Diagnostic yield of tuberculosis investigations on bone marrow biopsy samples in HIV positive participants at Groote Schuur Hospital

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Key words:

HIV; tuberculosis; HIV-associated tuberculosis; molecular diagnostic techniques; bacteriological methods; Xpert-Ultra; bone marrow

DECLARATION

I, …***Xikombiso Siphiwe Mandla Baloyi***……, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Abstract:

[246 /250 words]

Background:

Acid fast bacilli (AFB) staining on bone marrow samples has low sensitivity for diagnosing HIV-associated tuberculosis and Tuberculosis (TB) culture results may be delayed. The GeneXpert® MTB/RIF Ultra assay may provide a more sensitive diagnostic test on bone marrow biopsy samples.

Methods:

We conducted a two-stage study in a tertiary hospital in South Africa, initially assessing the retrospective yield of TB diagnoses on bone marrow biopsies in adult HIV-positive participants retrospectively from 01-01-2019 to 31-07-2020. Subsequently, determining the prospective yield and diagnostic performance of the GeneXpert® MTB/RIF Ultra assay on bone marrow aspirate and peripheral blood samples in adult HIV-positive participants undergoing bone marrow biopsy from 11-08-2020 to 31-01-2021.

Results:

One hundred and twenty-two biopsies were analysed, of which 59/122 were performed for haematological malignancy staging. Granulomata with AFB were detected in six samples, and nine new lymphoma diagnoses were made. Bone marrow TB culture detected only one non-tuberculous mycobacterial infection. All 17 participants who had TB diagnosed from another clinical site were bone marrow TB culture negative.

TB treatment was confirmed in 11/33 participants recruited prospectively. One trace positive GeneXpert® MTB/RIF Ultra result on peripheral blood was detected. All TB cultures on bone marrow aspirates and peripheral blood were negative.

Conclusion:

In a tertiary care hospital in South Africa, the utility of TB culture and GeneXpert® MTB/RIF Ultra on bone marrow aspirate specimens in HIV-positive participants was limited. We postulate that the initiation of empiric anti-tuberculosis treatment could have resulted in false negative results.

INTRODUCTION

[453 words]

Tuberculosis (TB) is a leading cause of death from infectious diseases in South Africa.[1] Participants with TB and HIV co-infection experience diagnostic delays and have high early mortality.[1] Participants with disseminated TB may present with cytopenias, and bone marrow biopsy may be

used as part of the diagnostic evaluation in the hospital setting. *Mycobacterium tuberculosis* (MTB) can be identified on bone marrow trephine biopsies using the Ziehl-Neelsen (ZN) stain for acid fast bacilli (AFB), however, this has inadequate sensitivity and specificity.[2] TB culture of bone marrow aspirates may take up to 6 weeks for a result. Bone marrow biopsy is an invasive investigation with associated costs. Assessing the yield and diagnostic performance of TB investigations on bone marrow samples is therefore important.

The GeneXpert® MTB/RIF assay (Cepheid) is a rapid, cartridge based, real-time polymerase chain reaction (PCR) assay used for the diagnosis of TB on sputum as well as certain extra-pulmonary samples. Its sensitivity on sputum is reduced in participants with HIV co-infection as their sputum may be paucibacillary.[3] Its performance on extra-pulmonary samples is variable, with some sample types, such as serous effusions, showing poor sensitivity and yield. A previous study using the GeneXpert® MTB/RIF on bone marrow aspirate samples reported sensitivity to be only 11% when compared to histological findings.[4] The World Health Organization (WHO) recently recommended the GeneXpert® MTB/RIF Ultra, which has higher sensitivity, to replace the GeneXpert® MTB/RIF in diagnostic algorithms [3]. South Africa has transitioned to the routine use of the GeneXpert® MTB/RIF Ultra. [5]

The GeneXpert® MTB/RIF Ultra assay utilises the same platform as the GeneXpert® MTB/RIF assay, but with a different cartridge and software. The Ultra cartridge makes use of molecular probes to simultaneously detect MTB and rifampicin resistance by targeting two different multi-copy amplification targets (the IS6110 and IS1081 insertion elements) and the Rifampicin Resistance Determining Region of the MTB *rpoB* gene. [3]

Peripheral blood and bone marrow aspirate samples contain high numbers of red blood cells which may inhibit PCR reactions. Recent studies have described techniques which address sample preparation to optimise these types of samples for PCR.[6,7] A protocol has been validated to remove haem and lysis-concentrate blood without significant loss of bacilli, allowing blood samples to be analysed using the GeneXpert® MTB/RIF Ultra. [7]

We conducted a two-stage study at Groote Schuur Hospital in South Africa. First, we evaluated the yield of TB cultures on bone marrow aspirate samples taken from HIV-positive participants retrospectively, over an 18-month period. We then prospectively assessed the yield and diagnostic performance of the GeneXpert® MTB/RIF Ultra assay on bone marrow aspirate and peripheral blood samples from HIV-positive participants undergoing bone marrow biopsy in the routine clinical services at the hospital.

