

https://dspace.org/

The usefulness of the extended differential parameters as a biomarker of bacteraemia at Chris Hani Baragwanath Academic Hospital

Lemkus, Lauren

2021
https://hdl.handle.net/10539/33695

Downloaded from a DSpace repository. https://dspace.org/

THE USEFULNESS OF THE EXTENDED DIFFERENTIAL PARAMETERS AS A BIOMARKER OF BACTERAEMIA AT CHRIS HANI BARAGWANATH ACADEMIC HOSPITAL

Dr Lauren Lemkus

A research report (in the format of a "submissible" paper) submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for degree of Master of Medicine in the branch of Haematology.

Johannesburg 2021.

Table of Contents

DECLARATION i
CONTRIBUTION OF THE CANDIDATE TO THE PAPER ii
ACKNOWLEDGEMENTS iii
LIST OF FIGURES iv
LIST OF TABLES v
NOMENCLATURE vi
RESEARCH ARTICLE IN THE FORMAT OF 'SUBMISSIBLE' PAPER 1
TITLE PAGE 2
ABSTRACT 3
1. INTRODUCTION 5
2. METHODS 10
2.1 Sample collection and processing 10
2.2 Sample classification 11
2.3 Statistical analysis 12
2.4 Ethics 13
3. RESULTS 14
3.1 Study population 14
3.2 Receiver operating characteristic curve analysis 17
3.3 Cohen's Kappa co-efficient 18
3.4 Analysis of the correlation between the NE-SFL and MO-Y and neutrophil and monocyte CD64 expression respectively 19
3.5 Analysis of discordant automated immature granulocyte counts compared to manual counts, particularly in HIV positive patients 20
4. DISCUSSION 21
5. CONCLUSION 24
REFERENCES 25
SUPPLEMENTARY METHODS 30
SUPPLEMENTARY DATA 35
APPENDIX I 36
Ethics approval 36
APPENDIX II 38
Research protocol 38
APPENDIX III 62

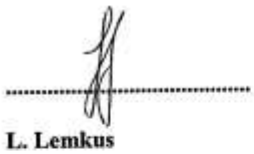
Turn-it-in report **62**

APPENDIX IV **71**

PLOS ONE submission guidelines **71**

DECLARATION

I, Lauren Lemkus, declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Medicine in the branch of Haematology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



L. Lemkus

10 May 2021

CONTRIBUTION OF THE CANDIDATE TO THE PAPER

Declaration: Student's contribution to the article and agreement of co-authors

I, Lauren Lemkus, student number 457987, declare that this Research Report is my own work and that I contributed significantly towards the research findings presented in the paper intended for the publication below.

Signature of student: 

Date: 10/5/2021

Primary supervisor: Dr J Vaughan **Signature:** 

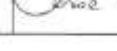
Date: 12/05/21

Co-supervisor: Dr D Lawrie **Signature:** 

Date: 12/5/2021

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of her Research Report.

Article title: The usefulness of the extended differential parameters as a biomarker of bacteraemia at Chris Hani Baragwanath academic hospital.

Author	Name	Signature	Date
1st author	Lauren Lemkus		10/5/2021
2nd author	Jenifer Vaughan		12/5/21
3rd author	Denise Lawrie		12/5/2021

ACKNOWLEDGEMENTS

I would like to thank the following people for their dedication and support in the creation of this manuscript:

- Dr Jenifer Vaughan, “Mentoring is a brain to pick, an ear to listen and a push in the right direction.” – John Crosby.
- Dr Denise Lawrie, “Make it simple, but significant.” – Don Draper.
- Professor Debbie Glencross, “Tell me and I forget, teach me and I may remember, involve me and I learn.” ― Benjamin Franklin.
- The National Health Laboratory Services for funding the study.
- Staff of the flow cytometry laboratory at Charlotte Maxeke Johannesburg Academic Hospital for their guidance and help with the running of samples.

LIST OF FIGURES

Figure 1. Graphs depicting the linear regression analysis between NE-SFL and the ratio of neutrophil to lymphocyte CD64 expression (A) and between MO-Y and the ratio of monocyte to lymphocyte CD64 expression (B)……20

Supplementary Figure 1. Live gating strategy for identifying populations of interest, namely granulocytes, monocytes and lymphocytes using CD45 versus side scatter (B)……31

Supplementary Figure 2. Flow cytometry live gating strategy to identify populations of interest, namely granulocytes, monocytes and lymphocytes (b). These feed into CD64 expression histograms for neutrophils (a) and lymphocytes (c), from where the ratio of CD64 expression was calculated…….33

LIST OF TABLES

Table 1. Biometrics and extended differential parameter data…….…....16

Table 2. Receiver operating characteristic curve analysis assessing the various biomarkers among patients with bacteraemic infection compared to those with no evidence of infection……...…….18

Supplementary Table 1. Comparison of extended differential parameters and flow cytometric ratio results for the three sample groups, namely bacteraemic, non-bacteraemic and no infection samples…….....…….32

Supplementary Table 2. Receiver operating characteristic curve analysis assessing the various biomarkers among patients with bacteraemic infection compared to those with non-bacteraemic

infection……...……..……...35

NOMENCLATURE

AUC - Area under the curve

CRP- C-reactive protein

FBC - Full blood count

HIV - Human immunodeficiency virus

ICU - Intensive care unit

MO-Y - Monocyte fluorescence

NE-SFL - Neutrophil fluorescent light intensity

NE-WY - Neutrophil fluorescent light distribution width

ROC – receiver operating characteristic

TB – Tuberculosis

RESEARCH ARTICLE IN THE FORMAT OF ‘SUBMISSIBLE’ PAPER

TITLE PAGE

The usefulness of the extended differential parameters as a biomarker of bacteraemia at Chris Hani Baragwanath Academic Hospital.

Authors: Lauren Lemkus, MBBCh; Jenifer Vaughan, FcPath (Haem), MMed Haem (Wits); Denise Lawrie PhD, MSc Medicine (Wits)

Affiliations: Department of Molecular Medicine and Haematology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa; and National Health Laboratory Services, Johannesburg, South Africa.

Corresponding author: Lauren Lemkus (e-mail: lauren.lemkus@nhls.ac.za / laurenlemkus@gmail.com; telephone: +2711-489-8568 / +27724294433)

Acknowledgements: The authors would like to thank the National Health Laboratory Services for funding the study, Professor D Glencross for her input, as well as the staff of the flow cytometry laboratory at the Charlotte Maxeke Johannesburg Academic Hospital for their guidance and help with the running of samples.

Author contributions: L Lemkus collected the data, performed data analysis and wrote the manuscript. J Vaughan designed the study, advised on data analysis and critically reviewed the manuscript, and D Lawrie assisted with the flow cytometry optimisation and analysis, and critically reviewed the manuscript.

ABSTRACT

Introduction:

Extended differential parameters are generated with the automated differential count by Sysmex XN-series automated haematology analysers. Within the extended differential parameters generated are the immature granulocyte count, the neutrophil fluorescent light intensity (NE-SFL) and the neutrophil fluorescent light distribution width (NE-WY), which have been proposed as early biomarkers of bacteraemia. This study aimed to evaluate the NE-SFL, NE-WY and immature granulocyte percentage in comparison to neutrophil CD64 expression, a high quality but expensive sepsis biomarker, among patients with suspected bacterial sepsis at the Chris Hani Baragwanath Academic Hospital in Johannesburg, South Africa.

Methods:

A daily search of the laboratory information system from the 22/08/2019 to 08/10/2019 identified samples submitted for a blood culture and a concurrent full blood count. An automated differential count using a Sysmex XN-9000 haematology analyser and neutrophil CD64 expression by flow cytometry were run on the residual sample.

Results:

A total of 151 samples were collected, of which 87 were excluded due to equivocal results with regards to the presence of bacterial infection. The remaining 64 samples included 21 with bacteraemia, 19 with evidence of non-bacteraemic infection and 9 with no evidence of infection. The extended differential parameters were all significantly higher among patients with bacteraemia as compared to those without infection, and on receiver

operating characteristic (ROC) curve analyses, all showed good performance (Area under the curve (AUC) >0.8). Overall, the NE-SFL performed best, with an AUC of 0.87, sensitivity of 85.7% and specificity of 89.1% (cut-off value 49.75). In comparison to neutrophil CD64 expression, the NE-SFL showed moderate agreement (kappa=0.51).

Conclusion: In this study, the NE-SFL showed promise as an inexpensive biomarker of bacteraemia in the resource constrained setting.

Keywords:

Sepsis biomarkers, Extended differential parameters, Sysmex, Tuberculosis, bacteraemia.

1. INTRODUCTION

Bacteraemia is defined by the presence of live bacteria circulating within the bloodstream.[1] Bacteraemia develops when bacteria succeed in evading the immune response of the host or when the immune response is insufficient to halt bacterial spread due to intrinsic or acquired immune defects that are associated with susceptibility to infection [2]. Sepsis is defined as severe organ dysfunction caused by a dysregulated response of the patient to an infection. If bacteraemia and sepsis are not identified early and rapidly managed, they can result in septic shock, multiple organ failure and eventual death [3]. Bacterial sepsis is estimated to affect >30 million people globally every year, being potentially lethal in up to six million cases.[4] A positive blood culture test is considered to be the gold standard for diagnosing bacteraemia. However, a significant limitation of the blood culture is the lengthy incubation period,[4] and as a result additional laboratory tests (septic biomarkers) are often used to screen for infection earlier in its course. Commonly used septic biomarkers include the full blood count (FBC) and differential count (to detect leucocytosis and neutrophilia), peripheral blood smear review (to detect morphological features of infection such as toxic granulation and band cells), C-reactive protein (CRP) and the soluble serum marker procalcitonin.[5] However, these tests are not specific to infective processes only. For example, procalcitonin reportedly has an excellent negative predictive value but may not be effective in the initial diagnosis nor monitoring of disease progress because levels may be elevated in both bacterial and non-bacterial infection and additionally in non-infectious disease processes.[5] Furthermore, septic biomarkers are sometimes costly tests (particularly procalcitonin) and often require serial monitoring.[6] Hence, finding a suitable and cost effective septic biomarker which is both highly specific and sensitive for detecting early bacterial

infection would be beneficial, both economically (in the resource limited setting) and therapeutically.[7]

Assessment by flow cytometry of the expression of CD64 on the surface of neutrophils in response to acute systemic inflammatory activation has shown promise as a potential septic biomarker.[5] CD64 is a high-affinity immunoglobulin Fc gamma receptor which is upregulated on activated neutrophils, with expression being low on resting neutrophils. In a meta-analysis by Yeh *et al.* the diagnostic accuracy of neutrophil CD64 expression as a marker of bacterial infection was found to be excellent, with a sensitivity of 87%, specificity of 89%, area under the curve of 0.94 and a diagnostic odds ratio of 53.[8] Furthermore, neutrophil CD64 expression out-performs both the CRP and Procalcitonin in sepsis detection.[8] As sepsis is an evolving process, where systemic inflammation is countered by an anti-inflammatory response,[9] some studies have augmented neutrophil CD64 expression testing by monitoring the concurrent decrease in monocyte HLA-DR expression (as a marker of this compensatory anti-inflammatory effect).[10] The downregulation of monocyte HLA-DR expression is more rapid when compared to the usual protracted rise of CRP or neutrophil CD64 expression.[10] In a study by Pradhan *et al.*, the ratio of neutrophil CD64 expression to monocyte HLA-DR expression was found to have similar sensitivity (although somewhat poorer specificity) as compared to the neutrophil CD64 expression among neonates with sepsis, and was potentially useful as a prognostic marker for predicting a poorer outcome.[10] A pilot study based in an adult intensive care unit (ICU) confirmed this, where the sepsis index (calculated as neutrophil CD64 expression to monocyte HLA-DR expression x 100) was shown to be useful for identifying and differentiating sepsis when compared to other available markers for

diagnosing sepsis.[11] Other studies have looked at monocyte HLA-DR expression as a prognostic marker of sepsis, and shown that lower expression is associated with a poorer outcome.[9,12,13] Unfortunately, a limitation to these flow cytometry-based tests is that they are expensive and require specialised laboratory facilities.

