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Evaluation of neutrophil CD64 in neonatal sepsis

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EVALUATION OF NEUTROPHIL CD64 IN NEONATAL SEPSIS

Matshediso Bernice Dhlamini



A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfillment of the requirements for the degree of

Masters of Medicine in the branch of Pathology (Haematology)

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DECLARATION

I, Matshediso Bernice Dhlamini declare that this research report is my own work. It is being submitted for the degree of Masters of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this University.

Parts of the study done by me included the following: informed the paediatricians at the three hospital about the study and asked them to take an extra EDTA tube when doing septic work up in the neonatal units, study enrolment of participants including completion of informed consent forms and laboratory work relating to sample preparation and flow cytometric analysis of neutrophil CD64 index.

Parts of the study not done by me: blood was taken by the paediatricians on call, the other blood tests (full blood count-FBC, C-reactive protein-CRP, White cell count-WCC including a differential count and blood culture) were done by routine clinical laboratories and a statistician was consulted for the statistical analysis.

Matshediso Bernice Dhlamini

Date: 13th October, 2011

Masters in the branch of Pathology (Haematology)

DEDICATION

In loving memory of my father and mother

Japhta Dinizulu Dhlamini

1941-2001

Mampakanyane Sophia Dhlamini

1942-1977

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

1. **Poster presentation** at South African Immunology Society Conference Programme, Vineyard Hotel, Newlands, Cape Town, South Africa 9-11 December 2009

Title: *Evaluation of the Leuko-64 kit (Trillium Diagnostics, LLC), for quantification of neutrophil CD64 expression in healthy adults.*

Authors: MB Dhlamini[1], M Suchard[1], T Wiggill[1], E Mayne[1], N Ramparsad[1], DE Ballot[2]

2**. Poster presentation** accepted for the European Society for Clinical Cell Analysis (ESCCA) 2011 Conference at Dublin, Ireland 13-17 September 2011

Title: *Evaluation of neutrophil CD64 for diagnosis of neonatal sepsis, the three hospital experience in Johannesburg, 2009-2010*

Authors: MB Dhlamini[1], MS Suchard[1], TM Wiggill[1], DE Ballot[2]

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ABSTRACT

Neonatal sepsis remains a global health problem due to its significant contribution to morbidity and mortality. The blood culture is the most reliable method for detection of bacterial infections. However, the sensitivity of the latter method is low and using it as a gold standard in diagnosis of bacteremia is fraught with difficulties. Neutrophil CD64 levels are upregulated in response to inflammation and tissue injury.

We quantitated neutrophil CD64 by flow cytometry in neonates with signs and symptoms suggestive of sepsis/infection within the 1st four weeks of life in a prospective observational study conducted at 3 hospitals in Johannesburg. Patients were classified into categories of infection namely definite, probable and possible according to signs and symptoms of infection and blood tests including blood culture results.

Of 76 neonates, there was 1 infant with definite infection, 5 infants with probable, 30 infants with possible and 32 infants with no infection. The PMN CD64 at cut off of 1.8 had a high negative predictive value in ruling out definite (100%) or probable + definite infection (95.2%). We recommend the inclusion of PMN CD64 index into the diagnostic algorithm for neonatal sepsis, as it has a high negative predictive value and can be used to rule out infection. As the positive predictive value of the test was low in confirming infection, PMN CD64 should be used as a screening rather than confirmatory test.

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ABBREVIATIONS USED IN THE TEXT:

ARDS	Acute respiratory distress syndrome
BD	Becton Dickinson
CSF	Cerebrospinal fluid
CRP	C-reactive protein
EOS	Early onset neonatal sepsis
ESR	Erythrocyte sedimentation rate
E. coli	Escherichia coli
FITC	Fluorescein Isothiocyanate
FBC	Full blood count
GBS	Group B Streptococcus
GCS	Glasgow coma scale
G-CSF	Granulocyte colony stimulating factor
IFN	Interferon gamma
IL-6	Interleukin-6
IL-12	Interleukin-12
IPSCC	International pediatric sepsis consensus conference
LOS	Late onset neonatal sepsis
LLC	Limited liability company
LSRII	Laser II
mRNA	Messenger ribonucleic acid
NIST	National Institute of Standards and Technology
NICU	Neonatal intensive care unit
PE	Phycoerythrin
PMN	Polymorphonuclear cell
PROM	Preterm rupture of membrane

ABBREVIATIONS USED IN THE TEXT:

PPROM	Prolonged preterm rupture of membrane
ROC	Receiver operating characteristic
SIRS	Systemic inflammatory response syndrome
SWU	Septic work up
SD	Standard deviation
SRM	Standard reference material
WCC	White cell count

1. INTRODUCTION

1.1 Neonatal sepsis - a global health problem, definitions and pathophysiology

Neonatal sepsis remains a global health problem due to its significant contribution to morbidity and mortality [1-2]. In developing countries neonatal sepsis is responsible for 30-50% of total neonatal deaths annually [3]. Infection in the neonate may be categorized as early onset sepsis (EOS) (<72hours of age) or late onset sepsis (LOS) (>72hours of age) [3-4].

1.1.1 Definitions

In the literature there are numerous definitions for neonatal sepsis with no universal agreement on one particular definition. In neonates, signs and symptoms of infection (implying a microbial organism as the aetiological agent) cannot be distinguished from signs and symptoms seen in other clinical conditions causing systemic inflammatory response syndrome (SIRS) [4]. The goal of the definition of sepsis by the International pediatric sepsis consensus conference (IPSCC) was to facilitate the performance of successful clinical studies in children with sepsis [5] . At the IPSCC twenty experts in sepsis and clinical research modified the current criteria used to define SIRS and sepsis in adults by incorporating pediatric physiological variables. Tables A1-4 (Appendix A) define pediatric sepsis in relation to specific vital signs, laboratory variables and organ dysfunction [5]. Lack of evidence for bacteremia or a focus of infection does not exclude sepsis where there is systemic response to a suspected infection [6]. The negative microbiological cultures do not always exclude the presence of bacterial sepsis [7]. However, continuation of antimicrobial therapy

for suspected infection can lead to longer hospital stay and emergence of multiresistant organisms [7]. We hypothesize that neutrophil CD64, could be a useful infection marker, in differentiating between infected and non-infected groups.

1.1.2 Pathophysiology

Early onset sepsis is caused by organisms prevalent in the maternal genital tract, infecting the neonate transplacentally or during passage through a colonised birth canal at delivery [6, 8]. The micro-organisms most commonly associated with EOS include group B *Streptococcus* (GBS), *Escherichia coli* (*E. coli*), *Haemophilus influenzae* and *Listeria monocytogenes*. The following risk factors are associated with increased risk of EOS: [3, 6]

- Certain procedures during pregnancy such as cervical cerclage, amniocentesis and transcervical chorionic villus sampling
- Low birth weight (<2500gms) or preterm baby
- Febrile illness in the mother within 2 weeks before delivery and during delivery
- Foul smelling and/or meconium stained amniotic fluid
- Prolonged rupture of membranes (>24hours)
- Prolonged and difficult delivery with instrumentation
- Perinatal asphyxia (Apgar score of <4 at 1minute of age) or difficult resuscitation

The LOS is acquired from the caring environment (hospital) [3, 8]. Micro-organisms associated with LOS include coagulase-negative *Staphylococci*, *Staphylococcus aureus*, *E coli*, *Klebsiella*, *Pseudomonas*, *Enterobacter*, *Candida*, GBS, *Serratia*,

Actinobacter, and anaerobes [8]. The risk factors implicated in LOS include the following: [3]

- Neonatal Intensive Care Unit (NICU) admission
- Poor hygiene
- Low birth weight
- Poor umbilical cord care
- Prematurity
- Bottle feeding
- Invasive procedure
- Superficial infection (pyoderma, umbilical cord sepsis)

In addition to the above maternal and neonatal risk factors, there are a number of host factors that predispose the neonate to sepsis [8]. An immature and compromised immune system in the neonate, especially in premature infant, at all levels of host defense, including barrier function, cellular and humoral immunity contributes to sepsis. [1, 8-9].

1.2 Introduction to diagnostic tools used in neonatal sepsis

1.2.1 Clinical signs and symptoms

The early diagnosis of neonatal sepsis presents a clinical dilemma because of the variable and non-specific clinical presentation of this condition [1, 9].

1.2.2 Laboratory tests

1.2.2.1 White cell count (WCC), platelet count and differential count

Despite being widely acknowledged to lack specificity and sensitivity the **white cell count, platelet count** and **white differential count** are still used for the screening of neonates with sepsis [2, 10]. Limitations of the **WCC** include the following [6, 8, 10]:

- May be normal, high or low in infection
- May be high due to many conditions, such as stress of delivery, following surgery or trauma
- There may be differences between arterial or venous sample values
- The differential count and assessment of the immature neutrophil cell count is operator dependent (subjective) and therefore not always reliable [2]

Thrombocytopenia (low **platelet count**) with counts of <100,00x10^9/l (normal neonatal reference range in our setting for platelets is 120-450x10^9/l, as obtained from Haematopathology Laboratory from Children Hospital, British Columbia, January 1990) may occur in neonatal sepsis but this is not specific to sepsis [8]. Increased mean platelet volume and platelet distribution width has been noted in neonates with sepsis within 2-3 days of life [8].

1.2.2.2 Acute phase reactants

Acute phase reactants are also frequently used in predicting neonatal sepsis. The most extensively used acute phase reactant is **C-reactive protein (CRP**). In a recent meta-analysis the sensitivity of this test was estimated to be 80%, but specificity was only 60% - 80% [10]. Several studies agree that serial measurements of **CRP** guide the duration of

antibiotic treatment in neonates managed for suspected sepsis before the final blood culture results are available [6, 11-12].

Interleukin-6 (**IL-6**), a pro-inflammatory cytokine, has been shown to be useful in the early diagnosis of neonatal infection [12]. The latter study also suggested that the best prediction of neonatal sepsis was obtained from the combined use of **IL-6** and **CRP**, that is **IL-6** initially and **CRP** at 24hours. However, **IL-6** is the main stimulus involved in the induction of the acute phase reaction and enhancement of **CRP** synthesis. Hence, serial measurement of **CRP** , which is much simpler to do and more cost effective, is probably of more value than expensive and time consuming determination of **IL-6** plasma concentration in the evaluation of neonatal sepsis [13].

One of the newer acute phase markers of infection is **Procalcitonin** (**PCT**), the prohormone of calcitonin, which occurs in very low concentrations in the serum of healthy people [14]. **PCT** is claimed to be more specific for bacterial infections than viral infections, but it is not universally accepted as an improved diagnostic assay of infection [15]. In the literature diagnostic accuracy of **PCT** appears to be superior to that of CRP [10]. Although **PCT** is reasonably predictive of neonatal sepsis, it is not sufficiently reliable to be used as a sole marker in evaluation of neonatal sepsis [14].

1.2.2.3 Microbiological cultures

Microbiological cultures are currently used as the gold standard for diagnosis of infection/sepsis and help in therapeutic decision-making, especially in choosing the appropriate antibiotics [2, 13, 16]. In this study the **microbiological culture** equals to

blood culture. Virological cultures were not routinely performed in our setting. The **microbiological cultures** are fraught with difficulties, which include the following:

- There may be delay in final culture results for 48-72 hours after collection. This results in unnecessary exposure to antibiotics in neonates with clinical suspicion of sepsis and creates an environment for emergence of bacterial resistance [1, 11, 13].
- Genuine bacteremia may remain undetected in a significant proportion of infected cases because of the small volume of blood taken from preterm infants [2].
- In neonatal sepsis **blood cultures** are often negative in some cases of pneumonia and meningitis [13]. The negative **blood cultures** may even occur in fatal generalized bacterial infection [13].
- The possibility of sepsis in the presence of negative **blood culture** has been noted in neonates who had been exposed to antibiotics in utero, presumably due to antibiotic interference with growth of the organism *in vitro* [1].
- Bacteremia may often be transient or intermittent, especially during the early stages of infection [2].