METHODS

[946 words]

Study design and population

Retrospective review of bone marrow biopsies

For the retrospective cohort study, all bone marrow biopsies performed between 1 January 2019 and 31 July 2020 at Groote Schuur Hospital, a tertiary hospital in Cape Town, South Africa were analyzed. Data was collected from the National Health Laboratory Service (NHLS) laboratory records. No clinical folder reviews were performed for the retrospective study. The requirement for informed consent was waived by the ethics committee for this component of the study.

Prospective evaluation of the GeneXpert® MTB/RIF Ultra assay

The GeneXpert® MTB/RIF Ultra assay was evaluated on bone marrow aspirate and peripheral blood samples in a cohort of consecutively enrolled adult HIV-positive participants undergoing bone marrow biopsy over a 6-month period (11 August 2020 to 31 January 2021), at the same hospital. The six-month period was chosen for feasibility as this study was conducted as part of a mini dissertation. The decision to conduct the bone marrow biopsy was made by the attending clinicians and not the study investigators.

Informed consent was obtained from all participants successfully recruited into the prospective study. Adult (>18 years old) HIV-positive participants admitted in or referred to Groote Schuur Hospital for a bone marrow biopsy, irrespective of the indication, were eligible for the study. Participants who were already on TB treatment were included in the study.

Participants were excluded from the study if they were younger than 18 years of age, or where aspirate samples could not be obtained or were of insufficient volume. HIV-positive participants booked for bone marrow biopsy as part of the routine initial staging and standard of care for known haematological condition were included, however follow-up bone marrow biopsies were excluded.

The University of Cape Town Human Research Ethics Committee approved both components of the study (HREC REF: 032/2020). Research activities were performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Approval from Groote Schuur Hospital management was obtained before data collection.

Data collection

Study data were collected and managed using the REDCap electronic data capture tool (REDCap 9.5.36 - © 2021 Vanderbilt University, Nashville, Tennessee, USA) hosted at the University of Cape Town. [8]

Retrospective review of bone marrow biopsies

Participant data including demographic information, clinical characteristics, HIV results (including recent viral load and CD4 count), tissue histology, staging bone marrow biopsy, all TB culture and GeneXpert® MTB/RIF Ultra results from any specimen within one month (before or after) of the bone marrow biopsy, and information on previous TB (including treatment, start date, duration, and treatment outcome) if available on the laboratory information system, were collected.

Prospective evaluation of the GeneXpert® MTB/RIF Ultra assay

Participants in the prospective cohort had full blood count values, results of radiological investigations (e.g., abdominal ultrasound, chest radiographs, Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) scan reports) and information on TB treatment (including start date, duration, and treatment outcomes if available) recorded, in addition to similar data collected as described for participants in the retrospective study.

To minimise experimental bias, individuals performing the GeneXpert® MTB/RIF Ultra assay (laboratory scientists at UCT Division of Medical Microbiology) were blinded to the results of the culture and other TB test results.

Sample collection and processing for the prospective study

We collected bone marrow aspirate and peripheral blood samples from participants who were HIV-positive and referred to our unit for bone marrow biopsy. We used residual bone marrow aspirate samples and collected an additional peripheral blood sample in EDTA tubes. These EDTA tubes were stored at -20 °C and batch-processed on the GeneXpert® MTB/RIF Ultra (Cepheid, Sunnyvale, CA, USA) within 3 weeks of sample collection (Figure 1). We removed red blood cells (RBC) using 0.22µm filtered RBC lysis buffer, followed by 2 wash steps and vortexing at 3000-4200g for 25-30 minutes (see supplementary material).

GeneXpert Ultra testing was performed using the GeneXpert DX System Version 4.8 (Cepheid, Sunnyvale, CA). Samples for GeneXpert were processed in accordance with a previously validated protocol [7] (see supplementary material).

We attempted to collect 5 to 10 ml of bone marrow aspirate and 5 to 10 ml of peripheral blood in a mycobacterial culture bottle (Myco/F Lytic bottles, Becton Dickinson BD, New Jersey USA) provided by the NHLS. The Myco/F Lytic bottles were placed in the BACTEC incubator unit (Becton Dickinson BD, New Jersey USA) within 6 hours of sample collection, where they were monitored for 42 days, and positive bottles screened for mycobacterial growth as per the laboratory standard operating procedure (SOP) (see supplementary material).

Definitions and statistical analysis

Descriptive analysis was used to summarise the study population (e.g. means, medians and interquartile ranges). A TB case was defined as: a positive TB culture or positive GeneXpert®

MTB/RIF Ultra on any specimen collected within one month (before or after) of bone marrow sample collection, morphological features of TB on the bone marrow trephine biopsy (the presence of granulomata and AFB on ZN staining which are also negative for PAS), or a strong clinical suspicion of TB. Strong clinical suspicion of TB was defined as the clinical diagnosis of TB made by a medical doctor and a decision made to start TB treatment.

Our original aim in the prospective study was to assess and compare the sensitivity and specificity of the GeneXpert® MTB/RIF Ultra on bone marrow aspirate and peripheral blood samples, using TB culture as a reference standard. Because all cultured samples were negative, this was not possible, and therefore report the yield instead. We include the index test on blood and bone marrow when calculating the diagnostic yield. Diagnostic yield was calculated as a proportion with a 95% confidence interval.

