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STUDY PROTOCOL

REVISED Investigating neutrophil cell death in TB pathogenesis

[version 2; peer review: 2 approved]

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Abstract

Background: Neutrophils are one of the major early role players in antimycobacterial immunity. Upon infection, neutrophils can undergo NETosis, a cell death characterized by release of neutrophil extracellular traps (NETs). The role of NETosis in TB progression remains poorly characterized. We aim to characterize mechanisms underlying NETosis during TB pathogenesis by identifying genes that drive the cell death, and to determine their potential as markers of disease progression in high-risk individuals. Finally, we intend to evaluate neutrophil associated genes as targets for host directed therapy to reduce pathological damage caused by NETosis. **Methods:** Quantitative PCR will be used to quantify expression of specific genes identified in the blood of individuals with active lung disease (n=30), compared to those from healthy (n=30) and latently infected individuals (LTBI) (n=30). In addition, temporal events associated with NETosis will be measured using live microscopy in a neutrophil in vitro model of *Mycobacterium tuberculosis* (Mtb) infection. Candidate genes found to be associated with NETosis will be targeted with pharmaceutical inhibitors. **Conclusion:** Genes associated with neutrophil mediated cell death may serve as potential biomarkers of pathological damage and disease progression, as well as targets for host-directed therapy.

Keywords
Neutrophils, Tuberculosis, NETosis

Open Peer Review

Approval Status ✓ ✓

	1	2
version 2 (revision) 29 Apr 2022	✓ view	
	↑	
version 1 07 Dec 2021	✓ view	✓ view

1. **Ashwani Kumar** [iD], Institute of Microbial Technology, Chandigarh, Chandigarh, India

2. **Delia Goletti** [iD], National Institute for Infectious Diseases (INMI), Rome, Italy

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: Fisher KL: Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Rajkumar-Bhugeloo K**: Investigation, Methodology, Writing – Review & Editing; **Moodley D**: Investigation, Methodology, Writing – Review & Editing; **Mpotje T**: Investigation, Methodology, Writing – Review & Editing; **Ramsuran D**: Data Curation, Visualization, Writing – Review & Editing; **Ndung'u T**: Resources, Supervision, Writing – Review & Editing; **Marakalala MJ**: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED **Amendments from Version 1**

No major differences were made from the original version of the manuscript, except for the clarification on study participants and samples in the method sections.

Any further responses from the reviewers can be found at the end of the article

Background

Tuberculosis is a disease caused by *Mycobacterium tuberculosis* (Mtb) and is responsible for approximately 1.5 million deaths annually[1]. Understanding lung immunity during TB progression is important in identifying biomarkers. During initial TB infection, an immune structure known as a granuloma forms to control and prevent the spread of *Mycobacterium tuberculosis*[2]. The granuloma is a highly organized immune structure composed of macrophages surrounded by a layer of epithelioid cells and multinucleated giant cells, with lymphocytic cuff[3]. Neutrophils are also found in the regions adjacent to caseum or necrotic regions of the granuloma[4]. Understanding the contribution of neutrophils to lung associated tissue damage is important in determining mechanisms that lead to TB associated pathology. Neutrophils are the most abundant cell subsets in the lung and are also amongst the first cells that are infected with Mtb[4]. Neutrophils play an important role in bacterial control during acute infection, however, they can release reactive oxygen species (ROS), deoxyribonucleic acids (DNA), myeloperoxidase, cathelicidins and S100 proteins that can result in excessive inflammation and damage to surrounding tissue[5,6]. Netosis is the release of nuclear DNA and associated proteins i.e. histones, called neutrophil extracellular traps (NETS) from the cell in response to bacterial, viral or stress induced conditions[7]. The size of the NETS released is dependent on the size of the pathogen[8], which is important in combatting infection. However, netosis has also been shown to contribute to tissue damage in various diseases[9–11]. Studying neutrophils in a clinically relevant environment is challenging due to the short life-span and the various complex triggers of activation, including respiratory stress, damage-signalling or prior activation to pathogenic stimuli resulting in ROS associated activities[12].

