



# Proceeding

THE 3<sup>rd</sup> INTERNATIONAL  
SYMPOSIUM ON  
TUBERCULOSIS AND  
MYCOBACTERIA:  
A MULTIDISCIPLINARY  
APPROACH



# ISTM 2026

2 - 4 FEBRUARY 2026  
Arnoma Grand Hotel Bangkok, Thailand

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## Proportion of pathogenic nontuberculous mycobacteria identified by partial-gene sequencing among presumptive tuberculosis patients in Indonesia

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The burden of Non-Tuberculous Mycobacteria (NTM) cases in Indonesia remains unknown due to diagnostic challenges that lead to under- and misdiagnosis. The paucity of data concerning this disease contributes to the neglect of NTM in the country. This study aimed to identify NTM species using partial superoxide dismutase A (*sodA*) gene sequencing among presumptive tuberculosis (TB) patients. Retrospective samples from two distinct cohorts, a community-based and a hospital-based cohort, were included. The stored isolates from individuals suspected of pulmonary NTM infection were re-cultured, and extracted genomic DNA was subjected to polymerase chain reaction (PCR) and sequencing. Sequences were processed using DNA Baser and compared with NCBI GenBank reference sequences for species identification. Among 3,426 enrolled patients presenting for pulmonary TB, 1,713 (50.0%) were positive on either solid or liquid culture for Mycobacteria. Of these, 55 (3.2%) were classified as NTM suspects based on a negative MPT64 antigen test. The proportion of NTM among culture-positive cases was higher in the hospital-based cohort (7.9%) than in the community-based cohort (2.2%). Sequencing results of *sodA* in 44 samples showed that *M. fortuitum* was predominant (25.0%), followed by *M. abscessus* (13.6%), and *M. chelonae* (11.4%). Several slow-growing NTM species were also identified among samples. Interestingly, 10 samples (22.7%) initially suspected as NTM were identified as *M. tuberculosis* via *sodA* sequencing. This discrepancy suggests potential mixed infection or the limited sensitivity of the MPT64 antigen test, particularly against *M. tuberculosis* mutants harboring *mpt64* mutations. Species identification was not possible for 11 samples due to failed re-culture or sequencing. The proportion of predominant species was not different between settings. These findings highlight the ongoing challenges in NTM screening within Indonesia's TB-endemic environment. Incorporating molecular identification methods offers a promising direction for enhancing NTM research and surveillance in Indonesia.

**Keywords:** nontuberculous mycobacteria, species identification, *sodA* gene, sequence-based analysis

### Introduction

Mycobacterial infections encompass a broad clinical spectrum, ranging from tuberculosis (TB), caused by the *M. tuberculosis* complex, and leprosy (*M. leprae*) to opportunistic infections caused by non-tuberculous mycobacteria (NTM) [1]. To date, the NTM groups, which are widely present in the environment, such as soil and water, consist of more than 270 species, with more than 60 species known to be opportunistic pathogens that

can cause pulmonary and skin diseases, lymphadenitis, and disseminated diseases [2]. The species are also divided into two categories, the rapid-growing NTM (< 7 days), and slow-growing NTM (> 7 days) [1].

In Indonesia, which currently ranks second in the world for TB burden, clinical vigilance has historically focused almost exclusively on TB. This focus is supported by a robust national TB program monitoring case notifications and treatment outcomes

[3,4] Conversely, NTM-associated diseases remain outside the primary focus of public health authorities. Despite their emergence as significant opportunistic human pathogens, limited data make the burden unclear [5,6] Although NTM cases often arise following unsuccessful TB treatment or alongside multidrug-resistant TB (MDR-TB), the case report was largely undocumented and likely underestimated [7]. In Indonesia, a study conducted in Surakarta reported NTM prevalence of 15% among positive mycobacterial cultures obtained from patients in the lung hospital [8]. Another study in Surabaya reported 5.78% NTM positivity rate among TB suspect patients in a general hospital [9]. Such variability highlights the diverse distribution of NTM species across distinct cohorts, which has hitherto been poorly characterized in broader epidemiological frameworks.

Consequently, NTM infections, particularly pulmonary forms that share similar symptoms with TB, remain epidemiologically neglected. Due to significant clinical and radiological overlap with bronchiectasis, chronic obstructive pulmonary disease (COPD), and pulmonary TB, NTM infections, especially NTM pulmonary diseases, are frequently misdiagnosed as TB or MDR-TB, resulting in treatment failures [10,11]. Furthermore, antimicrobial regimens for NTM are highly species-specific, necessitating precise identification to ensure treatment efficacy [12]. Despite this clinical requirement, routine diagnostic and speciation services for NTM are currently unavailable in Indonesia. This lack of

specialized laboratory infrastructure represents a significant limitation in the clinical management of NTM diseases. Diagnostic accuracy remains a critical challenge, as the therapeutic management of NTM infections differs fundamentally from that of TB.

Molecular identification methods have emerged as a promising approach for the precise characterization of NTM species. Among these, the *rpoB* and *hsp65* genes are widely utilized due to their recognized discriminatory power. However, several studies have identified limitations in these markers, particularly regarding their ability to differentiate closely related species within the *M. fortuitum* group and the *M. abscessus-chelonae* complex. The *sodA* gene, which encodes metalloenzymes essential for defending against oxidative stress, offers a robust alternative [13]. Previous research has shown that *sodA* sequence analysis successfully resolved discordant results in strains where *rpoB* and *hsp65* failed to provide definitive speciation [14]. Notably, partial *sodA* sequencing has been instrumental in defining the taxonomic status of species such as *M. mageritense* within the *M. fortuitum* group [15]. Consequently, *sodA* is increasingly recognized as a vital molecular target, serving either as a primary or supplementary marker to enhance the taxonomic resolution of NTM identification [16].

To address gaps in diagnostic and epidemiology, this study aimed to determine the proportion of pathogenic NTM in two different cohort populations and to characterize species diversity using *sodA* gene sequence-based analysis as another widely used molecular target for alternative molecular approach for NTM species identification among presumptive TB patients in Indonesia

## Material and Methods

### Clinical specimens and selection of isolates

This study involved retrospective specimens from two previous studies conducted in different cohorts in Bandung City, Indonesia; TB suspects from hospital-based and from community healthcare-based groups. Specimens were in the form of isolates stored in -80 °C and located in the Universitas Padjadjaran Faculty of Medicine Biobank. During the screening process, isolates were selected based on the availability of relevant information such as demographic data, confirmed positive Acid-Fast Bacilli (AFB) smear results, and documented culture and MPT64 antigen test results. Isolates with a record of positive mycobacterial culture but negative MPT64 antigen results were characterized as NTM suspect.

### Mycobacterial culture and DNA extraction

Selected isolates were re-cultured on Ogawa solid medium and incubated for 21 days to detect Mycobacterial growth. Following the appearance of visible colonies, genomic DNA was extracted using a Cetyltrimethylammonium bromide (CTAB)-based method, adapted from the protocol by Somerville et al. in 2005 [17]. An amount of 200 µL Tris-EDTA buffer was added to the isolates, the mixture was boiled at 85°C to kill the bacteria, followed by incubation at -20°C for 15 minutes and addition on 40 µL lysozyme (20 mg/mL). After another 1-hour incubation at 37 °C, 250 µL SDS 1% and Proteinase K were added to the mixture to break down the bacterial membrane and protein with continuous agitation and incubation at 65 °C. The pellet and

suspension were separated using CTAB reagent and NaCl. Extracted DNA was eluted in 60 µL of nuclease-free water and stored at -20°C for subsequent analysis

### PCR and targeted sequencing of the *sodA* gene

PCR was performed on the extracted genomic DNA using HotStar Taq Master Mix Kit (Qiagen, Hilden, Germany). A total of 20 µL of reaction consists of master mix, 7.4 µL of Nuclease Free Water (NFW), 0.15 µM of each forward and reverse primer, and 2 µL of template DNA. The *sodA* primers (SODLGF: 5'-GAA GGA ATC TCG TGG CTG AAT AC-3' and SODLGR: 5'-AGT CGG CCT TGA CGT TCT TGT AC-3'), which have been previously reported [15], aim for a 541 bp sequence product. The PCR was performed using LA1060 Wee 32 Thermal Cyclers (HiMedia Labs Pvt. Ltd., India) with the following conditions: initial activation at 95 °C for 5 minutes, 35 amplification cycles with 40 sec at 96 °C for denaturation, 50 sec at 60 °C for primer annealing, and 1 minute at 72 °C for elongation. Reaction ended with termination at 72 °C for 2 minutes.