Other possible infectious biomarkers include those generated by automated haematology analysers, namely extended differential parameters. These reflect differences detected in the characteristics of the different types of white cells (such as their size and fluorescence) during white cell counting and differentiation, including changes in the neutrophils, which play an important role in acute inflammation and infections.[4] Available extended differential parameters differ across the various haematology analysers, as their generation depends on the technology employed for white cell counting. Despite this, they hold appeal as biomarkers of sepsis in resource constrained environments, as they are routinely performed with the automated differential count and therefore incur no additional costs. Sysmex haematology analysers (Sysmex, Kobe, Japan) are widely used in Africa, and generate a number of extended differential parameters (including the automated immature granulocyte count, the neutrophil fluorescent light intensity (NE-SFL) and the neutrophil fluorescent light distribution width (NE-WY) parameters). The immature granulocyte count is performed by Sysmex XT-, XE- and XN-series full blood count analysers, and enumerates granulocyte precursors (including promyelocytes, myelocytes and metamyelocytes).[14,15] A study by Ansari-Lari *et al* [4]., originally demonstrated its utility in detecting bacterial infection, [4] demonstrating a specificity for detecting bacteraemia among samples submitted for a blood culture greater than 90% at an immature granulocyte count >3%.[4] However, the

sensitivity of this test was low in this study. The immature granulocyte count has since been shown to have utility in either diagnosing or stratifying sepsis severity in a number of clinical contexts, including in the ICU,[4,16-18] the emergency department,[19,20] burn victims[21] and patients admitted in haematology and infectious diseases departments.[22] The ideal cut-off value for interpretation of immature granulocyte results is not well standardised, with different studies reporting superior performance with counts >0.5%,[19,23] >2%,[21] >3%[4] or with elevation of the absolute immature granulocyte count.[16,20,21] The NE-SFL and NE-WY parameters, which are defined as the fluorescent light intensity of the neutrophil area and the fluorescent light distribution width of the neutrophil area on the white cell differential fluorescence scattergram respectively, are measured by Sysmex XN-series analysers and are speculated to reflect neutrophil activation. [23] They were shown by Park *et al.* to have good sensitivity and specificity for the detection of bacteraemia at a cut off of >51.6 for NE-SFL and >641.0 for NE-WY, as well as a positive correlation with an immature granulocyte count >0.5%.[23]

To date, no South African studies have evaluated any of the above extended differential parameters as sepsis biomarkers, however, a local study showed that the automated immature granulocyte count was inaccurate in a proportion of patients, particularly in those with underlying human immunodeficiency virus (HIV) infection.[24] As South Africa has a high burden of HIV-infection, with an estimated 7.97 million people (~13.5% of the population) living with HIV,[25] the utility of the immature granulocyte count as a sepsis biomarker may be questionable in the South African context. The immature granulocyte count is measured based on cell size and intracytoplasmic fluorescence of a nucleic acid binding dye. Among HIV positive patients, bizarre giant neutrophils are

common, and these cells are often miscounted by the analyser as immature granulocytes (probably due to their increased cell size and nucleic acid content). However, not all samples with inaccurate automated immature granulocyte counts have morphologically abnormal neutrophils, and it is possible that the inaccurate immature granulocyte counts in these cases may rather be a product of neutrophil activation. If this is the case, the immature granulocyte count may still be of value as a sepsis biomarker among people living with HIV infection, even if the count is not technically accurate in enumerating immature granulocytes.

Also of interest, in the South African context, is the performance of the extended differential parameters in differentiating bacterial sepsis from Tuberculosis (TB) (which is highly prevalent in South Africa), as these entities can mimic one another clinically. Like bacterial sepsis, TB is typified by a pronounced acute phase response, and has been shown to be associated with neutrophil activation.[26] However, in contrast to bacterial sepsis, TB is associated with a more prominent monocyte response, with both the ratio of monocytes to lymphocytes as well as expression of monocyte CD64 being increased in individuals with active TB.[27] Assessing for evidence of monocyte activation may thus be of additional value among patients with suspected bacterial sepsis in settings with a high prevalence of TB. The Sysmex extended differential parameter which assesses monocyte fluorescence (MO-Y) may be of value in this regard, as the MO-Y level is proportional to the amount of DNA and RNA in the cell, which is in turn believed to reflect the degree of monocyte activation. To date, none of the extended differential parameters have been assessed among patients with TB.

In this study, we aimed to assess the usefulness of extended differential parameters as biomarkers of bacteraemia in the South African setting, and then to assess how they perform in comparison to the CRP, procalcitonin and neutrophil CD64 expression, a very high quality but expensive biomarker of sepsis. As secondary objectives, we also aimed to evaluate whether erroneous elevation of the immature granulocyte count reflects neutrophil activation; whether NE-SFL and MO-Y levels reflect neutrophil and monocyte activation respectively and whether concomitant monocyte activation may be of value in discriminating bacterial sepsis from TB.

2. METHODS

2.1 Sample collection and processing

Samples submitted to the National Health Laboratory Service for a blood culture in standard aerobic bottles from patients admitted to the Chris Hani Baragwanath Academic Hospital were identified by a daily search of the National Health Laboratory Service laboratory information system from the 22/08/2019 to 08/10/2019. A search for an FBC sample submitted within 12 hours of the blood culture request was made and an automated differential count performed using Sysmex XN-9000 haematology analyser. Samples were excluded if they had an insufficient volume (<1 ml) following routine testing or if the analyser failed to perform a differential count. Two slides were then made and stained with May-Grünwald-Giemsa and a manual differential count performed by two competent morphologists on 200 cells each if the automated immature granulocyte count was >1.0%. A third competent morphologist performed a manual differential count in cases where the original morphologists had discrepant counts. The automated and mean

manual results were compared by means of a Rümke table and were classified as grossly discordant if the analyser immature granulocyte count fell ≥2% beyond the range specified in the Rümke table.

Neutrophil CD64 expression was measured on the same sample by flow cytometry using an adaptation of a previously described protocol[10] on a Beckman Coulter Navios flow cytometer (Beckman Coulter, Inc, Brea, California, USA). Analysis was performed within 36 hours after blood sampling to ensure viability of cellular components. A full description of flow cytometry methods can be found in Supplementary methods.

The mean fluorescent index for CD64 and HLA-DR was recorded for neutrophils, lymphocytes and monocytes and the ratios of CD64 expression for neutrophils and monocytes to lymphocytes, respectively were calculated. In addition, neutrophil CD64 expression to monocyte HLA-DR expression was also calculated. Lymphocyte CD64 is an internal negative control, and the ratios of CD64 expression of neutrophils and monocytes to lymphocytes was therefore assessed to compensate for minor variances of mean antigen mean fluorescence index between samples.

2.2 Sample classification

The sample results were then divided into three groups, namely a bacteraemic or blood culture positive group, a group with non-bacteraemic infection and an infection-negative group. Blood culture positive samples were taken as those with blood cultures that grew a pathogenic organism. Included in the non-bacteraemic infection group were patients with additional cultures of urine or sputum, as requested by the attending physician, that

grew a pathogenic organism (including TB (grown in Myco/F lytic bottle)) without a positive blood culture. Infection negative samples were defined as those with a negative blood culture, negative additional cultures and the absence of other laboratory or clinical evidence to support the presence of infection. Cases with negative or contaminated cultures with elevated septic biomarkers (CRP or procalcitonin) were regarded as equivocal and excluded from the extended differential parameter and neutrophil CD64 expression performance analysis, as elevated biomarkers are not specific for bacterial infection.[5,28]

Additional pertinent information, including HIV status, CRP, procalcitonin, relevant clinical details (where available) and other culture results, was recorded from the laboratory information system.

2.3 Statistical analysis

Continuous data are represented using their median and interquartile range whilst categorical data are reported as frequencies and percentages. The diagnostic performance for the extended differential parameters and flow cytometric indices (neutrophil and monocyte CD64 expression and monocyte HLA-DR expression) in identifying either non-bacteraemic infection or bacteraemia among samples were calculated by means of the area under a receiver operating characteristic curve. The Likelihood ratio was used to identify the best cut-off point. The sensitivity, specificity and positive and negative predictive values are reported.

The agreement between the extended differential parameters and the presence of an increased neutrophil CD64 expression to monocyte HLA-DR expression ratio (as the biomarker which showed the best diagnostic performance) was assessed by Cohen's kappa co-efficient in all samples. The optimal cut-off values for detection of bacteraemia (as determined on the ROC analysis) were used in this analysis. Values of <0.2 were considered to show no to slight agreement, 0.21 – 0.4 fair agreement, 0.41 – 0.6 moderate agreement and values of 0.61 – 0.8 substantial agreement. The Mann-Whitney U-test was used to compare continuous variables of interest, including the ratio of neutrophil CD64 expression to lymphocyte CD64 expression, among samples with erroneously elevated immature granulocyte counts as compared to those with concordant low counts. The correlation between the NE-SFL and MO-Y and neutrophil and monocyte activation (as evidenced by the ratios of these values to lymphocyte CD64 expression respectively) was assessed by linear regression analysis. Statistical significance was accepted at a p-value <0.05.

Statistical analysis was performed using Prism software, version 5 (GraphPad Software, San Diego, California, United States).

2.4 Ethics

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (M-190524).

3. RESULTS

3.1 Study population

A total of 151 samples were collected, with a median age of 36 years and a male: female ratio of ~0.5:1. The patients were admitted to a variety of wards, including medical (46%), surgical (10%), paediatric (11%), intensive care/high care (13%), maternity / gynaecology (13%), burns (2%), psychiatry (<1%) and oncology (5%) units. The HIV status was documented in 71 of the total number of samples, with a seropositivity rate of 60%. Unequivocal results were present in 64 samples, including 21 in the blood culture positive group, 34 in the non-bacteraemic infection group and 9 in the infection negative group. In addition, 4 patients were proven to have TB, all of whom were HIV positive. Relevant biometric information for these cases is shown in Table 1.

The biomarkers assessed were generally significantly higher among patients with bacteraemia, non-bacteraemic infection or TB as compared to those without infection. Exceptions to this included the NE-WY (which was not significantly increased in patients with either non-bacteraemic infection or TB), the MO-Y (which was only significantly increased in patients with TB) and the ratio of monocyte to lymphocyte CD64 expression (which was not significantly increased in any of the groups) (Table 1). Notably, the MO-Y was the only biomarker which was significantly higher among patients with TB as compared to those with bacteraemia ($p = 0.026$). When comparing patients with TB and non-bacteraemic infection, both the ratio of neutrophil to lymphocyte CD64 expression and the MO-Y were significantly higher among those with TB ($p = 0.0059$ and 0.0223 respectively). Statistically significant differences in the procalcitonin levels between the

groups was not seen, most likely because a procalcitonin result was available for only 23 cases.

Table 1. Biometrics and extended differential parameter data.