Neutrophilia, which is a characterized by an abundance of neutrophils above what is considered to be a normal count, has been associated with TB and it is therefore important to understand mechanisms underlying neutrophil mediated contribution to disease pathogenesis[13,14]. In previous findings, we reported that across cellular, caseous and cavitary granulomas, proteins associated with neutrophils were abundant in necrotic regions[15] and in the border of necrotizing tissue[5]. However, whether neutrophils are directly causing caseation and how it is mediated remains a gap in our knowledge. Our project is therefore aimed at determining expression profiles of neutrophil associated genes in blood from healthy, LTBI and TB participants as potential markers of the disease. We also intend to investigate the role of neutrophils during TB pathogenesis by exploring, *in-vitro*, the dynamics of NETosis, in neutrophils infected with Mtb and to target drivers of the cell death as host directed therapies (HDT).

Materials and methods
Study design and setting

This is a prospective study that will include participants recruited from healthcare facilities that service the eThekwini District in KwaZulu-Natal (KZN), South Africa. Participants will be recruited from Kwadabeka Clinic and Prince Zulu Communicable Disease Centre in Durban. South Africa has a TB prevalence rate of 737 in 100 000 in 2017[16] with eThekwini described as having the highest TB prevalence rate in KZN. This study was approved by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (BE022/13 and BE00003365/21).

Study size and ethics

A qualified nurse will enrol participants into the study after explaining the informed consent procedure according to good clinical health practices (Figure 1). Individuals who are newly diagnosed with active TB, determined as GeneXpert positive and who have not received treatment, will be recruited to the TB group of the study (n=30). Individuals who are healthy will be recruited to the healthy group as determined by their quantiferon (QFT) results. If the QFT result is positive those individuals will be recruited to the LTBI group (n=30) and those who are QFT negative will be recruited to the healthy group (n=30). Participants will be incentivised for participating in the study according to the ethics that have been approved through the University of KwaZulu-Natal. Participants will be informed that their biological samples will be stored for future use, including their genetic information, which will be used for research. A volume of twenty millilitres of blood will be used for neutrophil isolation. Plasma will be collected and stored at -80°C (Figure 2).

Sample processing

Samples obtained will be assigned a unique participant identification. A maximum of 40 ml of blood will be drawn from a participant. 10 ml of blood will be stored for paxgene processing in order to isolate RNA for genetic studies. 20 mls of blood will be collected and used to isolate peripheral blood mononuclear cells (PBMCs) and the remaining 10ml of blood will be used to collect plasma.