The PCR product was confirmed using 2% agarose gel electrophoresis and subsequently sequenced using the Applied Biosystems BigDye® Terminator Cycle Sequencing Kit v3.1, targeting the *sodA* gene. The resulting sequence data, in.abi and FASTQ format, were processed using DNA Baser software ([www.dnabaser.com](http://www.dnabaser.com)). This involved sequence trimming and base editing, with alignment and adjustment verified on sequencing chromatograms. Species identification was performed by comparing the edited sequences against the GenBank database. Sequences were queried through the nucleotide BLAST (BLASTn) suite to determine species identity, the sequence query cover, and percentage identity based on

*sodA* reference were recorded to ensure its reproducibility.

The study was approved by the Ethics Committee, Faculty of Medicine, Universitas Padjadjaran (434/UN6.KEP/EC/2020)

## Result

From two cohorts, a total of 3,426 patients (2,476 from the community healthcare-based group, and 950 from the hospital-based group) presenting for pulmonary TB suspects were subjected for data screening. The demographic characteristics of the study population are summarized in Table 1.

Mycobacterial growth was observed in 50% of patients (n=1,713; consisting of 288 from the hospital-based cohort and 1,424 from the community healthcare-based cohort) using liquid or solid culture methods. Of these culture-

positive samples, a total of 55 (3.2%) were classified as suspected NTM based on a negative MPT64 antigen test. However, the proportion of NTM among culture-positive cases was higher in the hospital-based cohort (23/288, 7.9%) versus 2.2% (32/1,424) in the community healthcare-based cohort. The MPT64-negative cohort was predominantly female (n = 32; 58.2%), with a median age of 39 years. Notably, 41.8% of the patients (n = 23) had a documented history of previous TB treatment.

The *sodA* gene fragments were successfully amplified in 44 out of 55 isolates, with 21 samples from the community healthcare-based cohort and 23 samples from the hospital-based cohort. However, species identification could not be performed for the remaining 11 samples (20%) due to a lack of viable growth during subculture or insufficient DNA quality and quantity for successful PCR amplification and sequencing.

**Table 1.** NTM Subject's Sociodemographic Characteristics (N = 55)

Characteristics	
Age (Median)	39
<b>Sex</b>	<b>N (%)</b>
Male	23 (41.82%)
Female	32 (58.18%)
<b>Category</b>	<b>N (%)</b>
Patient with TB history	23 (41.82%)

The result showed species identified from both cohorts are mostly rapid-growing non-tuberculous mycobacteria (RGM) such as *M. fortuitum* and members from the same group (*M. peregrinum*, *M. mucogenicum*, *M. porcinum*, *M.*

*farcinogens*), species from members of *M. chelonae-abscessus* complex (*M. abscessus*, *M. chelonae*). There were also some slow-growing non-tuberculous mycobacteria (SGM) species identified, such as *M. terrae*, *M. celatum*, and

members of *M. avium* complex (*M. marseilense*, *M. colombiense*) and *M. gordonae*. *M. fortuitum* was the most prevalent NTM species overall, appearing at comparable rates in both the community health care (28.6%) and hospital (21.7%) groups. The species from *M. chelonae-abscessus* complex (*M. abscessus* (14.3% and 13.0%, respectively) and *M. chelonae* (9.5% and 13.0%, respectively)) was also prominently represented across both cohorts. Interestingly, species identification via *sodA*

sequence analysis revealed that some isolates previously classified as NTM suspects, due to negative antigen MPT64 results, were actually members of the *M. tuberculosis* complex (MTBC), specifically *M. tuberculosis* and *M. bovis*. (Table 2). All of the samples have > 90% sequence query coverage aligned with the database, and the majority showed percentage of identity > 90% with *sodA* reference species database, except one sample (Table 3).

**Table 2.** Identified NTM Species based on Sequence-based Analysis of *sodA* gene in two Cohorts

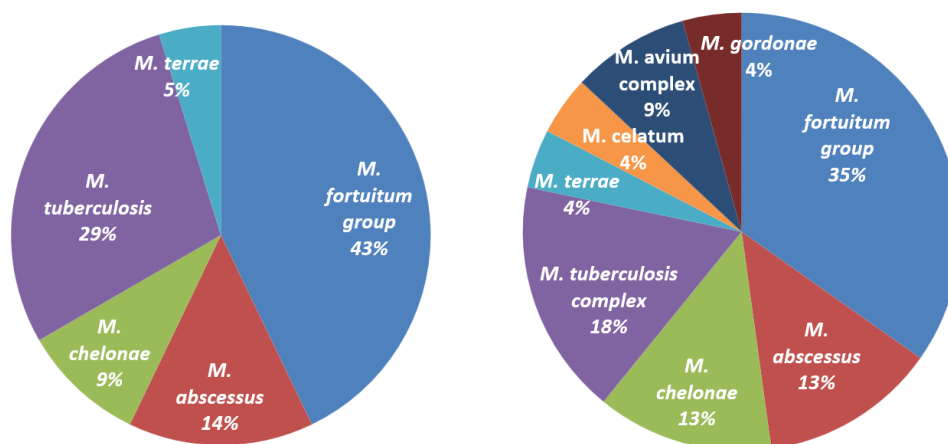
Name of species identified	Community healthcare-based cohort N (%)	Hospital-based cohort N (%)	Total N (%)	Note
<i>M. fortuitum</i>	6 (28.6%)	5 (21.7%)	11 (25.0%)	<i>M. fortuitum</i> group
<i>M. peregrinum</i>	1 (4.8%)	2 (8.7%)	3 (6.8%)	
<i>M. mucogenicum</i>	1 (4.8%)	-	1 (2.3%)	
<i>M. porcinum</i>	1 (4.8%)	-	1 (2.3%)	
<i>M. farcinogenes</i>	-	1(4.3%)	1 (2.3%)	
<i>M. abscessus</i>	3 (14.3%)	3 (13.0%)	6 (13.6%)	<i>M. chelonae-abscessus</i> complex
<i>M. chelonae</i>	2 (9.5%)	3 (13.0%)	5 (11.4%)	
<i>M. terrae</i>	1 (4.8%)	1 (4.3%)	2 (4.5%)	
<i>M. celatum</i>	-	1 (4.3%)	1 (2.3%)	
<i>M. marseilense</i>	-	1 (4.3%)	1 (2.3%)	<i>M. avium</i> complex (MAC)
<i>M. colombiense</i>	-	1 (4.3%)	1 (2.3%)	
<i>M. gordonae</i>	-	1 (4.3%)	1 (2.3%)	
<i>M. tuberculosis</i>	6 (28.6%)	1 (4.3%)	7 (15.9%)	<i>M. tuberculosis</i> complex (MTBC)
<i>M. bovis</i>	-	3 (13.0%)	3 (6.8%)	
<b>TOTAL</b>	<b>21</b>	<b>23</b>		<b>44</b>

