



OPEN Benchtop NMR urine metabolomics for diagnosing pulmonary tuberculosis

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Tuberculosis (TB) remains a major global health issue, especially in resource-limited settings, where rapid and accurate diagnosis is critical yet often lacking. Nuclear magnetic resonance (NMR)-based metabolomics has emerged as a promising tool for identifying disease-specific biomarkers. This study assessed the diagnostic potential of urinary metabolomics using high-resolution (HR-)NMR and benchtop NMR (bNMR) in Nigerian adults with presumed pulmonary TB, including individuals with and without HIV. Urine samples were analysed by HR-NMR and bNMR. Multivariate analysis was used to develop classification models based on confirmed TB status. Key discriminant metabolites were identified. In HIV-negative individuals, HR-NMR achieved 84% sensitivity and 86% specificity, while bNMR reached 83% and 84%, respectively. In people living with HIV, HR-NMR reached 75% sensitivity and 70% specificity; bNMR achieved 90% and 46%. Both models met or exceeded WHO sensitivity criteria for non-sputum-based TB diagnostics. Thirteen discriminatory metabolites were identified, with eight consistent with previous studies. These findings suggest common metabolic signatures of TB, regardless of HIV status. Urinary NMR-based metabolomics shows strong potential for TB diagnosis in high TB/HIV burden settings. HR-NMR provides excellent accuracy, while bNMR offers a cost-effective alternative. Further studies are needed to validate and optimise this approach for clinical use.

Tuberculosis (TB) remains a major global health challenge, particularly in low- and middle-income countries (LMICs) where the highest burden of disease resides and resources for diagnosis and treatment are limited. The high TB burden is further compounded by the high prevalence of Human Immunodeficiency Virus (HIV) and TB co-infection, which further complicates diagnosis and management due to overlapping clinical manifestations and limitations of current diagnostic methods. In 2024, the World Health Organization (WHO) estimated that 10.8 million people fell ill with TB, with Africa bearing the largest burden of HIV-associated TB¹. Nigeria, as one of the high TB burden countries, accounting for 4.6% of global cases in 2023, faces substantial challenges

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in controlling the disease, particularly considering the high rates of HIV-TB co-infection, which exacerbate diagnostic issues. It is estimated that last year, nearly a quarter of annual TB cases were not diagnosed (2.7 million), while 1.25 million people died due to this disease, of whom more than 1 million were HIV co-infected.

Accurate and timely diagnosis is crucial for effective treatment and interruption of transmission². However, current diagnostic methods face limitations, particularly in resource-limited settings. Smear microscopy, while widely available, lacks sensitivity, especially in people living with HIV and those with paucibacillary forms of TB. Culture, the gold standard, requires a long turnaround time and specialised infrastructure³. WHO-recommended molecular (mWRD) tests are rapid and sensitive, but they are expensive and require trained staff and the use of expensive laboratory reagents that frequently experience prolonged stock outs. Furthermore, the availability of sputum samples limits the sensitivity of these methods in populations with difficulty expectorating, with lower bacillary loads or extrapulmonary manifestations of TB. The lateral flow urine lipoarabinomannan (LAM) assay (Alere Determine™ TB LAM Ag) is rapid and can be performed at the point of care but is only recommended for people living with HIV^{4,5}. Newer generations of LAM assays, such as the Fujifilm SILVAMP TB LAM (FujiLAM) prototype, demonstrated improved diagnostic accuracy and may be effective regardless of HIV status, although the first version experienced sensitivity variations among batches⁶. However, limitations remain, and there is an urgent need for accurate, non-invasive diagnostic approaches suitable for LMICs.

Metabolomics, which comprehensively studies metabolites in biological systems, has emerged as a promising approach to understand the immune responses and identify diagnostic biomarkers in various diseases, including TB⁷. Within this field, Nuclear Magnetic Resonance (NMR) stands out as one of the most reproducible and robust techniques, with the potential to identify unique metabolic fingerprints associated with specific pathologies. With the development of compact benchtop platforms suitable for clinical laboratories, this technique becomes especially relevant in LMICs, where the burden of disease is greatest⁸. In this regard, NMR-based metabolomics has enabled a deeper understanding of the interactions between the host and *Mycobacterium tuberculosis* during disease, through the analysis of serum, plasma, and urine samples⁹. In previous studies, we established a characteristic urinary metabolic fingerprint for TB, highlighting the potential of NMR-based metabolomics to improve TB diagnosis¹⁰. Expanding our approach, we also managed to differentiate between different levels of diagnostic certainty of TB in a paediatric population in Haiti¹¹. However, the applicability of this approach in high TB burden settings, especially those with high HIV prevalence, has not yet been explored. The metabolic profiles of individuals in these regions may differ due to factors such as genetic diversity, environmental influences, and comorbidities like HIV. Understanding these differences is crucial for the development of effective TB diagnostic tools in diverse populations.

This study aimed to evaluate the discriminatory potential of urine NMR-based metabolomics using both high-resolution (HR-NMR) and benchtop NMR (bNMR) spectroscopy for diagnosing pulmonary TB in Nigerian adults with presumed disease, including HIV-positive and HIV-negative individuals. The performance of these metabolomic models will be compared with that of conventional diagnostic methods to assess their efficacy in high disease prevalence settings. Through this research, we aim to contribute to the development of more sensitive, non-invasive, and accessible diagnostic tools for TB that are suitable for use in high-burden countries, ultimately supporting global efforts to reduce TB incidence and mortality.

Results

Study population

The study included 206 patients, of whom 54 (26.2%) had bacteriologically confirmed pulmonary TB (TB group), and 152 (73.8%) did not (non-TB group) (Table 1). The mean age was 37.4 years (SD = 13.2), with no significant difference between the TB and non-TB groups ($p = 0.532$). A higher proportion of males was observed in the TB group (55.6%) compared to the non-TB group (31.6%), though this difference was not statistically significant ($p = 0.105$). HIV-negative participants were more frequent among TB patients (77.8%) than non-TB patients (62.5%), but again, the difference did not reach statistical significance ($p = 0.097$). Overall, 137 (66.5%) participants were HIV-negative, 66 (32.0%) were HIV-positive, and three (1.5%) had unknown HIV status.

Clinical characteristics and diagnostic testing

Significant differences between the TB and non-TB patients were observed in the onset of symptoms ($p = 0.008$), weight loss ($p = 0.049$), total number of symptoms ($p = 0.033$), and duration of symptoms ($p = 0.006$) (Table 1). TB patients exhibited a longer duration of symptoms, with 79.2% experiencing symptoms for more than two weeks, compared to 57.0% in the non-TB group ($p = 0.006$). TB patients were also more likely to present with five or more symptoms (75% vs. 57.7%, $p = 0.003$). Self-reported comorbidities were uncommon in both groups, with no significant differences ($p = 0.681$).

Among the 54 patients in the TB group, 37 (68.5%) had positive *M. tuberculosis* cultures, with an average time to positivity of 28.2 days (Table 2). Xpert MTB/RIF and Xpert MTB/RIF Ultra detected TB in 39 patients (72.2% and 79.6%, respectively), with a higher proportion showing “medium” and “high” cycle threshold (CT) values. Truenat-TB MTB-RIF detected TB in 38 patients (79.2%). Smear microscopy was positive in 22 cases (45.8%), with 40.9% of the cases classified as 2+ ($p = 0.325$).

When considering combinations of diagnostic tests, 20 TB patients (37%) tested positive across all tests, whereas 15 (27.8%) tested positive with only one test. Three (5.6%) culture-positive patients were negative for mWRD and smear microscopy, whereas 17 (31.5%) mWRD-positive patients had either negative or contaminated culture and smear microscopy results. All 22 smear-microscopy-positive patients also had at least one positive test result. No significant association was observed between HIV status and the results of any individual diagnostic tests ($p > 0.05$) (Table 2).