1.2.3 Cell surface antigens as biomarkers of infection

Recently numerous cell surface antigens have been studied as potentially promising biomarkers of infection, including CD11b, CD69 and CD64 [2]. CD64 is a 72-kDa glycoprotein, known as FC gamma receptor 1 (FC□ RI), that binds immunoglobulin (Ig) G with high affinity [17]. CD64 is part of three major classes of leukocyte FC□ receptors including FC□ RII (CD32) and FC□ RIII (CD16)[10, 17]. The FC receptors, as ligands

for immunoglobulin constant regions, play a coordinating role in immunity and mediate functions such as endocytosis, phagocytosis, antibody dependent cell mediated cytotoxicity (ADCC), and cytokine production [18].

CD64 is constitutively expressed on antigen presenting cells (monocytes, macrophages and dentritic cells), to a lesser extend to eosinophils, but only to a very low extent on resting neutrophils [10, 17-19]. Several studies have indicated that quantitation of the neutrophil /polymorphonuclear cell (PMN) CD64 is a worthwhile candidate for evaluation as a more sensitive and specific indicator of sepsis than the other available diagnostics tests [15]. During neutrophil activation, under the influence of inflammatory cytokines (Interleukin-12, Interferon gamma-INF and Granulocytecolony stimulating factor-G-CSF), there is upregulation of PMN CD64 [15]. So far only one study has indicated that PMN CD64 has very low diagnostic sensitivity of 25.8% with high specificity 96.8% [13]. The majority of the studies agree that PMN CD64 has high diagnostic specificity and sensitivity [15, 20-22].

There are many advantages of using PMN CD64 expression as a diagnostic indicator of neonatal infection/sepsis, including the following:
Upon neutrophil activation upregulation of PMN CD64 expression occurs within a relatively short time scale : 4-6hours for cell surface expression and 1-3 hours for detectable mRNA increase by Northern Blot Analysis [23]. The quantitation of PMN CD64 is rapid (<60minutes) and only minimal blood volume is used, which is a real advantage in neonates [1]. Exposure of neutrophils, in vitro and in vivo to INF-γ has been

shown to upregulate PMN CD64 expression resulting in enhanced antibody-mediated functional responses (such as phagocytosis and oxidative activity) [20, 24].

In contrast to other activation-related antigenic changes on neutrophils such as CD11b, CD18, CD16, CD45RA and CD62L, PMN CD64 expression is very low in healthy individuals. This makes normal ranges easy to define and independent of ethnic background, genetic influences or gender [23].

PMN CD64 expression can reportedly discriminate between acute inflammatory auto-immune disease or systemic infections where both erythrocyte sedimentation rate (ESR) and CRP are elevated [16].

Bhandari *et al*. [1] have shown that the CD64 index has highest area under the Receiver Operating Characteristic curve (ROC curve), compared to commonly used haematological parameters (including band cells and immature/total neutrophil ratio) in diagnosis of neonatal sepsis.

1.3 Objectives of this MMED research report

In the light of above background information we prospectively evaluated the usefulness of PMN CD64 expression in diagnosing neonatal sepsis in our setting.

1.3.1Primary Objective:

Quantitation of PMN CD64 by flow cytometry in all neonates with signs and symptoms suggestive of sepsis/infection within the 1st four weeks of life.

1.3.1.1 Secondary Objectives:

Firstly, to assess the utility of PMN CD64 as an early marker of neonatal sepsis and to compare this marker with other currently used infection markers including CRP, WCC, platelet count and blood culture.

Secondly, to assess if PMN CD64 is valuable in helping the physician to start antibiotics early enough, and in indicating when not to start antibiotics.

Choice of kit methodology for CD64 enumeration:

A Leuko64 kit from Trillium Diagnostics, Liability Limited Company (LLC) has been selected for use in this study for the following reasons:

The method uses a small amount of blood: only 50µL.

It is quick to do, under one hour of hands on time.

Reportedly has high correlation with presence of infection, with manufacturer reported sensitivity of 90.5%, specificity of 96.3%, positive predictive value of 95% and negative predictive value of 92.9% [25].

It is a standardized assay with internal calibrator and controls.

The automated user-friendly software allows for excellent reproducibility, with manufacturer reported coefficient of variation of less than 5%.

2. MATERIALS AND METHODS

2.1 Research design and site

This prospective observational study was conducted at 3 hospitals in Johannesburg, South Africa, namely Rahima Moosa, Chris Hani Baragwanath and Charlotte Maxeke Johannesburg Academic Hospital. Consecutive neonates with suspected infection or sepsis were enrolled from June 2009 to July 2010.

Permission was granted by the chief executive officers of the above mentioned hospitals to perform the study (see Appendix C for the permission letters). Ethical clearance was approved by the Human Research Ethics Committee of the University of Witwatersrand, Johannesburg (see Appendix D for the clearance certificate), under **protocol number M081106**. Written informed consent was granted by the parent/s of the neonates (see Appendix E for all informed consent forms).

2.2 Inclusion criteria and study definitions

All neonates (day 1-28) suspected of having infection/sepsis were eligible for enrollment in the study. Suspicion of infection included non-specific signs and symptoms such as respiratory distress, tachypnoea, tachycardia, hypoglycaemia, hyperglycaemia and maternal risk factors (please refer to Appendix B for more information).

Neonates with severe congenital or chromosomal abnormalities were excluded from the study. The septic work up was done at the clinician's discretion but variables (see Table 3.1) recorded for the purposes of the study. The septic work up included the following tests: Full blood count (FBC), differential count (not done for every patient), CRP and

blood culture. The CRP in the participants of this study was done on the second day (after 24-48 hours of suspicion of sepsis, while other tests were done on the first day of suspicion of sepsis).

Neonates who had been on antibiotics for 48 hours or more, but clinically appeared septic and needed re-evaluation (another septic work up) were also included in the study. Categories of infection were defined using previously published criteria: [11]

- **Contamination**: positive microbiological cultures with less than three signs and symptoms of infection and normal white cell count (WCC), differential count, platelets and CRP.
- **Possible infection**: one or more of abnormal platelet count, WCC, differential count or CRP with negative microbiological cultures and less than three signs or symptoms of infection.
- **Probable infection:** one or more of abnormal platelets, WCC, CRP, differential count with negative microbiological cultures with three or more signs or symptoms of infection.
- **Definite infection:** positive microbiological cultures with any clinical signs or symptoms of infection and one or more of abnormal WCC, differential count, platelets or CRP.
- **No infection:** normal CRP, WCC, differential count and platelet count with negative microbiological cultures and less than three symptoms or signs of infection.
- **Unclassified:** do not fit into any of the other categories

In this study a **differential count** was not used to categorize infection as this test was not done in majority of the patients.

2.3 Sample collection and storage:

The attending paediatrician collected an additional tube of 0.3 ml peripheral blood (purple top with Ethylenediaminetetraacetic acid-EDTA anticoagulant) for the purposes of this study concurrently with the other septic work-up blood investigations. This blood was stored in the fridge (4^{o}C) until informed consent was obtained from the parents for participation in the study. In the event that the consent was refused within 48 hours, the blood was discarded. This method was approved by the ethics committee previously.
As per manufacturer's guidelines, the specimens remain acceptable for up to 24 hours after collection when held at room temperature (18-22^{o}C) and for 48 hours when refrigerated (2-8ºC) [26].
Patients were managed by clinicians according to existing protocols.

2.4 Validation

Twenty adult blood samples were used to validate the suitability of the kit's reference ranges to the local population. We evaluated the Leuko64 kit using peripheral blood samples from healthy adult volunteers prior to assessing the utility of this kit in neonatal sepsis. The reason for using adult volunteers was due to the ethical dilemma of accessing peripheral blood from healthy neonates. With informed consent, 3-5ml EDTA anticoagulated blood was drawn from 20 adult healthy volunteers (see Appendix E for informed consent). All participants completed a questionnaire to confirm their health

status (Appendix F for the questionnaire). Pregnant women were excluded from the study. The bloods were then processed on the Laser (LSR) II flow cytometer (Becton-Dickinson-BD, San Jose) using the Leuko64 kit.

Please see Appendix H for the letter requesting approval from ethics committee for addition of adult control group**.**

2.5 Collation of clinical and laboratory data

For each neonate entered in the study:

Clinical history was retrieved from the hospital medical file, both neonatal and maternal (where available) and a data sheet filled (please refer to the Appendix B)

Available laboratory results were retrieved from the laboratory information technology system: FBC, differential count and blood culture at the time of the septic work-up and CRP the next day.

2.6 Neutrophil / PMN CD64 Index

2.6.1 Contents of the kit [26]

The CD64 kit includes three monoclonal antibodies (Reagent A): two with specificities to CD64 (both Fluorescein Isothiocyanate- FITC conjugated) and one recognizing CD163 (Phycoerythrin-PE conjugated). The use of two antibodies to different epitopes of CD64 is for two reasons. The first is to enhance the signal to noise ratio of the assay. The second reason is the provision of a mechanism to minimize lot to lot variation in the reagent fluorescence signal. The CD163, a monocyte-specific antigen, increases the specificity of leukocyte subpopulation identification, thus facilitating the Leuko64

QuantiCALC software's cluster-finding algorithm and allowing for identification of internal "positive and negative" control populations. In addition the kit also contains 10x concentrate of red cell lysis buffer (Reagent B), as this assay is a no-wash format.

A fluorescent polysterene bead suspension (Reagent C) is included for instrument calibration and standardization of the cellular CD64 quantitation and allows traceability to the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1932 (RM 8640). The microbeads serve as reference beads to properly adjust the sensitivities and gains of all the flow cytometer instrument fluorescence and light scatter signals. These beads are given an arbitary fluorescence value assignment within the lot-specific software of the Trillium Leuko64 kit, which is used to calibrate the fluorescence scales for both the FITC signal of the anti-CD64 antibodies and the PE signal of anti-CD163 antibody.

The calculation of the PMN CD 64 performed by the lot specific Leuko64 is as follows: **CD64 index** = Bead Calibration Factor x (Mean fluorescence intensity (MFI) of PMN's / MFI of Beads). The Bead Calibration Factor value is lot-specific for the Leuko64 assay and part of the patent. This factor allows for minimal lot to lot variations (Coeffecient of variation <10% among 5 lots) with the Leuko64 assay kits.

2.6.2 Sample and reagent preparation

The specimens were handled according to the kit instructions as below: [26].

The preparation of 10x Trillium Lyse (Reagent B) 1:10 dilution, was done by mixing 1 part of the concentrated Reagent B with 9 parts of filtered distilled water. The diluted lyse when prepared, was stable for 1week at room temperature (20-23^{o}C) or 30 days refrigerated (2-8^{o}C). The temperature of diluted lyse must be between 20-37 ^{o} C when used, and 1.0mL is required for each sample.

12x75mm polysterene tubes were used for the mixing of blood and reagents.