Sample group	Bacteraemic infection	Non bacteraemic infection	Proven TB	Infection Negative
Number of samples	21	30	4	9
Male: Female	0.25:1	0.65:1	01:01	01:01
Median Age [IQR] (years)	32 (28 - 47)	34 (23.5 – 47)	36 (29 - 43)	24 (5,3 – 39.0)
HIV positive samples (pos/neg) %	7/8 (87.5)	12/18 (66.6)	4/4 (100)	0/1 (0)
nCD64:lCD64				
Median	12.65	5.26	20.15	1.000
IQR (25%-75%)	5.69 - 37.84	2.55 - 9.40	13.38 - 98.75	1.00 - 2.45
p-value*	0.0005	0.0007	0.0055	Not applicable
mCD64:lCD64				
Median	40.49	46.13	66.81	35.69
IQR	20.59 - 66.13	23.19 - 61.89	30.05 - 131.2	22.79 - 42.11
p-value*	0.4	0.28	0.10	Not applicable
nCD64:mHLA-DR				
Median	1.474	0.52	3.9	0.07445
IQR	0.73 - 4.34	0.21 - 1.25	1.76 - 5.75	0.033 - 0.27
p-value*	< 0.0001	0.005	0.003	Not applicable
NE-WY				
Median	840.0	765	827.5	693.0
IQR	782.0 - 998.0	662.3 - 895.3	733.3 - 865.5	647.0 - 791.5
p-value*	0.0050	0.24	0.16	Not applicable
NE-SFL				
Median	57.80	51.4	54.15	44.80
IQR	54.70 - 67.15	46.05 - 54.5	48.7 - 60.58	42.30 - 47.50
p-value*	0.0019	0.014	0.034	Not applicable
MO-Y				
Median	104.2	104.2	124.5	96.0
IQR	94.6 - 113.7	98.7 - 110.4	117.4 - 125.8	82.1 - 107.0
p-value*	0.96	0.97	0.016	Not applicable
Automated IG%				
Median	1.900	1.45	3.0	0.50
IQR	0.8 - 2.7	0.68 - 4.4	1.88 - 4.95	0.35 - 0.85
p-value*	0.0053	0.0123	0.0133	Not applicable
Abs auto IG (x109/L)				
Median	0.1400	0.18	0.26	0.05
IQR	0.10 - 0.47	0.07 - 0.69	0.15 - 0.56	0.04 - 0.11
p-value*	0.0352	0.017	0.030	Not applicable
CRP				
Median +	228.0	117	139	8.00
IQR	92.5 - 314.0	68 - 197	68 - 341	5.00 - 10.00
p-value*	0.0002	0.0001	0.022	Not applicable
Pct				
Median ++	67.25	1.63	Unable to analyse	0.08
IQR	42.76 - 99.04	0.37 - 11.44	owing to too few	0.06 - 0.10
p-value*	0.0556	0.0677	samples	Not applicable

*The p-values are derived from comparison with the infection negative cases. N=17 (bacteraemic infection), N=27 (non-bacteraemic infection), N=7 (infection neg), N=3 (TB). nCD64:lCD64, ratio of neutrophil to lymphocyte CD64 expression; mCD64:lCD64, monocyte CD64 expression: lymphocyte CD64 expression; nCD64: mHLA-DR, neutrophil CD64 expression: monocyte HLA-DR expression; NE-WY, fluorescent light distribution width of the neutrophil area; NE-SFL, fluorescent light intensity of the neutrophil area; IG%, immature granulocyte percentage; CRP, C-reactive protein; Pct, procalcitonin.

3.2 Receiver operating characteristic curve analysis

For the purposes of ROC curve analysis, patients with TB were included among the cases with non-bacteraemic infection owing to the small sample size of this group.

With the exception of the absolute immature granulocyte count, all the parameters showed good performance (AUC>0.8) for distinguishing patients with bacteraemia from those without infection. The best performance seen was for the ratio of neutrophil CD64 expression to monocyte HLA-DR expression at a cut-off value of 0.34 (Table 2). Among the automated parameters, the NE-SFL showed the best performance (AUC 0.87) (Table 2) at a cut-off value of 49.75. The CRP also demonstrated excellent performance, but with lower specificity compared to the ratio of neutrophil CD64 expression to monocyte HLA-DR expression and NE-SFL (Table 2). None of the parameters demonstrated good performance for distinguishing patients with bacteraemia from those with non-bacteraemic infection (AUC <0.8) (Supplementary Table 1). The performance of procalcitonin could not be accurately assessed given the very small sample numbers (documented in only 23 of the unequivocal cases).

Table 2. Receiver operating characteristic curve analysis assessing the various biomarkers among patients with bacteraemic infection compared to those with no evidence of infection.

Parameter	AUC	95% CI	p-value for AUC	LR LR	Sensitivity (%)	Specificity (%)	Cut off value	NPV (%)	PPV (%)
nCD64:lCD64	0.91	0.80 -1.01	0.0005	6.86	76.19	88.89	> 4.22	67.00	94.00
nCD64:mHLA-DR	0.96	0.89 - 1.03	< 0.0001	8.57	95.24	88.89	> 0.34	88.89	94.74
NE-WY	0.83	0.67 - 0.99	0.0047	3.86	85.71	77.78	> 750.5	70.00	90.00
NE-SFL	0.87	0.71 - 1.02	0.0018	7.71	85.71	88.89	> 49.75	72.73	94.74
Automated IG%	0.83	0.68 - 0.98	0.005	3.43	76.19	77.78	> 0.85	58.33	88.89
Abs auto IG	0.75	0.56 - 0.94	0.0335	3.21	71.43	77.78	> 0.11	53.85	88.24
CRP+	1	1.00 - 1.00	0.0002	7	100	85.71	> 12.5	100.00	94.44

[+] N= 24. AUC, area under the curve; CI, confidence interval; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; nCD64:lCD64, ratio of neutrophil to lymphocyte CD64 expression; nCD64:mHLA-DR, ratio of neutrophil CD64 expression to monocyte HLA-DR expression; NE-WY, fluorescent light distribution width of the neutrophil area; NE-SFL, fluorescent light intensity of the neutrophil area; IG%, immature granulocyte percentage; CRP, C-reactive protein; Abs auto IG, absolute automated IG count.

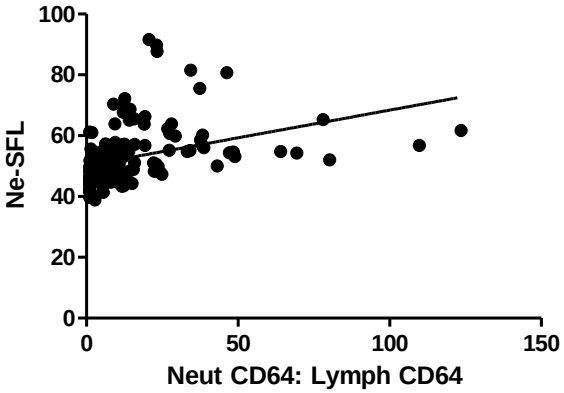
3.3 Cohen's Kappa co-efficient

Agreement between the extended differential parameters and the ratio of neutrophil CD64 expression to monocyte HLA-DR expression (as the sepsis biomarker demonstrated to have the best overall performance) was determined by Cohen's kappa co-efficient on all the tested samples (N=151) using the cut-off values determined by ROC analysis above. This revealed moderate agreement between the NE-SFL and the neutrophil CD64 expression to monocyte HLA-DR expression ratio in bacteraemic samples (kappa=0.51).

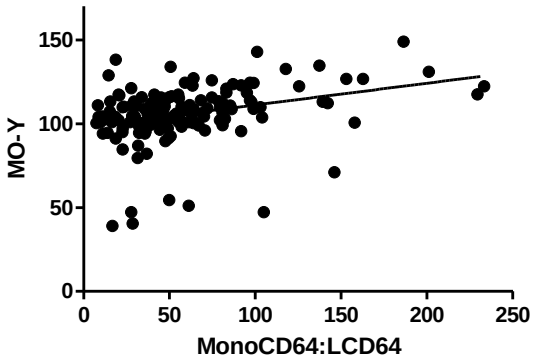
Fair agreement was noted with the automated immature granulocyte count (kappa =0.38) and NE-WY (kappa=0.34). Interestingly, the CRP showed only slight agreement with ratio of neutrophil CD64 expression to monocyte HLA-DR expression (kappa=0.18), which is likely to reflect the non-specific nature of the CRP, being an acute phase reactant elevated in many other inflammatory conditions.[29] In contrast, the ratio of neutrophil CD64 expression to monocyte HLA-DR expression may be more specific to the immune response to infection.[30]

3.4 Analysis of the correlation between the NE-SFL and MO-Y and neutrophil and monocyte CD64 expression respectively

In order to assess whether the extended differential parameters NE-SFL and MO-Y correlated with neutrophil and monocyte activation respectively, we assessed the expression of CD64 flow cytometrically for ratios of neutrophil and monocyte to lymphocyte CD64 expression, respectively. We then performed linear regression analysis and found weak positive correlations ($r2 = 0.14$, $p<0,0001$ for the NE-SFL and $r2 = 0.10$, $p<0,0001$ for the MO-Y), (Figure 1), suggesting that there is an association between these parameters and neutrophil and monocyte activation, respectively.



(A) Y=0.82x+50



(B) Y=0.13x+98

Figure 1. Graphs depicting the linear regression analysis between NE-SFL and the ratio of neutrophil to lymphocyte CD64 expression (A) and between MO-Y and the ratio of monocyte to lymphocyte CD64 expression (B).

3.5 Analysis of discordant automated immature granulocyte counts compared to manual counts, particularly in HIV positive patients

A local study showed that the automated immature granulocyte count was inaccurate in a proportion of patients, particularly in those with underlying HIV infection.[24] On comparison of the manual and automated differential counts in this study population, 85 samples had an automated immature granulocyte count ≥1% and of these, 8 (9.4%) were grossly discordant with the manual immature granulocyte count (median manual count 1.25% vs median automated count 12.4%). HIV-status was documented in 6 of these, with a seropositivity rate of 83.3%. Among all HIV-positive patients, 5 of the 43 samples (11.6%) had gross erroneous elevation of their immature granulocyte counts. In order to assess whether erroneous elevation of these counts reflects neutrophil activation (and is

therefore useful as a sepsis biomarker although technically inaccurate), neutrophil CD64 expression was assessed in those with grossly discordant elevation of the automated immature granulocyte counts. The ratios of neutrophil to lymphocyte CD64 expression among the grossly discordant cases with low manual immature granulocyte counts (≤1%) appeared substantially higher as compared to those cases with counts <1%, where the analyser and manual counts were in agreement (22.11 versus 11.67, where the former is a median ratio of neutrophil to lymphocyte CD64 expression in erroneously elevated immature granulocyte count and the latter for cases with concordantly low immature granulocyte count). However, this finding was not statistically significant ($p = 0.45$), possibly owing to the small number of cases assessed.

4. DISCUSSION

This study assessed extended differential parameters for the detection of bacteraemic infection in a South African laboratory with a high HIV-seropositivity rate. All extended differential parameters evaluated were performed concurrently with the differential white cell count on Sysmex haematology analysers, and therefore hold appeal in the resource constrained setting.

The NE-SFL and NE-WY performed well when comparing bacteraemic against infection negative samples, both with positive predictive values over 90%. The sensitivity and specificity of NE-SFL and NE-WY were 85.71% and 88.89% and 85.71% and 77.78% respectively, when cut off values of NE-SFL >49.75 and NE-WY >750.5 were applied. NE-SFL performed slightly better than NE-WY in differentiating samples with bacteraemia from those with no evidence of infection. These results are similar to those

of Park *et al.*, who showed that the NE-SFL and NE-WY had a sensitivity and specificity of 71.3% and 96.8%, and 86.2% and 84.2% at cut off values of NE-SFL >51.6 and NE-WY >641.0, respectively in a study comparing samples from patients with sepsis to normal controls. Furthermore, a significant correlation was found between the NE-SFL and the ratio of neutrophil to lymphocyte CD64 expression, which suggests that an elevated NE-SFL reflects neutrophil activation as hypothesized by Park *et al*.[23] Thus, although based on a small sample size, our findings are promising for the use of this parameter as a sepsis biomarker in the resource constrained setting. Interestingly, the NE-SFL was also significantly elevated amongst patients with TB, as was the MO-Y (which is thought to reflect monocyte activation). Notably, the MO-Y was increased only among patients with TB, and near normal among patients with bacterial infection (both bacteraemic and non-bacteraemic). TB is a notoriously difficult infection to diagnose, with the TB culture (the gold standard test) taking up to six weeks to perform. The possibility of a sensitive and specific extended differential parameter biomarker is consequently of great interest, and further studies on a larger number of patients with TB would be of value.