**Table 3.** Identified NTM Species Based on *sodA* Partial Gene Sequencing with comparison to NCBI GenBank reference

No	Code	Name of NTM Species Identified based on <i>sodA</i> sequence alignment	Query Cover	<i>sodA</i> Percentage Identity (PID)
1	41101080	<i>M. terrae</i>	100.0%	95.1%
2	41100213	<i>M. porcinum</i>	100.0%	100.0%
3	41100967	<i>M. peregrinum</i>	97.0%	98.5%
4	41100841	<i>M. mucogenicum</i>	100.0%	98.1%
5	41100318	<i>M. fortuitum</i>	100.0%	99.3%
6	41100560	<i>M. fortuitum</i>	100.0%	100.0%
7	41100593	<i>M. tuberculosis</i>	100.0%	100.0%
8	41100459	<i>M. fortuitum</i>	100.0%	99.8%
9	41100116	<i>M. fortuitum</i>	100.0%	94.4%
10	41100125	<i>M. fortuitum</i>	100.0%	100.0%
11	41100105	<i>M. fortuitum</i>	100.0%	100.0%
12	41100194	<i>M. chelonae</i>	100.0%	100.0%
13	41100864	<i>M. abscessus</i>	100.0%	100.0%
14	41100689	<i>M. abscessus</i>	100.0%	100.0%
15	41100246	<i>M. abscessus</i>	100.0%	100.0%
16	41100174	<i>M. tuberculosis</i>	100.0%	100.0%
17	41100721	<i>M. chelonae</i>	100.0%	99.3%
18	41100525	<i>M. tuberculosis</i>	100.0%	100.0%
19	41101057	<i>M. tuberculosis</i>	100.0%	100.0%
20	41100709	<i>M. tuberculosis</i>	100.0%	100.0%
21	41101072	<i>M. tuberculosis</i>	100.0%	100.0%
22	42000017	<i>M. fortuitum</i>	100.0%	100.0%
23	42000018	<i>M. fortuitum</i>	100.0%	99.6%
24	42000009	<i>M. abscessus</i>	100.0%	100.0%
25	42000074	<i>M. terrae</i>	100.0%	92.5%
26	42000019	<i>M. farcinogenes</i>	100.0%	100.0%
27	42000040	<i>M. tuberculosis (variant bovis)</i>	100.0%	100.0%
28	42000052	<i>M. fortuitum</i>	100.0%	100.0%
29	42000112	<i>M. peregrinum</i>	93.0%	99.8%
30	42000113	<i>M. fortuitum</i>	100.0%	100.0%
31	42000225	<i>M. celatum</i>	100.0%	98.9%

**Table 3.** (Continued.) Identified NTM Species Based on *sodA* Partial Gene Sequencing with comparison to NCBI GenBank reference

No	Code	Name of NTM Species Identified based on <i>sodA</i> sequence alignment	Query Cover	<i>sodA</i> Percentage Identity (PID)
32	42000179	<i>M. peregrinum</i>	91.0%	99.8%
33	42000202	<i>M. tuberculosis (variant bovis)</i>	100.0%	100.0%
34	42000271	<i>M. abscessus</i>	100.0%	100.0%
35	42000313	<i>M. chelonae</i>	100.0%	100.0%
36	42000131	<i>M. marseillense</i>	100.0%	98.3%
37	42000203	<i>M. fortuitum</i>	100.0%	100.0%
38	42000162	<i>M. chelonae</i>	100.0%	100.0%
39	42000204	<i>M. chelonae</i>	100.0%	99.8%
40	42000310	<i>M. colombiense</i>	100.0%	98.3%
41	42000161	<i>M. abscessus</i>	100.0%	99.8%

**Figure 1.** Proportion of Species Identified from NTM suspects in Two Cohorts; (Left) Community healthcare-based and (Right) Hospital-based cohort.

Molecular analysis based on the *sodA* gene sequences revealed similar distribution but differences in the diversity of NTM species between the two cohorts (Figure 1). Both cohorts were predominantly characterized by the same NTM groups, specifically the *M. fortuitum* group and the

*M. chelonae-abscessus* complex. While both groups also showed a substantial prevalence of MTBC, the specific species composition within this complex varied between the hospital and community healthcare-based settings. The community healthcare-based cohort displayed a relatively

narrow species profile consisting of RGM and MTBC. In contrast, the hospital-based cohort was characterized by broader species diversity, including the presence of several SGM species, which were absent in the community healthcare-based group.

## Discussion

Despite the high burden of TB in Indonesia, epidemiological data regarding non-tuberculous mycobacterial (NTM) infections remain limited. In TB-endemic regions, the probability of NTM infections is expected to rise [3]. Existing studies in Indonesia report an NTM prevalence of 3.3% to 22.72% among TB suspects – without species identification, with variations across different clinical settings remaining less characterized [8,9].

Previous research in Jakarta utilized 16S rRNA sequence-based analysis to identify 15 isolates, revealing a distribution of eight RGM isolates (*M. abscessus*) and seven slow-growing mycobacteria (SGM) isolates (*M. avium*, *M. intracellulare*, and *M. cookii*) [18]. Our previous work employed the *hsp65* gene and ITS 16S-23S rRNA sequencing to identify pathogenic NTM species, suggesting that a multigene sequence-based approach is essential for high-accuracy speciation [19,20]. More recently, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to identify 94 isolates from three referral laboratories in Java [21]. Collectively, these studies indicate that RGM species, particularly *M. abscessus* and *M. fortuitum*, are the predominant NTM taxa in Indonesia.

To expand upon these findings, the present study utilized partial *sodA* gene sequencing to characterize NTM species diversity and proportions across two distinct cohorts—hospital-based and community-based—in Bandung, Indonesia. The *sodA* gene, which encodes the enzyme superoxide dismutase, has demonstrated high discriminatory power for differentiating mycobacterial species and defining the taxonomic status, particularly within the RGM group [15,22].

To the best of our knowledge, this is the first study in Indonesia to compare the proportion of NTM suspects among culture-positive mycobacterial cases across two distinct clinical settings: hospital and community healthcare. We observed a significantly higher NTM suspect rate in the hospital-based cohort (7.9%) compared to the community-based cohort (2.2%), with an aggregate rate of 3.3% across both groups. This disparity in NTM proportions likely reflects the clinical profile of patients with TB suspects seeking tertiary care. Those who come to the hospital typically present with greater symptom severity and chronic, progressive respiratory conditions [23]. Given that the clinical manifestations of NTM, especially NTM pulmonary disease, closely mimic those of pulmonary TB, patients with a history of TB and persistent symptoms are more likely to seek specialized hospital care, later classified as NTM suspects, as their sputum resulted in a positive mycobacterial culture but negative antigen MPT64 test [9,24].

Notably, a total of 10 (22.72%) isolates from both cohorts initially classified as NTM suspects were definitively identified as MTBC by *sodA* gene sequence-based analysis. While the

community healthcare-based cohort showed a higher frequency of *M. tuberculosis* (28.6%), the hospital-based cohort showed greater diversity within this complex, including the identification of *M. bovis*. These discordant results between phenotypic and genotypic assays suggest several possibilities, such as mixed infection or the limited sensitivity of the MPT64 antigen test, which may not detect *M. tuberculosis* mutants harboring *mpt64* mutations (further analysis in unpublished data) [25]. Significant *mpt64* mutations such as 63-bp deletion, may modulate *M. tuberculosis* virulence and compromise the diagnostic accuracy of MPT64 antigen tests in high-burden settings like Indonesia, which may impact the TB case finding [26]. These results underscore the necessity of refining MPT64-based immunochromatographic test (ICT) designs to improve its sensitivity [25]. Furthermore, we suggest that *mpt64* genetic diversity should be a critical consideration in the ongoing development of TB diagnostics and vaccine research.

Although the proportion of NTM among culture-positive cases was higher in hospital-based than in community health care-based settings, the distribution of predominant NTM species was similar across both settings. Result shows NTM species identified are mainly RGM, *M. fortuitum* group followed by *M. chelonae-abscessus* complex, with a few of them being species of SGM. This finding also highlights a different geographical distribution of NTM species (e.g. Europe, where SGM has a higher prevalence).

However, the hospital-based cohort featured a more diverse spectrum of NTM species with high clinical relevance, including members of the *M. avium* complex (9%), *M. celatum* (4%), and

*M. gordonae* (4%), all of which were absent from the community healthcare-based samples. This increased diversity likely reflects the higher clinical severity of patients seeking hospital care, as SGMs are more frequently associated with advanced pulmonary disease. The SGMs are known to exhibit more virulence, due to their ability to adapt in a poor environment and enhanced cellular defense mechanisms, which contribute to pathogenicity [27].