Variable	All (n = 206)	Non-TB (n = 152)	TB (n = 54)	P-value
Age (years)				
Mean (SD)	37.4 (13.2)	37.8 (13.1)	36.4 (13.6)	0.532
Sex				
Male	95 (46.1%)	65 (31.6%)	30 (55.6%)	0.105
Female	111 (53.9%)	87 (57.2%)	24 (44.4%)	
HIV				
Negative	137 (66.5%)	95 (62.5%)	42 (77.8%)	0.097
Positive	66 (32.0%)	54 (35.5%)	12 (22.2%)	
Unknown	3 (1.5%)	3 (2.0%)	0 (0.0%)	
HIV Viral Load				
Undetectable	10 (15.2%)	9 (16.7%)	1 (8.3%)	0.279
< 40 copies/mL	16 (24.2%)	12 (22.2%)	4 (33.3%)	
40–10 ⁷ copies/mL	29 (43.9%)	25 (46.3%)	4 (33.3%)	
Indeterminate	4 (6.1%)	4 (7.4%)	0 (0.0%)	
Missing	7 (10.6%)	4 (7.4%)	3 (25.0%)	
Presence of TB Symptoms*				
Symptom onset (weeks)	2.4 (1.3)	2.2 (1.2)	2.8 (1.5)	0.008
Cough	190 (100%)	142 (74.7%)	48 (25.3%)	
Haemoptysis	24 (12.6%)	18 (12.7%)	6 (12.5%)	0.975
Fever	137 (72.1%)	98 (69.0%)	39 (81.3%)	0.102
Chest pain	154 (81.1%)	116 (81.7%)	38 (79.2%)	0.700
Weight loss	133 (70.0%)	94 (66.2%)	39 (81.3%)	0.049
Night sweats	70 (36.8%)	51 (35.9%)	19 (39.6%)	0.649
Weakness	142 (74.7%)	103 (72.5%)	39 (81.3%)	0.230
Loss of appetite	92 (48.4%)	68 (47.9%)	24 (50.0%)	0.800
Other non-TB symptoms [‡]	3 (1.6%)	2 (1.4%)	1 (2.1%)	1.000
Total TB symptoms*				
≤ 4 symptoms	60 (42.3%)	12 (25.0%)	72 (37.9%)	0.033
≥ 5 symptoms	118 (62.1%)	82 (57.7%)	36 (75.0%)	
Duration of TB symptoms*				
≤ 2 weeks	71 (37.4%)	61 (43.0%)	10 (20.8%)	0.006
> 2 weeks	119 (62.6%)	81 (57.0%)	38 (79.2%)	
Comorbidities*				
≥ 1 self-reported comorbidity	7 (3.7%)	6 (4.2%)	1 (2.1%)	0.681
Hypertension	5 (71.4%)	4 (66.7%)	1 (100%)	1.000
Diabetes mellitus	2 (28.6%)	2 (33.3%)	0 (0.0%)	
Sputum appearance*				
Saliva	33 (17.4%)	29 (20.4%)	4 (8.3%)	0.200
Mucoid	73 (38.4%)	50 (35.2%)	23 (47.9%)	
Mucopurulent	49 (25.8%)	36 (25.4%)	13 (27.1%)	
Purulent	35 (18.4%)	27 (19.0%)	8 (16.7%)	

Table 1. Demographic and clinical characteristics of the full study cohort (n = 206), prior to any exclusions for NMR or stratified analyses. *TB*: Tuberculosis; *HIV*, human immunodeficiency virus; The *p*-value indicate group comparisons between TB and non-TB patients, calculated using the chi-squared test, Fisher's exact test, or the Fisher–Freeman–Halton test with Monte Carlo simulation, as appropriate. *Missing rate: percentage of patients without available results for a given variable (7.8% for symptom onset, total number of TB symptoms, duration of TB symptoms, comorbidities, and sputum gross appearance). [‡]Includes gastrointestinal symptoms, hepatitis and headache. Significant values are in bold.

NMR analysis

Urine samples from all 206 participants were analysed using a bNMR spectrometer. In addition, a subset of 140 samples underwent HR-NMR analysis. The reduced number of samples analysed by HR-NMR was due to insufficient material to prepare two independent aliquots for both platforms.

Characterization of the TB metabolic fingerprint

Prior to statistical analysis, a total of seven HR-NMR and 45 bNMR spectra were excluded. Specifically, 26 bNMR spectra were removed due to acquisition artefacts or sample contamination. In each case, spectral quality

Variable	HIV-negative (n = 42)	HIV-positive (n = 12)	All (n = 54) ‡	p-Value
Culture				
Positive	30 (71.4%)	7 (58.3%)	37 (68.5%)	0.411
Time to positivity	28.5 (9.2%)	26.6 (17.4%)	28.2 (10.4%)	0.715
Colony count	1.7 (0.8%)	1.6 (1.0%)	1.7 (0.8%)	0.754
Xpert MTB/RIF				
Positive	33 (78.6%)	6 (50.0%)	39 (72.2%)	0.071
Ct category				
Very Low	7 (21.2%)	0 (0.0%)	7 (17.9%)	0.479
Low	2 (6.1%)	1 (16.7%)	3 (7.7%)	
Medium	13 (39.4%)	3 (50.0%)	16 (41.0%)	
High	11 (33.3%)	2 (33.3%)	13 (33.3%)	
Xpert MTB/RIF Ultra*				
Positive	33 (84.6%)	6 (60.0%)	39 (79.6%)	0.181
Ct category				
Trace	0 (0.0%)	1 (16.7%)	1 (2.6%)	0.095
Very Low	4 (12.1%)	0 (0.0%)	4 (10.3%)	
Low	6 (18.2%)	1 (16.7%)	7 (17.9%)	
Medium	7 (21.2%)	3 (50.0%)	10 (25.6%)	
High	16 (48.5%)	1 (16.7%)	17 (43.6%)	
Smear-microscopy*				
Positive	19 (48.7%)	3 (33.3%)	22 (45.8%)	0.478
Grade				
Scanty	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.584
1 +	6 (31.6%)	2 (66.7%)	8 (36.4%)	
2 +	8 (42.1%)	1 (33.3%)	9 (40.9%)	
3 +	5 (26.3%)	0 (0.0%)	5 (22.7%)	
Truenat MTB-RIF*				
Positive	30 (76.9%)	8 (88.9%)	38 (79.2%)	0.661
Ct value	27.1 (5.0)	28.8 (4.5)	28.0 (5.0)	0.665
Concordant positive test combinations				
All test positive	18 (42.9%)	3 (25.0%)	21 (38.9%)	0.221
Culture + mWRD	11 (26.2%)	2 (16.7%)	13 (24.1%)	
mWRD + Smear	1 (2.4%)	0 (0.0%)	1 (1.9%)	
Culture only	1 (2.4%)	2 (16.7%)	3 (5.6%)	
mWRD only	11 (26.2%)	5 (41.7%)	16 (29.6%)	
Total number of positives test per sample				
5 tests	17 (40.5%)	3 (25.0%)	20 (37.0%)	0.138
4 tests	7 (16.7%)	0 (0.0%)	7 (13.0%)	
3 tests	6 (14.3%)	2 (16.7%)	8 (14.8%)	
2 tests	1 (2.4%)	2 (16.7%)	3 (5.6%)	
1 test	11 (26.2%)	5 (41.7%)	16 (29.6%)	

Table 2. Diagnostic test results in bacteriologically confirmed pulmonary TB (n = 54) stratified by HIV status. TB Tuberculosis; HIV Human immunodeficiency virus; Ct Cycle Threshold value; Rif Rifampicin; The p-value indicates significant differences observed for each variable between HIV negative and positive patients.

was reassessed by repeating the acquisition up to two times, and only spectra that remained suboptimal were removed. Additionally, unsupervised Principal Component Analysis (PCA) identified 5 HR-NMR and 16 bNMR spectra as statistical outliers, which were also excluded. Finally, to avoid potential bias in the stratified analysis by HIV status, samples from three participants with unknown HIV status were excluded, two analysed by HR-NMR and all three by bNMR (Fig. 1).