In contrast to the NE-SFL and NE-WY (which are currently not universally available to all Sysmex users), the automated immature granulocyte count is reported as part of the six-part differential count on several Sysmex analysers. This parameter was found to be relatively sensitive and specific (74 - 77%) for detecting bacteraemia when compared to those without infection (AUC 0.83) at a cut-off value of 0.85%. However, it did not perform as well as the NE-WY and NE-SFL. As has been reported previously,[24] the immature granulocyte count was not found to be as reliable when interpreted in HIV

positive samples in this study population, with 11.6% of these samples showing gross erroneous elevation of this count. Owing to a small sample number, neutrophil CD64 expression could not be adequately analysed and the value of the immature granulocyte count among HIV positive patients therefore remains in question. Further studies in this regard would be of interest.

Results of neutrophil CD64 expression analysis among the bacteraemic samples are shown to be consistent with those published in a meta-analysis by Yeh *et al.*, showing that neutrophil CD64 expression (defined as a ratio to lymphocyte CD64 expression) is a good biomarker of sepsis. In this study population, the ratio of neutrophil CD64 expression to monocyte HLA-DR expression out-performed the former ratio, with a sensitivity, specificity and AUC of 95%, 88.9% and 0.96 (95% CI 0.89 - 1.03) respectively. The prognostic usefulness of the ratio of neutrophil CD64 expression to monocyte HLA-DR expression was not assessed in this study, however agreement between the extended differential parameters and this ratio (as the sepsis biomarker demonstrated to have the best overall performance) revealed moderate agreement for the NE-SFL. Given the high cost and specialised nature of flow cytometry-based tests, this finding further supports using the NE-SFL over the costly ratio of neutrophil CD64 expression to monocyte HLA-DR expression in resource limited settings.

When comparing extended differential parameters to CRP, the CRP showed a sensitivity of 100% and specificity of 85.71% when using a cut-off value of >12.50. The excellent performance of the CRP in this study is likely owing to the study design, which pre-selected a population that is highly likely to have infection. This lowers the possibility of

false positives and non-specific positivity of the CRP. Interestingly, there was very poor agreement between the CRP and the ratio of neutrophil CD64 expression to monocyte HLA-DR expression, which highlights the non-specific nature of a raised CRP. Notably, the weaknesses in this study's design (which are likely to have skewed the CRP result) also apply to the extended differential parameters, and further assessment in a broader sample (without pre-selection for clinical evidence of infection) is needed.

5. CONCLUSION

Extended differential parameters offer great potential as a marker of sepsis in that they are cost-efficient (run with routine tests) and readily available. In this study, the NE-SFL showed the greatest promise as a biomarker of bacteraemia. Further larger scale prospective studies in unselected patients would be of interest.

REFERENCES

1. Spraycar M eSsMDteB, Md: Lippincott Williams & Wilkins; 1995.
2. Christaki E, Giamarellos-Bourboulis EJ. The complex pathogenesis of bacteremia: from antimicrobial clearance mechanisms to the genetic background of the host. *Virulence.* 2014;5(1):57-65.
3. Singer M DC, Seymour CW, et al. . The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). . *JAMA.* 2016;315(8): 801-810.
4. Ansari-Lari MA, Kickler TS, Borowitz MJ. Immature granulocyte measurement using the Sysmex XE-2100. Relationship to infection and sepsis. *American journal of clinical pathology.* 2003;120(5):795-799.
5. Davis BH. Improved diagnostic approaches to infection/sepsis detection. *Expert review of molecular diagnostics.* 2005;5(2):193-207.
6. Smith KJ, Wateska A, Nowalk MP, et al. Cost-effectiveness of procalcitonin-guided antibiotic use in community acquired pneumonia. *Journal of general internal medicine.* 2013;28(9):1157-1164.
7. Cid J, Aguinaco R, Sanchez R, Garcia-Pardo G, Llorente A. Neutrophil CD64 expression as marker of bacterial infection: a systematic review and meta-analysis. *The Journal of infection.* 2010;60(5):313-319.
8. Yeh CF, Wu CC, Liu SH, Chen KF. Comparison of the accuracy of neutrophil CD64, procalcitonin, and C-reactive protein for sepsis identification: a systematic review and meta-analysis. *Annals of intensive care.* 2019;9(1):5.

9. Perry SE, Mostafa SM, Wenstone R, Shenkin A, McLaughlin PJ. Is low monocyte HLA-DR expression helpful to predict outcome in severe sepsis? *Intensive care medicine.* 2003;29(8):1245-1252.

10. Pradhan R, Jain P, Paria A, et al. Ratio of neutrophilic CD64 and monocytic HLA-DR: A novel parameter in diagnosis and prognostication of neonatal sepsis. *Cytometry Part B, Clinical cytometry.* 2016;90(3):295-302.

11. Sima C, Jagadish H. EARLY DIAGNOSIS OF SEPSIS THROUGH SEPSIS MARKERS AND SEPSIS INDEX THROUGH FLOW CYTOMETRY TECHNOLOGY. *Asian Journal of Pharmaceutical and Clinical Research.* 2017;10(11).

12. Monneret G, Lepape A, Voirin N, et al. Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock. *Intensive care medicine.* 2006;32(8):1175-1183.

13. Genel F, Atlihan F, Ozsu E, Ozbek E. Monocyte HLA-DR expression as predictor of poor outcome in neonates with late onset neonatal sepsis. *The Journal of infection.* 2010;60(3):224-228.

14. Fernandes B, Hamaguchi Y. Automated enumeration of immature granulocytes. *American journal of clinical pathology.* 2007;128(3):454-463.

15. Buoro S, Mecca T, Azzarà G, et al. Extended leukocyte differential count and C-reactive protein in septic patients with liver impairment: diagnostic approach to evaluate sepsis in intensive care unit. *Annals of translational medicine.* 2015;3(17):244.

16. Nierhaus A, Klatte S, Linssen J, et al. Revisiting the white blood cell count: immature granulocytes count as a diagnostic marker to discriminate between SIRS and sepsis--a prospective, observational study. *BMC immunology.* 2013;14:8.

17. van der Geest PJ, Mohseni M, Brouwer R, van der Hoven B, Steyerberg EW, Groeneveld ABJ. Immature granulocytes predict microbial infection and its adverse sequelae in the intensive care unit. *Journal of Critical Care.* 2014;29(4):523-527.

18. Buoro S, Mecca T, Azzarà G, et al. Extended leukocyte differential count and C-reactive protein in septic patients with liver impairment: diagnostic approach to evaluate sepsis in intensive care unit. *Annals of translational medicine.* 2015;3(17):244-244.

19. Ha SO, Park SH, Park SH, et al. Fraction of immature granulocytes reflects severity but not mortality in sepsis. *Scandinavian journal of clinical and laboratory investigation.* 2015;75(1):36-43.

20. Karon BS, Tolan NV, Wockenfus AM, et al. Evaluation of lactate, white blood cell count, neutrophil count, procalcitonin and immature granulocyte count as biomarkers for sepsis in emergency department patients. *Clinical biochemistry.* 2017;50(16-17):956-958.

21. Hampson P, Dinsdale RJ, Wearn CM, et al. Neutrophil Dysfunction, Immature Granulocytes, and Cell-free DNA are Early Biomarkers of Sepsis in Burn-injured Patients: A Prospective Observational Cohort Study. *Annals of surgery.* 2017;265(6):1241-1249.

22. Ayres LS, Sgnaolin V, Munhoz TP. Immature granulocytes index as early marker of sepsis. *International Journal of Laboratory Hematology.* 2019;41(3):392-396.

23. Park SH, Park CJ, Lee BR, et al. Sepsis affects most routine and cell population data (CPD) obtained using the Sysmex XN-2000 blood cell analyzer: neutrophil-related CPD NE-SFL and NE-WY provide useful information for detecting sepsis. *Int J Lab Hematol.* 2015;37(2):190-198.

24. Vaughan J, Alli N. Frequent inaccuracies in the Sysmex XN-series immature granulocyte percentage (IG%) among HIV positive patients and those on treatment for acute lymphoblastic leukaemia in samples flagged for smear review. *The Journal of Medical Laboratory Science and Technology of South Africa.* 2019;1(2):32-37.

25. South-Africa. Department of Statistics, Statistical release. (2019). *Mid-year population estimates.* 2019.

26. Alemán M, Beigier-Bompadre M, Borghetti C, et al. Activation of Peripheral Blood Neutrophils from Patients with Active Advanced Tuberculosis. *Clinical Immunology.* 2001;100(1):87-95.

27. La Manna MP, Orlando V, Dieli F, et al. Quantitative and qualitative profiles of circulating monocytes may help identifying tuberculosis infection and disease stages. *PLOS ONE.* 2017;12(2):e0171358.

28. Fan SL, Miller NS, Lee J, Remick DG. Diagnosing sepsis - The role of laboratory medicine. *Clinica chimica acta; international journal of clinical chemistry.* 2016;460:203-210.

29. Simon L, Gauvin F, Amre DK, Saint-Louis P, Lacroix J. Serum Procalcitonin and C-Reactive Protein Levels as Markers of Bacterial Infection: A Systematic Review and Meta-analysis. *Clinical Infectious Diseases.* 2004;39(2):206-217.

30. Leijte GP, Rimmelé T, Kox M, et al. Monocytic HLA-DR expression kinetics in septic shock patients with different pathogens, sites of infection and adverse outcomes. *Critical Care.* 2020;24(1):110.

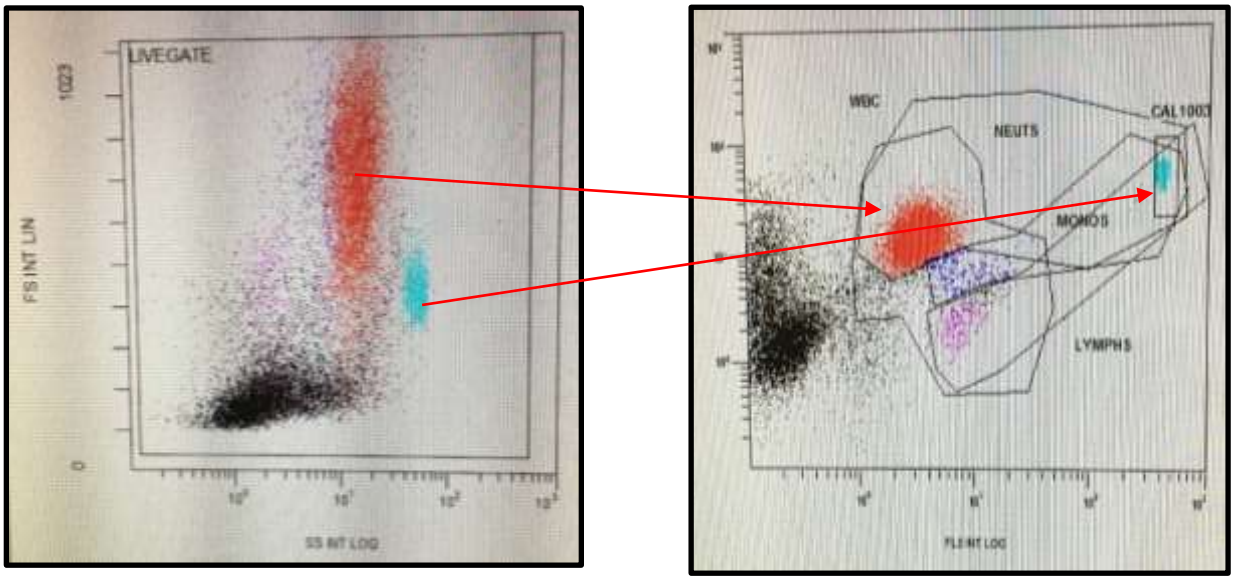
SUPPLEMENTARY METHODS

Neutrophil CD64 expression was measured on the same FBC sample by flow cytometry using an adaptation of a previously described protocol [8] on a Beckman Coulter Navios flow cytometer (Beckman Coulter, Inc, Brea, California, USA). Analysis was performed within 36 hours after blood sampling to ensure viability of cellular components. A lyse-no-wash preparation method using commercially produced fluorescence conjugated antibodies with specificity to white blood cells (CD45), monocytes (CD14), HLA-DR and CD64 and a fluorescence latex bead suspension was used. Briefly, 100 µL of whole blood was incubated for 15 minutes in the dark at room temperature with a mixture of the monoclonal antibodies followed by red cell lysis using the Beckman Coulter Immunoprep reagent system. Fluorescence beads (100µL) were then added (to monitor accuracy of CD64+ expression) and flow cytometer acquisition and analysis performed on a minimum of 50 000 leukocytes. Data analysis for fluorescence intensity was performed on the Beckman Navios flow cytometer. Live gating was performed according to light scatter for different populations (Supplementary Figure 1). CD64 mean fluorescence intensity (MFI) was measured as a linearized value of log scale on the lymphocytes (negative control), the monocytes (positive control), the neutrophils, and the beads.

