The usefulness of monocyte fluorescence as a biomarker of Tuberculosis infection at Chris Hani Baragwanath Academic Hospital

Dr Aamina Moosa

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.

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DECLARATION

I, Aamina Moosa, 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.

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A Moosa

08 November 2023

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LIST OF TABLES

ABBREVIATIONS

AFB - Acid fast bacilli

ART - Anti-retroviral treatment

AUC - Area under curve

CHBAH - Chris Hani Baragwanath Academic Hospital

CI - Confidence interval

CRP - C-reactive protein

CSF - Cerebrospinal fluid

CT - Cycle threshold

FBC - Full blood count

FNA - Fine needle aspirate

GXPU - Xpert MTB/RIF

Hb - Haemoglobin

HIV - Human Immunodeficiency Virus

IGFR - Interferon gamma release assay

IQR - Interquartile range

LAM - Lipoarabinomannan

LIS - Laboratory information system

LR - Likelihood ratio

MCV - Mean cell volume

M. tuberculosis - Mycobacterium tuberculosis

MO-Y - Monocyte fluorescence

NHLS - National Health Laboratory System

NE-SFL - Neutrophil fluorescent light intensity

NLR - Neutrophil:lymphocyte ratio

NPV - Negative predictive value

PCR - Polymerase chain reaction

PPV - Positive predictive value

PLWH - People living with HIV

RNA - Ribonucleic acid

ROC - Receiver operating characteristic

SA - South Africa

TB - Tuberculosis

WHO - World health organisation

RESEARCH ARTICLE IN THE FORMAT OF ‘SUBMISSIBLE’ PAPER

TITLE PAGE

The usefulness of monocyte fluorescence as a biomarker of Tuberculosis infection at Chris Hani Baragwanath Academic Hospital

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The usefulness of monocyte fluorescence as a biomarker of Tuberculosis infection at Chris Hani Baragwanath Academic Hospital

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Abstract

Introduction

South Africa has the 5th highest burden of Tuberculosis (TB) as well as coinfection with Human immunodeficiency virus (HIV) worldwide. Routine laboratory methods have varying sensitivity and specificity. The Xpert MTB/RIF (GXPU) (Cepheid, Sunnyvale, CA), has lower sensitivity in sputum smear negative cases and poor quality sputum samples. A robust, non-sputum based, inexpensive biomarker of TB would be of value in such cases.

Monocytes are the major leucocyte involved in the immune response to TB. The Sysmex haematology analysers (Sysmex, Kobe, Japan) measure monocyte activation via monocyte fluorescence (MO-Y). This study aimed to evaluate the MO-Y and other Sysmex extended differential parameters (EDPs) as biomarkers of TB infection in the local setting.

Methods

The MO-Y and EDPs were retrieved from the analyser for 121 adult cases (56 with TB, 65 controls). Further information was obtained from the laboratory information system,

including patient demographics and other laboratory results; TB culture, SARS-CoV-2 results, C-reactive protein level, HIV status, bone marrow biopsies and the cycle threshold (CT) values on positive GXPU analysis. The MO-Y, EDPs and full blood count (FBC) values were compared among patients with and without TB (HIV positive and negative). Statistical significance was assessed (P-value of <0.05).

Results

The MO-Y did not show utility in identifying patients with TB.

A sub-population of patients living with HIV (PLWH) with a CD4 <100 cells/ul showed significantly higher MO-Y levels, due to other opportunistic infections affecting monocytes.

Neutrophil surface fluorescence (a marker of neutrophil activation), was significantly higher in PLWH and with concomitant TB infection, possibly due to immune activation, worse illness, or increased bacterial infection.

Among the PLWH, those with TB had significantly lower CD4 counts, absolute lymphocyte counts and mean cell volume (MCV) values. The MCV (cut-off value 87 fL) showed the strongest diagnostic utility for discriminating PLWH with and without TB (AUC 0.79).

Conclusion

The MO-Y is not a useful biomarker of TB, but is significantly elevated in PLWH with low CD4 counts. The MCV showed adequate discriminatory power for differentiating patients with and without TB, at a cut-off level of 87fL.

Introduction

Tuberculosis (TB) is a communicable disease, caused by the microorganism *Mycobacterium tuberculosis* (*M. tuberculosis*). (1) Despite being a curable disease, TB is one of the top ten causes of death worldwide, and is one of the top three causes of death in South Africa (SA). (2,3) While great strides have been made in the diagnosis and treatment of TB in SA, it carries the 5th highest burden of TB in the world and is one of six countries contributing to 60% of the global TB burden. (2,4)

The majority of TB in SA is associated with human immunodeficiency virus (HIV) infection, and SA bears the highest burden of HIV and TB co-infection cases worldwide. (4)

A key factor in the fight against TB is early diagnosis, and forms the first pillar of the End TB Strategy by the World Health Organisation (WHO). (1) The current routine laboratory methods used to diagnose TB depend upon the identification of *M. tuberculosis*, most commonly from a sputum specimen. (5) Among the microbiological methods, microscopy is rapid, however sensitivity is limited to 5000–10,000 acid fast bacilli per ml (AFB/ml), and is particularly impaired in people living with HIV (PLWH). (6,7) In contrast, culture can require as few as 100 bacilli/ml and is the gold standard of TB diagnosis, however requires 2-6 weeks incubation. (6,8) The molecular method, Xpert MTB/RIF (GXPU) (Cepheid, Sunnyvale, CA), is based on polymerase chain reaction (PCR) amplification. This test has a rapid turnaround time (<2 hours), high sensitivity and specificity (88% and 96% respectively), and can identify *M. tuberculosis* and rifampicin resistance. (9,10) Samples can be of sputum and non-sputum origin, including but not restricted to fine needle aspirates (FNA) of serous effusions,

cerebrospinal fluid (CSF), and FNA of lymph nodes. (11) The GXPU test is not without limitations, being relatively less sensitive in smear negative cases and requiring a sample of adequate quality (which can be problematic in patients without a productive cough). (12,13) A robust, non-sputum based and inexpensive biomarker of TB would be of value to compliment available diagnostic test methodologies. (10)