After these exclusions, the final dataset consisted of 133 HR-NMR spectra (86 from HIV-negative participants and 47 from participants living with HIV [PLHIV]) and 161 bNMR spectra (108 from HIV-negative participants and 53 from PLHIV) which were used for further analyses (Fig. 1). In these subsets, additional significant differences not observed in the full cohort emerged (Supplementary Table 1), including a higher frequency of symptom onset, weight loss and fever among HIV-negative TB patients in the bNMR subset, and less frequent saliva-like sputum in TB cases across both subsets. Regarding diagnostic tests (Supplementary Table 2), HIV-

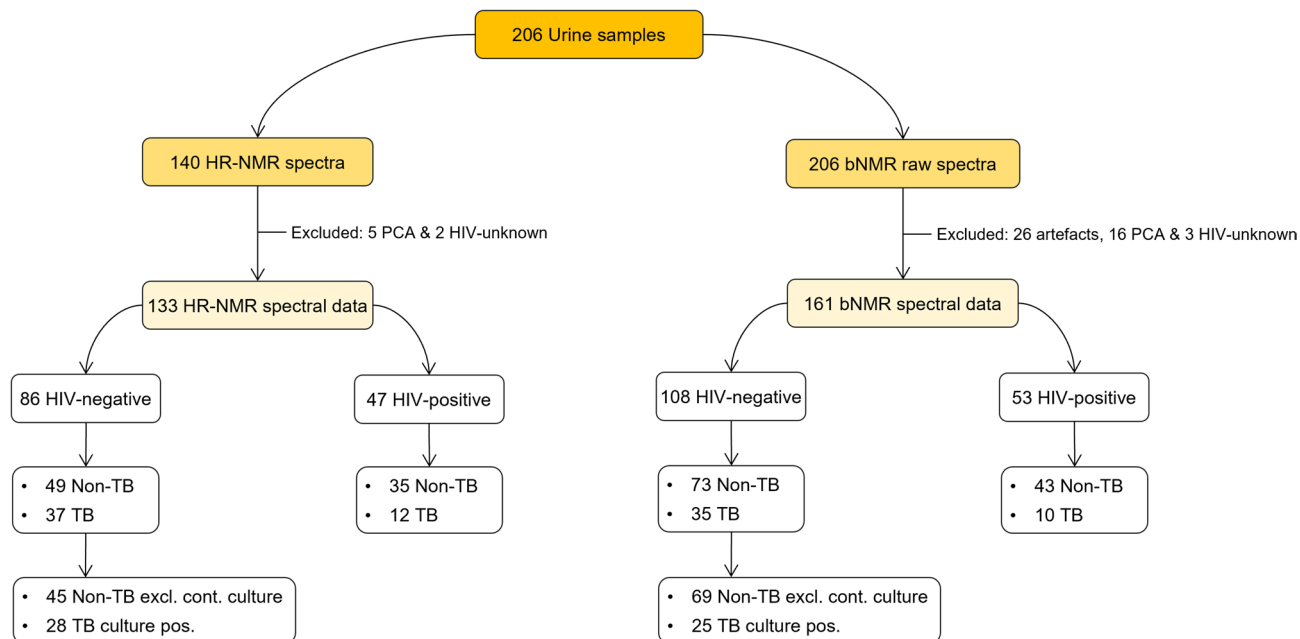


Fig. 1. Flowchart outlining the procedure followed for analysing urine samples from 206 participants using high-resolution (HR-NMR) and benchtop (bNMR) NMR spectrometers. A total of 7 HR-NMR and 45 bNMR spectra were excluded from the analyses. HR-NMR exclusions: 5 statistical outliers identified during unsupervised Principal Component Analysis (PCA) and 2 due to unknown HIV status. bNMR exclusions: 26 spectra affected by acquisition artefacts or contamination during sample processing, 16 removed as statistical outliers in PCA, and 3 due to unknown HIV status. *NMR*, nuclear magnetic resonance; *TB*, tuberculosis; *HIV*, human immunodeficiency virus; *PCA*, principal component analysis.

negative TB patients showed higher Xpert MTB/RIF positivity in both subsets, and higher Xpert Ultra positivity in the HR-NMR subset, while culture, smear and the number of positive tests per sample showed no significant differences.

To explore potential metabolic differences between the TB and non-TB groups, PCA was performed on both the ^1H HR-NMR and ^1H bNMR spectra. Although no clear group separation was observed, this analysis enabled the identification of relevant metabolic variability and contributed to quality control. To minimize the confounding effects of HIV and antiretroviral therapy (ART) on the detection of TB-specific metabolic signatures, initial analyses were conducted exclusively in the HIV-negative group. This allowed the models to focus on TB-related metabolic changes. These models were subsequently validated using samples from PLHIV to assess their broader applicability. PLS-DA was performed to identify metabolic patterns distinguishing the TB and non-TB groups within this subset. Both the HR-NMR and bNMR PLS-DA models effectively separated the two groups along the first component (Fig. 2). The robustness of these models was evaluated using fivefold cross-validation, demonstrating an accuracy of 79% for the HR-NMR spectra ($R^2=0.61$, $Q^2=0.33$) and 77% for the bNMR spectra ($R^2=0.63$, $Q^2=0.29$). VIP scores from each PLS-DA model (Supplementary Fig. 1) identified the spectral regions contributing to the observed metabolic differences. In the HR-NMR spectra, 39 spectral regions corresponding to 13 metabolites were identified, including aminoisobutyrate, fucose, glutamate, glutamine, 2-aminoadipic acid, citrate, creatine, creatinine, taurine, mannitol, hippurate, glucose, and phenylalanine (Fig. 3). In the bNMR spectra, 59 spectral regions, corresponding to 11 metabolites, were identified. However, some metabolic signals are challenging to confirm due to the limitations of the bNMR technique. Specifically, fucose cannot be reliably quantified due to its low signal intensity relative to other overlapping signals in the same spectral region. Additionally, while the spectral region corresponding to mannitol (3.62–3.66 ppm) was highlighted as significant for group discrimination, the resolution of the bNMR spectrometer was insufficient to clearly separate the mannitol signal from other more concentrated signals (e.g. glucose). Finally, the percentage changes and statistical significance of the 13 identified metabolites were calculated using HR-NMR spectroscopy (Table 3). Importantly, we also built separate models for male and female patients, which confirmed that the same set of 13 metabolites was consistently identified as discriminatory across sexes, with only minor variations in their relative VIP weights.

Classification models

Using the metabolic signals identified through VIP score analyses, we developed PLS-DA classification models to distinguish between TB and non-TB groups based on both HR-NMR and bNMR data. The HR-NMR model was constructed from the concentrations of aminoisobutyrate, fucose, glutamate, glutamine, 2-aminoadipic acid, citrate, creatine, creatinine, taurine, mannitol, hippurate, glucose, and phenylalanine. For the bNMR models, the same panel of metabolites was applied, with the exception of fucose and mannitol, which could not be reliably

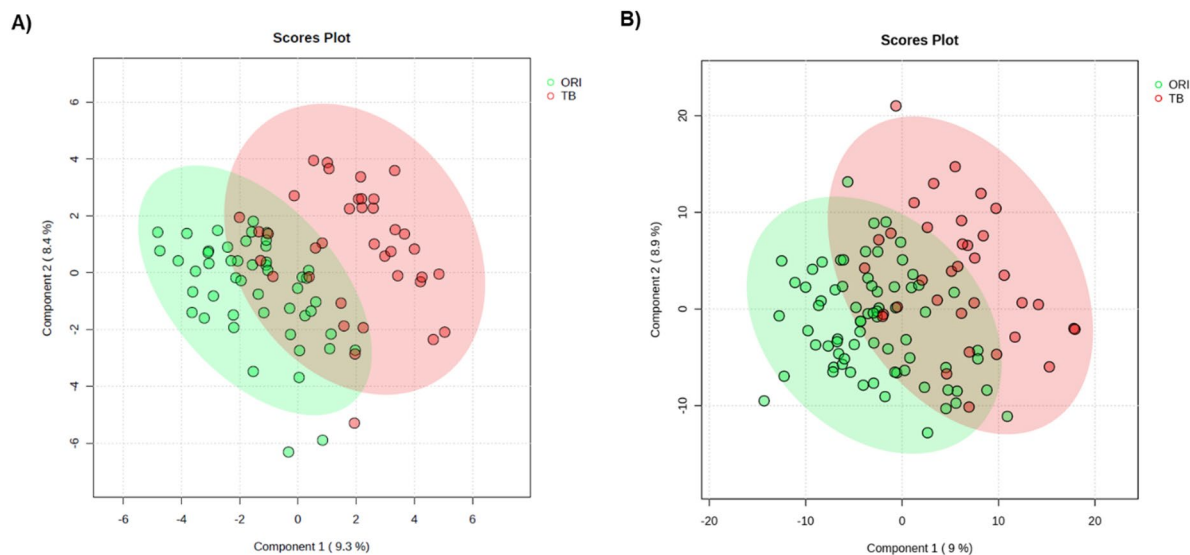


Fig. 2. Partial Least Squares Discriminant Analysis (PLS-DA) score plot of urine samples from tuberculosis (TB) and non-TB patients. **(A)** High Resonance-NMR; **(B)** benchtop NMR; Robustness parameter: **(A)** Accuracy = 0.79, $R^2 = 0.61$, $Q^2 = 0.33$; **(B)** Accuracy = 0.77, $R^2 = 0.63$, $Q^2 = 0.29$.