Neutrophil isolation

A volume of blood ranging between ten and twenty millilitres of blood will be collected in heparin collection tubes (Figure 2). The blood will be transferred to 50 ml conical tubes. A volume of ten millilitres of 0.01 M phosphate buffered solution (PBS) will be added to the blood collection tubes, to remove any blood remnants and then added to the 50 ml conical tube. A volume of twelve millilitres of the Cell-based Assay Neutrophil Isolation Histopaque (Sigma-Aldrich, Germany) will be placed in a new conical tube at room temperature (RT). The diluted blood will be pipetted on top of the histopaque and centrifuged at 800 x g for 20 minutes at RT, with the centrifuge setting of acceleration slow and deceleration off. The yellow and clear upper layers will be aspirated, leaving only the red blood cell (RBC) layer and
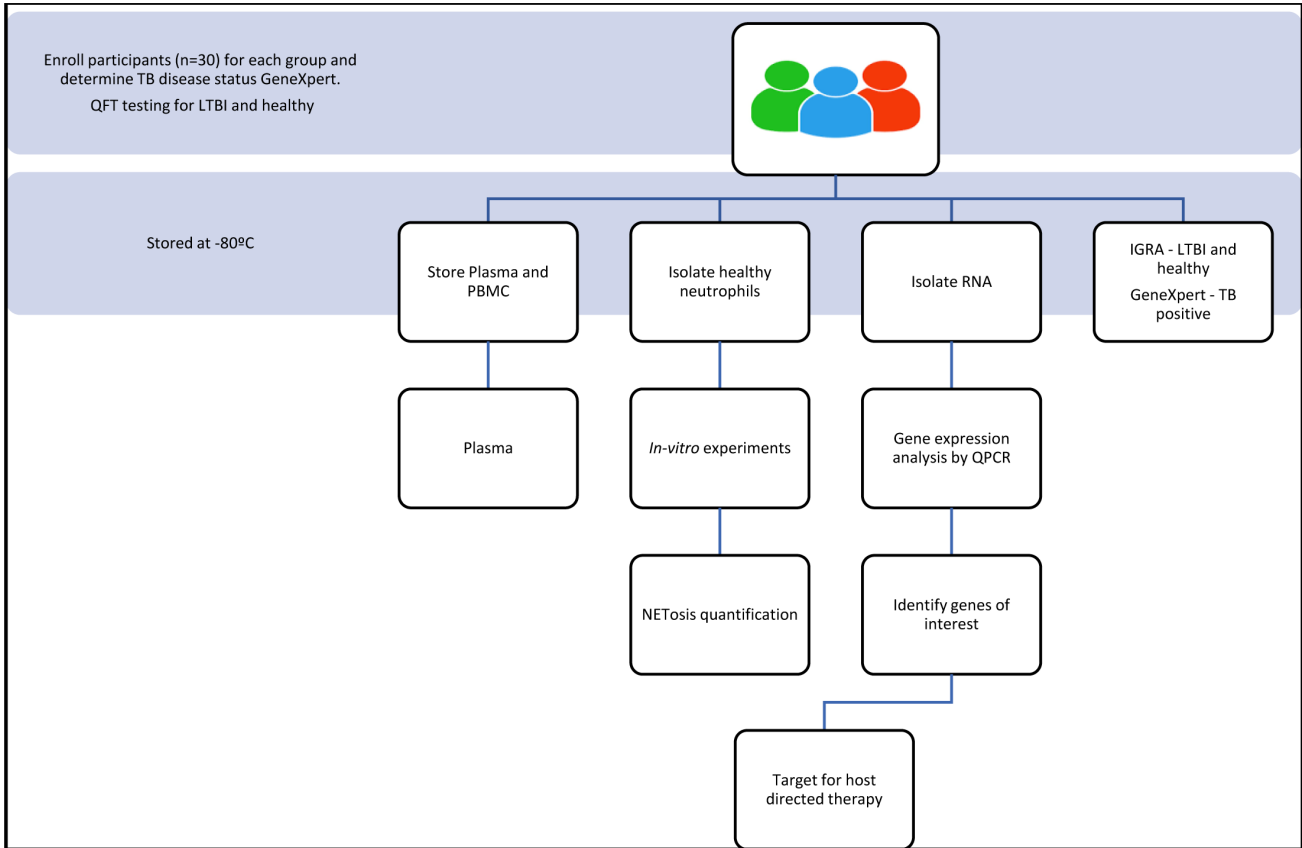
Figure 1. Schematic of the study design and experimental process that will be followed.
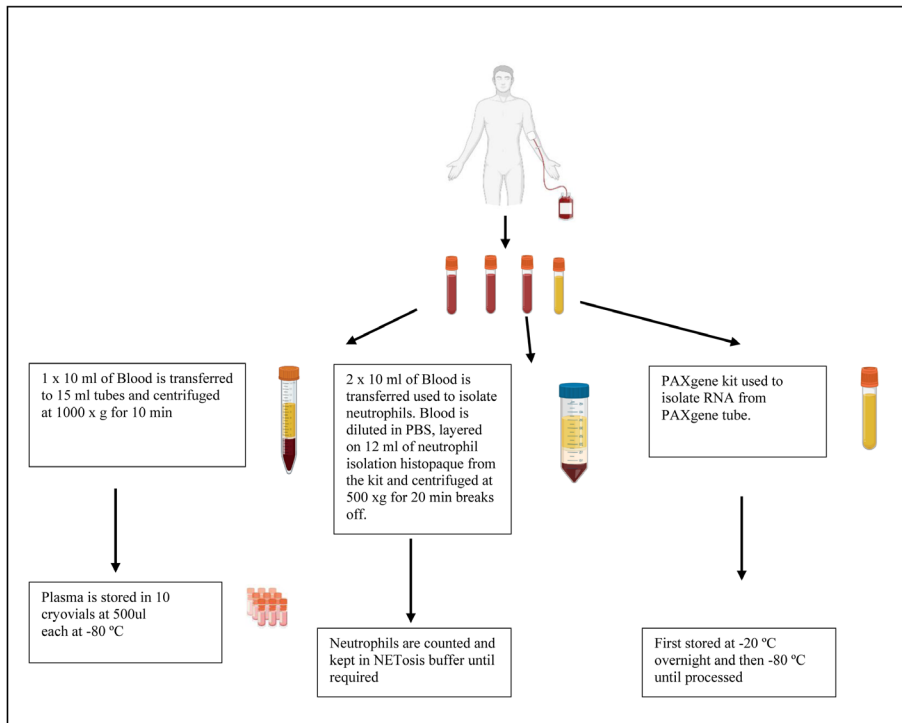