Monocytes are the major leucocyte involved in the immune response to TB. (14) Monocytes assist in controlling TB infection, and can become a reservoir for latent TB. (15) Monocytes and TB are a key area of research, with investigation having focused on the inflammatory profile of monocytes and the polarisation of monocytes, monocyte signal transduction, monocyte to lymphocyte ratios and expression of monocyte markers in individuals with TB.(14–17) The Sysmex XT-, XE- and XN haematology analysers (Sysmex, Kobe, Japan) measure the degree of monocyte fluorescence using flow cytometry whilst performing a differential white cell count, which correlates with activation of the cell. (18) A recent South African study evaluating novel white cell parameters generated by the Sysmex XN-9000 haematology analyser among patients with suspected bacterial sepsis found a significant association between elevated monocyte fluorescence (as reflected by the MO-Y parameter) and a diagnosis of TB. (19) As the MO-Y is measured with every differential count performed on the above Sysmex analysers (a test widely performed in the routine work-up of patients with suspected infectious diseases), this parameter holds strong appeal as an inexpensive biomarker of TB.

Monocyte activation may also be of value in the prognostication of patients with SARS-CoV-2 infection. Two studies have assessed extended differential parameters in the setting of SARS-

CoV-2 infection (20,21), one of which found increased monocyte fluorescence in critically ill patients. (20)

In this study, we aimed to evaluate the MO-Y parameter as a biomarker of TB infection among PLWH in the South African setting. As secondary objectives, we aimed to evaluate whether concomitant HIV-infection influences the utility of the MO-Y as a biomarker of TB infection, to assess the effects of TB on the peripheral blood counts and other EDPs, and to evaluate MO-Y levels in patients with documented SARS-CoV-2 infection.

Methods

Sample collection

The study was performed at the Chris Hani Baragwanath Academic Hospital, which is a large academic state-sector hospital in Johannesburg, SA, between the dates of 1st May and 31st August 2023. Adult specimens (defined as >18 years of age) submitted to the national health laboratory service (NHLS) for TB Gene Xpert MTB/RIF testing were identified by a daily search of the NHLS laboratory information system (LIS). The LIS was used to determine whether a recent full blood count (FBC) and automated differential count had been performed within the preceding 72 hours, on the Sysmex XN-9000 haematology analyser. If an automated differential count had not been performed, then refrigerated FBC samples that were less than 24 hours old, were retrieved and an automated differential count was performed. The monocyte fluorescence (MO-Y), neutrophil surface fluorescence (NE-SFL) and other extended differential parameters (EDP) for each differential count was obtained directly from the

analyser. The Sysmex XN-9000 measures the white cell count in the white cell chamber via impedance technology. (22) The differential count is measured in the WBC differential channel via flow cytometry. (22) Cells are permeabilised, intracellular content is stained and these are delivered to a laser point. White cell subpopulations are then identified via forward scatter (informs cell size), side scatter (informs cell complexity) and intracytoplasmic nucleic acid content. (22) The MO-Y and NE-SFL are determined via measurement of fluorescent light intensity of the monocyte and neutrophil areas on the white cell differential scattergram, respectively.

Additional information obtained from the LIS included patient demographics and other laboratory results if available, including HIV status, CD4 count, HIV viral load, TB culture, white cell and differential white cell counts, Haemoglobin (Hb), mean cell volume (MCV) platelet count, SARS-CoV-2 results, C-reactive protein (CRP) level, bone marrow biopsies and the cycle threshold (CT) values on positive GXPU analysis (where lower CT values indicate larger disease burden). As urine testing for the lipoarabinomannan (urine LAM) antigen is performed at the patient bedside, these results were not obtainable unless reported on in the bone marrow biopsy report. Since TB is very much more common among PLWH, for every individual living with HIV with a positive GXPU and an available FBC sample, a matching GXPU sample from an individual living with HIV with a negative result and available FBC was sought. Samples were matched for site of collection (sputum, fine needle aspirate and cerebrospinal fluid). As HIV negative patients were scarce, all samples from HIV negative patients with a GXPU request and an available FBC were included. Samples were consecutively included until the target sample size of approximately 100 cases (50 GXPU positive and 50 GXPU negative) had been

reached. In order to ensure a total HIV+ cohort of 100 patients, the sample size eventually reached 121.

Samples were excluded if there was no available differential count and the residual FBC sample was of insufficient volume to use or >24 hours old. Patients with inconclusive GXPU results or no available HIV result were excluded. The main analysis excluded cases with a positive test for the SARS-CoV-2 virus, however these were assessed as a subpopulation.

Sample size calculation

The sample size was calculated in order to ensure adequate power for detection of a significant difference in the MO-Y among PLWH with and without TB. In a previous study assessing the utility of EDPs in the detection of bacterial sepsis, the MO-Y was found to be marginally significantly higher in the small number of included patients who transpired to have TB as compared to those with and without bacterial sepsis. (19) In this study, all patients with TB were PLWH. The MO-Y did not differ significantly between TB-negative PLWH with and without bacteraemia. The mean difference in mean monocyte fluorescence between the MO-Y in PLWH with and without TB was 13.6 (E), and the standard deviation for the MO-Y among all PLWH patients was 23.3 (S). On the basis of these results, the estimated standardised effect size is ~0.6 (E/S). Given an α of 0.05, 1-β of 0.80 and allowing an additional 10% for any possible errors, the calculated sample size was 50 PLWH with and without TB (total HIV+ cohort of 100 patients).

Statistical analysis

TB positive samples were defined as those with positive GXPU or TB culture results. The median MO-Y levels, other EDPs and FBC values were compared among patients with and

without TB (HIV positive and negative) using a Mann-Whitney U-test. Statistical significance was accepted at a two-sided P-value of 0.05.