While our findings suggest a more complex species profile in the hospital-based cohort, these results must be interpreted with caution. Given the limited number of successfully sequenced isolates per group, the differences observed between the community and hospital settings may be influenced by sampling limitations rather than actual biological trends. As such, these observations should be considered exploratory, and larger multi-center studies are required to confirm whether significant differences in NTM diversity exist across various clinical settings in Indonesia.

The group or complex classification and growth times will facilitate better decision-making for therapy based on American Thoracic Society (ATS) guidelines, enabling timely and appropriate treatment decisions. It will help clinicians in selecting appropriate treatment strategies, reducing the risk of misdiagnosis with TB.

## Conclusion

Screening for NTM among presumptive TB cases remains challenging in Indonesia; incorporating molecular identification methods represents a promising approach for future NTM research and surveillance. This study demonstrates

that the proportion of NTM among culture-positive cases was higher in the hospital-based cohort than the community healthcare-based cohort. Despite the similarity in dominant species across both groups, the hospital cohort featured more SGM species, which typically exhibit greater virulence than RGM. Our findings suggest that partial *sodA* gene sequencing has potential as one of the first-line identification methods for NTM in presumptive TB cases. In this study, we were also able to identify a variety of significant, pathogenic NTM species through sequence-based analysis of the *sodA* gene. However, combining multiple gene targets improves the accuracy and reliability of mycobacterial speciation. Our findings suggest that the *sodA* gene should be included in the standard suite of diagnostic markers, as it effectively aids in the differentiation of NTM, particularly within the RGM group. Collectively, these results underscore the need to refine NTM case-finding strategies, identify patient groups at higher risk of NTM infection, and combine multiple biomarkers to improve species-level specificity.

### Acknowledgement

We thank Prof. Bachtı Alısjahbana, MD, PhD, for providing access to the samples used for this study. This study was financially supported by the Internal Research Grant of Universitas Padjadjaran (2203/UN6.3.1/PT.00/2022). The funding sources had no role in the study's design, conduct, analysis or reporting.

### Disclosure

The author reports no conflicts of interest in this work

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## The association study between DNA methylation on *CYP2D6* and *CYP2E1* gene promoter and anti-tuberculosis drug-induced liver injury in tuberculosis patients in Thailand

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Anti-tuberculosis drug-induced liver injury (ATDILI) is one of the most frequently reported adverse drug reactions among patients receiving first-line anti-tuberculosis (TB) therapy. This condition eventually contributes to treatment interruption and prolongation, presumably leading to treatment failure and liver failure, especially in highly predisposed patients. Importantly, the incidence of ATDILI is expected to increase continuously due to the widespread implementation of comprehensive TB treatment strategies across all affected populations. To prevent ATDILI and its related complications, regular monitoring of liver enzymes, together with the assessment of clinical symptoms, has been adopted as a primary diagnostic approach in many clinical settings. However, this method remains limited by its suboptimal sensitivity and specificity, thereby underscoring the need to identify more reliable biomarkers for early ATDILI detection. Given these concerns, this study aimed to investigate the association between DNA methylation (DNAm), a key epigenetic modification, at the promoter regions of drug-metabolizing enzyme genes, including *CYP2D6* and *CYP2E1*, and the occurrence of ATDILI in Thai TB patients. A total of 120 TB patients, divided into ATDILI cases (n=64) and non-ATDILI cases (n=56), and 52 age-matched healthy controls were enrolled. DNAm levels were quantified using pyrosequencing. Results demonstrated significantly higher DNAm levels at the *CYP2D6* promoter in patients with ATDILI compared with those without ATDILI. Without confounders adjusting, *CYP2D6* promoter hypermethylation was significantly associated with ATDILI occurrence. In contrast, no significant differences or associations were observed for *CYP2E1* promoter methylation. Collectively, *CYP2D6* promoter methylation was elevated in ATDILI patients and showed potential as a supportive biomarker; however, its independent association with ATDILI was not confirmed after adjustment for confounders. Therefore, considering the dynamic nature of epigenetic regulation, these findings warrant validation in independent cohorts with larger sample sizes before clinical application.

**Keywords:** Anti-tuberculosis drug-induced liver injury (ATDILI), DNA methylation (DNAm), Cytochrome P450 (*CYP2E1*, *CYP2D6*), Tuberculosis (TB), Epigenetic

### Introduction

Tuberculosis (TB) is airborne infectious disease caused by *Mycobacterium tuberculosis*. Regarding the widespread of this malicious disease at global scale, 10.8 million people experienced with TB and 1.25 million death cases, were reported in 2023. According to World Health Organization (WHO), Thailand was also classified as a country with high TB burden and confronting with TB-HIV situation

[1]. In order to overcome such diseases, high effective first-line anti-TB drugs regimen, which composed of intensive 2 months of concomitant isoniazid, rifampicin, pyrazinamide and ethambutol followed by 4 months of isoniazid and rifampicin, has been widely suggested by WHO and relevant organization to pharmacologically treat drug susceptible pulmonary TB [2,3]. Apart from favorable treatment outcome, similar to other drugs, adverse drug reactions can be

observed during this chemotherapeutic course especially anti-tuberculosis drugs-induced liver injury (ATDILI), which 11.50% incidence rate was exhibited and the incidence trend was predicted to be increasing [4]. According to the ongoing end TB strategy of WHO, which targeted to ultimately minimize TB incidence and death case within 2035, the boosting tendency of ATDILI incidence could be expected [1]. Once TB patients undergoing such therapy, clinical manifestation of ATDILI including abdominal pain, nausea, vomiting, jaundice, loss of appetite and notably increased liver enzymes level, could generally appeared and the latter has been greatly suggested to periodically monitor before and during drugs exposure [5]. Importantly, not only physical suffering, ATDILI could also lead to treatment interruption as drugs de-challenging and re-challenging strategies were recommended by treatment guidelines [5–7]. This discontinuation of treatment could eventually promote drug resistance and/or treatment prolongation [8]. In order to streamline these challenges, the rigorous understanding of ATDILI underlying mechanism is needed. Nevertheless, the mechanism of drug-induced liver injury is sophisticated and elusive as the occurrence of this condition could be elicited by several tracks including adaptive immune response, mitochondrial dysfunction, oxidative stress and excessive gathering of reactive metabolites, fatty acid as well as bile acid [9–13]. Moreover, ATDILI could be dominated by a wide variety of factors such as sex, age, nutrition status, alcohol consumption, metabolic disorders, viral infection, microbiome and genetic, indicating

ATDILI complexity [6,14,15]. On the basis of these facts, more effective ATDILI management and new insight are still warranted.

Currently, the detection of ATDILI is basically based on liver enzymes monitoring as previously described. However, their diagnostic performances were questioned and the abnormal levels could be detected after the pathological change has already occurred. Remarkably, there is prior study, which reported the positive association between the delaying of liver function test and liver failure [16]. To resolve these obstacles, a plethora of unprecedented biomarker for drug-induced liver injury were proposed including genetic, non-coding RNA, metabolomics, proteomic and histological biomarker. However, their intrinsic limitations were demonstrated. Technically, the instability could be shown among non-coding RNA, metabolomics and proteomic-based biomarker whilst, histopathological biomarker strictly requires invasive sample collecting [17–20]. Therefore, alternative biomarker such as DNA methylation-based biomarker should be considered.