The MFI for the beads remained stable throughout the study, with a mean of 642.6 (cv 5.08%) hence the values for MFI for patient samples could be taken directly from the printout.

The MFI for CD64 and HLA-DR was recorded for neutrophils, lymphocytes and monocytes. The ratio of CD64 expression for neutrophils to lymphocytes as well as the

ratio for neutrophil CD64 expression to monocyte HLA-DR expression were then calculated.



(A) **(B)**

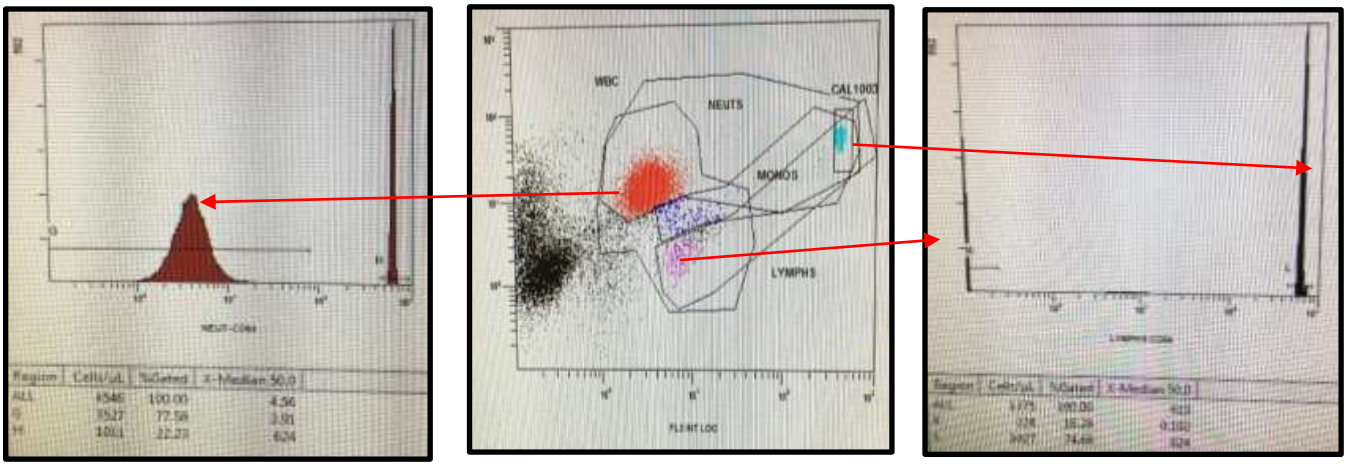
Supplementary Figure 1. Live gating strategy for identifying populations of interest, namely granulocytes (orange events), monocytes (blue events) and lymphocytes (pink events) using CD45 versus side scatter (B).

Supplementary Table 1. Comparison of extended differential parameters and flow cytometric ratio results for the three sample groups, namely bacteraemic, non-bacteraemic and no infection samples.

	Bacteraemic sample	Non bacteraemic sample	No infection sample
Blood culture	Staphylococcus aureus (STAAU)	Negative	Negative
Additional culture	None	Pneumocystis jirovecii (PCR result)	Negative
White cell count	8,28	10,38	7,04
NE-WY	914	793	648
NE-SFL	60,2	46.7	44.8
Auto IG%	0,7	1,5	0,4
Manual IG%	0,25	0,25	Not done
Neut CD64: lymph CD64	38.33	10.78	1
Neut CD64: mono HLA-DR	11.96	4.06	0.06
CRP	434	86	5

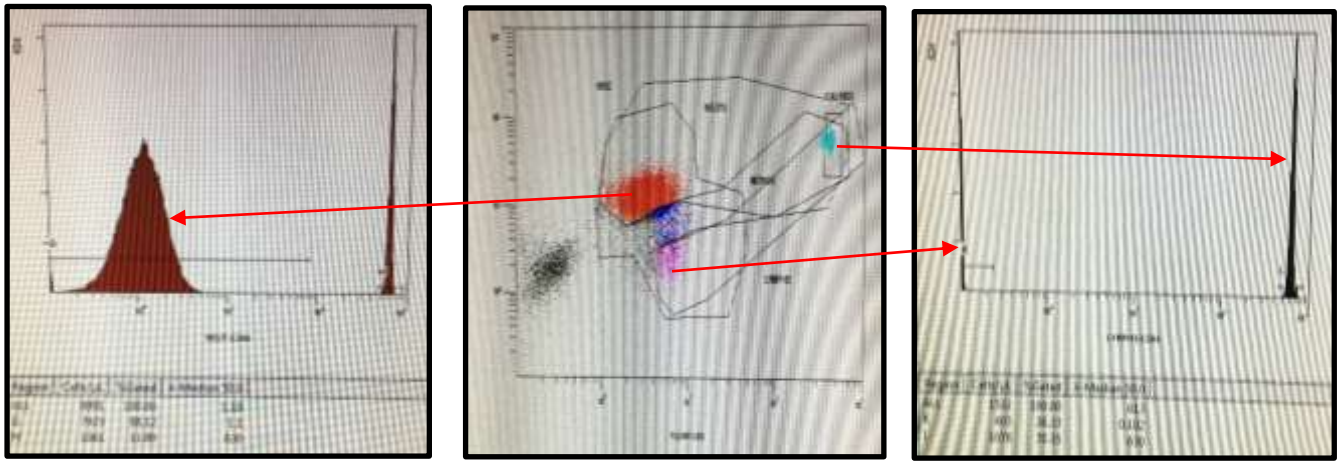
NE-WY, fluorescent light distribution width of the neutrophil area; NE-SFL, fluorescent light intensity of the neutrophil area; auto IG%, automated immature granulocyte percentage; manual IG%, manual immature granulocyte percentage; nCD64:lCD64, ratio of neutrophil to lymphocyte CD64 expression; nCD64:mHLA-DR, neutrophil CD64 expression:monocyte HLA-DR expression; CRP, C-reactive protein.

A



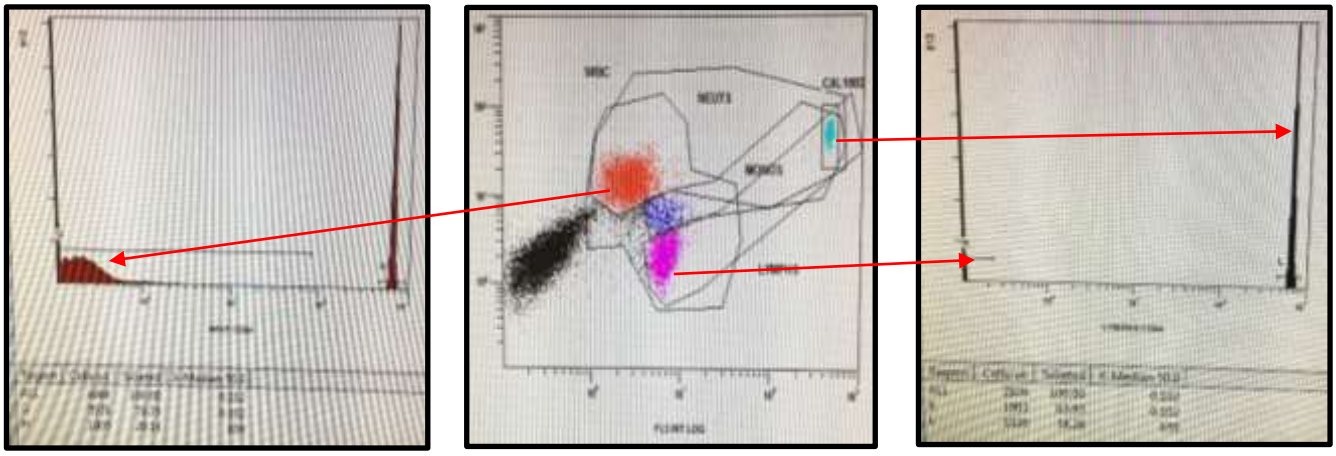
(a) (b) (c)

B



(a) (b) (c)

C



(a) (b) (c)

Supplementary Figure 2. Flow cytometry live gating strategy to identify populations of interest, namely granulocytes, monocytes and lymphocytes (b). These feed into CD64 expression histograms for neutrophils (a) and lymphocytes (c), from where the ratio of CD64 expression was calculated.

A. Bacteraemic samples - The MFI is read off the histogram plots, such that neutrophil CD64 expression is 3.91 (G) and lymphocyte CD64 expression is 0.102 (K). The beads remain constant in both the histograms (H and L respectively).

B. Non bacteraemic samples - The MFI is read off the histogram plots, such that neutrophil CD64 expression is 1.1 (G) and lymphocyte CD64 expression is 0.102 (K). The beads remain constant in both the histograms (H and L respectively).

C. Samples with no infection - The MFI is read off the histogram plots, such that neutrophil CD64 expression is 0.102 (G) and lymphocyte CD64 expression is 0.102 (K). The beads remain constant in both the histograms (H and L respectively).

SUPPLEMENTARY DATA

Supplementary Table 2. Receiver operating characteristic curve analysis assessing the various biomarkers among patients with bacteraemic infection compared to those with non-bacteraemic infection.

Parameter	AUC	95% CI	p-value for AUC	LR	Sensitivity (%)	Specificity (%)	Cut-off value	PPV (%)	NPV (%)
NE-SFL	0.77	0.63 - 0.91	0.0009	3.06	80.95	75.53	>54.5	65.38	86.21
nCD64:mHLA-DR	0.71	0.57 - 0.85	0.0099	2.08	85.71	58.82	>0.61	56.25	86.96
nCD64:lCD64	0.69	0.53 - 0.86	0.0165	2.59	76.19	70.59	>9.14	61.54	82.76
NE-WY	0.67	0.53 - 0.81	0.0369	1.62	76.19	52.94	>794.0	50.00	78.26
CRP	0.61	0.44 - 0.79	0.2145	1.24	82.35	33.33	>89.0	43.75	75.00
Absolute automated IG	0.55	0.39 - 0.71	0.5386	1.16	71.43	38.24	>0.36	33.33	59.46
Automated IG %	0.51	0.36 - 0.67	0.8829	1.25	80.95	35.29	>3.05	58.62	84.62

AUC, area under the curve; CI, confidence interval; LR, likelihood ratio; nCD64:lCD64, ratio of neutrophil to lymphocyte CD64 expression; nCD64:mHLA-DR, neutrophil CD64 expression : monocyte HLA-DR expression; NE-WY, fluorescent light distribution width of the neutrophil area; NE-SFL, fluorescent light intensity of the neutrophil area; IG%, immature granulocyte percentage; CRP, C-reactive protein; Pct, procalcitonin; Abs auto IG, absolute automated IG count.