Receiver operating characteristic (ROC) curve analysis was performed to determine the overall performance of the relevant assay (as evidenced by the area under the curve (AUC). Cut-off values with the best balance between test sensitivity and specificity for identifying TB (as evidenced by a high likelihood ratio (LR) with both high sensitivity and high specificity on ROC analysis) were selected for the EDPs and FBC parameters which were found to differ significantly from those of the control group.

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

Ethics

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (M-210337). Informed consent was not a requirement by the committee, as testing was performed on residual samples submitted for routine testing. Data was captured onto an excel spreadsheet and fully anonymized.

Results

Study population

A total of 121 samples were collected, 56 with TB, and 65 negative controls. Of the TB positive patients, there were 53 with a positive GXPU test, and 2 with a negative GXPU but positive TB culture (one unspecified aspirate and one blood sample). One patient was included in the TB positive group due to a positive Ziehl-Neelsen stain, performed on the bone marrow trephine biopsy. The TB blood culture subsequently revealed a *Mycobacterium avium complex* infection. The majority of the samples were sputum (82), with the remainder comprising fluid/aspirates (5 pleural fluid, 2 ascitic fluid, 1 pericardial fluid, 4 lymph node fine needles aspirates, 16 unspecified), 10 cerebrospinal fluid samples, and a single tissue sample. The median patient age was 39 years, females predominated comprising 53%. The patients were admitted to a variety of wards, including medical, surgical, and intensive care, and 2 samples were obtained from outpatients. The HIV seropositivity rate was 84% with a median CD4 count of 72 cells/ul. A CD4 count could not be traced for 7 patients.

In the TB negative group there were 7 patients who were found to be SARS-CoV-2 positive, and were thus excluded from the overall analysis. Of these patients, 4 were PLWH (57%). The median MO-Y in this group was 109.9 (IQR 105.2-112.9) with no significant difference in the MO-Y of these patients as compared to the control group (P = 0.64). Relevant biometric data is shown in Table 1.

MO-Y, extended differential parameters and blood count results

The MO-Y value did not differ significantly between the TB positive and TB negative patients, regardless of HIV status (Table 1). When the MO-Y was compared within the TB positive group, between those with high CT values and low CT values on GXPU analysis (indicating disease burden), there was no significant difference (P = 0.95). The possibility of a delay in processing affecting results was considered, however the time to process for the samples with the lowest MO-Y value (4 samples, MO-Y <100) was not found to be significantly prolonged (median 11.7 hours). Similarly, the monocyte to lymphocyte ratio did not differ between the TB positive and negative patients, regardless of HIV status.

In the PLWH, the MO-Y was significantly higher in patients with a CD4 count < 100 cells/ul (median MO-Y 118.4, IQR 108.8 - 124.2) as compared to those with a CD4 count > 100 cells/ul (median MO-Y 108.3, IQR 102.2 - 121.7 (P = 0.008)). This was found regardless of TB status, with a median MO-Y 119.4 versus 104.7 in patients with TB and CD4 counts >100 and <100 respectively (P = 0.024). Of note, one patient with a CD4 count of 1 cell/ul had a very high MO-Y (136.3) and a strong clinical suspicion of TB based on her chest radiography, however did not have a positive GXPU result. A second patient with a CD4 count of 34 cells/ul and a high MO-Y (125.3) was diagnosed with TB with difficulty, having three negative GXPU tests and two negative TB cultures, before being diagnosed with TB on a 3rd TB culture.

The NE-SFL was significantly higher in PLWH with TB compared to those without TB (P < 0.0001), however this finding was not replicated in the HIV negative group (P = 0.05) (Table 1).

Among the PLWH, the mean cell volume, absolute lymphocyte count and CD4 count were all significantly different in the TB positive versus TB negative groups (Table 2). The difference in MCV was most significant ($P < 0.0001$). With regard to other data collected, the CRP was significantly higher in the TB positive (median 139 mg/l, IQR 84 - 211 mg/l) as compared to the TB negative group (median 68 mg/l, IQR 41.50 - 125.5 mg/l ($P = 0.0001$)).

The MCV value showed the most utility in predicting TB, at a cut off value of 87 fL, with an AUC of 0.78.

Analysis of the differential counts and other EDP data in the HIV negative group did not yield any significant differences between patients with and without TB (Table 1).

A sub-analysis was performed on a further seven patients who tested positive for the SARS-CoV-2 virus. All patients in this group were negative for TB, four of the patients were PLWH. The median MO-Y in this group was 109.9 (IQR 105.2-112.9). There was no significant difference in the MO-Y of these patients as compared to the control group ($P = 0.63$).