Given that DNA methylation is the one machinery of epigenetic regulation, basically, this process enzymatically inserts methyl group from methyl donors to the fifth position of cytosine nucleotide, offering 5'-methylcytosine or methylated cytosine [21]. This modification mostly occurs on cytosine-guanine dinucleotide (CpG) rich loci such as gene promoters [22]. DNA methylation plays significant roles in many biological processes such as embryo development, X chromosome inactivation, genome stabilization and gene regulation [23]. To shortly explain gene modulation role, Promoter DNA methylation normally silences gene transcription, while DNA methylation on gene body often activates transcription [21, 22, 24]. Interestingly, DNA

methylation status on genomic DNA sequence can be transiently toggled to demethylation status (original cytosine or other demethylation mediator molecules) and vice versa under specific conditions or environmental factors [25–27]. According to these properties, DNA methylation has inspired research community to develop early diagnostic biomarkers based on this epigenetic modification. Although the association between DNA methylation status on pharmacokinetic and pharmacodynamics-related genes (or pharmacogenes) and ATDILI were previously revealed by many studies [28–32], the scarcity of such studies remains in Thailand with high TB burden.

In the context of drug biotransformation process, CYP2D6 and CYP2E1 are well known as the members of drug metabolizing enzyme CYP450 superfamily, which mainly express in liver to metabolize xenobiotic and endogenous substances. The role of *CYP2E1* in anti-TB drug metabolism pathway was definitely documented. This enzyme turns isoniazid and its metabolites, hydrazine and acetylhydrazine into reactive metabolites, which will lastly generate hepatotoxicity [33]. On the other hands, the unknown relation of CYP2D6 in anti-TB drug is still existing until now. However, 25% of clinical used drugs are metabolized by such enzyme [34]. Beside genetic factors, the expression of these enzymes could be defined by environmental factors such as smoking and nutrition, indicating the likelihood of epigenetic regulation of these potential enzymes [35,36]. Even though the correlation between *CYP2D6* and *CYP2E1* DNA methylation and ATDILI has already been published in Chinese population [31], but to

utilize precedent finding, confirmation study in independent populations should not be omitted as DNA methylation status could be distorted by different surrounding and genetic factor [37]. To the best of our knowledge, there is no research, which certainly explore DNA methylation profile on interested pharmacogenes in Thai TB patients with ATDILI.

Here, this study mainly aimed to investigate the association of DNA methylation level on *CYP2D6* and *CYP2E1* gene promoters, clinical data and the occurrence of ATDILI among Thai ethnic group.

## Material and Methods

### Study participants, DNA extraction and whole genome sequencing

This study combined 2 cohorts. For the first cohort, samples were leftover DNA samples belong to a previous study [38]. These samples were extracted from whole blood samples derived from TB patients of Nopparatrajathanee hospital, Ramathibodi hospital, Thaimuangchaipat hospital and Buddhachinaraj hospital by using QIAamp DNA Blood Mini Kit (Qiagen, CA, USA). The DNA samples of other the cohort were previously extracted from whole blood samples of TB patients in Borabue hospital, Roi Et hospital, Kalasin hospital, Kamalasai hospital, Phochai hospital and Chiangrai Prachanukroh hospital, by DNA extraction centre, Department of Medical Sciences, Ministry of Public Health, Thailand by using Chemagic 360 automated DNA extractor (Revvity, MA, USA). Blood samples of both cohorts as starting material for DNA extraction, were drawn from TB patients after 1-4 weeks of drug administration including isoniazid, rifampicin, pyrazinamide and ethambutol. Demographic and clinical data were also collected including age, gender, alcohol consumption, smoking status and biochemical parameters at first diagnosis and

1 - 4 weeks of drug administration. Totally, 120 age-matched DNA samples were retrieved. Among these, 64 samples of patients with ATDILI (based on following criteria; alanine transaminase (ALT) level/aspartate transaminase (AST) level > 5 x upper limit of normal (ULN) or ALT/AST level > 3 x upper limit of normal (ULN) with ATDILI - related symptoms or based on the judgement of clinicians) and 56 samples of patients without ATDILI (non – ATDILI) were defined as case and control group respectively. 52 samples of healthy individual were also included. This study was approved by Institutional Review Board of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University. All participants provided written informed consent, which included the use of their samples for further analysis as specified in protocol MU-DT/PY-IRB 2025/033.1505.

#### **Promoter sequences selection**

*CYP2D6* and *CYP2E1* promoter sequences were retrieved from 3 databases including NCBI, UCSC genome browser and eukaryotic promoter database. MATCHTM 1.0 was applied to select potential sequence performing as liver specific transcription factor binding site in vertebrate genome. Sequences are listed in Table 1.

#### **DNA methylation level quantification**

Sequentially, 250 ng of 172 DNA samples were chemically converted by following instruction of EZ DNA Methylation kit (Zymo

Research, CA, USA). PyroMark PCR kit (Qiagen, Hilden, Germany) and specific primers designed by PyroMark Assay Design Software 2.0.2 (Table 2) was employed to amplify *CYP2D6* and *CYP2E1* gene promoters under well optimized PCR conditions (Table 3). Next, 2% agarose gels were used to visually check amplified DNA samples by gel electrophoresis. Totally, 172 amplified DNA samples passed quality checking criteria regarding to gel electrophoresis. 10 uL of 172 Amplified DNA samples were eventually sequenced by PyroMark® Q48 Autoprep (Qiagen, Hilden, Germany) and DNA methylation levels were automatically calculated by PyroMark® Q48 Autoprep software.

#### **Statistical analysis**

General information of non-ATDILI and ATDILI were compared by Fisher's exact test and Mann Whitney test when data were categorical and continuous, respectively. The differences DNAm level on both gene promoters between non-ATDILI, ATDILI and HC were analyzed by Kruskal-Wallis test. Fisher's exact test and multiple logistic regression were deployed to explore the association between DNAm level on focused regions and ATDILI occurrence. The correlation between DNAm level, age, BMI and liver function – associated parameters were operated by Spearman correlation. Area under the curve (AUC), sensitivity and specificity of DNAm level on gene promoters, ALT and AST level were determined by Receiver Operating Characteristic Curve (ROC). The results would be statistically significant when P value < 0.05. GraphPad Prism version 10.3.1 (GraphPad Software, Boston, MA) was adopted as data analysis software.

**Table 1.** Consensus gene promoter sequences established based on sequences retrieved from 3 databases for DNA methylation analysis.

Target	Sequence to analyze
<b><i>CYP2D6</i> promoter</b>	<b>CGGGCGGT</b> CGGCGGTGTCCTCGCCGTGGGTCACCAGCGCCT CGCGCACGGCCAGCCCATTGAGCACGACCACGGCGTC CAGGCCAGCTGCAGGCTGAACA <b>CGTCCC</b> CGAAG <b>CGGCGCCG</b>
<b><i>CYP2E1</i> promoter</b>	GG <b>CCGGCG</b> AGG <b>CCGGG</b> CTGCTG <b>CGACG</b> CCAGGATA <b>ACC</b> GGG CTGG <b>CGGCC</b> AGATG <b>CGCACTCG</b> CTGGG <b>CGTCCG</b> CCTGTGTTT GCCAAAGCA <b>CGAGTTGAAA</b> CGTGAAGTGTGGG

**Table 2.** List of PCR primer and sequencing primer for template preparation and pyrosequencing reaction.

Target	Primer sequence (5' - 3')	Product size (bp)
<b><i>CYP2D6</i> promoter</b>	PCR primer Forward primer: AGGATTTGGGTGATGGGTATA	169
	Reverse primer: Biotin-CCCTAACCTCCCTCTACA	
	Sequencing primer GGGTGATGGGTATAG	
<b><i>CYP2E1</i> promoter</b>	PCR primer Forward primer: GGGTATGGGTATAG	144
	Reverse primer: Biotin-CCCAACACTTCACCTTCAACTCCTACTTTAAC	
	Sequencing primer GGTTGATGATGGGGA	

**Table 3.** PCR thermal profile for *CYP2D6* and *CYP2E1* promoter amplification

Step	Temperature (°C)	Time	Cycle
<b><i>CYP2D6</i> promoter</b>			
<b>Initial denaturation</b>	95	15 min	-
<b>Denaturation</b>	94	30 s	45
<b>Annealing</b>	60	30 s	
<b>Extension</b>	72	30 s	
<b>Final extension</b>	72	10 min	-