In instances where there was missing data, group totals were adjusted for analyses (indicated in the text where appropriate).

RESULTS

[959 words]

Retrospective review of bone marrow biopsies

A total of 760 bone marrow biopsies were performed between 01 January 2019 and 31 July 2020 at Groote Schuur Hospital. Of these, 122 bone marrow biopsies were from confirmed HIV-positive individuals with an accompanying bone marrow TB culture registered and were included in this study. Participant characteristics are summarised in Table 1.

Almost half of the included bone marrow biopsies were performed for the staging of a haematological malignancy (n=59), mostly lymphoma (Table 2 and Figure 2). Among these participants, classical Hodgkin Lymphoma (n=23; 38%) and Diffuse Large B-cell Lymphoma (DLBCL) (n=19; 31%) were the most common lymphoma diagnoses. The bone marrow was involved in six (23%) of Hodgkin Lymphoma (HL) participants and nine (24%) of non-Hodgkin Lymphoma (NHL) participants.

Forty percent (n=25) of the remaining 63 bone marrow biopsies, which were performed for diagnostic purposes, yielded a unique diagnosis. Of these, nine new cases of lymphoma were diagnosed on bone marrow biopsy and confirmed by immunohistochemical stains; the majority of these were classical HL (n=5) (Figure 3). No non-haematological malignancies were identified. There were four cases of pure red cell aplasia (PRCA).

There were 17 participants who had microbiologically confirmed tuberculosis in the cohort. Bone marrow granulomata were identified in seven of these participants, all of whom were on TB treatment before the bone marrow biopsy was performed, and three of them were positive for AFB on ZN

staining of the bone marrow trephine. None of these TB diagnoses were made on the bone marrow alone. Concurrent TB and lymphoma were found in five participants, four of whom had Hodgkin Lymphoma, and one who had a diagnosis of Diffuse Large B-cell Lymphoma (DLBCL).

When looking at the entire cohort, granulomata were observed in 15% (n=18) (Table 2). These participants had low CD4 counts (median 42 cells/uL), were mostly male (67%, n=12) and had bone marrow biopsies performed for diagnostic purposes. Four of these participants had lymphoma (HL (n=3) and DLBCL (n=1)) and one participant had Kaposi's Sarcoma. Eight of the 18 participants were on TB treatment prior to the bone marrow biopsy (Figure 2 and 3), only four of whom had microbiologically proven TB. No clear cause for the granulomata was identified in the other five participants.
Five (4%) participants had bone marrow granulomata on the trephine together with AFB on ZN staining which were negative on Periodic Acid-Schiff (PAS) staining. Only one new diagnosis of probable TB (positive for AFB on ZN staining) was made in the absence of current TB treatment. One participant was diagnosed with non-tuberculous mycobacterial (NTM) infection on bone marrow biopsy (curved bacilli identified on the trephine which were positive for both ZN and PAS staining), which was later confirmed on bone marrow aspirate TB culture.

Further testing of the single positive bone marrow NTM culture in our cohort led to the identification of *M. genavense / M. triplex* with a PCR/Line probe assay. The assay used (GenoType Mycobacterium AS, Hain Lifescience, Germany) is based on DNA STRIP technology, and due to the same banding pattern of both species, is unable to differentiate between *M. genavense* and *M. triplex*. [17] PCR and DNA sequencing and were unsuccessful in differentiating the two organisms.

No fungal infections were identified on bone marrow histology and only one participant had a diagnosis of lymphoma (HL) and concurrent TB in our cohort.

In the cohort of participants who had a diagnostic bone marrow biopsy, a quarter (n=15, 24%) of participants had a new unique diagnosis from the diagnostic bone marrow biopsy. The majority of these participants were on antiretroviral therapy (ART) (n=13) with age (median =35 years, IQR 32-39), a male predominance and median CD4 count of 226 cells/ul.

Bone marrow TB culture (n=122) only detected one active case of NTM, and none of the 17 microbiologically confirmed cases of TB in the retrospective cohort (Table 3).

In summary, there were 17 participants with microbiologically confirmed tuberculosis in our cohort, and two additional participants with presumed TB based on the presence of granulomata and AFB on ZN stain and negative PAS stain.

Prospective testing using GeneXpert® MTB/RIF Ultra assay

Fifty-two individuals were eligible for the study, but only 33 individuals were successfully recruited prospectively, with the others refusing study participation. The majority were inpatients (n=26; 79%), male (n=19; 58%), on ART and had had previous TB (n=18; 55%) (Table 4).