268 Table 1 Full blood count, differential count and extended differential parameter data of all patients

HIV status	**HIV Positive**			**HIV Negative**		
Sample group	**TB positive (n=46)**	**TB negative (n=55)**	**P-value***	**TB positive (n=10)**	**TB negative (n=10)**	**P-value***
Male: Female	0.6:1	1:1	-	1:1	1.5:1	-
Age (years) Median [IQR]	38 30-46	39 31-49	-	56 24-70	46 35-65	-
CD4 count (cells/ul) Median [IQR]	49 19-98	105 56 – 227	0.004	-	-	
HIV viral load (log(copies/ml)) Median [IQR]	5.4 3.2-6.1	4.8 2.9-5.6	0.15	-	-	-
Haemoglobin (g/dl) Median [IQR] [13.4 - 17.5]†	8.4 7.2 – 9.8	8.9 6.6-11.1	0.33	10.35 8.83-13.10	11.20 7.28-14.23	0.65
MCV (fl) Median [IQR] [83.1 - 101.6]†	84.5 80.1-87.5	92.0 87.4-96.8	< 0.0001	88.95 80.40-93.35	90.20 87.25-101.6	0.35
White cell count (x10^9/L) Median [IQR] [3.92 - 10.40]†	7.34 4.86-10.78	6.62 4.78-11.31	0.91	10.85 5.94-12.60	7.79 6.78-11.91	0.58
Neutrophil count (x10^9/L) Median [IQR] [1.60 - 6.98]†	5.87 3.76-9.14	5.20 3.14-9.83	0.66	8.31 4.87-10.55	6.23 3.80-8.51	0.32
Monocyte count (x10^9/L) Median [IQR] [0.30 - 0.80]†	0.30 0.15-0.57	0.40 0.20-0.62	0.11	0.56 0.33-0.83	0.57 0.33-0.81	0.97
Lymphocyte count (x10^9/L) Median [IQR] [1.40 - 4.20]†	0.51 0.29-1.00	1.01 0.56-1.62	0.002	0.92 0.56-1.52	1.40 0.96-2.63	0.12
Monocyte:Lymphocyte ratio Median [IQR]	0.50 0.32-1.08	0.44 0.26-0.62	0.07	0.54 0.37-1.04	0.40 0.22-0.74	0.44
Platelet count (x10^9/L) Median [IQR] [171 - 388]†	211 123-298	232 160-333	0.34	231 156-378	243 149-482	0.85
NE-SFL Median [IQR]	56.10 53.10-64.45	49.05 46.18-55.78	<0.0001	51.30 47.33-55.18	49.25 47.30-51.13	0.50

MO-Y Median [IQR]	117.20 106.7-125.4	114.50 106.2-121.3	0.44	108.5 104.4-115.8	109.2 107.2-113.0	0.68

†Reference range
HIV, human immunodeficiency virus; MCV, mean cell volume; NE-SFL, neutrophil fluorescent light intensity; MO-Y, monocyte fluorescence

Receiver operating characteristic (ROC) curve analysis

The results of the ROC curve analysis are presented in Table 2. The MCV value showed the most utility in predicting TB, at a cut off value of 87 fl, with an AUC of 0.78. The NE-SFL also showed good prediction of the presence of TB at a cut-off value of >52.35. The CD4 count and absolute lymphocyte count were comparatively poorer biomarkers, at cut off levels of of 59 cells/ul and 0.84 x 10^9/L, respectively.

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

Parameter	AUC	95% CI	P-value for AUC	LR	Sensitivity (%)	Specificity (%)	Cut off value	NPV (%)	PPV (%)
MO-Y	0.55	0.42 - 0.67	0.44	1.18	59.0	50.0	>115.6	58.5	50
NE-SFL	0.73	0.63 - 0.83	<0.0001	3.37	64.1	81.0	>52.35	82.2	67.9
MCV	0.78	0.68 - 0.90	<0.0001	2.80	79.0	72.0	<87 fl	78.2	73.8
CD4 count	0.68	0.51 - 0.77	0.004	1.74	69.4	60.0	<59 cells/ul	69.8	65.9
Lymphocyte count	0.68	0.57 - 0.79	0.002	2.00	66.7	66.7	<0.84 x 10^9/L	68.0	58.8

MO-Y, monocyte fluorescence; NE-SFL, neutrophil fluorescent light intensity; MCV, mean cell volume; AUC, area under curve; CI, confidence interval; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value

Discussion

This study assessed the use of the MO-Y value, as measured by the Sysmex XN-9000 haematology analyser, for the detection of TB in the setting of a South African laboratory. Our cohort of patients demonstrated a high HIV seropositivity rate (84%), thus reflecting the well-documented increased risk of TB in PLWH, as well as the high index of clinical suspicion for TB in these patients.

The MO-Y did not show utility as a biomarker for TB, irrespective of the HIV status. Of note, PLWH with CD4 <100 cells/ul showed a higher MO-Y value as compared to those with a CD4 count >100 cells/ul, regardless of TB status. The raised MO-Y in patients in this group of immunocompromised patients may be due to the presence of other opportunistic infections or chronic infections, resulting in monocyte activation. Alternatively, the MO-Y may still be a biomarker of TB in this subpopulation of patients, since CD4 counts < 100 cells/ul, have been associated with lower rates of laboratory confirmed TB diagnosis. (23) This was illustrated by two of our patients within this group. One had a CD4 count of 34 cells/ul, and underwent repeated testing, with a single positive TB culture obtained out of the six samples submitted (3 GXPUs and 3 TB cultures). A second patient with a CD4 count of 1 cells/ul had strong radiological suspicion of TB but negative GXPU testing. These patients highlight that a proportion of the patients with very low CD4 counts in whom TB could not be confirmed may yet have active TB infection, and that the MO-Y may still be a biomarker of TB in this subpopulation of patients. A biomarker of TB would be particularly useful in these cases, in which there is concern for smear negative TB resulting in reduced sensitivity of the GXPU assay. (24) Further study would be of interest in this group, including complete integration of

urine LAM testing, microbiological, clinical, and radiological findings in order to better assess the value of the MO-Y as an indicator of TB in PLWH and CD4 counts < 100 cells/ul.

The NE-SFL was found to be significantly higher in PLWH patients with TB, but not in the HIV negative group. The NE-SFL has previously been shown to reflect neutrophil activation and to perform as a good biomarker of bacterial sepsis. (19) The findings here may be due to a greater degree of general immune activation (resulting in neutrophil activation), more severe illness, or a higher rate of concomitant bacterial infection in PLWH with TB.