Figure 2. Schematic of plasma, neutrophil and RNA isolation from whole blood obtained from study participants.

neutrophils. A volume of twenty-five millilitres of the RBC lysis buffer (1x solution) will be added to the RBC and neutrophil layer and mixed. The mixture will be incubated at RT for 10 minutes. Following incubation, the cells will be centrifuged at 250 x g for 5 minutes at RT to pellet the neutrophils. The supernatant will be aspirated without disturbing the pellet. The pellet will be resuspended in 25 ml of 0.01 M of PBS and neutrophils counted using a hemocytometer.

PBMC isolation

To isolate PBMC from the blood, 10 ml of blood will be added to twenty five millilitres of 0.01 M PBS (warmed to RT) (Sigma-Aldrich, USA). The diluted blood will then be layered onto fifteen millilitres of histopaque (warmed to RT) (Sigma-Aldrich, USA). A density gradient will be formed which will allow the cells to separate. The layered cells will be centrifuged at 800 x g for 30 minutes with the centrifuge setting of deceleration off and slow acceleration. Following centrifugation, the whitish layer (buffy layer) will be aspirated to a new 50 ml tube and topped up with PBS and centrifuged at 300 x g for 10 min, with acceleration and deceleration at maximum. The supernatant will be discarded, and the cells will be resuspended in PBS before being counted. The cells will be centrifuged at 300 x g for 10 min with maximum acceleration and deceleration. Freeze media will be prepared by adding 500 μl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) to four and half millilitres of fetal calf serum (FCS) (Sigma-Aldrich, USA). The supernatant will be removed, and the cells will be stored in 1 ml of freeze media at a cell concentration of 10 million cells per ml in a cryovial labelled with the PID and date and stored in liquid nitrogen. Plasma will be isolated by transferring ten millilitres of blood to a tube, which will be centrifuged at 1000 x g for 10 minutes, with maximum acceleration and with deceleration off. Ten cryovials labelled with the PID and date, are used to store plasma at -80°C at a volume of 500 μl.

RNA extraction

All surfaces and pipettes will be decontaminated with RNAse away before RNA isolation will be done. RNA will be extracted using the PAXgene kit and the corresponding protocol. Briefly, blood from TB, LTBI and healthy participants will be collected in PAXgene tubes which contain anticoagulant to ensure the stability of the ribonucleic acid (RNA). The blood will be stored at 18–25°C during transit and upon arrival will be stored at -20°C at an angle on a wired rack for 24 hours before being transferred to the -80°C freezer. The frozen blood in the PAXgene tubes will be thawed in a water bath at 22–23°C for 20 minutes and the PAXgene tube will be inverted 8–10 times to before RNA isolation proceeded. Once thawing and inverting of the tube is complete, the PAXgene tube will be left at room temperature (RT) for a further 2 hours to ensure complete cell lysis. RNA will be isolated from the blood using the PAXgene kit according to the manufacturer's instructions (PreAnalytiX, Hombrechtikon, Switzerland).

RNA quality

To determine the RNA quality, we will be using a Nanodrop Lite Spectrophotometer (Thermofisher™, Switzerland) and the absorbance will be read at 260 nm. The sample will be diluted (dilution factor 2) with 10 mM of Tris-hydrochloride, pH 7.5. The relationship between the absorbance and the RNA yield will be taken as A260 of 1 is equal to 44 ug/ml, according to the manufacturer's instructions.

cDNA synthesis

The iScript cDNA synthesis kit will be used for cDNA synthesis as per manufacturer's instructions (Biorad, California, USA). The 5 x iScript reaction mix will be left to thaw at room temperature. The reaction will be set-up in sterile PCR tubes as follows; 500 ng of RNA will be added to each tube, followed by 4 μl of 5x iScript reaction mix, 20 μl of RNase free water and 1 μl of iScript reverse transcriptase. The total reaction mix will be 20 μl per tube. The tubes will be placed in a PCR machine and run on the thermocycler (Biorad, California, USA) using the following runs, 5 min at 25°C, 30 min at 42°C, 5 min at 85°C and on hold at 4°C. The resultant cDNA will be diluted 10x by adding 1 μl of cDNA to 9 μl of nuclease free water.