Ten participants were on TB treatment for a median duration of 36 days (IQR 6.75-49.5, Range 1-50 days), before the bone marrow biopsy was performed. Only six of these participants had microbiologically proven TB: five of these participants had a positive GeneXpert® MTB/RIF Ultra result on sputum, and an additional participant was only sputum TB culture positive (and GeneXpert® MTB/RIF Ultra negative). One positive result (trace- semi-quantitation) was detected using the GeneXpert® MTB/RIF Ultra assay on a peripheral blood sample. This participant had a positive sputum GeneXpert® MTB/RIF Ultra and had been started on treatment 1 week prior to the bone marrow biopsy. All other GeneXpert® MTB/RIF Ultra assays performed on bone marrow aspirate samples were negative. There were four participants where only a bone marrow aspirate was collected without a peripheral blood sample for the GeneXpert® MTB/RIF Ultra assay. One participant's peripheral blood sample was clotted and could not be processed for the GeneXpert® MTB/RIF Ultra assay. No error results were noted on the bone marrow GeneXpert® MTB/RIF Ultra. The diagnostic yield on the GeneXpert® MTB/RIF Ultra assay using peripheral blood was nine percent (1/11), and zero (n=11) using bone marrow aspirate. The diagnostic yield after excluding participants already on TB treatment before samples were collected is still zero using bone marrow aspirates (n=2).

All of 66 TB cultures on bone marrow aspirate and peripheral blood samples were negative.

DISCUSSION:

[855 words]

We have shown there remains a role for bone marrow biopsy in HIV participants in the era of widespread ART use. Diagnoses other than tuberculosis was identified frequently enough in the bone marrow biopsies of participants with cytopenias to warrant routine use of the investigation in clinical practice. In the prospective study, we found only one positive GeneXpert® MTB/RIF Ultra using peripheral blood and none using bone marrow, which may have been the results of the fact that many participants commenced TB treatment prior to sample collection.

A previous retrospective review reported on 147 bone marrow biopsies in HIV-positive participants at our hospital who underwent a bone marrow biopsy for the investigation of fever and/or cytopenias (bone marrow biopsies performed for staging of known haematological malignancies were excluded). [9] One-fifth of these participants were on TB treatment before the bone marrow biopsy, compared to 12% in our cohort. [9] It is worth noting that this analysis was performed in a different era, where not all participants with HIV were eligible for initiation of ART. New bone marrow diagnoses of Classic Hodgkin Lymphoma in our study were comparable to the earlier cohort of participants at the same centre (n=5 vs 4). [9]

The cases of PRCA we diagnosed on bone marrow biopsy were thought to be drug-related, with common viral causes including Parvovirus B19 excluded. Recent reports have implicated emtricitabine as a cause of PRCA, which was thought to be the probable cause in our participants. [13,14]

There were a surprisingly low number of biopsies with granulomata, which was half the proportion reported in the previous study at our hospital (15%; n=18 vs 33%; n=49)[9] but similar to a recent study at another hospital in Cape Town, South Africa, that found similarly low levels of TB in their bone marrow samples with granulomas.[15] This was somewhat surprising in a setting that has a very high incidence of TB in communities, in participants who are immunocompromised, though a selection bias may contribute to these findings as only participants who have had several investigations including sputum GeneXpert® MTB/RIF Ultra and imaging (e.g. chest x-rays, abdominal ultrasounds) and still do not have a diagnosis would be referred for a bone marrow biopsy. Bharuthram *et al*., showed similarly low findings of TB in their Johannesburg cohort.[16] In a recent large retrospective series from Western China- a region with a high prevalence of TB, over 11 000 cases were reviewed, with only 0.97% of cases displaying bone marrow granulomata.[17]

Bone marrow granulomata were identified in seven participants who had microbiologically confirmed TB and were on treatment before the bone marrow however, none of these tuberculosis diagnoses were made on the bone marrow findings alone. Despite the higher percentage of participants on TB treatment before the bone marrow biopsy, in the prior study at our hospital, they still reported 12% of bone marrow TB cultures positive for MTB.[9] We found that the yield of TB culture on bone marrow aspirate samples was limited in our contemporary study population. This may have been due to the number of participants who were on TB treatment before the bone marrow biopsy, and those whose diagnosis was made purely on radiological and clinical findings.

In the prospective arm of the study, we intended to recruit approximately 100 participants, however, we were not able to achieve these numbers due to insufficient referrals. Recruitment for the study began during COVID, and we believe that this did contribute by increasing the threshold for referrals to minimize risk to those performing the procedures. In addition, a few (19) participants did not consent to participation in the study. Among the 33 participants included in this assessment, none had a positive TB culture on bone marrow or blood. We were therefore unable to assess the diagnostic performance of GeneXpert® MTB/RIF Ultra on bone marrow aspirate or peripheral blood, as originally planned, given the lack of positive reference standard tests. Furthermore, these findings suggest that TB culture on bone marrow aspirate samples has limited yield in highly selected participants (those who are hospitalized and have had multiple previous investigations for TB which were negative) or those already on TB treatment. We would have expected TB DNA to persist.