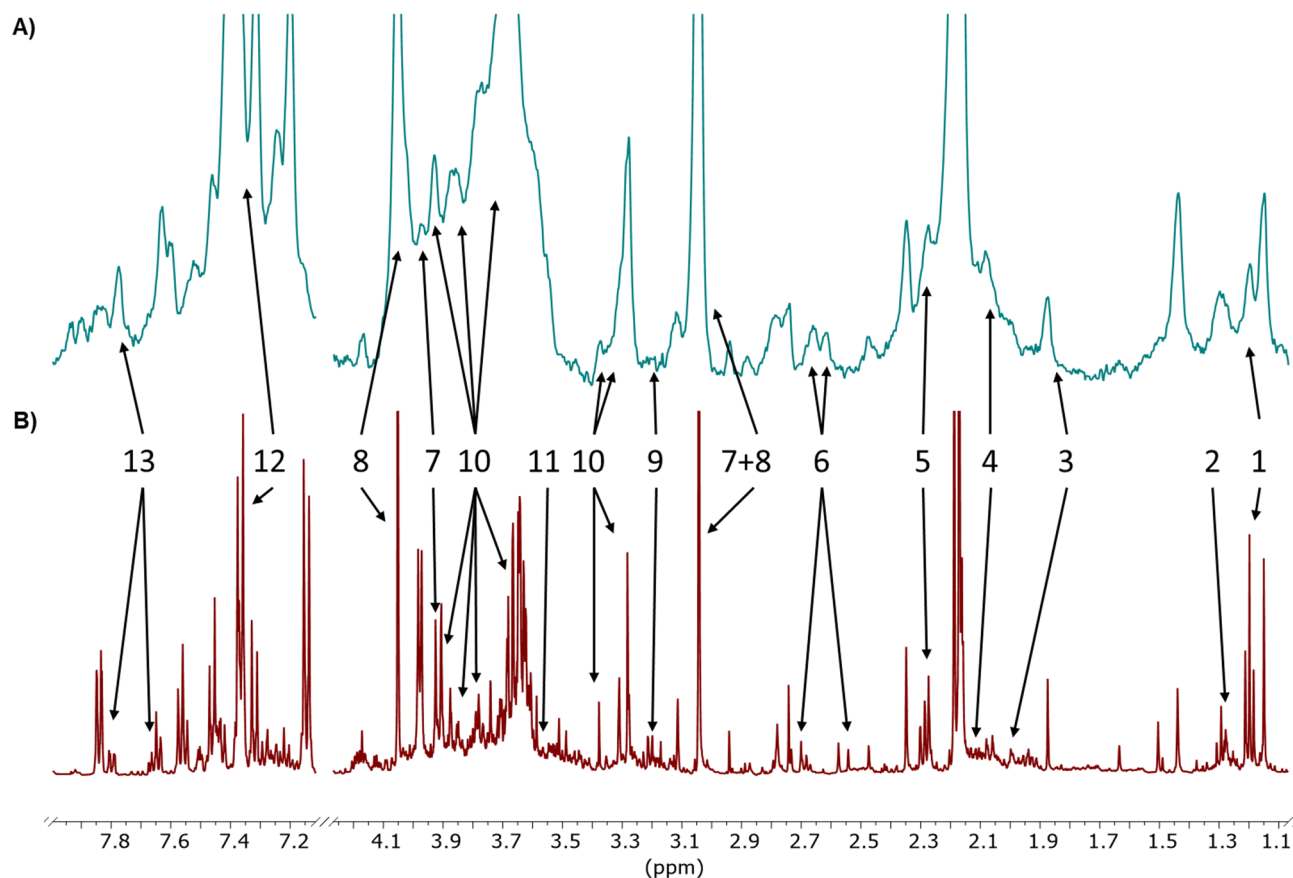


Fig. 3. Spectral regions in HR-NMR contributing to observed metabolic differences, identifying 13 metabolites across 39 regions: 1- Aminoisobutyrate, 2-fucose, 3-glutamate, 4-glutamine, 5-Aminoadipate, 6-citrate, 7-Creatine, 8-Creatinine, 9- taurine, 10-glucose, 11- mannitol, 12-phenylalanine, 13-hippurate. **(A)** High Resonance-NMR; **(B)** benchtop NMR.

Metabolite	VIP score	<i>p</i> -Value (Bonferroni-adjusted)	Change in concentration (TB vs non-TB) (%)
3-Aminoisobutyrate	3.1	<0.001	212.2
Fucose	5.6	0.023	– 59.0
Glutamate	2.3	<0.001	84.4
Glutamine	3.1	1.00	177.9
2-Aminoadipate	2.0	<0.001	91.5
Citrate	2.5	<0.001	– 62.2
Creatinine	4.6	1.00	– 7.6
Taurine	2.6	1.00	– 31.5
Mannitol	3.89	0.146	31.1
Glucose	2.7	0.113	29.0
Creatine	2.4	1.00	27.4
Phenylalanine	3.3	<0.001	156.7
Hippurate	2.7	<0.001	– 65.1

Table 3. The *p*-values after Bonferroni correction and percentage change in concentration of the of the VIP-score spectral regions corresponding to 13 metabolites that contributed to the observed metabolic differences between TB and non-TB groups in HR-NMR urine spectra. Metabolites with Variable Importance in Projection (VIP) scores > 2 were identified as potential markers contributing to group discrimination. *p*-values in bold indicate statistical significance (Two-sided *t*-tests < 0.05) before Bonferroni correction. Positive percentages correspond to higher levels in the TB group.

quantified due to the lower spectral resolution. The HR-NMR model achieved an AUC-ROC of 0.89 (95% CI: 0.75–0.98), with a sensitivity of 0.84 and specificity of 0.86. Similarly, the bNMR model yielded an AUC-ROC of 0.89 (95% CI: 0.78–0.99), with a sensitivity of 0.83 and specificity of 0.84 (Fig. 4). These models were further validated using samples from PLHIV, who were initially excluded to avoid potential confounding effects of HIV on the urinary metabolomic profile. For this validation cohort, the HR-NMR-based model achieved a sensitivity of 0.75 and a specificity of 0.70, while the bNMR-derived model demonstrated a higher sensitivity of 0.90 but a lower specificity of 0.46.

To enable a direct and fair comparison with other diagnostic techniques, classification models for HR-NMR and bNMR were built using only HIV-negative participants. Sensitivity was calculated from culture-positive TB cases, and specificity from non-TB cases with negative cultures. Samples with contaminated or missing culture results were excluded, even if they tested positive by other methods. This approach resulted in a dataset of 73 HR-NMR spectra (45 non-TB and 28 TB) and 94 bNMR spectra (69 non-TB and 25 TB) (Fig. 1). The HR-NMR-based model achieved an AUC-ROC of 0.96 (95% CI: 0.90–1), with a sensitivity of 0.88 and specificity of 0.96. In contrast, the bNMR-derived model yielded an AUC-ROC of 0.87 (95% CI: 0.75–0.98), with a sensitivity of 0.88 and specificity of 0.85 (Fig. 4). To further evaluate potential sex-related effects, we also built separate HR-NMR models for male and female patients. The predictive performance was comparable across sexes, with an AUC-ROC of 0.92 for males and 0.99 for females (Supplementary Fig. 2), compared to 0.96 for the combined analysis. These correspond to deviations of 4.16% and 3.12%, respectively. We consider these variations to be minimal, and they reinforce the interpretation that the discriminatory metabolic profile is common across sexes rather than sex-specific.

Using the same dataset, we calculated the sensitivity and specificity of other established diagnostic methods performed during recruitment, including Xpert MTB/RIF, Xpert MTB/RIF Ultra, Truenat MTB-RIF, smear microscopy, and FujiLAM (prototype). Analyses were restricted to HIV-negative patients within each NMR cohort who had results available for all diagnostic tests. This resulted in subsets of 42 non-TB and 27 TB patients for HR-NMR, and 62 non-TB and 24 TB patients for bNMR, reduced from the original totals due to missing results for smear microscopy, Xpert Ultra (*n* = 3 and *n* = 6, respectively), and Truenat MTB-RIF (*n* = 4 and *n* = 7, respectively). This ensured that all performance values in Table 4 were based on identical patient groups for consistent head-to-head comparison. In the HR-NMR cohort, Xpert MTB/RIF Ultra showed the highest sensitivity (96.3%), followed by Xpert MTB/RIF (92.6%) and the HR-NMR urine model (92.3%). In the bNMR cohort, Xpert MTB/RIF and Xpert MTB/RIF Ultra achieved identical sensitivities (95.8%), which were higher than that of the bNMR urine model (70.0%). Regarding specificity, all conventional methods reached 100% in both cohorts. The HR-NMR urine model achieved 93.0% specificity, while the bNMR model reached 94.6%. Detailed performance characteristics, including 95% confidence intervals, are provided in Table 4.