Initially 50 µl of Leuko64 Reagent A (Reagent A- anti-CD64 antibodies to different epitopes, clone22 and 32.2 and CD163 antibody) was mixed with 50µl of well mixed EDTA anticoagulated blood with white cell count <25 x10^9 cells/l (diluted as needed). The mixture was gently mixed and then incubated for 10 minutes in the dark room temperature (18-22^{o}C).

The second step was addition of 1ml of diluted lyse (Reagent B) to the above mixture. The mixture was gently mixed and then incubated for 15 minutes at room temperature. Intermittent vortexing before the incubations enhanced lysis.

The final step was addition of 5µl of Leuko64 beads (Reagent C) to the above mixture. The mixture was gently mixed and then analyzed on the flow cytometer. Prepared samples could be held at 2-8 ^{o}C, in the dark until analyzed (up to 6hours after preparation) if not analyzed immediately .

2.6.3 Flow cytometer Set-up.

Before analyzing the samples an acquisition protocol was set up on the LSR II flow cytometer (Becton-Dickson, San Jose) using FACSDIVA software according to manufacturer instructions.

Four 2-parameter histograms were set up:

- Forward scatter -FS (linear) vs Side scatter-SS (log)
- CD64 FITC vs SS (log)
- CD163 PE vs SS (log)
- CD163 PE vs CD64 FITC

And 3 one-parameter histograms:

- FL1 – CD64 FITC
- FL2 – CD163 PE
- FL3 (or Photomultiplier tube-PMT with filter setup able to detect 685nm).

All compensation settings were turned off.

A 12x75polysterene tube was prepared containing 5µl of Reagent C and 0.5ml of diluted Reagent B. This was used to establish PMT voltage and scatter settings.

The above bead suspension was run to make adjustments on the one parameter histograms.

The adjustments were as follows:

- The centre of the peak on the FL1 (FITC) axis was set at the end of the second decade of the fluorescence intensity. The MFI= about 0.90

- The centre of the peak on the FL2 (PE) axis was set at ~20 (the first tic in the second decade of fluorescence intensity). MFI = ~0.20
- The center of the peak on the FL3 (PerCP-Cy5-5 –A) axis was set at midscale in second decade.

On the FS vs log SS histogram, the bead population was positioned at the start of the third decade on log side scatter signal and at channel 85-100 on forward or low angle scatter signal (on 256 scale use channel 20-25).

On the cytometer the threshold to exclude platelets and red cell debris was set on log SS using lymphocytes. FSC 27,546 and SSC 600

The discriminator on log SS was set to allow for collection of 50, 000 ungated events.

Statistics were selected to report mean and median of FITC-A, PE-A and PerCP-Cy5-5-A channels (respectively FL1, FL2 and FL3).

Each day, before running the samples, the bead suspension was run to set the PMT's and parameter settings made so that the centre of the peak in histogram FL1 MFI was 525-645 and the center of the peak in histogram FL3 MFI was 790-970. This standardization was necessary for the use of the TrilliumQuantiCALC software.

Once the beads sample was run, the FCS files were exported at FSC 2.0 data files.

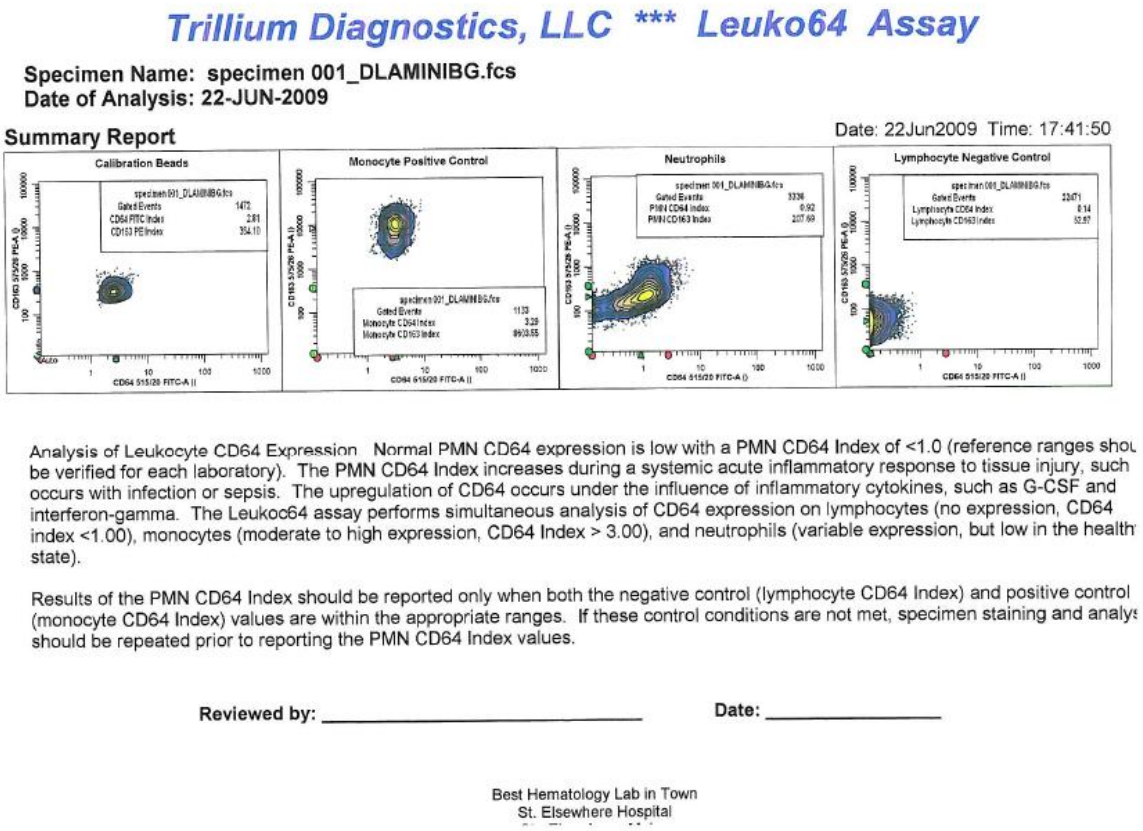
With each new lot of Leuko64 or following instrument service, positioning of the bead population on the FS vs SS histogram, the threshold to exclude platelets and red cell debris and confirmation of 50, 000 ungated events had to be repeated.

2.6.4 Listmode file analysis

Leuko64™ QuantiCALC software (Trillium Diagnostics, LLC) was used for data analysis.

This software is lot specific and protocols for use are instrument model-specific. Initially we used QuantiCALC Software protocol designed for BD FACS Canto instruments which were confirmed by the manufacture (Trillium Diagnostics, LLC) to be suitable for use with FACS Diva Software on the LSR II. However, with our last Leuko64 kit, we used QuantiCALC Software with protocol designed specifically for LSR II instrument.

See below **FIGURE 2.1 and 2.2** for the examples of the result sheets for PMN CD64 index.



Trillium Diagnostics, LLC *** Leuko64 Assay

Specimen Name: specimen 001_DLAMINIBG.fcs
Date of Analysis: 22-JUN-2009

Summary Report

Date: 22Jun2009 Time: 17:41:50

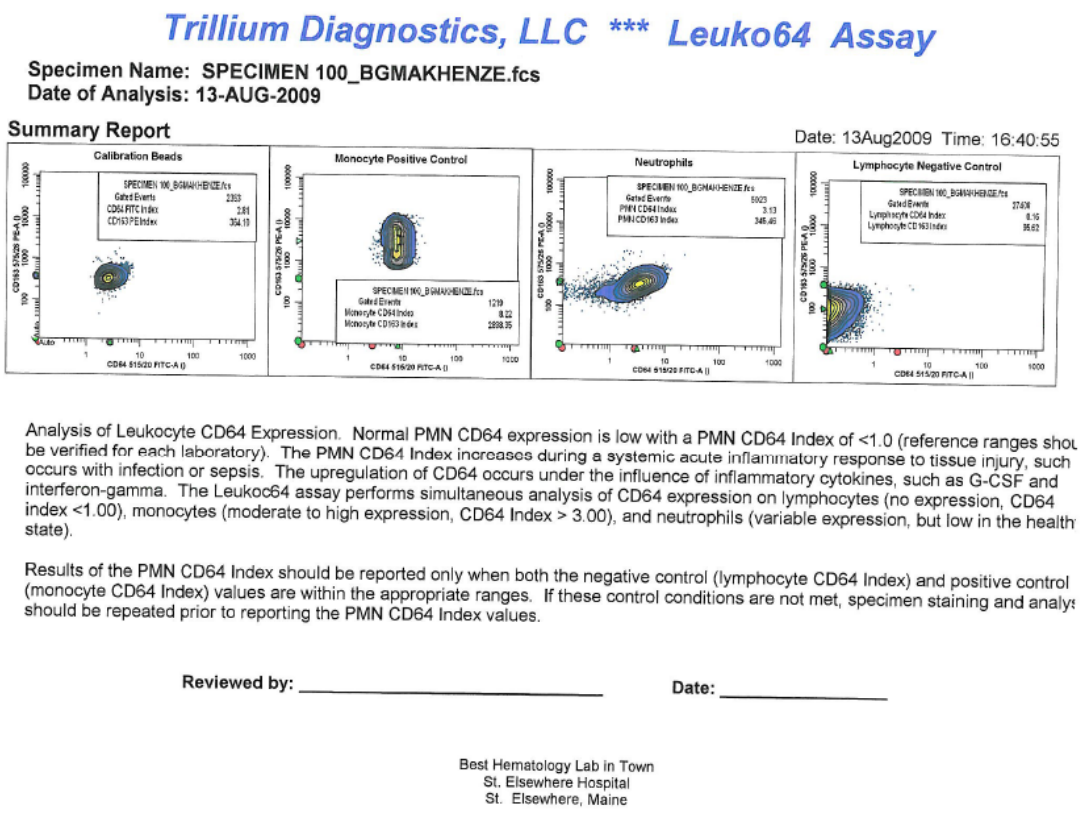
Analysis of Leukocyte CD64 Expression Normal PMN CD64 expression is low with a PMN CD64 Index of <1.0 (reference ranges shou be verified for each laboratory). The PMN CD64 Index increases during a systemic acute inflammatory response to tissue injury, such occurs with infection or sepsis. The upregulation of CD64 occurs under the influence of inflammatory cytokines, such as G-CSF and interferon-gamma. The Leukoc64 assay performs simultaneous analysis of CD64 expression on lymphocytes (no expression, CD64 index <1.00), monocytes (moderate to high expression, CD64 Index > 3.00), and neutrophils (variable expression, but low in the health state).

Results of the PMN CD64 Index should be reported only when both the negative control (lymphocyte CD64 Index) and positive control (monocyte CD64 Index) values are within the appropriate ranges. If these control conditions are not met, specimen staining and analys should be repeated prior to reporting the PMN CD64 Index values.

Reviewed by: _____ Date: _____

Best Hematology Lab in Town
St. Elsewhere Hospital

FIGURE 2.1: Normal PMN CD64 index result sheet, showing a monocyte positive control and lymphocyte negative control.



Trillium Diagnostics, LLC *** Leuko64 Assay

Specimen Name: SPECIMEN 100_BGMAKHENZE.fcs
Date of Analysis: 13-AUG-2009

Summary Report

Date: 13Aug2009 Time: 16:40:55

Calibration Beads

Monocyte Positive Control

Neutrophils

Lymphocyte Negative Control

Analysis of Leukocyte CD64 Expression. Normal PMN CD64 expression is low with a PMN CD64 Index of <1.0 (reference ranges shou be verified for each laboratory). The PMN CD64 Index increases during a systemic acute inflammatory response to tissue injury, such occurs with infection or sepsis. The upregulation of CD64 occurs under the influence of inflammatory cytokines, such as G-CSF and interferon-gamma. The Leukoc64 assay performs simultaneous analysis of CD64 expression on lymphocytes (no expression, CD64 index <1.00), monocytes (moderate to high expression, CD64 Index > 3.00), and neutrophils (variable expression, but low in the health state).