We considered that inhibition with haem and/or EDTA could have interfered with the GeneXpert® MTB/RIF Ultra assay performance and thus the diagnostic yield. However, this is unlikely given the negative TB cultures as well as the work done by Boloko et al validating the sample preparation protocols used in the study.[7,18] A pilot study performed at our institution (data not shown) showed minimal inhibition from haem and EDTA in bone marrow samples without the RBC lysis treatment, displaying similar PCR cycle threshold (Ct) values with varying volumes of bone marrow aspirate in EDTA. Comparable results were found in another South African study.[4] Digital droplet PCR

(ddPCR) has shown promise in detecting low levels of circulating *Mycobacterium tuberculosis* DNA in whole blood, noting that ddPCR is highly resistant to the PCR reaction inhibitors such as EDTA, and may be a direction in which future research will be focused.[19]

Strengths and limitations of the study

[242 words]

The retrospective review was conducted over a brief time (18 months) and did not include a clinical folder review which may have limited this study. The clinical information for participants included in the study was variable and based solely on the data provided by the requesting clinician at the time of scheduling the procedure. No consistent criteria for the requesting of diagnostic bone marrow biopsies could be found, and thus procedures were performed based on the clinical decision of the requesting physician in consultation with the haematopathologist.

For the prospective study, we could not calculate the performance of the GeneXpert® MTB/RIF Ultra assay in this study due to the lack of positive TB cultures. Sample volumes were not formally measured for the TB cultures, and this may have influenced the diagnostic yield of the tests. Increasing the sample volume of the TB blood cultures may have increased diagnostic yield.[2, 18] The average sample volume processed for the GeneXpert® MTB/RIF Ultra assay was 2.5 ml. The volume recommended by the manufacturer is 1ml for sputum,[20] and the recommended minimum volume is 3-5ml according to the protocol by David Barr (see supplementary material). Boloko et al were able to show that using 3–7 mL whole blood in EDTA was sufficient, and the GeneXpert® MTB/RIF Ultra assay was positive in 37% (n= 165 of 447) participants with confirmed tuberculosis by the microbiological reference standard in their cohort.[7]

Groote Schuur Hospital is a large referral hospital based in Cape Town, which is affiliated to the University of Cape Town, and participants are mostly referred for advanced specialist care. Its participant population is unlikely to represent the general HIV population as seen in the community, and thus these results may only be generalizable to other tertiary care hospitals in the region.

Conclusion

[259 words]

The retrospective study highlighted the contemporary utility of the bone marrow biopsy in HIV positive participants, with 17 new unique diagnoses made based on the biopsy, though there were fewer new diagnoses of TB than expected. In the prospective study, TB culture on bone marrow aspirate samples yielded no positive results in this highly selected cohort, one third of whom were already established on TB treatment. In keeping with this, the GeneXpert® MTB/RIF Ultra on bone marrow aspirate and peripheral blood was negative in almost all participants in the prospective study. We postulate that the reason for these findings is that participants with cytopenias due to HIV-associated TB in our setting are diagnosed on other clinical specimens or empirically started on TB

treatment, often at primary or secondary levels of care, and therefore not referred for biopsy or only referred if they deteriorate on TB treatment. The diagnoses made on bone marrow biopsy are therefore mainly non-TB diagnoses.

Future research on the diagnostic performance and yield of GeneXpert® MTB/RIF Ultra on bone marrow aspirate could involve prospectively recruiting HIV-positive participants with cytopaenias who are admitted to hospital and are being investigated for TB, but in whom initial sputum and urine-based TB diagnostics are negative, and who have not yet been started on TB treatment. This may provide a higher yield of diagnoses using this assay on bone marrow samples.

Our findings suggest that routine conduct of TB culture and GeneXpert® MTB/RIF Ultra on bone marrow samples in HIV positive participants where TB is not suspected, may not be warranted.

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Competing interests

The authors have no competing interests (financial, personal, or otherwise).

Authors' contributions

C.S and C.M devised the project, G.M., G.M., D.B and E.V assisted with developing the main conceptual ideas and proof outline. C.S. supervised the project. C.M. assisted with measurements and helped carry out the experiments. S.B performed the numerical calculations and data analysis. S.B. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Data availability statement

Data will be governed by a Creative Commons license, and researchers will have privileged use of the data for two years to enable the publication of research results, after which it will be made freely available under this license. The other data will be retained for 10 years.

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20. Xpert MTB/RIF Ultra Package Insert 301-5987, Rev. K March 2021.

TABLES AND FIGURES

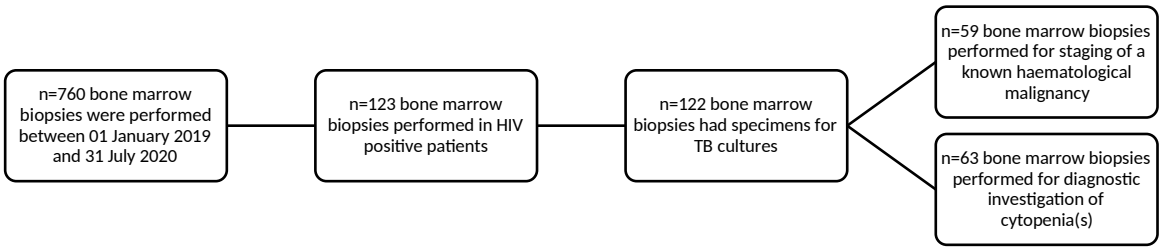

Figure 1: Consort diagram of retrospective review

We undertook a retrospective review of all bone marrow biopsies performed between 01 January 2019 and 31 July 2020. One hundred and twenty-three bone marrow biopsies were performed in confirmed HIV positive individuals. Of these, only one participant did not have a bone marrow TB culture registered. Just over half (n=63; (52%)) of all the bone marrow biopsies were performed to investigate the cause of cytopenias in HIV positive individuals.