**Table 3.** (Continued.) PCR thermal profile for *CYP2D6* and *CYP2E1* promoter amplification

Step	Temperature (°C)	Time	Cycle
<b><i>CYP2E1</i> promoter</b>			
<b>Initial denaturation</b>	95	15 min	-
<b>Denaturation</b>	94	30 s	45
<b>Annealing</b>	60	30 s	
<b>Extension</b>	72	30 s	
<b>Final extension</b>	72	10 min	-

## Result

### General information of study participants

A total of 120 patients were analyzed, divided into non-ATDILI group (n = 56) and ATDILI group (n = 64). According to Fisher's exact test and Mann Whitney test, there were no statistically significant differences between the two groups regarding age, gender distribution, BMI, alcohol consumption. However, the proportion of smoking status was considerably higher in non-ATDILI ( $P = 0.0161$ ). Before the commencement of anti-tuberculosis drugs, most liver function markers were comparable between the groups. However, baseline ALB levels were significantly lower in ATDILI ( $P = 0.0236$ ). Liver function parameters quantified during post-treatment revealed that ATDILI group exhibited marked elevations in ALT, AST, ALP, TB and DB compared to non-ATDILI group ( $P < 0.0001$ ;  $P < 0.0001$ ;  $P = 0.0052$ ;  $P = 0.0015$  and  $P < 0.0001$  respectively). Whereas, TB and ALB significantly declined in ATDILI ( $P = 0.0079$  and  $P < 0.0001$  respectively), (Table 4).

### DNAm level on *CYP2D6* and *CYP2E1* promoter in 3 groups

Regarding to Kruskal-Wallis test, DNA methylation level on *CYP2D6* promoter was significantly higher in ATDILI when compared to non-ATDILI and healthy control ( $P = 0.0003$ ;  $P = 0.0007$  respectively) (Figure 1A). Contrastingly, DNAm level on *CYP2E1* promoter were similarly low in 3 groups and posed no significant differences (Fig 1B).

### The association between DNAm on *CYP2D6* and *CYP2E1* promoter and ATDILI occurrence

Fisher's exact test revealed that individuals with *CYP2D6* hypermethylation (Determined by the mean of DNAm level in 3 groups. Samples with DNAm level not less than 79% and 26% were classified as *CYP2D6* and *CYP2E1* promoter hypermethylation respectively) significantly related to ATDILI risk (OR 2.54; 95% CI 1.17, 5.47;  $P = 0.016$ ). In contrast, no significant association was observed between *CYP2E1* hypermethylation and the incidence of ATDILI (OR 1.36; 95% CI 0.64, 2.75;  $P = 0.4621$ ). Furthermore, smoking also showed significant association with

ATDILI (OR 0.31; 95% CI 0.13, 0.77;  $P = 0.016$ ). However, further analysis of *CYP2D6* DNA hypermethylation and smoking adjusted confounding factors as variables showed no significant association with ATDILI risk (OR 1.20; 95% CI 0.34, 4.16;  $P = 0.7749$ ; OR 0.52; 95% CI 0.09, 2.82;  $P = 0.3923$ , respectively). Likewise, the risk remained non-significant for *CYP2E1* hypermethylation (OR 2.02; 95% CI 0.65, 6.63;  $P = 0.2291$ ) as demonstrated by multiple logistic regression analysis (Table 5).

### Correlation between *CYP2D6* and *CYP2E1* promoter DNAm level and clinical parameters

Spearman correlation analysis demonstrated that significantly negative correlations between DNAm level on *CYP2D6* gene promoter at post-treatment and BMI as well as TP and ALB were detected ( $r = -0.259$ ,  $P = 0.004$ ;  $r = -0.541$ ,  $P < 0.0001$ ;  $r = -0.236$ ,  $P = 0.009$  respectively) and significantly positive correlations between DNAm level, ALT and AST were obviously observed after treatment ( $r = 0.285$ ,  $P = 0.002$ ;  $r = 0.264$ ,  $P = 0.004$  respectively). Meanwhile, only significantly inverse correlations between *CYP2E1* DNA methylation level and ALP, TP and ALB, were

detected ( $r = -0.294$ ,  $P = 0.001$ ;  $r = -0.328$ ,  $P < 0.0001$ ;  $r = -0.333$ ,  $P < 0.0001$  respectively). Additionally, DNAm level on *CYP2D6* and *CYP2E1* promoter firmly displayed direct relation to each other ( $r = 0.248$ ,  $P = 0.006$ ) (Figure 2).

### Feasibility assessment of DNAm on *CYP2D6* and *CYP2E1* promoter as supportive biomarker for ATDILI diagnosis

To evaluate the potential of DNAm on *CYP2D6* and *CYP2E1* promoter as diagnostic tools, ROC curves were established. DNAm level on *CYP2D6* promoter quantified during 1-4 weeks at post-treatment notably posed highest AUC, sensitivity and specificity. However, the performances depleted when incorporated with other biomarkers (Figure 3). In addition, DNAm level on *CYP2E1* promoter measured at same time points demonstrated inferior AUC, sensitivity and specificity when compared to combined ALT and AST level at pre-administration (Baseline level). Nevertheless, AUC of combined DNAm on *CYP2E1* and *CYP2D6* promoter was higher and sensitivity was comparable to the combination of baseline ALT and AST level (Figure 4).

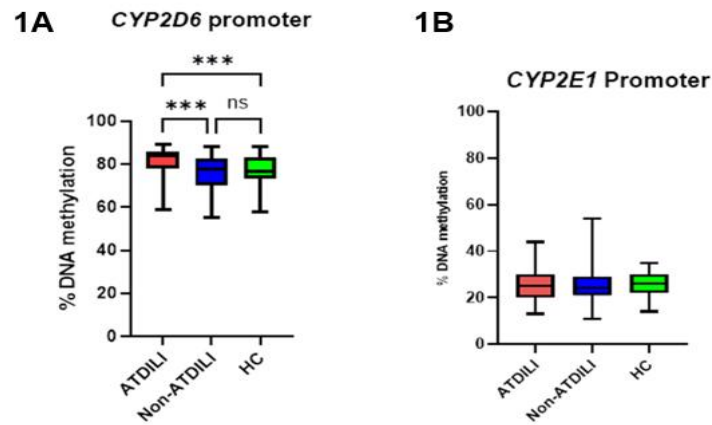
**Table 4.** General information of Non-ATDILI and ATDILI group.

	Non-ATDILI	ATDILI	<i>P</i> value
Age	47 (35.50, 62.00)	54 (42.50, 65.00)	0.0581
Gender			0.0848
Male	40 (72.73%)	36 (56.25%)	
Female	15 (27.27%)	28 (43.75%)	
BMI	18.94 (17.02, 20.86)	19.94 (16.65, 22.96)	0.2321
Alcohol consumption			0.0704
Yes	24 (53.33%)	28 (73.68%)	
No	21 (46.67%)	10 (26.32%)	

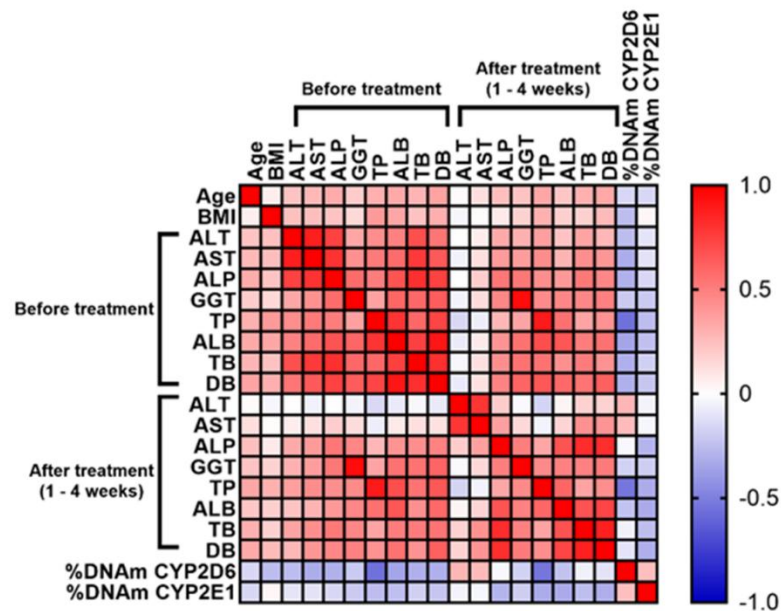
**Table 4.** (Continued.) General information of Non-ATDILI and ATDILI group.