Discussion

This study evaluated the discriminatory potential of NMR-based urine metabolomics for diagnosing pulmonary TB in a cohort of Nigerian adults with suspected TB, including HIV-positive and HIV-negative individuals. In contrast to our previous research^{10,11}, which characterized a urinary metabolic fingerprint for TB in a low TB burden adult cohort, this is the first study to apply NMR-based metabolomics in Nigeria, a country with a high prevalence of both TB and HIV co-infection. The unique epidemiological context of this study, combined with the inclusion of PLHIV, adds a critical dimension to our understanding of how metabolic profiles vary across

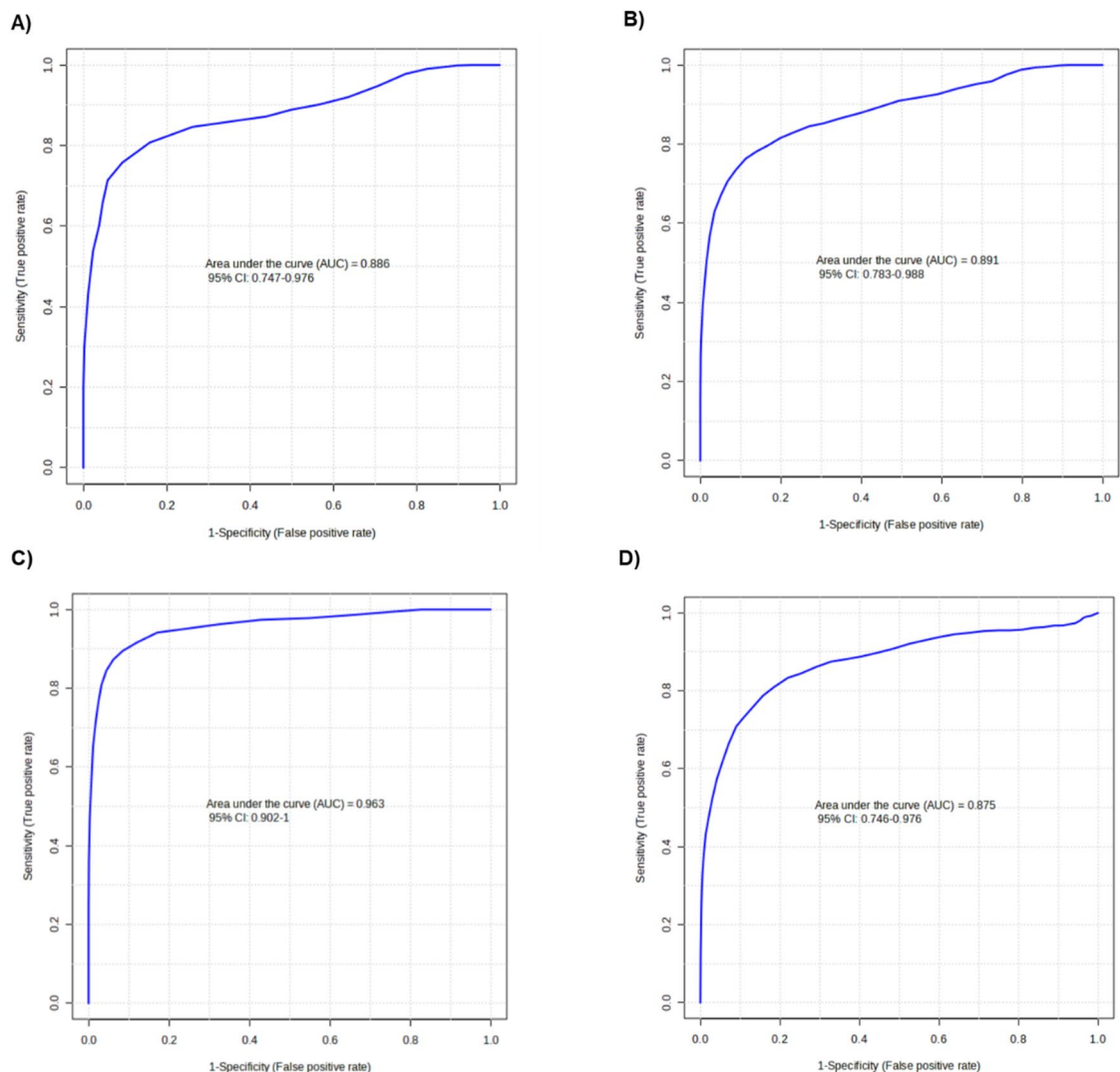


Fig. 4. Area Under the Receiver Operating Characteristic Curve (AUC-ROC) results from the Partial Least Squares Discriminant Analysis (PLS-DA) classification models developed to distinguish between the tuberculosis (TB) and non-TB groups based on (A) High Resonance-NMR; (B) benchtop NMR data; and between the subset of the tuberculosis (TB) group with confirmed positive *M. tuberculosis* cultures and non-TB groups based on (C) High Resonance-NMR; (D) benchtop NMR data.

populations with different TB and HIV burdens. This broader application underscores the potential utility of this approach in resource-limited and high-burden settings.

To reflect the clinical context and participant characteristics, unlike earlier studies with highly controlled cohorts, this investigation included all individuals presenting with TB-like symptoms and able to produce sputum. This design more accurately reflects real-world diagnostic scenarios in low- and middle-income countries (LMICs), where limited infrastructure and resources pose significant challenges for accurate and timely diagnoses. The inclusion of such a diverse cohort enhances the external validity of our findings, as it mirrors the complex real-world scenarios in district hospitals.

One notable difference in this study was the higher prevalence of weight loss among bacteriologically confirmed TB patients compared to the non-TB group. While weight loss has been previously associated with the catabolic state induced by TB^{12,13}, our findings suggest that it may also serve as an indicator of severity^{14,15}, especially in regions with high TB prevalence. In this cohort, TB patients more often reported weight loss, presented with a greater number of symptoms (≥ 5), had symptoms for longer (> 2 weeks), and experienced a later onset of the first symptom compared with non-TB participants. These differences were statistically significant in the overall study population and were supported by stratified analyses. In the subset of HIV-negative patients from the bNMR cohort, TB cases more frequently showed fever, later symptom onset, and less frequent saliva-like sputum. They also had higher Xpert MTB/RIF positivity in both HR-NMR and bNMR subsets, and higher Xpert Ultra positivity in the HR-NMR subset. However, the larger sample size in the bNMR HIV-negative group may have increased the statistical power to detect certain associations not observed in the

Diagnostic test	TP n	FP n	FN n	TN n	SE [95% CI]	SP [95% CI]
HR-NMR cohort						
Urine HR-NMR	24	3	2	40	92,3% (73.4–98.7)	93,0% (79.9–98.2)
Xpert MTB/RIF	25	0	2	42	92,6% (74.2–98.7)	100% (89.6–100)
Xpert MTB/RIF Ultra	26	0	1	42	96,3% (79.1–99.8)	100% (89.6–100)
Truenat-TB	21	0	6	42	77,8% (57.3–90.6)	100% (89.6–100)
Smear-microscopy	16	0	11	42	59,3% (39.0–77.0)	100% (89.6–100)
FujiLAM	19	1	8	41	70,4% (49.7–85.5)	97,6% (85.9–99.9)
bNMR cohort						
Urine bNMR	21	3	9	53	70,0% (50.4–84.6)	94,6% (84.2–98.6)
Xpert MTB/RIF	23	0	1	62	95,8% (76.9–99.8)	100% (92.7–100)
Xpert MTB/RIF Ultra	23	0	1	62	95,8% (76.9–99.8)	100% (92.7–100)
Truenat-TB	19	0	5	62	79,2% (57.3–92.1)	100% (92.7–100)
Smear-microscopy	15	0	9	62	62,5% (40.8–80.4)	100% (92.7–100)
FujiLAM	18	0	6	62	75,0% (52.9–75.0)	100% (92.7–100)