Among the PLWH, those with TB had significantly lower CD4 counts and absolute lymphocyte counts. TB infection is independently associated with a lymphopenia, owing to lymphocyte involvement in the immune-response to TB and augmented by HIV co-infection. (25) This finding was observed in a previous study conducted at the NHLS at Chris Hani Baragwanath Academic Hospital, evaluating the changes in the full blood count in PLWH and TB co-infection. (26)

Anaemia is a common haematological manifestation of TB, and has been suggested as the first indication of TB due to iron dysregulation. (26,27) A previous comparative cross-sectional study showed that HIV co-infection worsens the haematological abnormalities associated with pulmonary TB, including a significantly lower Hb level. (28) In our study, PLWH and TB co-infection had marginally lower haemoglobin levels and significantly lower MCV values, as compared to matched TB negative controls. On ROC analysis, the parameter with the strongest diagnostic utility for discriminating PLWH with and without TB was the MCV (AUC of 0.78 at a cut-off value of 87 fl ($P < 0.0001$)). This group also demonstrated significantly

higher CRP values, thus concurring with a study performed by Rohini et al, who also demonstrated significantly lower haemoglobin levels and higher CRP values in patients with TB as compared to normal controls. (29) It is likely that the lower MCV values in our cohort of patients are related to underlying anaemia of chronic disease. This hypothesis is supported by previous studies, one of which showed an improvement in both the CRP and haemoglobin levels during treatment of TB. (30) Another supportive study investigated the haematological parameters in patients with sputum smear positive TB. The investigators detected a microcytic anaemia in patients with TB, which improved with treatment. (31) An alternative explanation for the lower MCV in patients with TB is greater anti-retroviral therapy (ART) coverage in the TB negative group. This theory is less favoured, as the HIV viral load did not differ between these groups.

Notably, there were no significant differences in any of the blood count parameters in the HIV-negative group with and without TB. This is in direct contrast with several previous studies which have investigated haematological manifestations of TB in patients that are not immunocompromised. (27,31–34) This is possibly explained by the smaller cohort of HIV-negative patients in this study, or the fact that many previous studies have used healthy controls. Yoon et al is one of the exceptions to the latter statement, and used the neutrophil:lymphocyte ratio (NLR) to differentiate pulmonary TB from community acquired pneumonia. (32)

Interferon-gamma release assays (IGRA) are performed on whole blood specimens and measure the cellular immune response to the *M. tuberculosis* antigen, however these have not been used in the clinical setting in SA. (35) Luo et al combined the IGRA test and full blood

count results to construct a diagnostic model which would differentiate between active and latent TB. The MCV, Hb, and lymphocyte count significantly decreased in patients with active TB vs latent TB. (34) The investigators also postulated that using routine blood testing as well as interferon-gamma release assays specifically in immunocompromised patients may assist in identifying active TB. (34) While our study compared patients with active TB to negative controls, this is still an area of possible further research. The diagnostic utility of our significant results may be improved in combination with interferon-gamma release assays, however there are logistical and financial difficulties involved in implementation in the clinical setting.

Limitations

This study has several limitations. Diagnosis of TB in PLWH is challenging, and is associated with smear negative TB resulting in reduced sensitivity of the GXPU assay, and lower rates of laboratory confirmed TB. (24) As illustrated in one patient, diagnosis may require repeated investigation and TB cultures. Our cohort showed an 84% HIV seropositivity rate, with a median CD4 count of 72 cells/ul. This raises concern for false negative results within our control group, which would alter results. Access to the bedside urine LAM test results, clinical details, radiology findings would be of assistance in these cases.

The study was conducted during the COVID-19 pandemic. All patients included had a negative polymerase chain reaction test for the SARS-CoV-2 virus, however the possibility of false negatives cannot be excluded, which may have affected results.

Lastly, the COVID-19 positive sub-group comprised only seven patients, and the sub-analysis performed was very limited.

Conclusion

The MO-Y is not a useful biomarker for the diagnosis of TB, however was significantly elevated in a subgroup of PLWH with CD4 counts < 100 cells/ul. The significance of this finding is not clear, and requires further study. There were a number of other parameters within this group which showed a significant difference in patients with TB as compared to the control group. These were the MCV, lymphocyte count and CD4 count. Of these, the MCV showed the best discriminatory power for differentiating patients with and without TB, at a cut-off level of 87 fl. The diagnostic utility of these parameters may be improved by combining them with an interferon-gamma release assays, and further study in this area would be of interest.

Conflicts of interest

Dr Moosa, Dr Vaughan, and Dr Hodkinson report no conflicting interests.

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APPENDIX I

Ethics approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO:	Dr A Moosa School of Pathology Department of Molecular Medicine and Haematology Medical School University E-mail: aami.m21@gmail.com
CC:	Supervisor: Drs J Vaughan and K Hodkinson <Jenifer.Vaughan@nhls.ac.za> and <HREC-Medical Research Office@wits.ac.za>
FROM:	Mr Iain Burns Human Research Ethics Committee (Medical) Tel: 011 717 1252 E-mail: Iain.Burns@wits.ac.za
DATE:	2021/07/13
REF:	R14/49
PROTOCOL NO:	**M210337** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)
PROJECT TITLE:	*The usefulness of monocyte fluorescence as a biomarker of Tuberculosis Infection 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 Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Dr A Moosa

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210337

NAME: (Principal Investigator)	Dr A Moosa
DEPARTMENT:	School of Pathology Department of Molecular Medicine and Haematology Medical School University
PROJECT TITLE:	*The usefulness of monocyte fluorescence as a biomarker of Tuberculosis Infection at Chris Hani Baragwanath Academic Hospital*
DATE CONSIDERED:	2021/03/26
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Drs J Vaughan and K Hodkinson
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/07/13

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat 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 **March** and therefore reports and re-certification will be due in the month of **March** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

A Moosa — Signature of Principal Investigator

14/7/2021 — Date

APPENDIX II

Research Protocol

The usefulness of monocyte fluorescence as a biomarker of Tuberculosis infection at Chris Hani Baragwanath Academic Hospital