RT-QPCR for gene expression

Qualitative polymerase chain reaction (qPCR) will be done on candidate genes as per the manufacturer's instruction to determine the expression of the candidate genes in TB, LTBI and healthy participants. Briefly, half a microlitre of reverse primer, half a microlitre of the forward primer, three microlitres of nuclease free water and five microlitres of iTaq Universal SYBR green supermix (Biorad, California, USA) will be added to each well. A volume of nine microlitres of the mastermix will be added to each well. One microlitre of the diluted sample will be added to each well. The plate will be run on the Biorad CFX 96 thermocycler (Biorad, California, USA) according to the following protocol, 30 sec at 95°C, 5 sec at 95°C, 30 sec at 59°C, for 39 cycles. The melt curve analysis will be done at 65–95°C at 0.5°C increments.

NETosis quantification

A NETosis imaging kit (Cayman Chemical, Michigan, USA) will be used to quantify the amount of NETosis occurring in TB patients (n=4) compared to healthy participants (n=4). The assay will be performed as per manufacturer's instructions. Briefly, 6×10^6 neutrophils isolated on the same day, will be transferred to a clean tube and adjusted in 0.01 M PBS to 1×10^6 cells/ml. A volume of six microlitres of Permeable Nuclear Red Reagent (5 mM) will be added to each well and incubated in the dark at 37°C for 15–30 minutes. A volume of 25 ml of PBS will be added to the cells, centrifuged at 250 x g and the supernatant aspirated. The cells will be resuspended in twelve microlitres (0.5×10^6 cells/ml) of NETosis Imaging buffer (1x). A volume of twelve microlitres of Extracellular Nuclear Green Reagent (5 mM) will be added to the tube and mixed before the cells are seeded into the wells at 100 μl/well in a flat-bottom 96 well plate (Greiner Costar, Sigma-Aldrich, Germany). A volume of 100 μl of stimuli or NETosis Imaging Buffer (1x) will be added to each well for a final volume of 200 μl. The plate will be centrifuged at 250 x g to pellet the cells at the bottom of the plate. The BioTek cytation 5 (Biotek, Vermont, USA) which uses brightfield and fluorescence

microscopy will be used to image activity every 30 min-1 hour for six to twelve hours at a temperature of 37°C and 5% CO_2. Green fluorescence indicates NETosis activity and red fluorescence is a marker for the nucleus (Figure 3). The neutrophils will be treated with optimized concentrations of various NETosis inhibitors depending on the chosen gene targets.

Data analysis
Student t-tests will be used to analyse the difference in gene expression between healthy, LTBI and TB participants. The data will be reported with the median and interquartile range. Pearson R Correlation or a Spearman R Correlation analysis will be done to determine if there is an association between protein and gene expression of specific NETosis associated protein and genes. All data will be analysed using Graphpad Prism v 9 software.

Discussion
Proteomic analysis of lung tissue laser microdissections of various granulomas showed that proteins associated with neutrophils were abundant in necrotic regions of caseous and cavitary granulomas[15]. This indicated that neutrophils potentially contribute to necrotic damage observed in cavitary granulomas, due to their presence in these regions. This provides a potential link of the neutrophils with increased TB pathogenesis, hence the need to investigate their role during TB as potential target for therapy or as biomarkers. Identifying factors or mechanisms that contribute to lung tissue damage is vital in identifying markers of disease progression.

Neutrophils have been identified in the regions that border necrotizing tissue in other diseases[17] and whether this is true for TB infection and granulomas remains to be determined. Recent studies have also identified the role of DNA-MPO[11,16] or histones complexes[10,18], ROS pathways[9] and neutrophil elastase[19] in tissue damage associated with various respiratory infections and as

markers of disease progression. Studies in C3HeB/FeJ mice infected with Mtb have also shown that neutrophils contribute to necrosis and liquefaction of granulomas mediated by ROS events[20]. These studies suggest that similar mechanisms mediated by neutrophil derived proteins and cell death activities may be driving TB lung pathology in humans.

NETosis proteins have been detected in the serum of individuals with acute respiratory distress syndrome associated pathology (as reviewed by 21). However, whether NETosis directly contributes to damaging pathology observed in TB granulomas is not well known. To validate this, our initial aim is to establish whether NETosis associated genes are present in the blood and are differentially expressed in LTBI, TB and healthy participants. In addition, we aim to determine whether expression of specific genes associated with NETosis correlates with the spectrum of disease observed during TB infection (as reviewed by 22,23). In addition, these unique gene expression profiles may be used to identify potential biomarkers of disease progression that may be used to diagnose TB at point of care.