APPENDIX I

Ethics approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Dr L Lemkus
School of Pathology
Department of Molecular Medicine & Haematology
National Health Laboratory Service

E-mail: Lauren.Lemkus@nhls.ac.za

CC: Supervisor: Drs J Vaughan and D Lawrie <Jenifer.Vaughan@nhls.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2019/07/05

REF: *R14/49*

PROTOCOL NO: **M190524** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *The usefulness of the extended differential parameters as a biomarker of bacteraemia at Chris Hani Baragwanath Academic Hospital*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr L Lemkus

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190524

NAME: (Principal Investigator)	Dr L Lemkus
DEPARTMENT:	School of Pathology Department of Molecular Medicine & Haematology National Health Laboratory Service
PROJECT TITLE:	The usefulness of the extended differential parameters as a biomarker of bacteraemia at Chris Hani Baragwanath Academic Hospital
DATE CONSIDERED:	2019/05/31
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Drs J Vaughan and D Lawrie
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2019/07/05

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **May** and will therefore reports and re-certification will be due early in the month of **May** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES

APPENDIX II

Research protocol

The usefulness of the extended differential parameters as a biomarker of bacteraemia at Chris Hani Baragwanath Academic Hospital

Dr L. Lemkus

Department of Molecular Medicine and Haematology, University of Witwatersrand Medical School, Johannesburg, South Africa

Dr J. Vaughan

Department of Molecular Medicine and Haematology, University of Witwatersrand Medical School, Johannesburg, South Africa

Dr D. Lawrie

Department of Molecular Medicine and Haematology, University of Witwatersrand Medical School, Johannesburg, South Africa

Contents

ABBREVIATIONS 41

1. INTRODUCTION 43

1.1 The definition of sepsis 43

1.2 Laboratory tests to detect infection 43

1.2.1. Neutrophil CD64 expression as a septic biomarker 44

1.2.2 Extended differential parameters as septic biomarkers 45

1.3 South African experience using EPDs 46

2. STUDY HYPOTHESIS, AIMS AND OBJECTIVES 46

2.1 Hypothesis 46

EDPs are useful tests in the diagnosis of sepsis among South African patients, including HIV positive patients. 10

2.2 Study aims 47

2.2.1 Primary aim 47

2.2.2 Secondary aims 47

2.3 Objectives 47

3. METHODS 48

3.1 Study setting 48

3.2 Study population 48

3.2.1 Inclusion criteria 48

3.2.2 Exclusion criteria 49

3.3 Study design 49

3.4 Data collection 49

3.4.1 Clinical parameters to be recorded 49

3.4.2 Laboratory parameters to be recorded 49

3.4.3 Sample size 50

3.4.4 The variables to be assessed 50

3.4.5 Laboratory methods 50

4. DATA ANALYSIS 52

5. ETHICS 54

6. TIMING 54

Table 2. Timeline for projected completion of research thesis 55

7. FUNDING 56

REFERENCES 57

APPENDIX 1 60

ABBREVIATIONS

AUC - Area under the curve

CHBAH - Chris Hani Baragwanath Academic Hospital

CMJAH - Charlotte Maxeke Johannesburg Academic Hospital

CRP - C-reactive protein

DOR - Diagnostic odds ratio

EDPs - Extended differential parameters

EDTA – Ethylenediaminetetraacetic acid

FBC - Full blood count

FcgRI - Immunoglobulin Fc gamma receptor I

FSC - Forward scatter

HIV - Human immunodeficiency virus

ICU - Intensive care unit

IG - Immature granulocyte

IG# - Absolute immature granulocyte count

IG% - Immature granulocyte percentage

INR – International normalised ratio

LIS - Laboratory information system

MFI - Mean fluorescence intensity

NE-SFL - Fluorescent light intensity of the neutrophil area

NE-WY - Fluorescent light distribution width of the neutrophil area

NHLS - National Health Laboratory Service

Pct – Procalcitonin

PTT – Partial thromboplastin time

SFL - Side fluorescence

SROC - Summary receiver operating characteristic

SSC - Side scatter

WDF - White cell differential fluorescence

1. INTRODUCTION

1.1 The definition of sepsis

Bacteraemia develops when bacteria succeed in evading the immune response of the host or when the immune response is insufficient to halt bacterial spread due to intrinsic or acquired immune defects that are associated with susceptibility to infection (1). Sepsis is defined as severe organ dysfunction caused by a dysregulated response of the patient to an infection. If bacteraemia and sepsis are not identified early and rapidly managed, they can result in septic shock, multiple organ failure and eventual death (2). The epidemiological burden of sepsis worldwide is difficult to accurately determine, but it is estimated to affect >30 million people globally every year, being potentially lethal in up to 6 million cases (3).

1.2 Laboratory tests to detect infection

In the appropriate clinical setting, bacteraemia is best confirmed by a positive blood culture, which is considered to be the "gold standard" test. However, a significant downfall of this test is the lengthy incubation period that is required for a definitive diagnosis to be made (4). Therefore, additional laboratory tests have been used as septic biomarkers to screen for infection earlier in its course. Various laboratory tests in this regard have not changed greatly since the 1970s, with the full blood count (FBC) and differential count (to detect leucocytosis and neutrophilia), peripheral blood smear (to detect morphological features of infection such as toxic granulation and band cells), C-reactive protein (CRP) as well as other acute-phase-reactants (5) being utilised currently as the major diagnostic tools. The limitation with these tests however, is that they are not specific to infective processes. For example, CRP may be raised with tissue injury

following any disease process, not just sepsis (6). Other tests such as the soluble serum marker procalcitonin (Pct) has an excellent negative predictive value but may not be effective in the initial diagnosis or monitoring of disease progress because levels have been elevated in bacterial, non-bacterial, as well as non- infectious disease processes (5). Furthermore, Pct is costly and often requires serial monitoring (7). Hence, finding a suitable and cost effective septic biomarker (which is both highly specific and sensitive for detecting early bacterial infection) would be beneficial, both economically and therapeutically (6). This has the potential to enable the initiation of early and appropriate antimicrobial therapy, reducing morbidity and mortality whilst concurrently lowering costs of redundant testing in uninfected patients (4, 6).

1.2.1. Neutrophil CD64 expression as a septic biomarker

Among the many potential septic biomarkers which have been investigated, the expression of CD64 on the surface of neutrophils in response to early systemic acute inflammatory activation has shown promise (5). This marker is a high-affinity immunoglobulin Fc gamma receptor I (FcgRI) and is upregulated on activated neutrophils, with expression being low on resting neutrophils. In a meta-analysis by Yeh *et al.*, the diagnostic accuracy of the expression of CD64 on polymorphonuclear neutrophils as a marker of bacterial infection was found to be excellent (sensitivity of 87% and specificity 89%, with summary receiver operating characteristic (SROC) area under the curve (AUC) of 0.94 and a diagnostic odds ratio (DOR) of 53) (8). Furthermore, neutrophil CD64 expression was found to out-perform both the CRP and Pct in sepsis detection (8). However, a downfall to this test is that it is expensive and requires specialised laboratory facilities.

1.2.2 Extended differential parameters as septic biomarkers

Other possible infectious biomarkers include those generated on white cell analysis by automated haematology analysers (extended differential parameters (EDPs)). These detect changes in the neutrophils, which play an important role in acute inflammation and infections (4). EDPs hold appeal in resource constrained environments, as they are performed as part of each differential count, which is inexpensive. EDPs include the automated immature granulocyte (IG) count performed by Sysmex XT-, XE- and XN-series full blood count analysers (Sysmex, Kobe, Japan), which enumerates granulocyte precursors (measuring promyelocytes, myelocytes and metamyelocytes). When compared to a manual immature granulocyte count, the automated IG count has been shown to be more precise and accurate when using the XE-2100 (Sysmex, Kobe, Japan) (9). Its utility in detecting bacterial infection was originally demonstrated in a study by Ansari-Lari *et al*. (4), which established that the specificity was greater than 90% for an IG count >3% for bacteraemia among samples submitted for a blood culture (4). The sensitivity of this test was however low in this study. This parameter has since been shown to have utility in either diagnosing or stratifying sepsis severity in a number of clinical contexts, including patients in the intensive care unit (ICU) (4, 10-12), the emergency department (13, 14), burn victims (15) and patients admitted in the haematology and infectious diseases departments (16). The ideal cut-off value for interpretation of IG results is not well standardised, with different studies reporting superior performance with counts >0.5% (13, 17), >2% (15), >3% (4) or with elevation of the absolute IG count (10, 14, 15). Additional newer EPDs with possible value as sepsis biomarkers are the NE-SFL and NE-WY parameters (which are defined as the fluorescent light intensity of the

neutrophil area and the fluorescent light distribution width of the neutrophil area on the white cell differential fluorescence (WDF) scattergram respectively) measured by Sysmex XN-series analysers. These parameters were shown by Park *et al.* to have good sensitivity and specificity for the detection of bacteraemia at a cut off of >51.6 for NE-SFL and >641.0 for NE-WY, as well as a positive correlation with an IG count >0.5% (17).

1.3 South African experience using EPDs

To date, no South African studies have evaluated any of the above EPDs as sepsis biomarkers, however, a local study (currently in press) showed that the automated IG count was inaccurate in a proportion of patients, particularly in those with underlying human immunodeficiency virus (HIV) infection. Its utility as a sepsis biomarker may thus be questionable in the South African context. Sysmex analysers measure the IG count based on cell size and intracytoplasmic fluorescence of a nucleic acid binding dye. Among HIV positive patients, bizarre giant neutrophils are common and these cells are often miscounted by the analyser as IGs, probably due to their increased cell size and nucleic acid content. However, not all samples with inaccurate automated IG counts have morphologically abnormal neutrophils, and we hypothesize that the inaccurate IG counts in these cases may rather be a product of neutrophil activation. Hence, it is possible that a raised automated IG count could still be an appropriate sepsis biomarker, even though the IG count may not be technically accurate.

2. STUDY HYPOTHESIS, AIMS AND OBJECTIVES

2.1 Hypothesis

EDPs are useful tests in the diagnosis of sepsis among South African patients, including HIV positive patients.

2.2 Study aims

2.2.1 Primary aim

To evaluate EDPs as biomarkers of sepsis in the South African setting.

2.2.2 Secondary aims

1) To evaluate whether erroneously elevated IGs reflect neutrophil activation.

2) To evaluate whether the novel EDPs (NE-SFL and NE-WY) reflect neutrophil activation (as has been previously speculated).

2.3 Objectives

1. To compare the EDPs (the automated IG count, the NE-SFL and the NE-WY) in samples with and without bacteraemia among patients with clinical suspicion of sepsis (as evidenced by a blood culture request).

2. To determine cut off values for the EDPs with optimal diagnostic performance (sensitivity, specificity, positive- and negative predictive values) for detection of bacteraemia among patients with clinical suspicion of sepsis.

3. To assess EDPs as markers of infection in comparison to conventional sepsis biomarkers (CRP, Pct and neutrophil CD64 expression (as a high-quality septic biomarker)) among patients with clinical suspicion of sepsis.

4. To assess whether there is a relationship between erroneously elevated automated IG counts and neutrophil activation (as evidenced by neutrophil CD64 expression).

5. To assess whether there is a relationship between the novel EDPs (NE-SFL and NE-WY) and neutrophil activation (as evidenced by neutrophil CD64 expression).

3. METHODS

3.1 Study setting

National Health Laboratory Service (NHLS) haematology and flow cytometry laboratories at Chris Hani Baragwanath Academic Hospital (CHBAH) and Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) respectively.

3.2 Study population

Samples submitted from patients admitted to the CHBAH with suspected bacterial sepsis (as evidenced by a blood culture request) will be evaluated. Samples will be identified by a daily search of the NHLS laboratory information system (LIS) (TrakCare) for all blood cultures received in the prior 24 hours. A search for an FBC submitted within 12 hours before or after the blood culture request will be sought for each blood culture received. Samples from patients of all ages will be eligible for inclusion and will be consecutively included until the target sample size of approximately 150 has been reached.

3.2.1 Inclusion criteria

All cases where a blood culture and FBC have been requested within 12 hours of each other will be eligible for inclusion.

3.2.2 Exclusion criteria

Samples will be excluded if:

1) They are of insufficient volume (<1 ml) following routine testing.
2) The Ethylenediaminetetraacetic acid (EDTA) tube submitted for an FBC is >24 hours old if a differential count was requested with the original FBC, or >12 hours old if a differential count was not originally requested.
3) The analyser fails to perform an automated differential count.