Results of the PMN CD64 Index should be reported only when both the negative control (lymphocyte CD64 Index) and positive control (monocyte CD64 Index) values are within the appropriate ranges. If these control conditions are not met, specimen staining and analys should be repeated prior to reporting the PMN CD64 Index values.

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St. Elsewhere Hospital
St. Elsewhere, Maine

FIGURE 2.2 Abnormal PMN CD64 index result sheet, showing monocyte positive control and lymphocyte negative control.

2.7 Statistical analysis of the data

Data was analysed by a statistician in the following manner:

Stata 10.0 software was used to analyse the data from a total of 76 neonates. Normality of data was tested using Shapiro-Wilk test. Birth weight of participants, PMNCD64 and WCC showed a positively skewed distribution. Descriptive statistics for categorical (proportions and percentages) and continuous variables (median) were reported.

Logistic regression models were used to determine the association between selected neonatal factors (sex and birth weight) and infection. The logistic model considered infection (blood culture positive) as the outcome variable of interest and neonatal factors as independent factors. Logistic regression models were also used to determine the association between selected neonatal factors (sex, gestational age, and birth weight) and PMNCD64. Abnormal PMNCD64, that is, > 1.8 [26] was considered as outcome of interest with the selected neonatal factors as independent variables.

ROC curves were generated for PMNCD64 at various cut offs, by plotting sensitivity versus 1-specificity. PMN CD64 index of >1.8, >1.6 and >2.0 were considered as outcome of interest.

P values were reported as statistically significant if <0.05 or 5%.

3. RESULTS

3.1 Results of validation study

We established that the expression of CD64 in leukocytes in the local population fell within the normal ranges suggested by the manufacturers (normal adult reference range ≤1.0 PMN CD64 index; and 1-1.50 zone of diagnostic uncertainty - repeat within 12-24 hours and clinical correlation suggested to confirm or exclude infection/sepsis) [26]. The mean PMN CD64 index of the samples was 0.84 (range of samples was 0.55 – 1.99), with a standard deviation of 0.31.

19 /20 samples fell within the normal adult range of PMN CD64. We had one (1/20) outlier with a PMN CD64 of 1.99, suggestive of possible undiagnosed infection.

Reproducibility was tested by setting up a sample from one adult volunteer ten times. The CV% was noted to be 2.1% for the PMN CD64 index.

Statistical analysis using Spearman correlation coefficient revealed r = 0.6657 (p = 0.0014) for neutrophils and monocytes CD64 indices, indicating a good linear relation between the two. Using linear regression, for every unit increase in the monocyte CD64 index, there was increase in PMN CD64 index by 0.1347199 (R^2 = 0.7333; p = 0.0000).

Our conclusion was that, the PMN CD64 results for healthy adult volunteers were within the normal range as described by the Leuko64 kit, Trillium Diagnostics, LLC, and that monocyte CD64 and PMN CD64 are linearly related.

3.2 Study population

The total number of neonates screened to be enrolled in the study was 141. Participants who met inclusion criteria were 76 neonates. Please refer to limitations of the study (4. Discussion), for further explanation as to why the rest of neonates could not be enrolled. Twelve mothers refused consent.

3.3 Patient characteristics

According to the clinical findings and blood culture results, patients were classified into the following categories of infection as defined in Section2.2.The number of the participants finally assigned to each category was as follows:

definite infection (1);

probable infection (5);

possible infection (30);

no infection (32);

unclassified (5)

contamination (0)

Table 3.1 shows the demographic data in this study, using infection classified as negative versus positive blood culture (that is definite infection), we found that infection was not statistically associated with sex, (odds ratio 1.62 ; 95% CI 0.07 – 38.61; P-0.764) or birth weight (odds ratio 0.99; 95% CI 0.97 – 1.01; P-0.181).

Similarly, no other sepsis class (no infection versus definite+probable+possible infection); was statistically associated with sex, (odds ratio 0.83; 95% CI 0.16-4.39; P-

0.824); birth weight, (odds ratio 0.99; 95% CI 0.99- 1.00; P-0.502) and gestational age, (odds ratio 1.07; 95% CI 0.08-15.06; P-0.958).

Using PMN CD64 index classified as normal (<1.8) versus high (>1.8), PMN CD64 was not statistically associated with sex, odds ratio 5.66 (95% CI 0.84 - 38.0; P-0.074); birth weight, odds ratio 1.00 (95% CI 0.99 - 1.00; P-0.974); and gestational age, odds ratio 2.83 (95% CI 0.166 - 48.35; P-0.472).

The one neonate who had definite infection (positive blood culture) was female, preterm and had a low birth weight (890 grams). This neonate was born via the caesarean section route, and was four days old at the time of enrolment. The blood culture was positive for gram-negative bacilli (*Klebsiella pneumoniae*, extended spectrum beta lactamase producer). Her CRP was 29.0 and PMN CD64 was index 3.70.

The most frequent clinical sign in the study was respiratory distress 92% (70/76), followed by tachypnoea ~20% (15/76). Other signs and symptoms included the following hypoglycaemia 6.6% (5/76); tachycardia 4.0% (3/76); hypothermia 2.6% (2/76); failure to wean oxygen 2.6% (2/76) and hyperglycaemia 1.3% (1/76).

TABLE 3.1: Demographic data

Variable	Frequency (%)
Sex	
Female	29 (38.2)
Male	47 (61.8)
Birth weight (Range is 800-4660g, Median 1710g)	
Less than 1000g	6 (7.9)
Equal or greater than1000g but less than 1500g	21 (27.6)
Equal or greater than 1500g but less than 2500g	25 (32.9)
Greater than 2500g	24 (31.6)
Mode of delivery	
Caesarian section	46 (60.5)
Normal vaginal Delivery	30 (39.5)
Gestational age	
Preterm	54 (71.0)
Term	22 (29.0)

Demographic data of participants including sex, birth weight, mode of delivery and gestational age.

TABLE 3.2: Summary of blood results

Platelets (x10^9/l)	**Frequency /%**
Normal (120-450)	66 (90.4)
High (>450)	2 (6.9)
Low (<120)	5(2.7)
WCC (x10^9/l)	
Normal (9-30)	45 (60.8)
High (>30)	2 (2.7)
Low (<9)	27 (36.5)
PMNCD64 index using cutoffs of 1.6; 1.8 or 2.0	
Normal (<1.6)	38 (50)
High (>1.6)	38 (50)
Normal (<1.8)	42 (55.7)
High (>1.8)	34 (44.3)
Normal (<2.0)	46 (60.5)
High (>2.0)	30 (39.5)
Blood Culture	
Negative	70 (92.1)
Positive	1 (1.3)
Not done	4 (5.3)
Contamination	1 (1.32)*
CRP (mg/L)	
Normal	51 (75.0)
High	17 (25.0)

Summary of the blood platelet count, white cell count, PMN CD64 index, blood culture and CRP.
*This patient cultured Corynebacterium *spp* consistent with contamination, however, as patient had signs and symptoms of infection, the patient fell into the unclassified category of infection in Section 2.2.

3.4 Receiver operating characteristic (ROC) analysis

The ROC curve is a graphical presentation of trade-off between sensitivity and specificity, and the greater the area under the ROC curve (AUC), the more discriminatory the test is[27]. AUC measures the overall ability of the test (continuous variable) to discriminate between individuals with and without disease. If a test that is plotted against a gold standard and has AUC of 1.0 or closer to 1.0, it is a perfectly discriminatory test. However, if the AUC is 0.5 or less the results in terms of specificity and sensitivity are no better than flipping a coin (that is due to chance) [27].

In our study the ROC curves were done to see if PMN CD64 is a better discriminatory test in comparison to the current gold standard test for diagnosing infection and sepsis (microbiological blood culture). In addition the performance of the PMN CD64 was also compared to our sepsis classifications, using them as the gold standard. Figure 3.1, 3.2 and 3.3 shows ROC curves (using the cut-off value of 1.8 PMN CD64) in comparison to positive blood culture (definite infection), definite and probable (excluded possible infection from the all sepsis classification) and all sepsis (definite+probable+possible infection) respectively. The accompanying Tables 3.3; 3.; 4 and 3.5 shows the sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), and calculations for all sepsis classifications for the cut off value of 1.8 (PMN CD64). Table 3.6, 3.7 and 3.8 show the summary of the sensitivity, specificity, PPV, NPV, 95% confidence interval (CI) and AUC values of the different cut off values (1.6; 1.8 and 2.0) for the PMN CD64 in different sepsis classes.

TABLE 3.3: PMN CD64 cut off value 1.8 plotted against definite infection

Gold standard (blood culture)

Test (PMNCD64)	Blood Culture Positive	Blood culture Negative	Total
High	1	30	**31**
Normal	0	40	**40**
	1	**70**	**71**

Table of CD64 results at cut off of 1.8 as a diagnostic test, using only patients with definite infection as gold standard positive

Sensitivity: 1/1 = 100%

Specificity: 40/70=57.1%

PPV: 1/31=3.2%

NPV: 40/40=100%

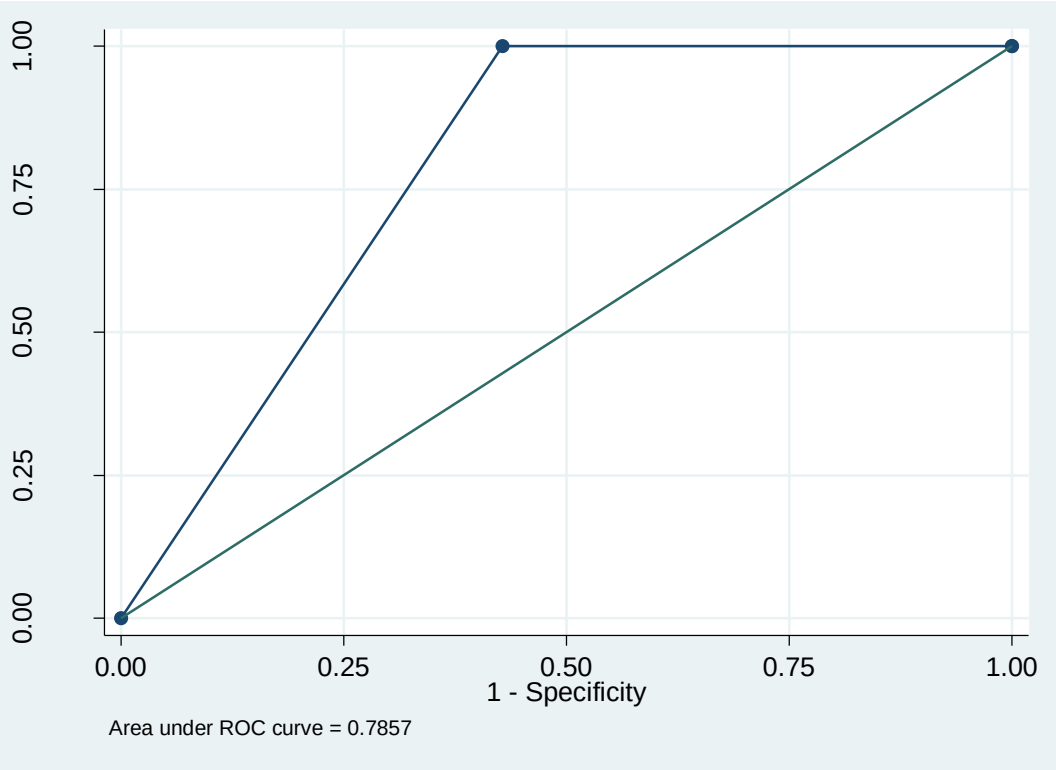


FIGURE 3.1: ROC curve of PMN CD64 at cut off value 1.8 versus definite infection. Area under the curve is 0.7857