Ongoing clinical trials are investigating the use of NETosis specific gene inhibitors to reduce tissue damage associated with NETotic events[24–28]. Therefore, identifying potential targets for HDTs that may serve as adjunct therapies to current antibiotic regimens remains vital in combating the disease.

Due to the heavy TB disease burden in KZN, the study participants that will be enrolled in this study will provide important and relevant information regarding the tissue destruction mechanism that contributes to disease progression. A limitation of this study is that we will not be able to obtain matched blood samples from lung tissue donors. Another limitation of this study is that when performing the NETosis experiments it is difficult to distinguish between LTBI volunteers
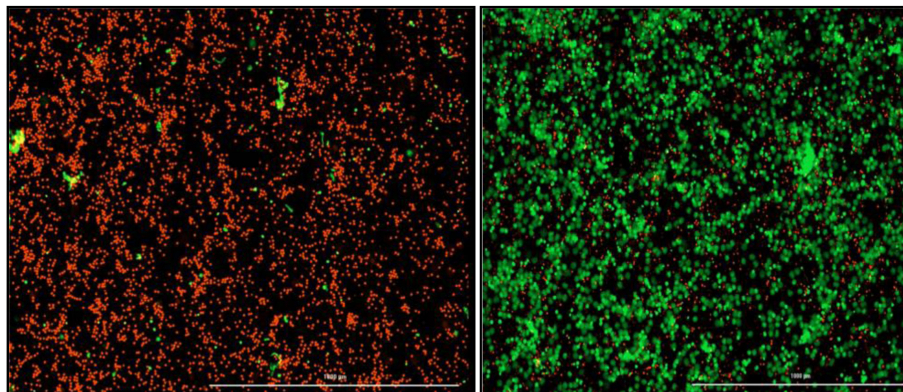

Figure 3. Images acquired with the Biotek Cytation 5 (1000 μM) to visualize and quantify NETosis. Red indicates intact neutrophils. Green indicates neutrophils undergoing NETosis and extruding their cellular DNA. (Left) Negative control which is only healthy neutrophils with no stimulation. (Right) Positive control, PMA stimulated neutrophils.

and those who are healthy in a timeous manner. This limits the ability of us to obtain a healthy blood volunteer to have an appropriate control.

The aim of this study is to identify potential biomarkers of TB disease progression that can lead to better diagnosis and ultimately, treatment of TB. We hope to characterize drivers of neutrophil mediated damage by developing a neutrophil *in-vitro* infection model. These findings may contribute to a better understanding of the neutrophil driven mechanisms that lead to damage often observed in the lungs of individuals with TB disease.

Data availability

Data will be stored and available through the Africa Health Research Institute according to the institutes protocols which insure participant privacy and rights protection.

Author contributions

KLF and MJM wrote the manuscript. MJM conceived the study. KLF optimized the protocol and performed experiments. KRB, DM and KLF were responsible for developing and optimizing protocols for this study. TM provided helpful discussion in data analysis. DR assisted with instrumentation and imaging. MJM and TN supervised KLF and obtained funding for the study. All authors read and approved the manuscript in its current version.

Acknowledgements

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Open Peer Review

Current Peer Review Status: ✓ ✓

Version 2

Reviewer Report 09 May 2022

https://doi.org/10.21956/gatesopenres.14919.r31993

✓   **Ashwani Kumar** (iD)

Council of Scientific and Industrial Research, Institute of Microbial Technology, Chandigarh, Chandigarh, Punjab and Haryana, India

My suggestions have been incorporated into the revised study protocol.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: TB Pathogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 07 April 2022

https://doi.org/10.21956/gatesopenres.14730.r31865

✓   **Delia Goletti** (iD)

Translational Research Unit, National Institute for Infectious Diseases (INMI), Rome, Italy

This study is on a project that is going to be done on the evaluations of genes associated with neutrophil mediated cell death. The hypothesis is that their study might be useful as potential biomarkers of pathological damage and disease progression, as well as targets for host-directed

therapy.

The hypothesis and protocol of the study are well written.

Few comments

Methods:
- Please comment on how the number of patients and controls was chosen.

