range. Also, Skrzeczynska et al.[26] reported that, the individual variations were significant, indicating that single measurements of CD14$^+$CD16$^+$ MO proportion are of limited diagnostic value. In contrast, repeated determinations proved useful. This statement was proved in our study when we measured CD14 and CD16 expression on monocytes twice in the group of early sepsis, on admission and after 48 hours. We found that mean fluorescence intensity of CD16 expression on the CD14$^+$ monocytes (MFI) after 48 hours is significantly higher than the initial results. However, there was no significant difference between the results after 48 hours and initial results as regards CD14$^+$CD16$^+$ MO%, CD14^{dim} CD16$^+$ MO% and CD^{high} CD16$^+$ MO%. This suggests that MFI of CD16 expression on monocytes increases with progression of sepsis.

According to Murphy and Reen[27] ,the majority of cord and adult monocytes expressed CD14 at a high density (CD14^{high}) while approximately 15% of monocytes expressed this antigen at a lower level (CD14^{dim}). The CD14^{dim} monocytes expressed CD16 (FcγRIII) three times as did the CD14^{high}

monocytes in both cord and adult preparations, while its level of expression was significantly reduced on cord $CD14^{dim}$ monocytes relative to adult $CD14^{dim}$ monocytes[27].

In our series, we found that $CD14^{high}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$ cells were significantly higher in studied patients in comparison with the control group. $CD14^{high}$ $CD16^{+}$ MO accounted for 44.39± 23.71% and 46.61 ± 10.81% of all monocytes in the group of proven sepsis and early sepsis respectively, and accounted for 2.36 ± 1.58% in the control group. Skrzeczynska et al.[26] reported that the $CD14^{high}$ $CD16^{+}$ population comprised 10-70% of all $CD16^{+}$ MO. Both populations were significantly increased in proven sepsis group, while $CD14^{dim}$ $CD16^{+}$ MO were also significantly elevated in the group of possible infection, when compared to controls.

We also found that the percentage of $CD14^{high}$ $CD16^{+}$ MO was higher than that of $CD14^{dim}$ $CD16^{+}$ MO in 93% of patients and in all controls. In agreement with our results, Skrzeczynska et al.[26] stated that in blood samples of studied children, the majority of $CD16^{+}$ MO showed high expression of CD14. This was in contrast to adult blood donors where reverse proportions were observed (J. Skrzeczynska, unpublished observation; quoted from Skrzeczynska et al.[26]). The highest proportion of $CD14^{high}$ $CD16^{+}$ MO was observed in patients with symptoms of sepsis and in particular in patients with positive blood cultures. This was the novel finding of Skrzeczynska et al.[26] as the increased proportion of $CD14^{high}$ $CD16^{+}$ MO in sepsis has not been reported previously. The question remains whether this observation reflects general phenomenon or is characteristic for sepsis in neonates and small children. The increase of $CD14^{high}$ $CD16^{+}$ MO level may reflect the mobilization of this subset from the bone marrow during the first year of life, because MO of similar phenotype were observed after treatment with granulocyte colony-stimulating factor (G-CSF) or macrophage colony-stimulating factor (M-CSF)[28,29].

$CD14^{high}$ $CD16^{+}$ MO include population with high expression of CD163 scavenger receptor[30]. In vivo, the development of cells which express this scavenger receptor was associated with the healing phase of acute inflammatory response and was shown to produce anti-inflammatory and angiogenic factors[31,32]. Based on the studies of healthy blood donors, $CD14^{dim}$ $CD16^{+}$ MO are believed to represent a pro-inflammatory, macrophage-like subpopulation[15,16].

Concerning the phagocytic activity of MO of children with sepsis, Skrzeczynska et al.[26] reported that the phagocytic activity of $CD14^{+}$ $CD16^{+}$ MO which prevail in the blood of septic patients was comparable with or higher than that of $CD14^{+}$ $CD16^{-}$ MO. However, in their control samples, the proportion of cells which phagocytosed bacteria within $CD14^{+}$ $CD16^{+}$ MO was significantly lower than in $CD14^{+}$ $CD16^{-}$ MO.

In the current study, neither $CD14^{+}$ $CD16^{+}$ MO% nor MFI of $CD16^{+}$ MO were correlated with the age and sex of neonates neither in the patients nor in the control group. $CD14^{+}$ $CD16^{+}$ MO % did not correlate with markers of neonatal sepsis (Hb, TLC, ANC, I/T ratio, platelets and CRP), whereas MFI of $CD16^{+}$ MO showed significant positive correlation with the CRP and negative significant correlations with platelets count. In agreement with our results, Fingerle et al.[23] reported that $CD14^{+}$ $CD16^{+}$ cells did not correlate to sex, age, infection focus, leukocyte count or platelet count. Only $CD14^{+}$ $CD16^{+}$ percentage and body temperature showed a striking correlation. However, Skrzeczynska et al.[26] stated that in neonates and small children, the increased proportion of $CD14^{+}$ $CD16^{+}$ MO correlates with clinical symptoms of sepsis. Furthermore, this increase was noted 1-2 days before blood culture became positive. Nockher and Scherberich[18] stated that no correlation was found between the absolute monocyte blood count and the percentage of $CD14^{+}$ $CD16^{+}$ monocytes.

The sensitivity and negative predictive value of $CD14^{+}$ $CD16^{+}$ MO% and MFI of $CD16^{+}$ MO were higher than that of CRP. Specificity and positive predictive value of $CD14^{+}$ $CD16^{+}$ MO% were similar to those of CRP, whereas those for MFI of $CD16^{+}$ MO were less than those of CRP. This excellent negative predictive value can reduce the unnecessary use of antibiotic therapy and early discharge of at-risk non-infected neonates from hospital to reduce nosocomial infection.

In addition, blood culture which is the gold standard for diagnosis of infection has a high false negative results and only acquires excellent negative values at 36 to 48 hours and the results may not be available for 24 to 48 hours after treatment decisions must have been made[33].

This agrees with Messer et al.[34] who reported that CRP determination was not valuable for early diagnosis of infection as its sensitivity in the first 12 hours was only 51%. Nevertheless, its sensitivity improved to 83% after 24 hours. De Bont et al.[35] reported that CRP has a short half life (4-6 hours) and a variable lag period between the clinical onset of the disease and elevated serum concentration.

We found that MFI of $CD16^{+}$ MO was significantly high after 48 hours in comparison with

the initial results in the group of early sepsis suggesting that as the infectious process proceeded, the expression of CD16 on CD14$^+$ monocytes increased with progression of the systemic inflammatory process.

In conclusion, measurement of CD16 expression on CD14$^+$ monocytes is a rapid and efficient test that helps in the diagnosis of neonatal sepsis at its early stage and valuable in excluding neonatal infection in neonates who are at a risk to develop sepsis. So, it helps to reduce the unnecessary antibiotics use and to discharge the at-risk but healthy neonates. Future studies including a larger sample size with repeated determinations of CD14$^+$ CD16$^+$ MO% in both fullterm and preterm neonates at risk or having sepsis are recommended.

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