	Non-ATDILI	ATDILI	<i>P</i> value
<b>Smoking status</b>			<b>0.0161</b>
Yes	24 (50.00%)	10 (23.81%)	
No	24 (50.00%)	32 (76.19%)	
<b>Clinical parameters (at first diagnosis/before drugs commencement)</b>			
ALT	24.00 (14.75, 32.50)	19.90 (11.00, 36.75)	0.5293
AST	31.00 (23.00, 44.75)	27.00 (16.00, 45.50)	0.2151
ALP	93.50 (59.50, 125.50)	99.00 (79.50, 113.50)	0.435
GGT	3.85 (3.73, 4.35)	3.90 (3.60, 4.80)	0.4771
TP	7.55 (7.30, 8.08)	7.40 (6.73, 7.68)	0.2809
ALB	3.60 (3.20, 4.25)	3.05 (2.63, 3.60)	<b>0.0236</b>
TB	0.65 (0.30, 0.95)	0.58 (0.42, 0.92)	0.9085
DB	0.20 (0.20, 0.29)	0.21 (0.10, 0.40)	0.846
<b>Clinical parameters (1 - 4 weeks after drugs administration)</b>			
ALT	20.00 (14.00, 34.00)	102.00 (35.00, 163.00)	<b>&lt;0.0001</b>
AST	26.00 (21.00, 37.00)	148.00 (82.00, 246.00)	<b>&lt;0.0001</b>
ALP	90.00 (67.00, 134.00)	135.00 (91.00, 171.00)	<b>0.0052</b>
GGT	4.25 (3.68, 4.70)	4.20 (4.00, 4.30)	0.8541
TP	8.20 (7.30, 8.73)	7.20 (6.25, 7.88)	<b>0.0079</b>
ALB	4.00 (3.55, 4.30)	3.00 (2.38, 3.43)	<b>&lt;0.0001</b>
TB	0.60 (0.40, 1.00)	0.90 (0.60, 2.50)	<b>0.0015</b>
DB	0.20 (0.10, 0.37)	0.61 (0.35, 1.60)	<b>&lt;0.0001</b>

Categorical data and continuous data were expressed as percentage and inter quartile range (IQR) respectively. Bold *P* value denoted *P* value with statistical significance (*P* value < 0.05). BMI Body Mass Index, ALT alanine aminotransferase, AST aspartate aminotransferase, ALP alkaline phosphatase, GGT gamma-glutamyl transferase, TP total protein, ALB albumin, TB total bilirubin, DB direct bilirubin.



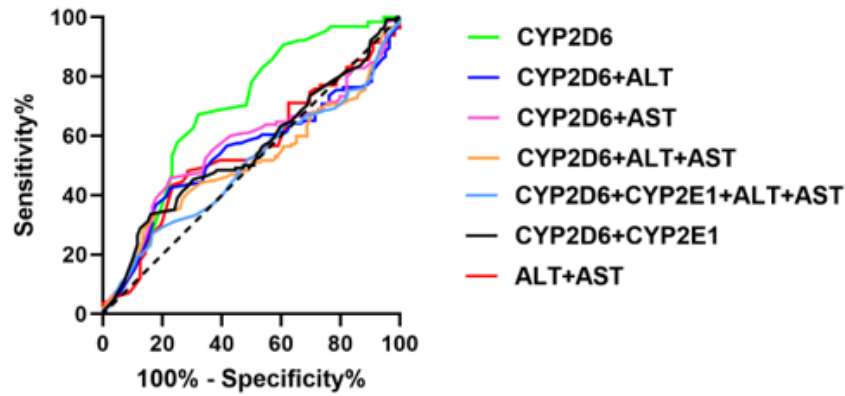
**Figure 1.** Comparison between DNAm level on *CYP2D6* and *CYP2E1* promoter between non-ATDILI, ATDILI and healthy control group. \*\*\*  $P$  value  $< 0.05$ . ns non-significant ( $P$  value  $\geq 0.05$ ).



**Figure 2.** Heatmap displayed correlations between DNAm level on *CYP2D6* and *CYP2E1* promoter and biochemical parameters measured before treatment and 1 – 4 weeks after treatment. ALT alanine aminotransferase, AST aspartate aminotransferase, ALP alkaline phosphatase, GGT gamma-glutamyl transferase, TP total protein, ALB albumin, TB total bilirubin, DB direct bilirubin.

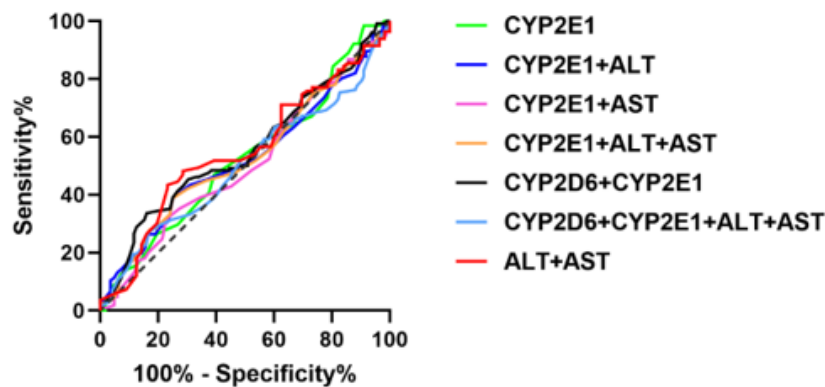
**Table 5.** Multivariate logistic regression analysis between DNA methylation status on *CYP2D6* and *CYP2E1* promoter and ATDILI occurrence. OR odds ratio. <sup>a</sup> adjusted for age, gender, BMI, alcohol consumption and smoking status.

Variables	Univariate Analysis		Multivariate Analysis	
	OR (95%CI)	P value	OR (95%CI)	P value <sup>a</sup>
<b><i>CYP2D6</i> hypermethylation</b>	2.54 (1.17, 5.47)	<b>0.0160</b>	1.20 (0.34, 4.16)	0.7749
<b><i>CYP2D6</i> hypomethylation</b>	1 (Ref)	-	1 (Ref)	-
<b><i>CYP2E1</i> hypermethylation</b>	1.36 (0.64, 2.75)	0.4621	2.02 (0.65, 6.63)	0.2291
<b><i>CYP2E1</i> hypomethylation</b>	1 (Ref)	-	1 (Ref)	-
<b>Age</b>				
<b>≥ 60 years</b>	1.41 (0.67, 3.12)	0.4413	1.48 (0.39, 6.01)	0.9400
<b>&lt; 60 years</b>	1 (Ref)	-	1 (Ref)	-
<b>Gender</b>				
<b>Female</b>	2.07 (0.98, 4.34)	0.0848	1.46 (0.43, 4.97)	0.5465
<b>Male</b>	1 (Ref)	-	1 (Ref)	-
<b>BMI</b>				
<b>&lt; 18.5 Kg/mm<sup>2</sup></b>	0.72 (0.30, 1.67)	0.5185	0.35 (0.10, 1.13)	0.0935
<b>≥ 18.5 Kg/mm<sup>2</sup></b>	1 (Ref)	-	1 (Ref)	-
<b>Alcohol consumption</b>				
<b>Yes</b>	0.41 (0.17, 1.06)	0.0704	0.38 (0.06, 2.21)	0.2960
<b>No</b>	1 (Ref)	-	1 (Ref)	-
<b>Smoking</b>				
<b>Yes</b>	0.31 (0.13, 0.77)	<b>0.0161</b>	0.52 (0.09, 2.82)	0.3923
<b>No</b>	1 (Ref)	-	1 (Ref)	-



Biomarker	Cut off	AUC	Sensitivity (%)	Specificity (%)	P value
CYP2D6	> 80.50	0.6903	67.19	67.86	0.0003
CYP2D6+ALT	> 74.50	0.5420	56.60	58.33	0.3205
CYP2D6+AST	> 75.50	0.5677	57.14	60.71	0.1098
CYP2D6+ALT+AST	> 62.50	0.5111	51.02	50.00	0.7599
CYP2D6 + CYP2E1	> 42.50	0.5538	51.56	49.11	0.1508
CYP2D6 + CYP2E1 + ALT + AST	< 30.50	0.5008	52.13	51.19	0.9782
ALT + AST	< 23.50	0.5499	51.81	51.79	0.3191

**Figure 3.** Comparison between diagnostic performance of DNAm on *CYP2D6* promoter as stand-alone and combined diagnostic biomarker for ATDILI. ALT and AST level were obtained before drug administration.