TABLE 3.4: PMN CD64 at cut off value 1.8 plotted against definite+probable infection

Gold standard (Sepsis)

Test (PMNCD64)	Sepsis group:definite+probable infection Positive	Sepsis group: definite+probable infection Negative	Total
High	6	13	**19**
Normal	1	20	**21**
	7	**33**	**40**

Table of CD64 results at cutoff of 1.8 as a diagnostic test, using patients with definite and probable infection as gold standard positive

Sensitivity: 6/7=85.7%

Specificity: 20/33=60.6 %

PPV: 6/19=31.6%

NPV: 20/21=95.2%

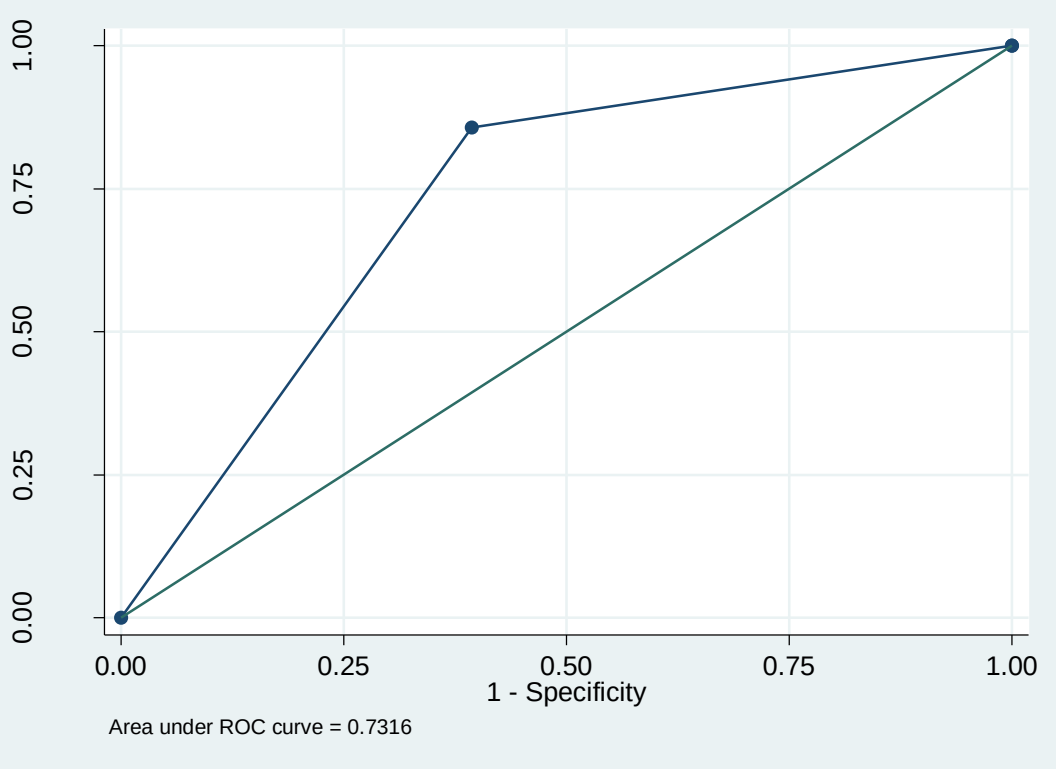


FIGURE 3.2: ROC curve of PMN CD64 at cut off value 1.8 versus definite+probable infection. Area under the curve is 0.7316

TABLE 3.5: PMN CD64 cut off value 1.8 plotted against definite+probable+possible infection

Gold standard (Sepsis)

Test (PMNCD64)	Sepsis group: definite+probable+possible Positive	Sepsis group: definite+probable+possible Negative	Total
High	18	13	**31**
Normal	19	20	**39**
	37	**33**	**70**

Table of CD64 results at cut off of 1.8 as a diagnostic test, using patients with definite, probable and possible infection as gold standard positive

Sensitivity: 18/37=48.6%

Specificity: 20/33=60.6%

PPV: 18/31=58.1%

NPV: 20/39=51.3%

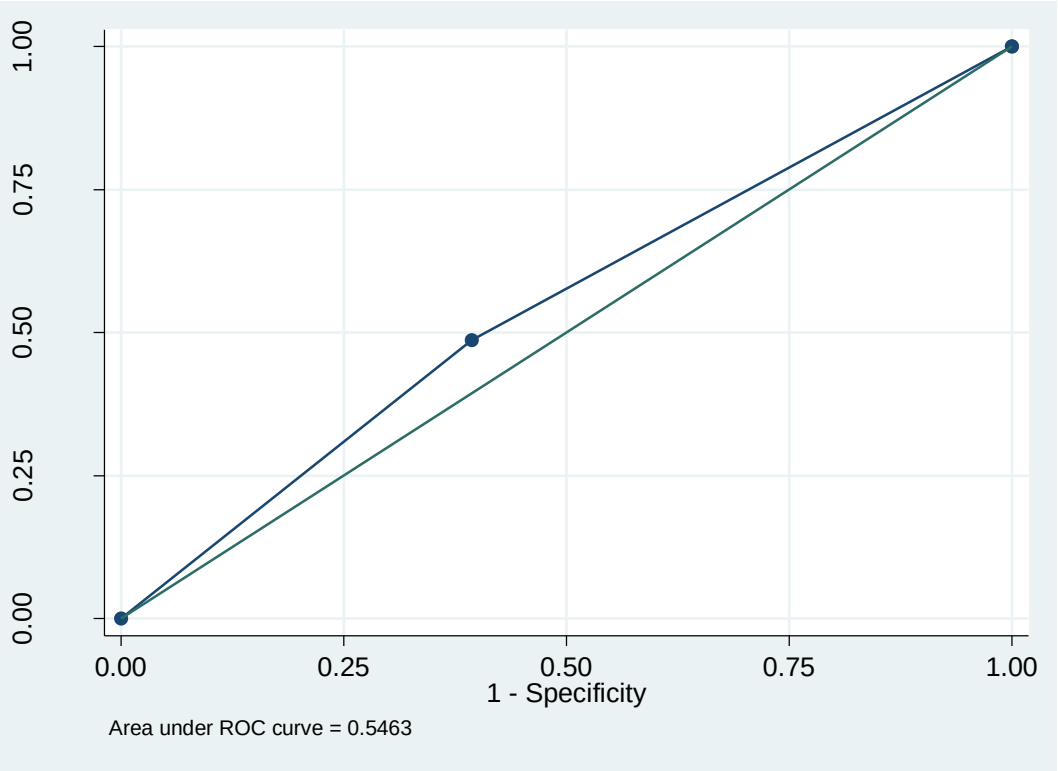


FIGURE 3.3: ROC curve of PMN CD64 cut off value 1.8 versus definite+probable+possible infection. Area under ROC curve is 0.5463.

Similar to above, the sensitivity, specificity, positive and negative predictive values and areas under the ROC curves were calculated using a cut off of 1.6 or a cut off of 2.0. The data is summarized below.

TABLE 3.6: Summary of PMN CD64 index using definite infection as gold standard

	1.8 cut off value	1.6 cut off value	2.0 cut off value
Sensitivity	100%	100%	100%
Specificity	57%	51.4%	62.9%
PPV	3.2%	2.8%	3.7%
NPV	100%	100%	100%
AUC	**0.7857**	0.7571	0.7571
95% CI	0.67560 – 0.87660	0.64455 – 0.85390	0.70727 – 0.89874

The table shows sensitivity, specificity, positive and negative predictive values of CD64 index at differing cut offs. Highest area under the ROC curve when patients with only definite infection were considered as gold standard positive was achieved using 1.8 as the cutoff value.

TABLE 3.7: Summary of PMN CD64 index using definite and probable infection as gold standard

	1.8 cut off value	1.6 cut off value	2.0 cut off value
Sensitivity	85.7%	85.7%	85.7%
Specificity	60.6%	51.5%	33.3%
PPV	31.6%	27.3%	21.4%
NPV	**95.2%**	94.4%	**91.6%**
AUC	0.7316	0.6861	**0.7619**
95% CI	0.56800 – 0.89520	0.5154 – 0.85075	0.59983 – 0.92398

The table shows sensitivity, specificity, positive and negative predictive values of CD64 index at differing cut offs. Highest area under the ROC curve when patients with both definite and probable infection were considered as gold standard positive was achieved using 2.0 as the cutoff value. Notably, with this patient classication, the negative predictive value of the CD64 index was 95.2% at cutoff of 1.8 and 91.6% at cutoff of 2.0.

TABLE 3.8: Summary of PMN CD64 index using definite, probable and possible infection as gold standard

	1.8 cut off value	1.6 cut off value	2.0 cut off value
Sensitivity	48.6%	51.4%	43.2%
Specificity	60.6%	51.5%	66.7%
PPV	58.1%	54.3%	59.3%
NPV	51.3%	48.6%	51.2%
AUC	0.5463	0.5143	**0.5495**
95% CI	0.42867 – 0.66387	0.39534 – 0.63333	0.43459 – 0.66451

The table shows sensitivity, specificity, positive and negative predictive values of CD64 index at differing cut offs. Highest area under the ROC curve when patients with definite, probable and possible infection were considered as gold standard positive was achieved using 2.0 as the cutoff value.

3.5 Correlation of PMN CD64 with other markers of infection and sepsis

There was no statistically significant correlation between the abnormal WCC, PLT and CRP with the high PMN CD64 index in this study. Thrombocytopenia was observed in 7% of the neonates enrolled for the study. Thirty six percent of the participants in the study had a low white cell count (<9.0X10^9/l). Twenty five percent of the neonates enrolled in this study had a CRP of >10 mg/L (abnormal).

4. DISCUSSION

4.1 Assessment of the utility of PMN CD64 index as early marker of infection compared to currently used infection markers.

Diagnosis of neonatal sepsis is a challenge, as there is no single reliable test for the early confirmation of definite sepsis [2, 11]

Currently blood culture is the most reliable method for detection of bacterial infections. However, the sensitivity of this method is low and using it as a gold standard in diagnosis of bacteremia is fraught with difficulties [2, 28].A study done by Magudumana *et al*. [11] revealed that less than 10% of neonates they evaluated and treated for sepsis had definite infection. It is clear that to properly manage neonates with sepsis, a single reliable marker of infection is needed, to avoid unnecessary antibiotic therapy. Several published studies have indicated that PMN CD64 is an ideal candidate for evaluation as a more sensitive and specific marker of infection [1, 10, 15, 29].

In our study, after enrolling 76 participants suspected to have sepsis by clinical signs, only one child had a positive blood culture result. An additional 5 patients had probable infection, according to categories of infection classification system, and a further 30 had possible infection.