3.3 Study design

Prospective cross-sectional analytical study.

3.4 Data collection

3.4.1 Clinical parameters to be recorded

Age, sex, ward, date of admission and available clinical information will be documented from the LIS.

3.4.2 Laboratory parameters to be recorded

Results available in the LIS to be recorded include the FBC, available automated differential count, CRP and Pct results within 72 hours, prior culture results, HIV status, CD4 count, HIV viral load (where applicable), as well as recent international normalised ratio (INR)/partial thromboplastin time (PTT), urea, creatinine and liver function test results.

3.4.3 Sample size

Approximately150 samples will be included. The sample size is comparable with several prior studies evaluating various parameters as sepsis biomarkers (4, 17-19). Using the following formula: $N=4(1.65)^2P(1-P)/(0.2)^2$ and assuming a positive blood culture rate of 30%, this sample size will allow us to detect a minimum test sensitivity of 80% (+/-10%) at a 90% confidence level.

3.4.4 The variables to be assessed

1) The automated differential count (which includes the IG percentage (IG%), absolute IG count (IG#), NE-SFL and the NE-WY)
2) A manual differential count
3) Neutrophil CD64 expression by flow cytometry
4) Blood culture results as well as other pertinent laboratory results and clinical data from the LIS

Please see APPENDIX I for the data collection sheet.

3.4.5 Laboratory methods

1. An automated differential count will be performed on all included samples (if one was not already performed during routine testing) using the Sysmex XN-9000 haematology analysers at the NHLS laboratory at CHBAH. White cell analysis is performed on XN-series analysers using flow cytometry technology in the white cell differential (WDF) channel (20). Following red cell lysis, cells are permeabilised and exposed to a polymethine dye which binds to nucleic acid and proteins in the cytoplasmic organelles. Cellular differentiation is then performed on the basis of cell size (reflected by forward scatter (FSC)), intracytoplasmic nucleic acid content (as

evidenced by side fluorescence (SFL)) and intracellular contents (reflected by laser light side scatter (SSC)). IGs are located above the neutrophils on the SSC/SFL plot (reflecting their larger size), and the NE-SFL and NE-WY parameters are measured as the fluorescent light intensity of the neutrophil area and the fluorescent light distribution width of the neutrophil area on the WDF scattergram respectively (20).

Following the automated differential count, two slides will be made and stained with May-Grünwald-Giemsa, and a manual differential count performed by two competent morphologists on 200 cells each. Should these results be discrepant, a third morphologist will be asked to perform a manual differential count. The automated IG percentage (IG%), absolute IG count (IG#), NE-SFL, NE-WY, white cell count, platelet count, Haemoglobin, neutrophil count and the manual IG count (calculated as the average IG count from the two morphologists) will be recorded.

2. Neutrophil CD64 expression will be measured on the same sample by flow cytometry in the flow cytometry laboratory of the at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). The samples will be transported from CHBAH to CMJAH with the NHLS transport system. Neutrophil CD64 expression will be measured using an adaptation of a previously described protocol (21) on a Beckman Coulter Navios flow cytometer (Beckman Coulter, Inc, Brea, California, USA). A lyse-no-wash preparation method using commercially produced fluorescence conjugated antibodies with specificity to white blood cells (CD45), monocytes (CD14), HLA-DR and CD64 and a fluorescence latex bead suspension will be used. Briefly, 100 µL of whole blood, or diluted whole blood to adjust leukocyte concentration to less than 10 x $l0^9$/L, will be

incubated for 15 minutes in the dark at room temperature with a mixture of the monoclonal antibodies followed by red cell lysis using the Beckman Coulter Immunoprep reagent system.100µL of fluorescence beads will then be added (to monitor accuracy of CD64+ expression) and flow cytometer acquisition and analysis performed on a minimum of 50 000 leukocytes. Data analysis for fluorescence intensity will be performed on the Beckman Navios flow cytometer (Table 2). CD64 mean fluorescence intensity (MFI) will be measured as a linearized value of log scale on the lymphocytes (negative control), the monocytes (positive control), the neutrophils, and the beads. The CD64 index will be calculated as the ratio of linearized MFI of the cell population of interest to the signal from the beads. As an internal quality control, the CD64 index should be <1.0 for the lymphocyte CD64 index, >3.0 for the monocyte CD64 index. Flow cytometry will be performed within 36 hours after blood sampling to ensure viability of cellular components.

4. DATA ANALYSIS

Positive samples will be defined as those with positive blood cultures (with pathogenic organisms).

The median EDP levels will be compared among patients with and without bacteraemia using a Mann-Whitney *U*-test.

Receiver operating characteristic (ROC) curve analysis will be performed for each of the parameters of interest to determine the optimal cut-off for interpretation and the overall performance of the assay (as evidenced by the AUC).

Sensitivity, specificity, positive predictive value and negative predictive value will be calculated for each parameter using the following formulas (22):

1) Sensitivity: A/(A+C) × 100
2) Specificity: D/(D+B) × 100
3) Positive predictive value (PPV): A/(A+B) x 100
4) Negative predictive value (NPV): D/(D+C) x 100

		Disease	
Test Result	Disease	Non-Disease	Total
Positive	A (True Positive)	B (False positive)	T Test Positive
Negative	C (False negative)	D (True Negative)	T Test Negative
	T Disease	T Non-Disease	Total

Table 1: Two-way table used to calculate sensitivity, specificity, positive and negative predictive values (22)

Agreement between the EDPs and the presence of increased neutrophil CD64 expression (as the gold standard sepsis biomarker) will be determined by Cohen's kappa co-efficient.

Manual IG% counts will be compared to the automated IG% using a Rümke table, which assigns a 95% confidence interval for the true number of cells of interest present (depending both on the total number of cells counted and the percentage of the cells of interest observed). Results will be classified as grossly discordant if the analyser IG% falls ≥2% beyond the range specified in the Rümke table.

The Mann-Whitney *U*-test will be used to compare the neutrophil CD64 index among samples with erroneously elevated IG% counts/elevated NE-WY or NE-SFL levels to those with low IG% counts/low NE-WY or NE-SFL respectively. Statistical significance will be accepted at a p-value <0.05.

5. ETHICS

An application has been submitted to the Human Research Ethics Committee of the University of the Witwatersrand for approval to conduct the study. The risk of the study is negligible, as it will not impact patient care, and no identifying information will be included.

6. TIMING

Sample collection will commence once ethics approval has been granted and up until the time the sample number of approximately 150 has been reached. The time frame to reach this has been estimated at approximately 3 months.

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec
Literature review												
Protocol preparation												
Ethics application												
Protocol assessment												
Data collection												
Data analysis												
Writing up: thesis												
Writing up: article												To be done the following year

Table 2. Timeline for projected completion of research thesis

7. FUNDING

	Units	Unit cost	Total cost
CD64 PE monoclonal antibodies	3	R 3090.00	R 9270.00
HLA-DR FITC monoclonal antibodies	3	R 4 560.00	R 13680.00
CD14 PC-5	3	R916.00	R 2748.00
CD45 ECD	3	R2621.00	R 7863.00
Consumables			R 1000.00
Full blood count reagents			To be covered by the laboratory
Publication costs			R 10000.00
		Total	R44 561.00

Table 3. Proposed budget for research thesis

Funding applications will be submitted to the NHLS research trust and the Faculty Research Committee of the University of the Witwatersrand.

Should the above funding not come through, Prof. Debbie Glencross has committed to sponsor the reagents for the study.

LIMITATIONS

Samples with blood cultures which are contaminated with skin commensals will be excluded from analysis.

Patients clinical files will not be examined.

There may be difficulty in tracing all of a patient's blood results if the same name and hospital number were not used for all requests.

REFERENCES

1. Christaki E, Giamarellos-Bourboulis EJ. The Complex Pathogenesis of Bacteremia: From Antimicrobial Clearance Mechanisms to the Genetic Background of the Host. Virulence. 2014;5(1):57-65.

2. Singer M DC, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). The Journal of the American Medical Association. 2016;315(8): 801-10.

3. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlattmann P, et al. Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations. American Journal of Respiratory and Critical Care Medicine. 2016;193(3):259-72.

4. Ansari-Lari MA, Kickler TS, Borowitz MJ. Immature Granulocyte Measurement Using the Sysmex XE-2100. Relationship to Infection and Sepsis. American Journal of Clinical Pathology. 2003;120(5):795-9.

5. Davis BH. Improved Diagnostic Approaches to Infection/Sepsis Detection. Expert Review of Molecular Diagnostics. 2005;5(2):193-207.

6. Cid J, Aguinaco R, Sanchez R, Garcia-Pardo G, Llorente A. Neutrophil CD64 Expression as a Marker of Bacterial Infection: A Systematic Review and Meta-analysis. The Journal of infection. 2010;60(5):313-9.

7. Smith KJ, Wateska A, Nowalk MP, Raymund M, Lee BY, Zimmerman RK, et al. Cost-Effectiveness of Procalcitonin-guided Antibiotic use in Community Acquired Pneumonia. Journal of General Internal Medicine. 2013;28(9):1157-64.

8. Yeh CF, Wu CC, Liu SH, Chen KF. Comparison of the Accuracy of Neutrophil CD64, Procalcitonin, and C-reactive Protein for Sepsis Identification: A Systematic Review and Meta-analysis. Annals of Intensive Care. 2019;9(1):5.

9. Fernandes B, Hamaguchi Y. Automated Enumeration of Immature Granulocytes. American Journal of Clinical Pathology. 2007;128(3):454-63.

10. Nierhaus A, Klatte S, Linssen J, Eismann NM, Wichmann D, Hedke J, et al. Revisiting the White Blood Cell Count: Immature Granulocytes Count as a Diagnostic Marker to Discriminate Between SIRS and Sepsis- A Prospective, Observational Study. BMC Immunology. 2013;14:8.

11. van der Geest PJ, Mohseni M, Brouwer R, van der Hoven B, Steyerberg EW, Groeneveld ABJ. Immature Granulocytes Predict Microbial Infection and its Adverse Sequelae in the Intensive Care Unit. Journal of Critical Care. 2014;29(4):523-7.

12. Buoro S, Mecca T, Azzarà G, Apassiti Esposito S, Seghezzi M, Vavassori M, et al. Extended Leukocyte Differential Count and C-reactive Protein in Septic Patients with Liver Impairment: Diagnostic Approach to Evaluate Sepsis in Intensive Care Unit. Annals of Translational Medicine. 2015;3(17):244-.

13. Ha SO, Park SH, Park SH, Park JS, Huh JW, Lim CM, et al. Fraction of Immature Granulocytes Reflects Severity but not Mortality in Sepsis. Scandinavian Journal of Clinical and Laboratory Investigation. 2015;75(1):36-43.

14. Karon BS, Tolan NV, Wockenfus AM, Block DR, Baumann NA, Bryant SC, et al. Evaluation of Lactate, White Blood Cell Count, Neutrophil Count, Procalcitonin and Immature

Granulocyte Count as Biomarkers for Sepsis in Emergency Department Patients. Clinical Biochemistry. 2017;50(16-17):956-8.

15. Hampson P, Dinsdale RJ, Wearn CM, Bamford AL, Bishop JRB, Hazeldine J, et al. Neutrophil Dysfunction, Immature Granulocytes, and Cell-free DNA are Early Biomarkers of Sepsis in Burn-injured Patients: A Prospective Observational Cohort Study. Annals of Surgery. 2017;265(6):1241-9.

16. Ayres LS, Sgnaolin V, Munhoz TP. Immature Granulocytes Index as Early Marker of Sepsis. International Journal of Laboratory Hematology. 2019;1-5.

17. Park SH, Park CJ, Lee BR, Nam KS, Kim MJ, Han MY, et al. Sepsis Affects Most Routine and Cell Population Data (CPD) Obtained Using the Sysmex XN-2000 Blood Cell Analyzer: Neutrophil-related CPD NE-SFL and NE-WY Provide Useful Information for Detecting Sepsis. International Journal of Laboratory Hematology. 2015;37(2):190-8.