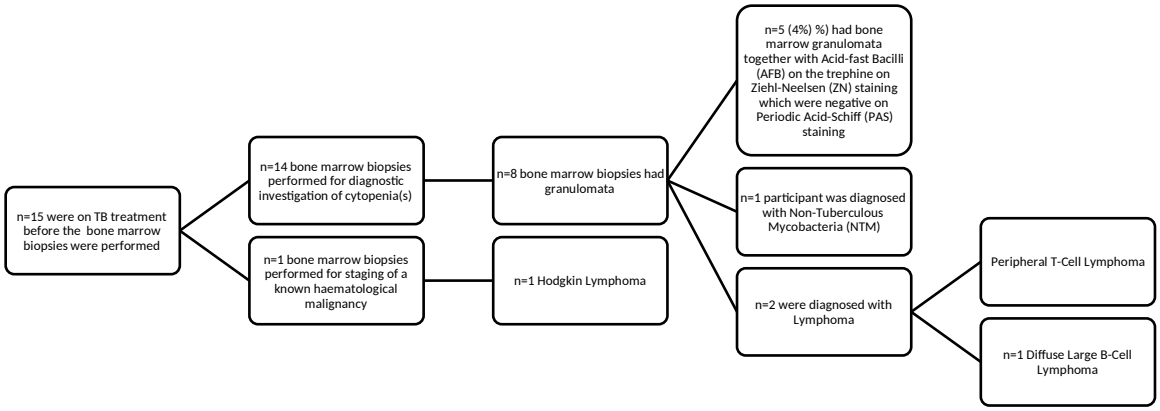

Figure 2: Participants who had been started on TB treatment before the bone marrow biopsy in the retrospective arm of the study

Most participants who were on TB treatment (n=15) before the bone marrow biopsy were referred for a diagnostic rather than staging bone marrow procedure. Of these participants, 5 (4%) had bone marrow granulomata together with Acid-fast Bacilli (AFB) on the trephine on Ziehl-Neelsen (ZN) staining which were negative on Periodic Acid-Schiff (PAS) staining. One participant was diagnosed with Non-Tuberculous Mycobacteria (NTM) on bone marrow biopsy (curved bacilli identified on the trephine which were positive for Ziehl-Neelsen (ZN) staining and Periodic Acid-Schiff (PAS) staining), which was later confirmed on TB bone marrow culture.

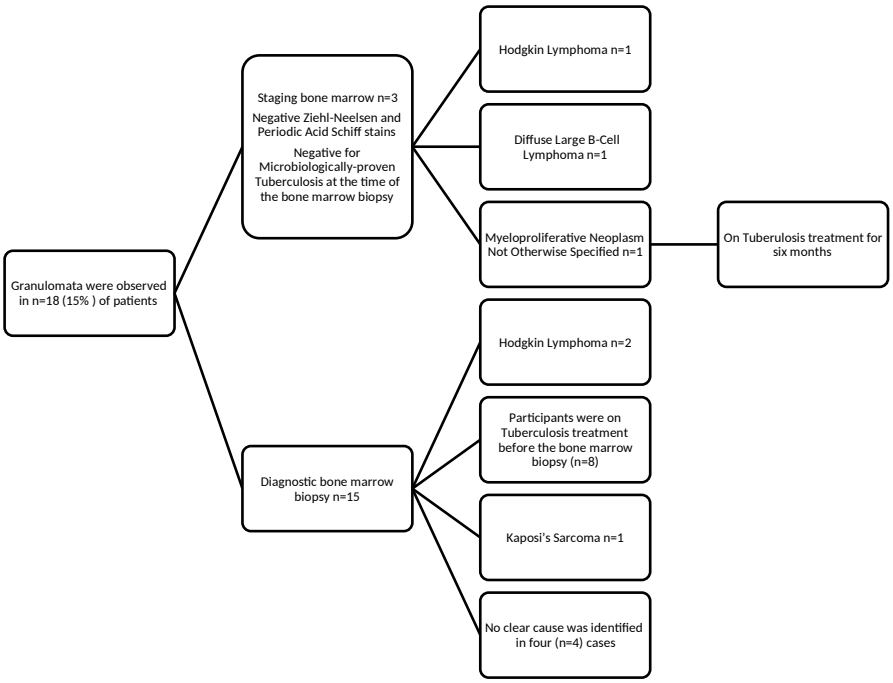

Figure 3: Participants who had granulomata on the bone marrow biopsy in the retrospective arm of the study.