- Please comment on the definition of patients with latent tuberculosis infection, and which tests/radiological tools you will use to define them

- Please comment on the definition of patients' active tuberculosis, and which tests/radiological tools you will use to define them

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunology of tuberculosis and COVID-19 and Echinococcosis.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

> Author Response 25 Apr 2022
>
> **Mohlopheni Jackson Marakalala**, Africa Health Research Institute, Durban, South Africa
>
> **Reviewer 2**
>
> - **Please comment on how the number of patients and controls was chosen.**
>
> - **Please comment on the definition of patients with latent tuberculosis infection, and which tests/radiological tools you will use to define them**
>
> - **Please comment on the definition of patients' active tuberculosis, and which tests/radiological tools you will use to define them**
>
> Response to reviewer 2:

We appreciate the useful comments by Reviewer 2 which we believe will help strengthen our manuscript.

We will not be using a radiological test to confirm TB infection but rather GeneXpert. Those individuals who are GeneXpert positive will be recruited to the TB group. Those individuals who will be recruited to the healthy group were further tested to determine whether they were perhaps latently infected with TB using the quantiferon (QFT) test. Those that have a positive QFT result will be recruited to the LTBI group (n=30) and those that have a negative QFT result will be recruited to the healthy group (n=30).

Please see below edits to the manuscript on lines 86-91:

"*Individuals who are newly diagnosed with active TB, determined as GeneXpert positive and who have not received treatment, will be recruited to the TB group of the study (n=30). Individuals who are healthy will be recruited to the healthy group as determined by their quantiferon (QFT) results. If the QFT result is positive those individuals will be recruited to the LTBI group (n=30) and those who are QFT negative will be recruited to the healthy group (n=30).*"

The size of the group was determined based on our discovery proteomics data. We decided to investigate the neutrophil specific protein responses in a small cohort that would allow us to determine if similar responses were seen in local populations within KZN. We acknowledge that the sample size is small and is a limitation of this study. However, the findings of this study could be validated in a larger cohort, should promising biomarkers of disease progression be discovered.

Competing Interests: No competing interests to disclose

Reviewer Report 02 February 2022

✓ **Ashwani Kumar** [ID]

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Study protocol titled "Investigating neutrophil cell death in TB pathogenesis" provides details of methods related to the assessment of differential gene expression in PBMCs and their association with latency of TB (if any). It also provides details of methods that could assist in deciphering genes involved in NETosis. Overall the manuscript is well written and provides sufficient details of methods. I would like to suggest a few minor changes to the manuscript.

1. Study size and ethics sub-headings of the Methods section shall provide sample size, expected numbers of individuals that will be enrolled in the study and its justification.

2. Background section, line 1, authors could use a recent TB report 2021 instead of Global TB report 2020.

3. There are a few grammatical errors in the methods sections, correcting these may improve the readability of the manuscript. As an example in the "study size and ethics" sub-heading, "plasma is collected' is used. It could be changed to "plasma will be collected".

4. Authors should use the convention of a space between numbers and units eg., "5 mM" instead of "5mM".

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: TB Pathogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 25 Apr 2022
Mohlopheni Jackson Marakalala, Africa Health Research Institute, Durban, South Africa

We appreciate the useful comments by Reviewer 1 which we believe will help strengthen our manuscripts.

Reviewer 1

1. Study size and ethics sub-headings of the Methods section shall provide sample size, expected numbers of individuals that will be enrolled in the study and its justification.

Response to question 1:

Please see below edits to the manuscript on line 86-91:

"Individuals who are newly diagnosed with active TB and are GeneXpert positive, will be recruited to the TB group of the study (n=30). Individuals who are healthy will be recruited to the healthy group as determined by their quantiferon (QFT) results. If the QFT result is positive those individuals will be recruited to the LTBI group (n=20) and those who are QFT negative will be recruited to the healthy group (n=20)."

2. Background section, line 1, authors could use a recent TB report 2021 instead of Global TB report 2020.

A more recent TB report has been used. Please see line 39 and 380.

3. There are a few grammatical errors in the methods sections, correcting these may improve the readability of the manuscript. As an example in the "study size and ethics" sub-heading, "plasma is collected' is used. It could be changed to "plasma will be collected".

The manuscript has been edited to change the tense to the correct grammatical tense.

4. Authors should use the convention of a space between numbers and units eg., "5 mM" instead of "5mM"

The manuscript has been edited to include a space between the number and units of measurements.

Competing Interests: No competing interests to disclose