18. Cornet E, Boubaya M, Troussard X. Contribution of the New XN-1000 Parameters NEUT-RI and NEUT-WY for Managing Patients with Immature Granulocytes. International Journal of Laboratory Hematology. 2015;37(5):123-6.

19. Nigro KG, O'Riordan M, Molloy EJ, Walsh MC, Sandhaus LM. Performance of an Automated Immature Granulocyte Count as a Predictor of Neonatal Sepsis. American Journal of Clinical Pathology. 2005;123(4):618-24.

20. Briggs C, Longair I, Kumar P, Singh D, Machin SJ. Performance Evaluation of the Sysmex Haematology XN Modular System. Journal of Clinical Pathology. 2012;65(11):1024-30.

21. Pradhan R, Jain P, Paria A, Saha A, Sahoo J, Sen A, et al. Ratio of Neutrophilic CD64 and Monocytic HLA-DR: A Novel Parameter in Diagnosis and Prognostication of Neonatal Sepsis. Cytometry Part B, Clinical Cytometry. 2016;90(3):295-302.

22. Harris M TG. Medical Statistics Made Easy: 3rd Edition. Scion Publications. 2014:116.

APPENDIX 1 (protocol)

DATA SHEET:

Sample number: …… Specimen laboratory episode number: ……

Patient age: …….. Patient sex: …….. Patient location (ward): ……..

Date of admission: ……. Date and time bloods taken: ……

Diagnosis / other clinical information from the LIS (if available): …….

……

……..

WCC..... HB……. MCV……. Plts……. Pct……. CRP…….

Automated differential count: Neut……. Lymph……. Mono……. Eos……. Baso……. IG% ……. Absolute IG …….

Manual differential count 1: Neut……. Lymph……. Mono……. Eos……. Baso……. IG% ……. Absolute IG …….

Manual differential count 2: Neut……. Lymph……. Mono……. Eos……. Baso……. IG% ……. Absolute IG …….

Average manual count: Neut……. Lymph……. Mono……. Eos……. Baso……. IG% ……. Absolute IG …….

NE-SFL …….. NE-WY ……..

Time and date of flow cytometric analysis …….…….

Neutrophil CD64 MFI …….. Monocytes CD64 MFI ……..

Neutrophil CD64 index ……..

Blood culture results:

.....

.....

.....

Previous blood culture results:

……

……..

HIV: Positive / Negative /Not tested

CD4 count (if available) …….. Viral load (if available) ……..

Other pertinent results (if available): U&E / LFT / INR / PTT
.....

APPENDIX III

Turn-it-in report

Chris Hani Baragwanath Hospital
Johannesburg
17/11/20

To whom it may concern,

Re: TURN-IT-IN report for the MMed research report entitled: The usefulness of the extended differential parameters as a biomarker of bacteraemia at Chris Hani Baragwanath Academic Hospital.

As per standard university protocol, this MMed thesis was analysed with TURN-IT-IN software. This has yielded a similarity index of 14%. As supervisors of this project, we are satisfied that the similarities detected are not due to plagiarism, and that this thesis represents the student's own wok.

Yours sincerely,

Dr Jenifer Vaughan

Dr Denise Lawrie
Supervisors of Dr Lauren Lemkus

457987:Thesis_457987.docx

ORIGINALITY REPORT

14%	11%	12%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	ajlmonline.org Internet Source	2%
2	Park, S. H., C.-J. Park, B.-R. Lee, K.-S. Nam, M.-J. Kim, M.-Y. Han, Y. J. Kim, Y.-U. Cho, and S. Jang. "Sepsis affects most routine and cell population data (CPD) obtained using the Sysmex XN-2000 blood cell analyzer: neutrophil-related CPD NE-SFL and NE-WY provide useful information for detecting sepsis", International Journal of Laboratory Hematology, 2014. Publication	1%
3	repub.eur.nl Internet Source	1%
4	hdl.handle.net Internet Source	1%
5	journals.lww.com Internet Source	1%
6	Sang Hyuk Park, Chan-Jeoung Park, Bo-Ra	<1%

	Lee, Mi-Jeong Kim, Min-Young Han, Young-Uk Cho, Seongsoo Jang. "Establishment of Age- and Gender-Specific Reference Ranges for 36 Routine and 57 Cell Population Data Items in a New Automated Blood Cell Analyzer, Sysmex XN-2000", Annals of Laboratory Medicine, 2016 Publication	
7	J L Vaughan, T M Wiggill, N Alli, K Hodkinson. "The prevalence of HIV seropositivity and associated cytopenias in full blood counts processed at an academic laboratory in Soweto, South Africa.", South African Medical Journal, 2017 Publication	<1%
8	link.springer.com Internet Source	<1%
9	Livaditi, O.. "Neutrophil CD64 expression and serum IL-8: Sensitive early markers of severity and outcome in sepsis", Cytokine, 200612 Publication	<1%
10	academic.oup.com Internet Source	<1%
11	annalsofintensivecare.springeropen.com Internet Source	<1%
12	journals.sagepub.com Internet Source	<1%

13	Cihan Bedel, Mustafa Korkut, Ali Avcı, Ahmet Uzun. "Immature Granulocyte Count and Percentage as New Predictors of Mortality in Patients with Upper Gastrointestinal Bleeding", Indian Journal of Critical Care Medicine, 2020 Publication	<1%
14	www.thefreelibrary.com Internet Source	<1%
15	Jenifer L. Vaughan, Nazeer Alli, Sandra Havyarimana, Estee Benade. "Assessment of International Consensus Group for Haematology smear review rules among patients with Plasmodium falciparum malaria in Johannesburg, South Africa", African Journal of Laboratory Medicine, 2018 Publication	<1%
16	Submitted to Academic Services Korea Student Paper	<1%
17	M Feng, S L Zhang, Z J Liang, Y L Wang, X C Zhao, C Gao, H Guo, J Luo. "Peripheral neutrophil CD64 index combined with complement, CRP, WBC count and B cells improves the ability of diagnosing bacterial infection in SLE", Lupus, 2019 Publication	<1%
18	Submitted to Nottingham Trent University Student Paper	<1%

19	www.journals.ac.za Internet Source	<1%
20	www.chp.gov.hk Internet Source	<1%
21	Submitted to University of Witwatersrand Student Paper	<1%
22	pedres.org.cn Internet Source	<1%
23	bmcpediatr.biomedcentral.com Internet Source	<1%
24	dissertations.ub.rug.nl Internet Source	<1%
25	kclpure.kcl.ac.uk Internet Source	<1%
26	S Danese. "Activated platelets are the source of elevated levels of soluble CD40 ligand in the circulation of inflammatory bowel disease patients", Gut, 2003 Publication	<1%
27	banglaforexacademy.com Internet Source	<1%
28	Schapkaitz, Elise, and Dashini Pillay. "Prolonged storage-induced changes in haematology parameters referred for testing",	<1%

	African Journal of Laboratory Medicine, 2015. Publication	
29	www.hindawi.com Internet Source	<1%
30	openmicrobiologyjournal.com Internet Source	<1%
31	M. Christ-Crain, B. Muller. "Biomarkers in respiratory tract infections: diagnostic guides to antibiotic prescription, prognostic markers and mediators", European Respiratory Journal, 2007 Publication	<1%
32	www.health.au.dk Internet Source	<1%
33	Fugui Zhang, Grace Hsu, Somdipto Das, Youbai Chen, Meredith August. "Independent Risk Factors Associated with Higher Mortality Rates and Recurrence of Brain Abscesses from Head and Neck Sources", Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology, 2020 Publication	<1%
34	"Abstracts from the 35th Annual Meeting of the Society of General Internal Medicine", Journal of General Internal Medicine, 2012 Publication	<1%
35	onlinelibrary.wiley.com	

	Internet Source	<1%
36	etheses.bham.ac.uk Internet Source	<1%
37	"Poster abstracts", International Journal of Laboratory Hematology, 2012. Publication	<1%
38	synapse.koreamed.org Internet Source	<1%
39	Ye Zhang, Yun Zhou, Wenjie Li, Veronica Lyons, Amanda Johnson, Amanda Venable, John Griswold, Dimitri Pappas. "Multiparameter Affinity Microchip for Early Sepsis Diagnosis Based on CD64 and CD69 Expression and Cell Capture", Analytical Chemistry, 2018 Publication	<1%
40	obgyn.onlinelibrary.wiley.com Internet Source	<1%
41	www.nature.com Internet Source	<1%
42	www.scielo.br Internet Source	<1%
43	R. J. Dinsdale, A. Devi, P. Hampson, C. M. Wearn et al. "Changes in novel haematological parameters following thermal injury: A	<1%

	prospective observational cohort study", Scientific Reports, 2017 Publication	
44	"Promoting Healthy Human Relationships in Post-Apartheid South Africa", Springer Science and Business Media LLC, 2021 Publication	<1%
45	www.bioscience.org Internet Source	<1%
46	asclepiusopen.com Internet Source	<1%
47	D. D. Danikas. "Prognostic value of phagocytic activity of neutrophils and monocytes in sepsis. Correlation to CD64 and CD14 antigen expression", Clinical & Experimental Immunology, 10/2008 Publication	<1%
48	"ESICM LIVES 2019", Intensive Care Medicine Experimental, 2019 Publication	<1%
49	Guillaume Monneret, Alain Lepape, Nicolas Voirin, Julien Bohé et al. "Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock", Intensive Care Medicine, 2006 Publication	<1%

50	G S Ogilvie. "Diagnostic accuracy of self collected vaginal specimens for human papillomavirus compared to clinician collected human papillomavirus specimens: a meta-analysis", Sexually Transmitted Infections, 6/1/2005 Publication	<1%
51	moam.info Internet Source	<1%

Exclude quotes	On	Exclude matches	< 5 words
Exclude bibliography	On		

APPENDIX IV

PLOS ONE submission guidelines

Style and Format

File format	Manuscript files can be in the following formats: DOC, DOCX, or RTF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.
Length	Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.
Font	Use a standard font size and any standard font, except for the font named "Symbol". To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.
Headings	Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.
Layout and spacing	Manuscript text should be double-spaced. Do not format text in multiple columns.
Page and line numbers	Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).
Footnotes	Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.
Language	Manuscripts must be submitted in English. You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.
Abbreviations	Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.
Reference style	PLOS uses "Vancouver" style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.
Equations	We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor or Microsoft's Insert→Equation function is acceptable. Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., "$a^2 + b^2 = c^2$"), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values.

		Do not use MathType, Equation Editor, or the Insert→Equation function for only a portion of an equation. Rather, ensure that the entire equation is included. Equations should not contain a mix of different equation tools. Avoid “hybrid” inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.
Nomenclature	Use correct and established nomenclature wherever possible.	
	Units of measurement	Use SI units. If you do not use these exclusively, provide parentheses after each value. Read more about SI units.
	Drugs	Provide the Recommended International Non-Proprietary
	Species names	Write in italics (e.g., *Homo sapiens*). Write out in full the both in the title of the manuscript and at the first mention paper. After first mention, the first letter of the genus nam full species name may be used (e.g., *H. sapiens*).
	Genes, mutations, genotypes, and alleles	Write in italics. Use the recommended name by consultin genetic nomenclature database (e.g., HGNC for human ge recommend using this tool to check against previously ap is sometimes advisable to indicate the synonyms for the g appears in the text. Gene prefixes such as those used for c cellular localization should be shown in roman typeface (MYC).
	Allergens	The systematic allergen nomenclature of the World Healt Organization/International Union of Immunological Socie Allergen Nomenclature Sub-committee should be used fo include the description or use of allergenic proteins. For n describing new allergens, the systematic name of the aller approved by the WHO/IUIS Allergen Nomenclature Sub- manuscript publication. Examples of the systematic allerg can be found at the WHO/IUIS Allergen Nomenclature si