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CONTENTS

List of Tables

ABBREVIATIONS

AUC - Area under curve

CHBAH - Chris Hani Baragwanath Academic Hospital

FBC - Full blood count

GXP - Xpert MTB/RIF

HIV - Human Immunodeficiency Virus

LAM - Lipoarabinomannan

LIS - Laboratory information system

LR - Likelihood ratio

M. tuberculosis - Mycobacterium Tuberculosis

MO-Y - Monocyte fluorescence

NHLS - National Health Laboratory System

PCR - Polymerase chain reaction

RNA - Ribonucleic acid

ROC - Receiver operating characteristic

TB - Tuberculosis

INTRODUCTION

Burden of Tuberculosis

Tuberculosis (TB) is a communicable disease, caused by the microorganism *Mycobacterium tuberculosis*. (36) Despite being a curable disease, TB is one of the top 10 causes of death worldwide, and is a leading cause of death in South Africa(SA). (37) While great strides have made in the diagnosis and treatment of TB in SA, SA still has the 5th highest burden of TB in the world and is one of six countries which contribute to 60% of the global TB burden. (37,38) The majority of TB in SA is associated with Human immunodeficiency virus (HIV) infection, and SA bears the highest burden of HIV and TB co-infection cases worldwide. (38)

Diagnosis of Tuberculosis

A key factor in the fight against TB is early diagnosis, which forms the first pillar of the End TB Strategy by the World Health Organisation. (36) The diagnosis of TB depends largely upon the identification of *M. tuberculosis*, usually from a sputum specimen. (39) Among the microbiological methods, microscopy is rapid, however is low in sensitivity, particularly in patients with HIV. (40) The composition of the mycobacterial cell wall makes staining difficult, and acid fast bacilli are detectable only at concentrations of 10,000 bacilli/ml. (41) Culture can require as few as 100 bacilli/ml, (41) and is the gold standard of TB diagnosis, however requires lengthy incubation. (40) The molecular method, Xpert MTB/RIF (GXP), is high in sensitivity (88%) (42) and specificity due to a polymerase chain reaction (PCR) based amplification. (43) Importantly, the production of an adequate sputum sample is often not possible in patients with proven TB (44), rendering the search for a biomarker of TB an imperative.

Biomarkers of Tuberculosis

Tests based on biomarkers of TB are a consideration in achieving the goals of the World Health Organisation End TB Strategy, especially as these can be employed as a point of care test. (43) A biomarker is defined as 'objective characteristics that indicate a normal or pathogenic biological process'. (43) A biomarker of TB should be necessary and specific to the underlying process of the disease, ideally would not require elaborate or expensive equipment, and would be utilised on easily accessible samples e.g. blood or sputum. (43) The detection of the lipoarabinomannan (LAM) antigen in the urine, as a marker of TB infection is widely used in SA, however this marker is adequately sensitive only in patients living with HIV, with a significantly low CD4 count. (43) The tuberculin skin test is often used in the primary health care setting, however cannot establish active TB versus BCG immunisation. (45) Interferon-γ release assays cannot differentiate latent TB from active TB. (45)

Peripheral Blood Biomarkers of Tuberculosis

Peripheral blood biomarkers are an attractive option due to easy accessibility of samples. Recent biomarkers that have been explored include Ribonucleic acid (RNA) profiles and circular RNA expression. (45–50) The drawback to these would be the inaccessibility of these tests for peripheral primary health care facilities. Immunological biomarkers explored thus far require flow cytometry analysis of B-lymphocytes and T-lymphocytes (39,51) or require detection of serum cytokines (52), likewise inaccessible options.

Monocyte Fluorescence in Tuberculosis

Monocytes are the major leucocyte involved in the immune response to TB. (53) Monocytes assist in controlling TB infection, and can become a reservoir for latent TB infection. (54) Monocytes and TB are a key area of research, with investigation having focused on the inflammatory profile of monocytes and the polarisation of monocytes, (54) monocyte signal transduction, (53) monocyte to lymphocyte ratios (55) and expression of monocyte markers (55,56) in individuals with TB. The Sysmex XT-, XE- and XN haematology analyzers (Sysmex, Kobe, Japan) utilise fluorescent dye which stains intracellular nucleic acid and proteins for cell differentiation. (57) The degree of monocyte fluorescence as measured by flow cytometry correlates with activation of the cell. (58) Notably, a recent South African study evaluating novel white cell parameters generated by the Sysmex XN9000 haematology analyser among patients with suspected bacterial sepsis found a significant association between elevated monocyte fluorescence (as reflected by the MO-Y parameter) and a diagnosis of TB (personal communication Dr J Vaughan). As the MO-Y is measured with every differential count performed on several Sysmex analysers (a test widely performed in the routine work-up of patients with suspected infectious diseases), this parameter holds strong appeal as an inexpensive biomarker of TB.

Monocyte Fluorescence in other infection and in SARS-CoV-2 infection

Monocyte fluorescence in the setting of infectious diseases has not been a major area of study, locally or internationally. A study by Buoro et al. assessed extended differential parameters in patients with sepsis, with and without liver impairment and found that monocyte fluorescence intensity may be useful in the prognostication of septic patients in the

ICU. (59) Two recent studies have found extended differential parameters to be potentially valuable in the prognostication of patients with SARS-CoV-2 infection, (60,61) one of which found increased monocyte fluorescence in critically ill patients. (60)

STUDY HYPOTHESIS, AIMS AND OBJECTIVES

Hypothesis

The Sysmex MO-Y parameter (as a reflection of monocyte fluorescence) is a useful biomarker for TB infection in South African patients living with HIV.

Study aims

Primary aim

To evaluate the MO-Y parameter as a biomarker of TB infection in patients living with HIV, in the South African setting.

Secondary aims

To evaluate whether concomitant HIV-infection influences the utility of the MO-Y as a biomarker of TB infection.

Objectives

To compare the MO-Y in samples collected for a routine full blood count (FBC) and differential count from patients with and without TB where there is a clinical suspicion of mycobacterial infection (as evidenced by a GXP request). Patients will be identified as having TB on the basis of the GXP or accompanying TB culture results.