Interpretation of the diagnostic utility of the CD64 index depends on which class of patients is used as the gold standard, and what is taken as the dichotomous "positive/negative" cut off. Using only blood culture confirmed patients as the gold standard positive group, the specificity and PPV were highest using 2.0 as a cut off level

on comparison to other cut off levels (1.6 and 1.8). Using this cut off value (2.0), PMN CD64 had high sensitivity (100%) when compared to blood culture in diagnosis of infection. The specificity was low however with this cut off value (2.0), 62.9% and this is likely due to the PMN CD64 being more sensitive than the existing gold standard (refer to Table 3.6) .

In neonatal sepsis a negative blood culture does not necessarily rule out infection. Using the patient group with both definite and probable infection as the gold standard positive group, PMN CD64 showed better specificity, sensitivity, PPV and NPV and AUC with the cut off value of 1.8 compared to other cut off values. Using this cut off, the negative predictive value of PMN CD64 was 95.2%. This is the patient group we feel most likely represents the true positive patient group, and the high negative predictive value of PMN CD64 in this group indicates that PMNCD64 may find its role in ruling out sepsis/infection when the PMN CD64 index is within the normal reference range. We believe that some of this group of babies, despite negative blood cultures, had infection/sepsis. As noted in literature, the reasons for the negative blood cultures are multiple, including possible effects of antibiotic therapy in utero [1].

Our findings concur with the outcome of numerous studies done on the diagnostic performance of PMN CD64 in sepsis in view of the high sensitivity compared to blood culture and high negative predictive value [15, 21, 30-31]. Most studies, however, report high positive predictive values which do not concur with our findings. The reason for low positive predictive value in our study could be the fact that, as this was a prospective analysis, we did not start off with patients who had proven infection like the other

studies. As a result, we had more patients without infection, and other studies had more patients with infection.

If the patients with possible infection are included with the definite and probable infection groups as the gold standard positive group (1.8 cut off), the sensitivity of PMN CD64 in diagnosis of infection decreases without an improvement in specificity. The PPV improved from 3.2 (definite infection- sepsis group 1.8 cut off value) to 58.1% (definite+probable+possible –sepsis group), but the NPV dropped to 51.3 from 100%. We, however, feel this is not the most likely representation of the true positive patient group (that is infected neonates). If we hypothesize that all patients with" possible" infection are really in need of antibiotics for microbial sepsis, then there will be no need for laboratory diagnostic markers of infection.

While our study was conducted in neonates with early onset infection ,the study done by Ng *et al*. [9] in neonates with late onset infection showed PMN CD64 had the highest sensitivity and specificity at the onset of suspected infection (95 and 88 % respectively) and at 24hrs (97 and 90 %) [10]. In the same study PMN CD64 had best overall performance compared to other infection markers.

In our study there was no statistically significant correlation between PMN CD64 and other markers of infection (CRP, WCC and PLT). This may suggests that, CD64 is not giving redundant information already given by CRP, WCC and PLT, but represents an independent variable with additional potential clinical impact.

4.2 Does PMN CD64 index have a role to play in helping physicians to commence or discontinue antibiotics

We could not answer the above question in this study, as the clinical follow up was a challenge. However, our study has shown that the PMN CD64 may be of value in ruling out infection, as evidenced by NPV of 92-100%. The high NPV was noted at all the different cut off values; that is 1.6; 1.8 and 2.0 using ROC curves, when the gold standard positive groups included definite or definite + probable infection. The above findings support the hypothesis that if the PMN CD64 is within the normal reference range, the paediatrician can likely discontinue antibiotics.

Current practice in the three hospitals where we conducted the study is to use serial CRP (at 24-48 hours), to help the paediatricians decide to stop or continue antibiotic therapy. In future studies, the PMN CD64 should be directly compared to serial CRP as an indicator of when to discontinue antibiotics. We could not directly assess the utility of once-off CRP as an independent predictor of sepsis, as it was one of the criteria used to stratify patients.

One study has reported that individuals of African ancestry have increased CD64 expression on their resting neutrophils, but this has not been confirmed [10]. In validation of the Leuko-64 kit using samples from healthy adult controls, we found that 3/20 (15%) of our volunteers had PMN CD64 index higher than 1, but less than 2. These donors had no history to suggest infection. There could be a genetic link for slightly

higher PMN CD64 in some of our healthy adult volunteers. The second reason for the higher PMN CD64 expression could be increased exposure to infectious diseases in our setting (developing country). In view of the above, our normal range for PMN CD64 expression might be slightly higher compared to first world countries.
As suggested by the manufacture of the Leuko-64 kit we used in this study, each laboratory should confirm its own reference range.

4.3 Limitations of the study

4.3.1 Neonatal healthy controls

Due to ethical dilemmas of neonatal research, we could not access healthy neonatal controls. Initially we received ethics clearance to use blood already taken for follow-up blood in neonate with no suspicion of infection but having physiological jaundice. We struggled to get the samples because in these neonates, paediatricians used heel prick sample, not venous blood. We therefore, could not use heel prick sample to determine a reference range for a venous sample. We used normal values as prescribed by the manufacture of the commercial kit. Therefore, we could not be absolutely sure if what we call normal was really normal for our setting

The next step we took was to review the blood results on the laboratory information system of all neonates with no evidence of infection and retrieve their blood specimen for PMN CD64 quantitation. We approached the Witwatersrand University to allow us to do this under research and development umbrella (meaning that we did not have to ask for

consent from the parents). Unfortunately with both methods we could only manage to get two healthy neonatal controls in a period of thirteen months.

The control from the first method had a PMN CD64 index of 1.87 (slightly higher than the normal reference range of <1.8. As we failed to access age-matched controls, this prevented us from confirming the manufacturer (Trillium Diagnostics, Leuko-64 kit) recommended neonatal reference range of PMN CD64 index (1.8) in our setting. However, as the sample population in whom the test is intended is not healthy neonates, but neonates with signs suggestive of infection, our control group of neonates who were eventually classified as "infection unlikely" is the more appropriate control group for evaluation of PMN CD64 as a diagnostic assay.

4.3.2 Reasons why not all neonates who were screened could not be enrolled to the study

A. Consent:

Twelve parents (either both mom and dad, or just one parent- mother or father), refused consent. Some mothers we not well enough to give consent at the time of screening. The dilemma here was that we could only use the sample within 2 days (48hours), and if we did not have the consent within that time, we lost the opportunity to use that sample.

B. Failure to locate the mother in within 48 hours of sample collection.

There were multiple reasons for failure to locate the care-giver within 48 hours of sample collection, including the following:

- Mother not at the hospital, and could not reach her telephonically
- Mother reached telephonically or by the cell, but by the time the mother comes to the hospital it was too late to use the sample.
- The caregiver was not available with neonates referred from other hospitals or primary health care centers.

C. Technical:

In total five samples were clotted and were unsuitable for use.

In one sample the monocyte positive control failed and another one, the lymphocyte negative control failed. The reason for both failures was not found.

4.3.3 Lack of follow up and clinical correlation

Due to logistic concerns, and involvement of many different clinicians and confounding multiple variables if patients are followed up longitudinally, such as later nosocomial infections, follow up data and final treatment decisions were not used as outcome variables in this study.

4.3.4 Failure to access the samples from neonates with late onset sepsis

The objective of this study was to assess the utility of PMN CD64 in diagnosis of early and late onset sepsis. We failed to acquire enough samples from suspected late onset sepsis to assess the utility of PMNCD64 in late onset sepsis; as this depended on the paediatrician on duty. The latter, we believe contributed to finding only one positive blood culture in our study.

4.3.5 PMN CD64 and Human immunodeficiency virus (HIV)

In our study we did not look a HIV status and PMN CD64. In our setting there is high prevalence of HIV. The confounding effects of HIV on the CD64 index cannot be excluded.

4.4 Strengths of the Study

This study was a prospective study conducted in paediatric patients, in whom venous blood samples are difficult to access. We assessed the utility of PMN CD64 index in the most diagnostically relevant cohort to study – a cohort of patients with suspected sepsis. We analysed the PMN CD64 results in comparison with well defined categories of infection based on objective variables such as temperature, respiratory rate, heart rate, white cell count and CRP rather than subjective physician opinion to start or stop antibiotic therapy.

5. CONCLUSIONS

We recommend the inclusion of PMN CD64 index into the diagnostic algorithm for neonatal sepsis. A negative PMN CD64 result had a high negative predictive value in ruling out definite (100%) or probable + definite infection (95.2%) with the 1.8 cut off value. Results of PMN CD64 should be available in a much shorter time than the blood culture. As the Positive predictive value of the test was low in confirming infection, PMN CD64 should be used as a screening rather than confirmatory test for infection. We believe PMN CD64 should be used in combination with other tests for infection such as FBC, DIFF, CRP and PCT.

6. APPENDICES

APPENDIX A

TABLE A1: PEDIATRIC AGE GROUP DEFINITIONS

Newborn	0-7 days
Neonate	7-28 days

Age range definitions of the newborn and the neonate.
Adapted from Pediatr Crit Care Med 2005 Vol. 6, No1 Page 3

TABLE A2: DEFINITION OF PEDIATRIC SEPSIS

SIRS – need at least 2 of the following 4 criteria, 1 of which must be abnormal temperature or white cell count (WCC)	1. Core temperature >38.5 or <36 ºC 2. Tachycardia >2 standard deviation (SD) above normal for age OR for children <1 year old bradycardia 3. Mean respiratory rate > SD above normal for age or mechanical ventilation 4. High or low WCC for age or >10% immature neutrophils
Infection	Suspected or proven (for example positive microbiological culture), caused by any pathogen or clinical syndrome with high probability of infection
Sepsis	SIRS in the presence of or as a result of suspected or proven infection
Severe sepsis	Sepsis plus one of the following: cardiovascular organ dysfunction, acute respiratory distress syndrome, or two or more of other organ dysfunction
Septic shock	Sepsis and cardiovascular organ dysfunction as defined in Table A4. Sepsis induced hypotension despite adequate fluid resuscitation (isotonic fluid ≥40mL/kg in 1 hr) or need for vasoactive drug to maintain blood pressure

Specific definitions of pediatric sepsis continuum; including systemic inflammatory response syndrome (SIRS), infection, sepsis, severe sepsis and septic shock.
Adapted from Pediatr Crit Care Med 2005 Vol. 6, No1 Page 4

TABLE A3: AGE-SPECIFIC VITAL SIGNS

	Heart Rate,	Beats/min	Respiratory Rate	WCC,	Systolic Blood
Age group	Tachycardia	Bradycardia	Breaths/min	Leukocytes x $10^3/mm^3$	Pressure (BP), mm Hg
0-7 days	>180	<100	>50	>34	<65
7-28 days	>180	<100	>40	>19.5 or <5	<75

Cut offs for abnormal vital signs for newborns and neonates.
Adapted from Pediatr Crit Care Med 2005 Vol. 6, No1 Page 4

TABLE A4: ORGAN DYSFUNCTION CRITERIA

Cardiovascular dysfunction, when despite adequate administration of isotonic intravenous fluid the following occur, that is A or B	A: 1. Hypotension 2. Need for vasoactive drug to maintain blood pressure in normal range
	B: Two of the following- 1. Unexplained metabolic acidosis 2. Increased arterial lactate 3. Oliguria 4. Prolonged capillary refill 5. Core to peripheral temperature gap
Respiratory – presence of one of the following:	❖ $PaO_2/F_{IO2}<300$ ❖ $PaCO_2$ >65 torr or 20mm Hg over baseline $PaCO_2$ ❖ Proven need or >50% F_{IO2} to maintain saturation ≥92% ❖ Need for nonelective invasive or non-invasive mechanical ventilation
Neurologic - presence of one of the following:	a. Glasgow Coma Scale (GCS) ≤11 b. Acute change in mental status with decrease in GCS ≥3 points from abnormal baseline
Hematologic – presence of one of the following:	1. Platelet count of <80,000/mm^3 or decline of 50% in platelet count from highest value recorded over the past 3 days 2. International normalized ratio >2
Renal	Serum creatinine ≥2 times upper limit of normal for age or 2-fold increase in baseline creatinine
Hepatic – presence of the one of the following:	▪ Total bilirubin ≥4mg/dl (NA for newborn) ▪ Alanine transaminase 2 times upper limit of normal for age