This figure illustrates those participants who had granulomata on their bone marrow. Four of these participants had Lymphoma (HL (n=3) and DLBCL), one participant had Kaposi's Sarcoma. No clear cause could be identified in four cases. Eight participants of the 18 were on TB treatment before the bone marrow biopsy. DLBCL= Diffuse Large B-Cell Lymphoma; HL=Hodgkin Lymphoma MPN NOS= Myeloproliferative Neoplasm Not Otherwise Specified; PAS= Periodic Acid Schiff stain; TB=Tuberculosis; ZN= Ziehl-Neelsen stain

Table 1: Participant characteristics in the retrospective review of bone marrow TB cultures in HIV positive participants (n=122)

This table describes the participant characteristics of participants in the retrospective arm of the study. They were mostly male (n=70), virally unsuppressed with a median CD4 count of approximately 200 cells/uL. The majority bone marrow biopsies performed in these participants were for diagnostic reasons (n=63). Thirteen percent (n=16) of participants had started TB treatment before the bone marrow biopsy was performed.

Characteristic	**n (%) or median (IQR)**
Median age in years (IQR)	38 (33-44)
Sex n (%)	
Male	70 (57)
Female	53 (43)
In/Outpatient Status n (%)	
Inpatient	91 (74)
Outpatient	31 (24)
Most recent CD4 count (IQR) n=122	187 cells/uL (42-271)
Most recent HIV viral load (IQR) n=101	569 651 copies/ml (0- 49 367)
Antiretroviral treatment (ART) status (%)	
Antiretroviral naive	12 (10)
Currently on Antiretroviral treatment	81 (67)
Interrupted Antiretroviral treatment	12 (10)
Unknown	17 (14)
Indication for the bone marrow n (%)	
Staging for a known haematological malignancy	59 (48)
Diagnostic investigation of cytopenia (s)	63 (52)
On TB treatment before the bone marrow n (%)	
Yes	15 (12)
No	107 (88)

IQR: interquartile range

Table 2: Bone marrow findings in the retrospective study

Bone marrow (BM) finding:	**n (%)**

Granulomata	
Present	18 (15)
Absent	102 (83)
Lymphohistiocytic aggregates	2 (2)
Ziehl-Neelsen stains	
Positive for AFB	6 (5)
Negative	25 (20)
Not done	91 (75)
Periodic Acid Schiff stains	
Positive	1 (1)
Negative	25 (20)
Not done	96 (79)

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507 **Table 3: TB culture results in the retrospective study**

Specimen type	Positive n (%)	Negative n (%)
TB culture on		
Sputum n=18	2 (11)	16 (89)
Bone marrow n=122	1 (0.8)	121 (99.2)
Blood n=31	3 (10)	28 (90)
Other n=73	4 (6)	69 (94)
CSF n=15		
Pleural fluid n=6	1 (17)	5 (87)
Urine n=12	1 (8)	11 (92)
Joint aspirate n=2	0 (0)	2 (100)
Tissue biopsy n=38	0 (0)	38 (100)
GeneXpert® MTB/RIF Ultra		
Sputum n=54	8 (15)	46 (85)
Other n=29	1 (3)	28 (97)
CSF n=12		

Pleural fluid n=4	0 (0)	4 (100)
Urine n=3	1 (33)	2 (67)
Joint aspirate n=2	0 (0)	2 (100)
Tissue biopsy n=14	0 (0)	14 (100)

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509 CSF= Cerebrospinal fluid, Tissue = Core needle biopsy or excision biopsy of any organ including liver and lymph nodes

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511 **Table 4: Prospective study participant characteristics**

Characteristics n=33	**n (%) or median (IQR)**
Sex	
Male	19 (58)
Female	14 (42)
Most recent CD4 count n=33	201 cells/uL (47-195)
Most recent HIV viral load n=28	493 861 copies/mL (0- 17 845)
Previous TB	18 (55)
ART (Anti-retroviral treatment) status Antiretroviral naive Currently on ART Interrupted ART	 4 (12) 18 (55) 11 (33)
In/Outpatient Status Inpatient Outpatient	 26 (79) 7 (21)
TB cases [a] at time the bone marrow was performed:	11 (33)
Median duration of TB treatment (days) before the bone marrow biopsy n=10 (30%); In one patient the treatment duration was unknown	36 (6.75-49.5)
Indication for the bone marrow Staging for a known haematological condition Diagnostic investigation of cytopenias	 14 (42) 19 (58)
GeneXpert® MTB/RIF Ultra positive: peripheral blood	1 (trace)[b] in a participant with TB on treatment

GeneXpert® MTB/RIF Ultra positive: bone marrow	0
TB culture positive (Bone Marrow/Peripheral Blood)	0

a: A TB case was defined as: a positive TB culture or positive GeneXpert® MTB/RIF Ultra on any specimen collected within one month (before or after) of bone marrow sample collection, morphological features of TB on the bone marrow trephine biopsy (the presence of granulomata and AFB on ZN staining which are also negative for PAS), or a strong clinical suspicion of TB. Strong clinical suspicion of TB was defined as the clinical diagnosis of TB made by a medical doctor and a decision made to start TB treatment.

b: Trace-mini semi-quantitation available on the GeneXpert® MTB/RIF Ultra

Appendix A-SOP for Xpert-ultra on blood

Developed and validated by David Barr david.barr@liverpool.ac.uk

Used with permission.