To determine cut off values for the MO-Y with optimal diagnostic performance (sensitivity, specificity, positive and negative predictive values) for detection of TB infection among HIV-positive patients with clinical suspicion of TB.

To compare the relationship between MO-Y and TB in HIV positive versus HIV negative patients.

METHODS

Study setting

National health laboratory service (NHLS) haematology laboratory at Chris Hani Baragwanath Academic Hospital (CHBAH).

Study population

Samples submitted from adult patients (defined as >18 years of age) admitted to the CHBAH with suspected TB (as evidenced by a GXP request) will be evaluated. Samples will be identified by a daily search of the NHLS laboratory information system (LIS) (TrakCare) for all GXP requests received in the prior 24 hours. All samples with a positive GXP result will be identified, and an available HIV result as well as a FBC submitted within 72 hours before or after the request will be sought. For every HIV positive patient with a positive GXP and an available FBC sample, a matching GXP sample from an HIV positive patient with a negative result and available FBC will be sought. Samples will be matched for site of collection (sputum, fine needle aspirate and cerebrospinal fluid). As HIV negative patients are anticipated to be scarce, all samples from HIV negative patients with a GXP request and an available FBC will be included. Samples will be consecutively included until the target sample size of

approximately 50 HIV positive, GXP positive and 50 HIV positive, GXP negative cases has been reached.

Inclusion criteria

Adult patients with an available HIV result in whom a GXP test has been performed and a recent FBC (requested within 72 hours of the GXP) is available will be eligible for inclusion.

Exclusion criteria

Cases will be excluded if:

There is no documented HIV status

The FBC sample is of insufficient volume following routine testing and a differential count was not requested with the original FBC.

The sample submitted for an FBC is >24 hours old and a differential count was not requested with the original FBC.

The analyser fails to perform an automated differential count.

GXP results are inconclusive.

Study design

Prospective cross-sectional analytical study.

Data collection

Clinical parameters to be recorded

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

Laboratory parameters to be recorded

Results available on the LIS to be recorded include the FBC, automated differential count, HIV status, CD4 count, HIV viral load (where applicable), SARS-CoV-2 PCR results, GXP results (including CT value), ESR, CRP, blood culture and TB culture results.

Sample size

The sample size has been calculated in order to ensure adequate power for detection of a significant difference in the MO-Y among HIV positive patients with and without TB. In a previous study assessing the utility of extended differential parameters in the detection of bacterial sepsis, the MO-Y was found to be marginally significantly higher in the small number of included patients who transpired to have TB as compared to those with and without bacterial sepsis. In this study, all patients with TB were HIV positive. The MO-Y did not differ significantly between HIV+ TB-negative patients with and without bacteraemia. The mean difference in mean monocyte fluorescence between the MO-Y in HIV+ patients with and without TB was 13.6 (E), and the standard deviation for the MO-Y among all HIV+ patients was 23.3 (S). On the basis of these results, the estimated standardised effect size is ~0.6 (E/S). Given an α of 0.05, 1-β of 0.80 and allowing an additional 10% for any possible errors, the calculated sample size is 50 HIV+ patients with and without TB (total HIV+ cohort of 100 patients). The documented HIV-seropositivity rate is estimated to be ~40% (based on findings from the afore-mentioned previous study), and the GXP-positivity rate is ~15%. It is therefore estimated that ~800 GXP results will need to be screened to reach the target sample size.

The variables to be assessed

The automated differential count (which includes the monocyte count and MO-Y)

TB GXP and culture results as well as other pertinent laboratory results and clinical data from the LIS

Please see APPENDIX I for the data collection sheet.

Laboratory methods

An automated differential count will be performed on all included samples (if one was not already performed during routine testing) using the Sysmex XN9000 haematology analysers at the NHLS laboratory at CHBAH. The Sysmex XN9000 measures the white cell count in the white cell chamber via impedance technology. (62) The differential count is measured in the WBC differential channel via flow cytometry. (62) Cells are permeabilised, intracellular content is stained and these are delivered to a laser point. White cell subpopulations are then identified via forward scatter (informs cell size), side scatter (informs cell complexity) and intracytoplasmic nucleic acid content. (62)

The MO-Y is determined via measurement of fluorescent light intensity of the monocyte area on the white cell differential scattergram.

DATA ANALYSIS

Positive samples will be defined as those with positive GXP or TB culture results.

The median MO-Y levels will be compared among patients with and without TB (HIV positive and negative) using a Mann-Whitney U-test.

Receiver operating characteristic (ROC) curve analysis will be performed to determine the overall performance of the assay (as evidenced by the area under the curve (AUC)). The Likelihood ratio (LR) will be used to identify the best cut-off point using the following formula: LR = Sensitivity/(1-Specificity).

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

Sensitivity= A/(A+C) × 100

Specificity= D/(D+B) × 100

Positive predictive value (PPV)= A/(A+B) x 100

Negative predictive value (NPV): D/(D+C) x 100

		Disease		
		Disease	Non-Disease	Total
Test Result	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 3. Two-way table used to calculate sensitivity, specificity, positive and negative predictive values

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.

TIMING

Sample collection will commence once ethics approval has been granted and up until the time the sample number of approximately 100 HIV positive patients has been reached. The time frame to reach this has been estimated at approximately 2 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												

Table 4. Timeline for projected completion of research thesis

FUNDING

	Units	Unit cost	Total cost
Full blood count reagents			To be covered by the laboratory
Publication costs			R 10 000.00
		Total	**R 10 000.00**

Table 5. Proposed funding for research thesis

A funding application will be submitted to the Faculty Research Committee of the University of the Witwatersrand to cover publication costs.

PROBLEMS

Limitations of this study include:

Patients' clinical files will not be examined.

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

False negative GXP tests need to be accounted for in analysis of data.

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APPENDIX

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):
……..
……
……..
HIV: Positive / Negative
WCC..... HB……. MCV……. Plts…….