Criteria for organ dysfunction used to classify categories of the severity of sepsis as represented by Table A2.
Adapted from Pediatr Crit Care Med 2005 Vol. 6, No1 Page 5

APPENDIX B

TABLE B1: Data sheet

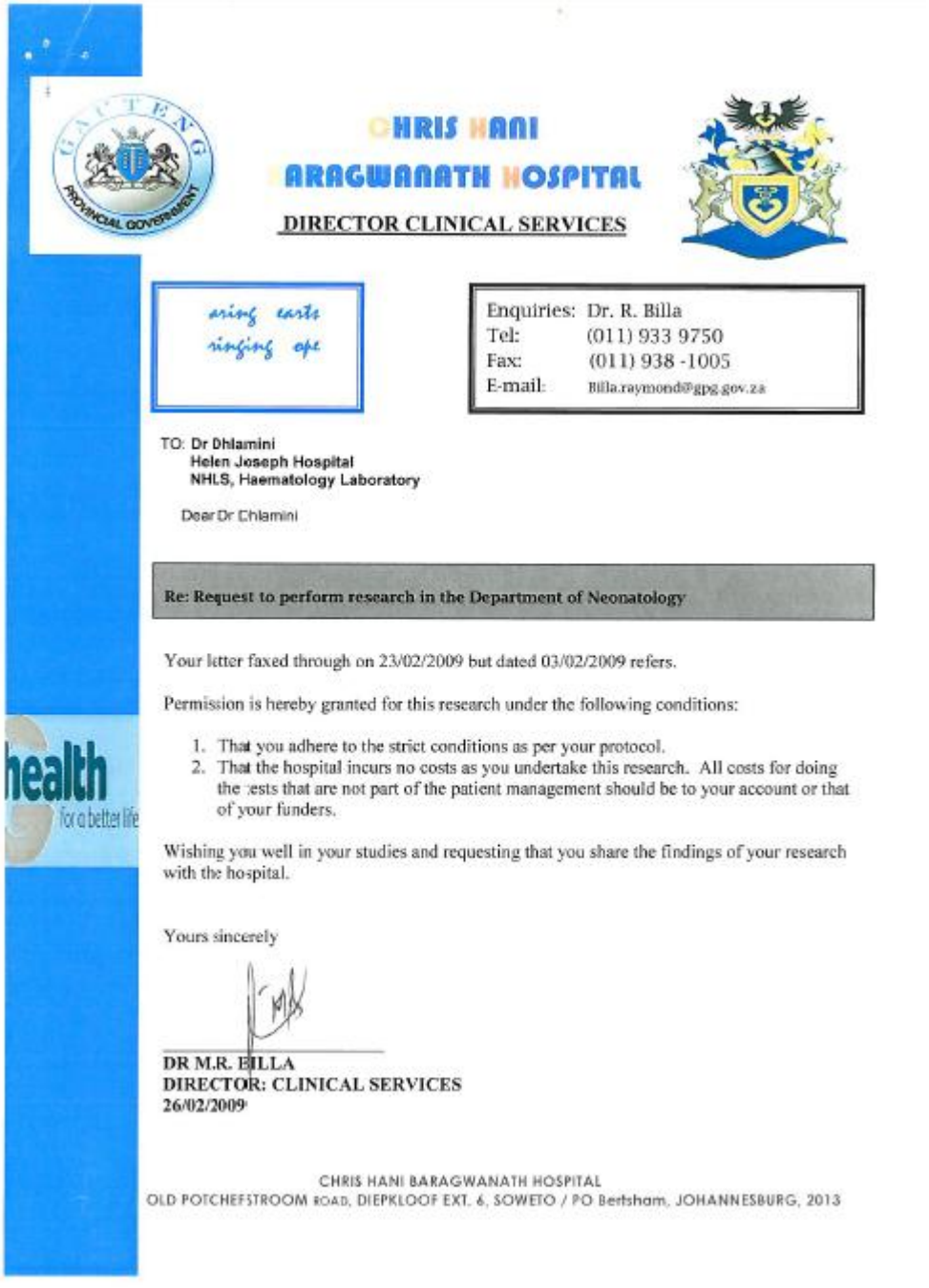
Study Number			
Birth Weight		Gestation age	
Gender		Mode of delivery	
Date of SWU			
Maternal risk factors			
PROM		PPROM	
Pyrexia		Chorioamnionitis	
Foul smelling liquor		GBS carrier	
Antibiotic therapy			
Indication for SWU			
Signs of infection			
Lethargy		Abdominal distension	
Vomiting		Aspirates	
Bloody stools		Loose stools	
Respiratory distress		Increasing oxygen requirements	
Seizures		Pyrexia	
Hypothermia		Hyperglycaemia	
Hypoglycaemia		Tachypnoea (>60bpm)	
Failure to wean oxygen		Apnoea	
Tachycardia (> 160 bpm)		Skin infection (abcess)	
Other (specify)			
Results			
Hb		WCC	
Platelets		Differential count	
CRP			
Blood culture			
CSF		CSF culture	
Urine		Urine culture	
Sepsis classification			
PMNCD64			

Clinical data recorded from each patient.

SWU: Septic work up (Full blood count, differential count, blood culture, CRP).
CSF and urine were not always done as part of the SWU in this study.
PMN: Polymorphonuclear cell
PROM: Preterm rupture of membranes
PPROM: Prolonged preterm rupture of membranes

APPENDIX C: 3 Letters from the three hospitals chief executives officers

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CHRIS HANI
BARAGWANATH HOSPITAL
DIRECTOR CLINICAL SERVICES

arising earth
ringing ope

Enquiries: Dr. R. Billa
Tel: (011) 933 9750
Fax: (011) 938 -1005
E-mail: Billa.raymond@gpg.gov.za

TO: Dr Dhlamini
Helen Joseph Hospital
NHLS, Haematology Laboratory

Dear Dr Dhlamini

Re: Request to perform research in the Department of Neonatology

Your letter faxed through on 23/02/2009 but dated 03/02/2009 refers.

Permission is hereby granted for this research under the following conditions:

1. That you adhere to the strict conditions as per your protocol.
2. That the hospital incurs no costs as you undertake this research. All costs for doing the tests that are not part of the patient management should be to your account or that of your funders.

Wishing you well in your studies and requesting that you share the findings of your research with the hospital.

Yours sincerely

DR M.R. BILLA
DIRECTOR: CLINICAL SERVICES
26/02/2009

CHRIS HANI BARAGWANATH HOSPITAL
OLD POTCHEFSTROOM ROAD, DIEPKLOOF EXT. 6, SOWETO / PO Bertsham, JOHANNESBURG, 2013

GAUTENG PROVINCIAL GOVERNMENT

DEPARTMENT OF HEALTH

health for a better life

Department of Health
Lefapha la Maphelo
Departement van Gesondheid
Umnyango wezeMpilo

RAHIMA MOOSA MOTHER AND CHILD HOSPITAL
PRIVATE BAG X20
NEWCLARE
2112

Dr. M. B. Chlamini
Pathologist
NHLS

Dear Dr. Dhlamini

Re: Permission to conduct a study re: "Neutrophil CD64 in Neonateal Sepsis" in the Department of Neonatology at Rahima Moosa hospital

Permission is granted for you to conduct the above research as indicated in you request provided:

1. The Rahima Moosa hospital will not in anyway incur or inherit costs as a result of the said study.
2. Your study shall not disrupt services at the study site.
3. Strict confidentiality shall be observed at all times.
4. Informed consent shall be solicited from patients participating in your study.
5. NO file should leave the records department and/or the hospital premises.

Arrangement will be made with recordkeeping clerks so that you could occupy a space in their department.

Kindly forward this office with the results of your study on completion of the research.

Yours sincerely,

CHIEF EXECUTIVE OFFICER
S./cj. 2009-06-04
(011) 470-9030
Cynthia.Jaars@gauteng.gov.za

Private bag X39, Johannesburg 2000, South Africa
Tel: +27 (0) 11 488 4911, Fax: +27 (0) 11 640 1602
www.johannesburghospital.org

Gauteng Department of Health

Office of the CEO
Enquiries: M. Motjelele
(011): 488-3792 / 3
(011) 488-3753

05th February 2009

Dr Matshediso B Dhlamini
Pathologist
NHLS

Dear Dr Dhlamini

RE: Permission to Conduct a Study re: "Neutrophil CD64 in Neonatal Sepsis" in the Department of Neonatology at Charlotte Maxeke Johannesburg Academic Hospital.

Permission is granted for you to conduct the above research as indicated in your request provided:

1. The Charlotte Maxeke Johannesburg Academic hospital will not in anyway incur or inherit costs as a result of the said study.
2. Your study shall not disrupt services at the study sites.
3. Strict confidentiality shall be observed at all times.
4. Informed consent shall be solicited from patients participating in your study.

Please liaise with the Head of Department and Unit Manager or Sister in Charge to agree on the dates and time that would suit all parties.

Kindly forward this office with the results of your study on completion of the research.

Yours sincerely

Dr. S. B. Mfenyana
Acting Chief Executive Officer

Charlotte Maxeke Johannesburg Academic Hospital

APPENDIX D: Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dhlamini

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M081106**
PROJECT	Evaluation of Neutrophil CD64 in Neontal Sepsis
INVESTIGATORS	Dr MB Dhlamini
DEPARTMENT	Department of Haematology
DATE CONSIDERED	08.11.28
DECISION OF THE COMMITTEE*	

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof D Ballot

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX E
Neonatal non-infection controls and Information Sheet

Dear Parent/Guardian

Hello, my name is Matshediso Bernice Dhlamini, a doctor in the Department of Molecular Medicine and Haematology at the National Health Laboratory Service, Helen Joseph Hospital.

Together with two supervisors Prof D Ballot, a principal neonatologist at Neonatal Intensive Care Unit of Charlotte Maxeke Johannesburg Academic Hospital and Dr Tracey Wiggill, acting principal pathologist, Department of Molecular Medicine and Haematology, we are performing a study on newborn babies with possible infection.

New babies are less able to fight infection than older children. At present it is difficult to prove infection in newborn babies as there is no simple reliable test to do this. The aim of this study is to look at a new test called Neutrophil /PMN CD64 which may help to prove infection as early as possible and can help show which newborn babies will need antibiotics.

If blood needs to be taken from your baby for any other condition and your baby is free of infection, your baby can be included in this study as a non-infective control. We would like to perform the quantitation of neutrophil/PMN CD64 on the blood of your baby as part of our neonatal non-infection controls to validate the Leuko CD64 kit used in this study. A single tube of EDTA blood (0.5ml) will be taken from your baby with your permission.

Taking part in the study is entirely up to you. If you do not want your baby to take part, this will not affect the care of your baby in any way. You are also free to remove your baby from the study at any time.

If you are happy to allow your child to take part in this study as a non-infection control, please sign the consent form below. Please contact me if you have any queries/questions about the study.