Reagents / consumables

- Sterile Ethylenediaminetetraacetic acid (EDTA) tubes
- RBC lysis buffer: [155mM NH_4Cl; 12mM $NaHCO_3$; 0.1 mM EDTA]. Made with 0.22µm Filtered water and 0.22µm filtered again prior to use.
- Sterile water: 0.22µm filtered deionised water.
- 50 and 15ml centrifuge (falcon) tubes.
- Pipettes and centrifuge.
- Xpert-ultra lysis buffer and cartridges

A note on volume of blood to collect

The volume to collect is arbitrary – larger volumes will increase sensitivity. The below SOP was optimised for 3-5ml (based on the maximum volume we could safely take from the patient in the experiment design which involved multiple other samples) but could be used for up to 10ml. If you are processing more than 10ml blood the volumes of lysis buffers etc might be too small, and it will affect the pelleting etc, so the simplest thing would be to split the sample, e.g., if you collect 15ml could run that as two 7.5ml samples but combine them at the end for one cartridge.

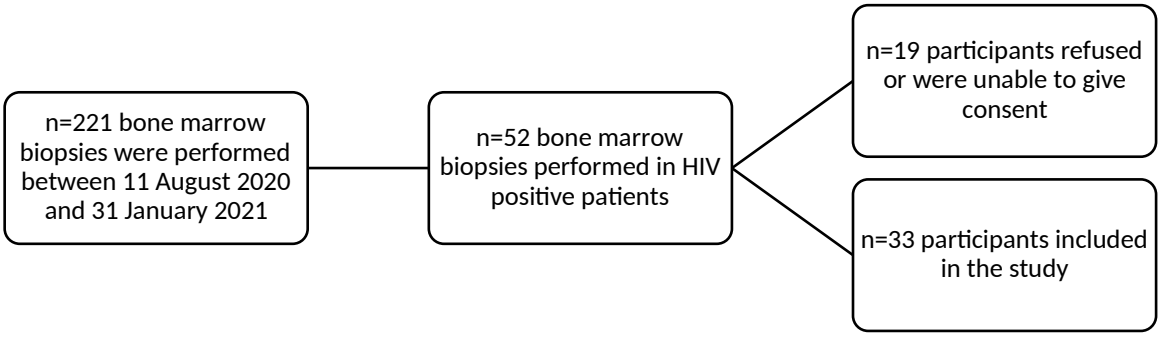
Method

1. Collect blood in a sterile EDTA tubes (purple top).
2. Transfer blood to a 50ml falcon tube. Wash out any residual blood in the EDTA tube using RBD lysis buffer and add this to the falcon tube (you do not want to miss any bacilli adhering to the sides of the tube). Make up the falcon tube volume to 45ml with RBC lysis buffer.
3. Incubate at room temperature for 30 minutes with regular inversions to mix, then pellet at 3000g for 30min. Remove the supernatant by incredibly careful pouring (into a NaOH waste pot in the hood). It is more important to not accidentally pour any of the pellet out than it is to remove all the supernatant. The pellet is soft and easily disperses so I would leave 4-6ml supernatant at the bottom.
4. Resuspend the pellet in the residual supernatant volume by repeated reverse pipetting (works better than vortexing). It is essential to resuspend in this smaller volume before moving to next step.
5. Make to volume up to 45ml using sterile water. Incubate at room temperature for 30 minutes with regular inversions to mix, then pellet at 3000g for 30min. The pellet should now not be red – should be pale pink or buff colour. Remove supernatant and resuspend in residual volume as above.

[Samples can be frozen for batching at this stage]

6. Add the sample to a 15ml falcon tube and make volume up to 10 ml using sterile water. Spin at 4200G for 25 minutes and remove 9.3 ml supernatant, leaving ~0.7ml sample. This should be resuspended in the 0.7ml by pipetting.
7. Add 1.5ml of the GeneXpert® MTB/RIF Ultra buffer to each (final volume ~2.2ml – *it should never be less than 2ml*. These are mixed by vigorous shaking 20 times (make sure lid is on) and then incubated at room temperature in hood for 10 minutes, followed by another 20 shakes and further 5-minute incubation.
8. Sample can now be added to the GeneXpert® MTB/RIF Ultra cartridge and processed as per manufacturer instructions.

Appendix B: Consort diagram of Prospective study



Consort diagram of Prospective study

The GeneXpert® MTB/RIF Ultra assay was evaluated on bone marrow aspirate and peripheral blood samples in a cohort of consecutively enrolled adult HIV-positive participants undergoing bone marrow biopsy over a 6-month period (11 August 2020 to 31 January 2021), at the same hospital. The six-month period was chosen for feasibility as this study was conducted as part of a mini dissertation. Two hundred and twenty-one bone marrow biopsies were performed during this period, 52 bone marrow biopsies performed in HIV positive patients and 33 participants were included in the final study. A total of 19 participants were unable to give informed consent or refused to participate in the study.