Table 4. Diagnostic performance of HR-NMR and bNMR urine metabolomics models compared with other TB diagnostic methods. *TP*, true positive; *FP*, false positive; *FN*, false negative; *TN*, true negative; *SE*, sensitivity; *SP*, specificity; *CI*, confidence interval. Analyses were restricted to the same subset of HIV-negative patients within each NMR cohort who had results available for all diagnostic tests shown. In the HR-NMR cohort, this subset comprised 42 non-TB and 27 culture-positive TB patients (reduced from the original 45 non-TB and 28 TB) due to missing results for smear microscopy and Xpert Ultra ($n=3$) and Truenat MTB-RIF ($n=4$). In the bNMR cohort, the subset comprised 62 non-TB and 24 culture-positive TB patients (reduced from the original 69 non-TB and 25 TB) due to missing results for smear microscopy and Xpert Ultra ($n=6$) and Truenat MTB-RIF ($n=7$). FujiLAM was included as a prototype test; all others are established diagnostic methods.

smaller HR-NMR and HIV-positive subsets. Together, these findings highlight the potential value of integrating symptom clustering, temporal patterns, sputum characteristics, and emerging diagnostic tools to improve TB diagnosis in high-prevalence context.

Regarding microbiological diagnosis, the classification of participants into the TB and non-TB groups was based on the positivity of cultures and/or mWRD. In this study, we observed notable variability in the performance of diagnostic tests for TB, especially when different methods were combined. The fact that only 37% of TB patients tested positive in all tests highlights the need to utilise multiple diagnostic methods to improve detection. Furthermore, the fact that 27.8% of patients tested positive in only one test underscores the limitations of individual methods and suggests that relying on a single test could lead to misdiagnosis or missed TB cases. The 17 patients who tested positive on mWRD but had negative or contaminated results in cultures and smear microscopy, provided evidence of potential limitations in the ability of these traditional methods (cultures and smear microscopy) to detect the disease in certain contexts. The lack of a statistically significant association between HIV status and diagnostic test results suggests that HIV-TB co-infection did not decisively impact test performance in this cohort. However, it is worth noting that, although not significant, the likelihood of positive results for culture, Xpert, and smear microscopy was approximately 20% higher in HIV-negative participants compared to PLHIV. Additionally, limitations in sample size and particularities of the study environment need to be considered. Current reference tests are imperfect and perform less well in patients with HIV, extrapulmonary TB, and disseminated TB, depending on the sample quality and whether the patient is excreting bacilli on the day of sampling.

As for the performance of the metabolomic models, the results were promising. The classification models for HIV-negative individuals showed a remarkable ability to distinguish between TB and non-TB groups, achieving sensitivities and specificities of 84.0% and 86.0% in HR-NMR and 83.0% and 84.0% in bNMR. These results underscore the effectiveness of the model in identifying TB cases while minimising false positives, which is an essential factor in resource-limited settings, where misdiagnoses can strain already limited healthcare resources. Both models produced an AUC-ROC value of 0.89, highlighting their consistency and reliability. The comparable performance of HR-NMR and bNMR suggests that bNMR, with its lower cost and operational simplicity, could serve as a practical alternative in LMICs, where access to advanced technologies such as HR-NMR is often inaccessible. Although the diagnostic accuracy in this Nigerian cohort was slightly lower than that in the cohort of our previous study, where sensitivities and specificities of 100% were reported, this discrepancy is likely due to the greater population heterogeneity and larger sample size in Nigeria, factors that can complicate model performance in diverse and resource-constrained environments.

Thirteen metabolites were identified in the HR-NMR spectra, eleven of which were also detected in the bNMR spectra. The absence of fucose and mannitol in the bNMR data is likely due to their low concentrations and lower spectral resolution of the benchtop instrument. Future advancements in benchtop NMR technology, particularly spectrometers operating at 90 MHz, may overcome these limitations¹⁶.

Eight metabolites matched our previous findings (2-aminoadipic acid, citrate, creatine, creatinine, mannitol, hippurate, glucose, and phenylalanine). In both cohorts, 2-aminoadipic acid, creatine, phenylalanine, and mannitol showed increased concentrations in patients with TB compared to controls, suggesting TB-induced changes in amino acid and carbohydrate metabolism^{17,18}. Likewise, decreased levels of citrate, creatinine, and hippurate were observed in patients with TB, indicating alterations in energy metabolism and muscle mass due to the disease^{19,20}. These consistent patterns suggest that certain metabolites may serve as TB markers across different populations.

Glucose levels exhibited opposite trends between the cohorts. In the Nigerian cohort, glucose levels were significantly increased in patients with TB, possibly because of inflammatory responses causing glucose intolerance and insulin resistance^{21,22}. In contrast, the previous study cohort showed decreased glucose levels in TB patients compared to uninfected controls, but increased levels compared to those with pneumococcal pneumonia. This discrepancy may stem from differences in the control group composition and underlying conditions, making direct comparisons challenging.

Additionally, this study found elevated levels of aminoisobutyrate, glutamate, and glutamine and decreased levels of fucose and taurine in TB patients. The increased amino acid levels may result from chronic inflammation and metabolic stress²³. The decrease in fucose could be linked to reduced complement activation during infection²⁴, although this contrasts with the findings of Conde et al. (2021), possibly due to differences in sample types or disease stages²⁵. Reduced taurine levels align with previous observations and may reflect limited availability due to metabolic stress²⁶.

To assess the impact of HIV co-infection, we analyzed the models in PLHIV. The HR-NMR model achieved sensitivity and specificity of 75.0% and 70.0%, respectively, effectively capturing subtle metabolic changes that may help differentiate TB in the context of HIV-TB co-infection. In contrast, the bNMR-based model demonstrated a higher sensitivity (91.0%), but with a significantly lower specificity (48.0%). This discrepancy is likely due to the lower spectral resolution of bNMR, which may limit its ability to detect complex metabolic interactions that arise from HIV-TB co-infection and ART^{8,27}. HIV and ART are known to induce significant changes in energy metabolism, inflammation, and immune response^{28,29}, which can complicate the detection of TB-specific metabolic signatures³⁰. These overlapping metabolic pathways may increase variability and reduce the accuracy of the model in distinguishing TB cases from non-TB cases in PLHIV. Future advancements in bNMR technology, such as instruments operating at higher frequencies (80–90 MHz), could improve resolution and enhance the detection of these metabolic interactions, potentially increasing the model's accuracy in this context⁸.

The remarkable sensitivity achieved in the bNMR model enables the identification of a greater number of potential TB cases in PLHIV, especially in those with difficulties in producing sputum, lower bacterial loads, or extrapulmonary forms of the disease. This is crucial to avoid missing cases that may be overlooked by less sensitive tests. However, the low specificity observed could result in a greater number of false positives, indicating the need for additional strategies to confirm diagnosis before initiating TB treatment.

In high-TB prevalence settings, where every potential case in a vulnerable population should be considered, this low specificity might be manageable if combined with additional tests of higher specificity. Integrating our high-sensitivity bNMR metabolomic approach with higher-specificity urine-based LAM assays, such as the Alere Determine TB LAM Ag assay, which has a sensitivity of 42% and specificity of 91% in PLHIV, or future generations, such as FujiLAM, could provide a rapid, non-invasive, and low-complexity solution to reduce false positives and ensure accurate treatment^{4,6}.

Additionally, C-reactive protein (CRP) has been proposed as a point-of-care biomarker for TB screening in people living with HIV, offering high sensitivity but moderate specificity (90% and 50%, respectively)⁶. Although its moderate specificity leads to a considerable rate of false positives, it is an example of a diagnostic tool that, despite these limitations, is still considered valuable in certain contexts due to its high sensitivity.

When benchmarked against existing diagnostic methods, both NMR models showed excellent performance (AUC-ROC of 0.96 and 0.87, respectively) compared to culture, the microbiological reference method for TB diagnosis. Both models achieved sensitivities of 88.0%, exceeding the minimum sensitivity requirements (65%) established by the WHO in 2014 for sputum-alternative TB diagnostic tests as well as the more stringent standards defined in the updated 2024 guidelines for near-POC (75% sensitivity) and low-complexity (80% sensitivity) tests³¹.