Automated differential count: Neut……. Lymph……. Mono……. Eos……. Baso…….
MO-Y ……..
TB GXP result…… CT value ……..
Site of collection ……
TB Blood and Sputum culture results:
.....
.....
.....
SARS-CoV-2 PCR result……
CD4 count (if available) …….. Viral load (if available) ……..
ESR…… CRP ……..
Other pertinent results (if available):
.....
.....
.....

APPENDIX III

Turn-it-in report

Department of Molecular Medicine and Haematology

Medical School: 7 York Road, Parktown, Johannesburg 2193, South Africa Private Bag3, Wits 2050 South Africa
Tel: +27 11 717 2561 or +27 11 489 8505

24/08/23

To Whom it May Concern,

Re: TURN-IT-IN report for the MMed research report entitled: The usefulness of monocyte fluorescence as a biomarker of Tuberculosis infection at the 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 19%, which is somewhat higher than desirable. However, on closer inspection, this is seen to be flagging general formatting and non-specific scientific terminology. As the body of the thesis shows no features concerning for plagiarism, we have deemed the findings acceptable.

Yours sincerely,

Dr Jenifer Vaughan

Dr Katherine Hodkinson

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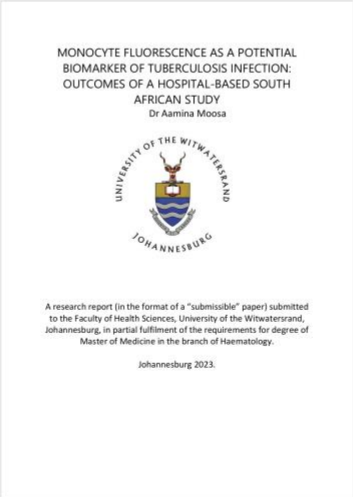


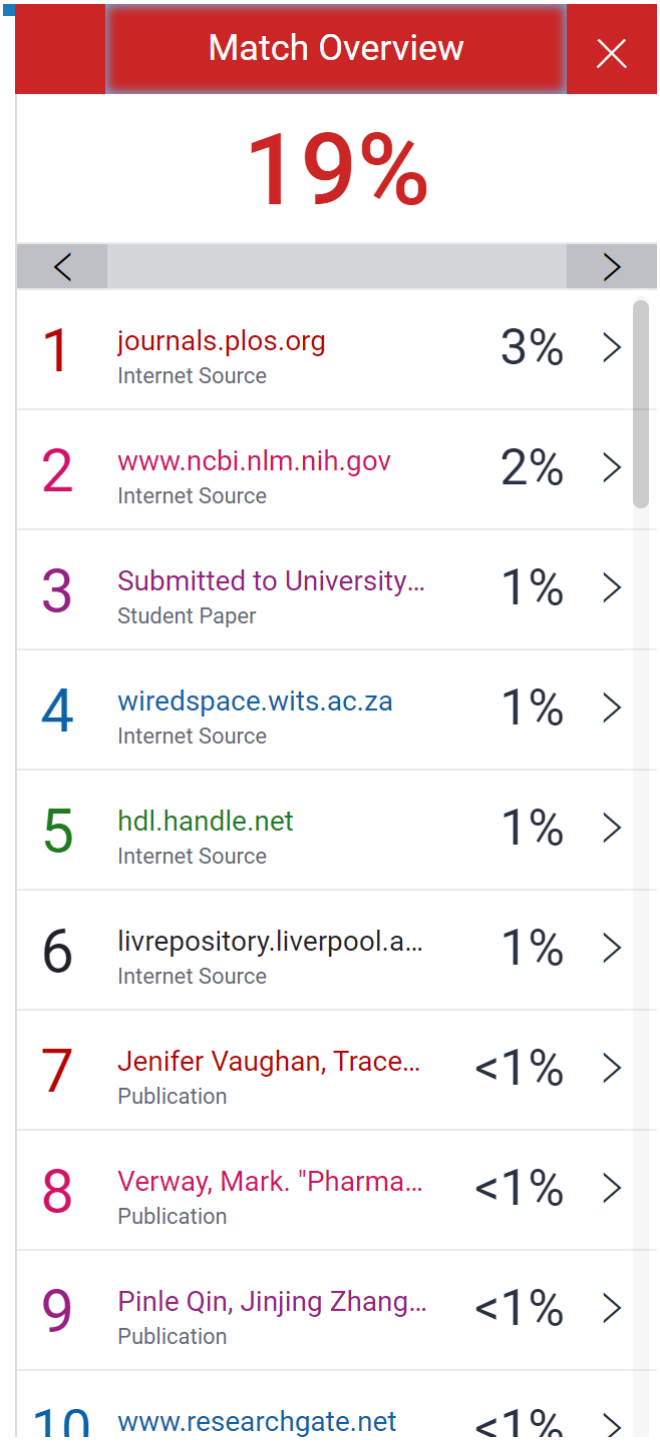
MONOCYTE FLUORESCENCE AS A POTENTIAL BIOMARKER OF TUBERCULOSIS INFECTION: OUTCOMES OF A HOSPITAL-BASED SOUTH AFRICAN STUDY

Dr Aamina Moosa

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

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 2023.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APPENDIX IV

Submission Guidelines

Submission Guidelines | PLOS ONE

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., "$a^2 + b^2 = c^{2n}$"), 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.

Nomenclature	Use correct and established nomenclature wherever possible.	
	Units of measurement	Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.
	Drugs	Provide the Recommended International Non-Proprietary Name (rINN).
	Species names	Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).

›mission-guidelines

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	Genes, mutations, genotypes, and alleles	Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HGNC for human genes; we strongly recommend using this tool to check against previously approved names). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).
	Allergens	The systematic allergen nomenclature of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee should be used for manuscripts that include the description or use of allergenic proteins. For manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-Committee prior to manuscript publication. Examples of the systematic allergen nomenclature can be found at the WHO/IUIS Allergen Nomenclature site.