Thank you, Dr MB Dhlamini.

Informed Consent Form

I hereby confirm that I have been informed by the study doctor, Matshediso Bernice Dhlamini and/or a medical representative, about the nature, conduct, benefits and risks of this study (Evaluation of neutrophil CD64 in neonatal sepsis).

I has also received, read and understood the above written information regarding the study. I am aware that the information about the study will be anonymously processed into a study report. I may at any stage, without prejudice, withdraw my consent and participation in the study.

I …….
Parent/ guardian/ caregiver of ……..agree to allow my child to participate in your study as a non-infection control .

Signature:
Date and time:
Place:

I, Dr MB Dhlamini, herewith confirm that the parent of the baby with physiological jaundice participating in this study has been fully informed about the nature, conduct and risks of the above study.

Signature of the study doctor:
Date and time:

Research person/s: Dr MB Dhlamini
Tel: 011 489 0732 / Cell 083 5785361
Dr T Wiggill Tel: 011 489 8533
Prof D Ballot Tel: 011 488 4232

Volunteer healthy adult controls Information Sheet

Dear Participant
Hello, my name is Matshediso Bernice Dhlamini, a doctor in the Department of Molecular Medicine and Haematology at the National Health Laboratory Service, Helen Joseph Hospital.

Together with two supervisors Prof D Ballot, a principal neonatologist at Neonatal Intensive Care Unit of Charlotte Maxeke Johannesburg Academic Hospital and Dr Tracey Wiggill, acting principal pathologist, Department of Molecular Medicine and Haematology, we are performing a study on newborn babies with possible infection.

New babies are less able to fight infection than older children. At present it is difficult to prove infection in newborn babies as there is no simple reliable test to do this. The aim of this study is to look at a new test called Neutrophil /PMN CD64 which may help to prove infection as early as possible and can help show which newborn babies will need antibiotics.

We would like to perform the quantitation of neutrophil/PMN CD64 on your blood as part of our adult healthy controls to validate the Leuko CD64 kit used in this study. To participate you need to be healthy and not currently pregnant. A single tube of EDTA blood will be taken and used in the study with your consent.

Please note that this study is completely anonymous and confidentially will be maintained. Participation in this study is voluntary.

To participate you will be given a short questionnaire (to ascertain your health).
If you are happy to participate in the study outlined above, please sign the consent form below.

Informed Consent Form

I hereby confirm that I have been informed by the study doctor, Matshediso Bernice Dhlamini, about the nature, conduct and discomforts of this study (Evaluation of neutrophil CD64 in neonatal sepsis).

I has also received, read and understood the above written information regarding the study. I am aware that the information about the study will be anonymously processed into a study report. I may at any stage, without prejudice, withdraw my consent and participation in the study.

I …….
Agree to participate in your study as a healthy adult control.

Signature:
Date and time:
Place:

I, Dr MB Dhlamini, herewith confirm that the above healthy adult participating in this study has been fully informed about the nature, conduct and risks of the above study.

Signature of the study doctor:
Date and time:

Research person/s: Dr MB Dhlamini
Tel: 011 489 0732 / Cell 083 5785361
Dr T Wiggill Tel: 011 489 8533
Prof D Ballot Tel: 011 488 4232

APPENDIX F
Healthy adult control Questionniare

Age :

Sex :

Race :

Are you currently healthy?

Have you had a recent flu-like illness?
If yes, please specify when?

Medical history- Do you have a history of any illness?

Current medication (including pain killers such as aspirin, panado, etc.)

APPENDIX G

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Academic & Research)

MEMORANDUM

TO: **Dr MB Dhlamini:**
Department of Haematology
EMAIL: matshediso.dhlamini@nhls.ac.za

FROM: Ms Anisa Keshav
Secretary: Human Research Ethics Committee (Medical)
Tel 717-1234 fax 011 717 1265
e-mail: anisa.keshav@wits.ac.za

DATE: 12 December 2008

REF: ***R14/49***

The protocol below was considered at a meeting of the Human Research Ethics Committee (Medical) on **Friday 28 November 2008.** The Committee requires the following amendments/corrections/information from you before your application can be approved.

M081106: Evaluation of Neutrophil CD64 in Neonatal Sepsis
-Complete the ethics application in full-5.2 fill in properly, 6.1 tick that records will be reviewed and blood sampling
-The information sheet-needs a title and the contact details of the Ethics Committee Chairman for participants
-On the data collection sheet-remove identifying information and use a code instead

Please let me have the amendments as soon as possible as protocols on which no action has been taken will be removed from the agenda without approval after two months.

Please highlight any changes made and send two copies to this office.

APPENDIX H

MB Dhlamini
Department of Molecu ar Medicine and Haematology
NHLS and WITS Medical School
4 May 2009

Prof Cleaton-Jones
Human Research Ethics (Medical)
Committee of the University of the
Witwatersrand, Medical School

Dear Prof and members of the committee

Re: Amendments to the ethics of Protocol Number M081106
Project: Evaluation of Neutrophil CD64 in neonatal sepsis.

We have previously received ethics approval for the above mentioned research.
Now we are requesting ethics approval for the following amendments:

1. Measuring of neutrophil/PMN CD64 in 20 healthy normal controls to validate the Leuko 64 kit we are using in this study. Participants will be given a questionnaire, information sheet and consent forms before being entered into the study.
2. As the neutrophil CD64 index is slightly higher in neonates, we are also planning to do age-matched controls, that is 20 neonatal controls without infection being followed up with blood sampling for other conditions such as physiological neonatal jaundice. Parents/care givers will be given a questionnaire, information sheet and a consent form before being entered into the study.

Your favourable consideration will be highly appreciated.

Kind regards,
Dr MB Dhlamini
Specialist Haematologist
National Health Laboratory Service
Helen Joseph Hospital
Tel : 011 489 0732
Cell : 0835785361

APPENDIX I

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))
Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5709
Private Bag 3, Wits 2050, South Africa

1 June 2009

Dr MB Dhlamini
Molecular Medicine & Haematology
National Health Laboratory Services
Helen Joseph Hospital
University

Dear Dr Dhlamini

RE: PROTOCOL M081106

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the following on the abovementioned protocol:

- Addition of control group
- 0.5ml extra blood taken during normal diagnostic venesection-no extra 'pricks'
- Collection of <5ml from health adults to validate kit
- All new consents provided

Thank you for keeping us informed and updated.

Yours sincerely,

Anisa Keshav
Secretary
Human Research Ethics Committee (Medical)

APPENDIX J

Department of Molecular Medicine
and Haematology
University of Witwatersrand
Johannesburg
South Africa
24th May 2010

Prof Cleaton-Jones
Human Research Ethics (Medical)
Committee of the University of the
Witwatersrand, Medical School

Dear Prof Cleaton-Jones

Re: An additional project under the Research and Development (R&D) Programme in the Department of Molecular Medicine and Haematology, University of the Witwatersrand

The department of Molecular Medicine and Haematology currently holds blanket ethics approval for its (M00-01-07), represented by Lesey Scott), which involve validation studies and the development of new diagnostic assays. This blanket approval covers projects that will make use routine specimens received in the NHLS for routine diagnostic testing. Once the routine testing is complete, R&D has access to these specimens. No patient demographics are required other than the result of the test being validated (or developed). No additional patient specimens are received for the R&D testing. To date this approval has been granted for projects such as CD4 validation, HIV viral load validation, serology validations, coagulation assay development etc.

We would like to add another R&D project to this list:
Project title: Evaluation of Neutrophil CD64 in neonatal sepsis.

This is in response to application for amendment to the ethics of Protocol Number M081106 with regards to age-matched controls for validation of the Leuko-64 kit (Trillium Diagnostics, LCC). The Leuko-64 kit is used to quantify neutrophil (PMN) CD64 levels which are upregulated in response to inflammation and tissue injury. Sepsis affects millions of individuals annually, with associated mortality rate, and it is among the top 10 causes of death. We evaluated the Leuko-64 kit using peripheral blood samples from healthy adult volunteers prior to assessing the utility of this kit in neonatal sepsis. However, as per manufacturer's instructions age-matched controls, that is 20 neonatal controls are required for proper validation of the kit, as the neutrophil CD64 index is slightly higher in neonates than in adults. R&D specimens (neonatal peripheral blood sent for routine testing) will be used for evaluation of neutrophil CD64. No additional patient information or specimens will be required other than age (confirm neonatal blood) and laboratory final result.

APPENDIX K: 2 MMED individual research grants

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Research Office, Faculty of Health Sciences

MMED INDIVIDUAL RESEARCH GRANTS

Awardee: DHLAMINI MATSHEDISO BERNICE

Department/School: Mol Med and Haematology

Project Title: Evaluation of Neutrophil CD64 in Neonatal Sepsis

Grant Number:
| 001 | 254 | 8468101 | 5121105 | 000000 | 4000000000 | 4678 |

Grant Awarded (R):	MMED
Note that any VAT incurred on either the running or equipment costs should be identified separately	R20 000,00
TOTAL (R)	**R20 000, 00**

Please Note:

1. The award of this grant does not override the requirement, in appropriate cases, to obtain prior approval from one or more of the University's three Ethics Committees, or the Biosafety Committee, as the case may be.
2. No grant may be used for purposes other than those specified in the application approved by the University without written consent of the Head of the Research Office.
3. The monies awarded to you must be spent in the year for which they were awarded. A written request for the carry forward of unspent funds must be submitted to this office not later than **31st January 2010.**
4. The support of the above grant must be acknowledged in any publications arising out of this research project.
5. A progress report must be submitted to the (Faculty Research Office) not later than **31 December 2009**, irrespective of whether continued funding is to be sought
6. Please contact Ms Nivien Subramany (Finance) 717 2055 with any queries re accessing your grant
7. Please note that your grant will not be activated until the following conditions have been met: N/A

C. Hlungwani
For: Chair of the FRC

Date: 29th May 2009

cc. Grantee
Head of School
Ms Nivien Subramany: Finance

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Research Office, Faculty of Health Sciences

FACULTY RESEARCH COMMITTEE INDIVIDUAL RESEARCH GRANTS

Awardee: Dr DHLAMINI MATSHEDISO BERNICE

Department/School: Molecular Med & Haem

Project Title: Evaluation of Neutrophil CD64 in Neonatal Sepsis

Grant Number:
001 254 8468101 5121105 000000 0000000000 4678

Grant Awarded (R):	**MMED**
Note that any VAT incurred on either the running or equipment costs should be identified separately	R 20,000.00
TOTAL (R)	**R 20,000.00**

Please Note:

1. The award of this grant does not override the requirement, in appropriate cases, to obtain prior approval from one or more of the University's three Ethics Committees, or the Biosafety Committee, as the case may be.
2. No grant may be used for purposes other than those specified in the application approved by the University without written consent of the Head of the Research Office.
3. The monies awarded to you must be spent in the year for which they were awarded. A written request for the carry forward of unspent funds must be submitted to this office not later than 31st **January 2011.**
4. The support of the above grant must be acknowledged in any publications arising out of this research project.
5. A progress report must be submitted to the (Faculty Research Office) not later than **31 December 2010**, irrespective of whether continued funding is to be sought
6. Please contact Ms Nivien Subramany (Finance) 717 2055 with any queries re accessing your grant
7. Please note that your grant will not be activated until the following conditions have been met: N/A

C. Hlungwani
Faculty Research Office
Tel: 011 717 2023

Date: 13th April 2010

For: Chair of the FRC

cc. Grantee
Head of School
Ms Nivien Subramany: Finance

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