Compared with other available diagnostic tests, the HR-NMR model showed higher sensitivity than Truenat (77.8%), smear microscopy (59.3%), and FujiLAM (70.4%), while the bNMR model outperformed smear microscopy (62.5%) and showed similar sensitivity to Truenat (79.2%) and FujiLAM (75.0%). The specificity achieved in both models (93.0% in HR-NMR and 94.6% in bNMR) was slightly below the most stringent standards defined for the target product profiles (specificity of 98%), but comparable to or higher than some conventional methods. While Xpert Ultra demonstrated slightly higher sensitivity (96.3% in HR-NMR and 95.8% in bNMR), the advantage of our metabolomic approach lies in its applicability to urine samples, offering a non-invasive alternative. Importantly, the NMR-based metabolomic approach is complementary to Xpert Ultra, as NMR reflects host metabolic responses rather than detecting bacterial DNA directly. This difference could enhance diagnostic accuracy if combined. Despite its high sensitivity, Xpert Ultra relies on sputum samples, which may be challenging to obtain from children, people living with HIV, or patients with low bacillary load. In contrast, urine is easy to collect and may facilitate diagnosis in such populations, particularly in decentralised or resource-limited settings.

When comparing our model to the FujiLAM assay in HIV-negative participants, FujiLAM demonstrated a sensitivity of 70.4% and a specificity of 97.6% in the HR-NMR cohort, and 75.0% sensitivity and 100% specificity in the bNMR cohort. The HR-NMR model achieved higher sensitivity and similar specificity, while the bNMR model showed slightly lower sensitivity but still high specificity. This suggests that our approach may complement

next-generation urine LAM tests, offering potentially improved diagnostic capabilities in certain contexts, particularly for enhancing TB detection rates in high-burden settings⁶. A higher sensitivity can be particularly beneficial for early diagnosis and treatment initiation, which are critical for controlling TB transmission and improving patient outcomes.

Despite these promising results, it is important to acknowledge the limitations of the present study. First, no longitudinal follow-up of participants was conducted, which could have led to the reclassification of some individuals initially classified as non-TB to TB, potentially affecting the specificity of the models. Second, the study focused on patients who were able to provide sputum samples, which could underestimate the potential of the urine metabolomic approach to identify patients with difficult-to-diagnose pulmonary TB, such as those with difficulties in producing sputum. Similarly, the limited number of bacteriologically confirmed TB cases in patients with HIV could reflect the diagnostic challenges in this population, where current methods may fail to provide accurate diagnoses. This limitation could have affected the evaluation of the urine metabolomic profile, potentially underestimating its true potential identifying TB in PLHIV.

In addition, a significant number of spectra had to be excluded from the bNMR dataset due to acquisition problems related to the limited shimming adjustment capabilities of the early benchtop NMR technology used (Magritek Spinsolve 60 MHz Ultra). Shimming difficulties, especially relevant in biological fluids with high salt content such as urine, resulted in poor spectral resolution that could bias the analysis if retained. Although the affected samples were reanalysed under different shimming modes, the problem could not be resolved, and their exclusion was necessary to maintain the robustness of the dataset.

Future studies should focus on validating these findings in larger and more diverse populations as well as exploring the interaction between TB and HIV in the context of metabolomics. The identification of urinary metabolites, which is consistent with our previous studies, reinforces the robustness of the TB metabolic fingerprint, suggesting its potential applicability across different regions. Consequently, assessing the transferability of these metabolic signatures to other geographical areas should be pursued in future research.

In parallel, efforts are underway to explore whether a reduced panel of key metabolites, identified through VIP-based selection, could sustain adequate diagnostic performance. This line of work could pave the way for simpler, faster assays, such as point-of-care formats, and enhance the scalability of metabolomics-based diagnostics in resource-limited settings.

Additionally, it is crucial to further investigate the underlying biological mechanisms that could explain the discrepancies observed in the levels of certain metabolites, such as glucose or fucose, between different studies and clinical contexts. The portable nature of bNMR, with the possibility of incorporating batteries in the future to eliminate dependence on the power grid, makes it a viable and more accessible alternative for TB diagnosis in clinical settings, where access to advanced technology such as HR-NMR is limited, thus promoting more equitable access to effective diagnostic tools⁸. Although the large-scale implementation of urine NMR-based metabolomics could contribute to earlier and more accurate detection, including the identification of subclinical TB cases as highlighted in the recent WHO report¹, further studies are needed to assess its cost-effectiveness, particularly in low-resource settings. Tiered pricing mechanisms could help address this challenge, ensuring broader accessibility to this technology.

In conclusion, this study reinforces the potential of NMR-based urine metabolomics as a non-invasive diagnostic tool for TB in settings with a high prevalence of the disease, demonstrating a significant capacity to differentiate between TB and non-TB groups, even in the presence of HIV-TB co-infection. While challenges exist, such as variability in metabolomic profiles and the need to improve specificity in certain contexts, the results suggest that this approach could complement current diagnostic tools and be used in countries with a high burden of TB and HIV. Integration of technologies such as bNMR in these settings could offer a more accessible and effective alternative, contributing to improved TB diagnosis and management on a global scale.

Methods

This cross-sectional retrospective study analysed urine samples from a Nigerian cohort of adults with compatible TB capable of producing sputum. Participants attended TB diagnosis and treatment clinics for disease evaluation or were referred from general outpatient departments and HIV clinics for pulmonary TB screening at one of the following three district hospitals in Abuja, Nigeria: the Keffi Federal Hospital, Nyanya General Hospital, and Asokoro District Hospital. Patients were consecutively included, regardless of their HIV status. Patients who had been previously diagnosed with TB and those who had received anti-TB treatment in the previous year were excluded. Written informed consent was obtained from all the participants. The study was approved by the research ethics committees of the Liverpool School of Tropical Medicine (protocol 17-014; 26 April 2017), the Nigerian National Ethics Committee (protocol NHREC/21/95/2005/00,463; 30 March 2017), and the Ethics Committee of Hospital Universitari Germans Trias i Pujol (Protocol PI-13-1546; 9 June 2015). All methods were carried out in accordance with relevant guidelines and regulations, including those set out in the Declaration of Helsinki.

Data and sample collection

After obtaining written informed consent, patients were interviewed to obtain demographic information (age, sex, and country of birth) and medical history (onset of first symptoms and comorbidities). The variables were collected at the first visit (baseline). Additionally, they were asked to provide blood samples, two sputum samples, and a midstream urine sample at the site for routine and study-specific analyses. The date and time of collection and delivery of the samples sent to the laboratory, as well as the volume and quality of the samples, were recorded. All samples, except the urine samples, were processed locally and used for patient management.

HIV status was routinely assessed using rapid diagnostic tests; for those testing positive, plasma viral loads were measured using the Xpert HIV-1 viral load (VL) assay (Cepheid, Sunnyvale, CA) according to the

manufacturer's instructions³². ART was administered to participants living with HIV (PLHIV), according to the 2016 national guidelines, which recommend initiating ART in all HIV-positive individuals regardless of age, clinical status, or disease stage.

Pulmonary TB was assessed using microscopy, culture (reference test) and mWRD. The staff evaluated the volume (a minimum of 2 mL each) and appearance of sputum specimens (saliva, mucoid, mucopurulent, purulent, or blood-stained); thus, they were blinded to the results. Sputum samples were cultured on duplicate solid media using Löwenstein-Jensen medium, while mWRD included Xpert MTB/RIF and Xpert MTB/RIF Ultra (Cepheid, Sunnyvale, United States of America) and the Truenat MTB-RIF Dx test (Molbio Diagnostics, Goa, India). The patients were divided into two groups based on the test results. Patients with any positive test were classified as bacteriologically confirmed TB cases according to the standardised definitions described by the WHO and were grouped into the TB group. Those with negative tests were classified as individuals with other respiratory infections (non-TB group).

Urine samples were collected in sterile plastic containers and stored in cold boxes until processing and storage on the same day. Each sample was aliquoted into two 2 mL cryovials and transported frozen (-20 °C) to the laboratory at the Institut d'Investigació Germans Trias i Pujol (IGTP) in Badalona, Spain.

A post hoc analysis using MetSizeR (FDR = 0.05, > 5% expected significant bins) estimated a required sample size of 32, well below our actual cohort (n = 206), confirming that the study is adequately powered³³.

Sample preparation for NMR analysis

Upon arrival at the IGTP, a 2 mL aliquot from each urine sample was thawed at room temperature. For both HR-NMR and bNMR measurements, 400 µL of urine was mixed with either 200 µL (for HR-NMR) or 250 µL (for bNMR) of a standard phosphate buffer solution (0.2 M) dissolved in 99.9% deuterium oxide (D₂O) to adjust the pH-7.4. This buffer contained 0.09% sodium azide and 0.3 mM trimethylsilyl propanoic acid (TMSp), following established protocols from previous studies^{10,11}.

NMR spectroscopy

The buffered urine samples were first analysed using a Magritek Spinsolve 60 MHz Ultra spectrometer at the IGTP. One-dimensional water suppression by presaturation pulse sequence (PRESAT) was employed, and the spectra were averaged over 64 acquisitions, as previously described. Data acquisition was performed at 25 °C and lasted a total of 11 min.

After bNMR analysis, the buffered urine samples were frozen (-20 °C) and transported to the Centre for Cooperative Research in Biomaterials (CIC biomaGUNE) in Donostia, Spain, for further HR-NMR analysis. At CIC BiomaGUNE, frozen samples were thawed and analysed using a Bruker Avance III 500 MHz spectrometer. One-dimensional ¹H-NMR spectra were collected at 25 °C using the first increment of a standard two-dimensional pre-saturation NOESY experiment. The acquisition parameters used included a 12-ppm sweep width, a four-second acquisition time, a 100-ms mixing time, a 10-ms recycle delay, and 990-ms saturation delay. The total data acquisition time was 13 min. Standard solvent-suppressed spectra comprised 32,000 data points averaged over 256 acquisitions. The free-induction decay (FID) signals were multiplied by an exponential window function corresponding to a line broadening of 0.3 Hz.

NMR data processing

NMR data were processed and edited using the MestReNova software (v.15; Mestrelab Research, Santiago de Compostela, Spain) according to previously described protocols^{10,11}. From the raw NMR spectra, the chemical shift regions from 5.65 to 6.20 parts per million (ppm) and from 4.85 to 5.15 ppm were excluded to remove random variations due to urea and water resonance suppression. Metabolite signals were shift-aligned using the regional spectral alignment tool in MestReNova, which adjusts for pH variations between the samples. Baseline correction was automatically performed using the 'Whittaker Smoother' algorithm. Binning (also known as bucketing) was applied to the NMR spectra, reducing the data to equal-length integral segments (bins) of $\delta = 0.01$ ppm to account for variations in resonance positions. All bins were normalised to the total spectral regions, meaning that each bin's integral was divided by the sum of all NMR integrals. This normalisation ensured that the relative concentration of each metabolite, expressed in arbitrary units, was adjusted for variations in urine concentrations, allowing for direct comparison between samples. This processing protocol was consistently applied to both HR-NMR and bNMR spectra to maintain the uniformity and accuracy of data analysis.

Data analysis

The study population was analysed and stratified according to the study group (TB vs. non-TB) and HIV status. Given the known metabolic alterations caused by both HIV infection and ART, which can confound the analysis of TB-related metabolic profiles, we opted to perform separate analyses for PLHIV and without HIV. By stratifying the dataset based on HIV status and accounting for ART, we aimed to minimize the potential confounding effects of these factors that are not directly related to TB. In this context, participants with inconclusive HIV status were excluded from the stratified metabolomic analyses to avoid potential misclassification and bias.

Demographic and clinical characteristics were summarised using descriptive statistics, consistent with established methodologies. Categorical variables are presented as frequencies and percentages, while continuous variables are presented as means and standard deviations. Group comparisons were performed using the appropriate statistical tests. For categorical variables, the chi-squared test was applied when expected frequencies were adequate; otherwise, Fisher's exact test was used. For larger contingency tables where, direct exact computation was not feasible, the Fisher-Freeman-Halton test with Monte Carlo simulation (10,000 samples, 99% confidence interval) was applied. For continuous variables, Student's t-test was used when data were normally distributed; and the Mann-Whitney U test was applied otherwise. Statistical significance was defined

as a p -value < 0.05 . All analyses were conducted using the SPSS software (version 29.0.2.0; SPSS, Inc., Chicago, IL, USA)³⁴. For diagnostic test accuracy, 95% binomial confidence intervals (CI) were computed using the VassarStats website for statistical computation (<http://vassarstats.net/>)³⁵. For multivariate model performance metrics, CIs were estimated by bootstrap resampling during cross-validation.

Metabolomic analysis

Data from the ^1H NMR spectra were analysed using MetaboAnalyst 6.0^{36,37}. Prior to the multivariate statistical analysis, the spectral data were scaled appropriately based on the NMR instrument used. HR-NMR spectra were scaled using Pareto scaling to reduce the impact of large intensity values, while preserving the data structure. This method ensures that moderate metabolite signals are comparable, which is crucial for high-sensitivity instruments where dominant peaks could otherwise overshadow smaller yet significant signals. In contrast, the bNMR spectra were autoscaled (standardised) to give equal weights to all signals. Autoscaling adjusts each variable to have a mean of zero and standard deviation of one, enhancing the visibility of lower-intensity metabolites that are closer to the noise level³⁸.

Unsupervised Principal Component Analysis (PCA) was applied to the data to identify patterns and differences between samples without prior knowledge of the group labels³⁹. PCA score plots were used to highlight statistical outliers by calculating the Mahalanobis distance for each sample, which measures the distance from the centre of the PCA space⁴⁰.

Supervised Partial Least Squares Discriminant Analysis (PLS-DA) was employed to identify metabolic patterns that could discriminate between predefined groups³⁹. The robustness of the PLS-DA models was validated using fivefold cross-validation, assessing the performance accuracy, R^2 , and Q^2 metrics. Confidence intervals for cross-validated performance metrics were estimated using bootstrap resampling with replacement during the cross-validation procedure. In addition, permutation testing was performed in MetaboAnalyst (Prediction accuracy during training: 100 permutations), which confirmed that the original models achieved significantly higher R^2 and Q^2 values than the permuted models (empirical $p < 0.01$). Variable Importance in Projection (VIP) scores were calculated to identify the most significant spectral regions for group differentiation. Spectral regions with VIP scores greater than two were selected for developing the classificatory models. The accuracy, sensitivity, specificity, and Area Under the Receiver Operating Characteristic Curve (AUC-ROC) were calculated to evaluate the performance of these models. ROC curves were constructed using the full set of discriminatory metabolites identified by VIP score analysis ($\text{VIP} > 2$). The models were therefore based on the combined concentrations of these metabolites rather than on individual metabolites considered separately. Confidence intervals were estimated using bootstrap resampling methods during cross-validation.

Metabolites corresponding to the significant spectral signals used in the classification models were identified using Chenomx software, the Human Metabolome Database, and the DrugBank database⁴¹. The statistical significance of these metabolites between the groups was determined using the Bonferroni-corrected Student's t -test for multiple comparisons, thereby reducing the likelihood of false positives. This conservative approach ensured that only the most robust and reliable differences were highlighted.

Comparison with FujiLAM assay

To contextualise the diagnostic performance of our NMR-based metabolomic models, we compared them with the FujiLAM assay, using data from the same subset of HIV-negative participants included in the comparative analysis of other established diagnostic tests (culture-confirmed TB as reference standard). The FujiLAM assay was performed as described in our previous study⁴², following the manufacturer's instructions. Sensitivity and specificity were calculated against culture results.

Data availability

The data presented in this study are available on request from the corresponding authors. The data are not publicly available due to privacy reasons.

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Author contributions

Study design and supervision: P.C.D.B., I.R., J.D., L.E.C., J.R.C., and J.L.I.G.; Recruitment of study subjects: R.A., J.S.B., O.O., and L.E.C.; Acquisition of data: P.C.D.B., R.A., P.A.M., Z.B.I., A.B.M.C., J.R.C., and J.L.I.G.; Analysis and interpretation of data: P.C.D.B., I.R., J.L.I.G.; Revising the manuscript critically for important intellectual content and final approval of the version to be published: all authors.

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Declarations

Competing interests

José Luis Izquierdo-García, Patricia Comella-del-Barrio, and José Domínguez are registered as inventors on a patent filed by the Institut d'Investigació Germans Trias i Pujol and CIBERES, which discloses the use of an NMR-based urine metabolomic profile for TB diagnosis. Ignacio Rodríguez, Ramota Alaran, John S Bimba, Okoedoh Osazuwa, Pilar Alonso-Moreno, Luis E Cuevas, Jesús Ruiz-Cabello, Zuriñe Blasco-Iturri, and Ana B Miguel-Coello declared no competing interests.

Additional information

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