Biomarker	Cut off	AUC	Sensitivity (%)	Specificity (%)	P value
CYP2E1	> 24.50	0.5248	51.56	53.57	0.6397
CYP2E1+ALT	< 24.50	0.5228	52.83	45.24	0.5897
CYP2E1+AST	< 24.50	0.5050	48.57	47.62	0.9052
CYP2E1+ALT+AST	< 24.50	0.5269	51.7	46.43	0.4577
CYP2D6 + CYP2E1	> 42.50	0.5538	51.56	49.11	0.1508
CYP2D6 + CYP2E1 + ALT + AST	< 30.50	0.5008	52.13	51.19	0.9782
ALT + AST	< 23.50	0.5499	51.81	51.79	0.3191

**Figure 4.** Comparison between diagnostic performance of DNAm on *CYP2E1* promoter as stand-alone and combined diagnostic biomarker for ATDILI. ALT and AST level were obtained before drug administration.

## Discussion

While genetic variations in the CYP450 superfamily contribute to ATDILI risk, the role of epigenetic regulation, specifically DNA methylation remains under-researched in high-burden regions like Thailand. Given that epigenetic markers are influenced by population-specific environmental factors, we investigated the association between *CYP2D6* and *CYP2E1* methylation levels and ATDILI to develop new-insight for addressing the knowledge gap in TB treatment. The findings suggest that baseline demographic factors were not primary drivers of liver injury in this specific cohort. A key observation is the significantly lower baseline Albumin in ATDILI group, which might represent the malnutrition as the risk factor of ATDILI [11].

Recent finding also observed significantly higher DNAm level on *CYP2D6* promoter in patient with ATDILI when compared to non-ATDILI and healthy control. According to transcription factor binding sites prediction, it revealed that our candidate CpG site on regulatory region of *CYP2D6* gene is spontaneously embedded close to binding site of hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ), indicating that transcription factor binding might interact with higher DNA methylation level on focusing sequences and plausibly contribute to ATDILI. To exclusively elaborate, it was known that HNF4 $\alpha$  mainly plays a role in liver regeneration process, liver fibrosis inhibition, bile acid synthesis and liver metabolism [39–42]. Downregulation of HNF4 $\alpha$  was mostly observed in many liver diseases with undesirable prognosis [43–45]. Importantly, the binding site of this transcription factor can be markedly observed on the regulatory sequence of

various drug metabolizing enzymes including CYP2D6 [46]. Taken together, DNA methylation on candidate CpG region might presumably interact with adjacent transcription factor binding site and eventually cause aberrant liver function, leading to ATDILI [47]. However, this hypothesis-generating must be confirmed by further investigation such as reporter assay and methylation-expression correlation study, to reveal the new insights in epigenetic mechanism underlying ATDILI. While higher DNAm level on *CYP2D6* promoter was observed among ATDILI, DNAm level on *CYP2E1* promoter were similarly low between 3 groups and exhibited no significant differences. Our findings were partially in line with previous study performed in Han Chinese population, which reported hypermethylation on both promoters as the risk of ATDILI. Owing to the discrepancy of study population, DNAm quantification technique and promoter sequences, these factors might determine inconsistency between the study.

As our study additionally investigated the potential association between the promoter methylation levels of *CYP2D6* and *CYP2E1* and the risk of ATDILI, the result revealed only a statistically significant correlation of *CYP2D6* hypermethylation with liver injury risk. However, this association did not reach statistical significance when adjusted by confounding factors, suggesting that DNAm might not be dependent ATDILI risk factor since our result also revealed that smoking associated with ATDILI protection. However, some study reported that smoking was ATDILI risk factor [48]. Several works also demonstrated other factors as ATDILI predispositions that might compromise

the effect of DNAm on *CYP2D6* promoter [4,5,49,50].

According to the prevailing literature which reported that, obesity generally characterized by BMI, associated with low *CYP2D6* mRNA level and another study reported inverse relation between DNAm level on *CYP2D6* promoter and *CYP2D6* mRNA expression, it could be collectively infer that there is positive correlation between DNAm level on *CYP2D6* promoter and BMI regarding to these evidences. However, our findings revealed an inverse correlation between BMI and DNA methylation at *CYP2D6* promoter [35,51]. Since *CYP2E1* was reported as obesity-related gene by epigenome-wide association study [52], however the correlation between *CYP2E1* promoter DNAm level and BMI, was not observed by our study. Regarding to significantly positive correlation between *CYP2D6* promoter DNAm level, ALT level and AST level as well as significantly negative correlation between *CYP2E1* promoter DNAm level and ALP, suggesting that higher DNA methylation level on *CYP2D6* promoter might relate to increasing of liver enzymes as the key biomarker of liver abnormality [53,54].

Besides association study, we also evaluate performance of DNAm as alternatively novel biomarker for ATDILI diagnosis. Even inferior performance of DNAm on *CYP2E1* promoter was reported in our study regardless separation or combination, intriguingly DNAm on *CYP2D6* promoter showed outstanding performance (AUC = 0.6903; 67.19% sensitivity; 67.86% specificity;  $P = 0.0003$ ) when compared to traditional aminotransferase enzymes level (AUC = 0.5499; 51.81% sensitivity; 51.79% specificity;  $P =$

0.3191). However, it should be noted that baseline ALT and AST level in our ROC analysis is not conventional biomarker for ATDILI diagnosis since post-treatment level are generally adopted in clinical practice. Moreover, sensitivity and specificity of proposing biomarker might be limited since DNAm level were analyzed by using blood samples. Using liver-specific exosome separated by immunoprecipitation as the sample for DNAm quantification could enhance the accuracy and reliability of prospective study focusing on pathogenesis of liver diseases [55].

Unfortunately, this study faced many hurdles. Firstly, associated data of some samples were incomplete. Secondly, owing to tissue and location specific nature of DNA methylation, we were not able to collect patient-derived liver tissue sample to accurately determine actual DNA methylation level and phenotype of liver injury due to ethical concern. Nevertheless, whole blood-derived DNA methylation level was analyzed by the majority of previous works focused on DNA methylation in ATDILI [28,31,56,57]. Similarly, the downstream effect of DNA methylation including both mRNA and protein expression level could not be observed due to limited process of sample collection. Even the high stability of DNA methylation marker is perceived when compared to other epigenetic marks, its dynamic state should be considered to provide the result with more accuracy and reliability. To address this concern, samples should be collected at several time points from first challenging of anti-TB drugs to ATDILI onset. However, this recommendation was unachievable. Moreover, since our samples had been stored at -20 degree Celcius for 18 years until date of analysis,

the plasticity of DNA methylation during distinct long-term storage conditions was uncovered. Nonetheless, the stability could be long lasting up to 20 years in cryopreserved conditions regarding an evidence [58]. In overall, these weaknesses strongly emphasize the necessity of sample collection pipeline improvement, single-cell and multi-omics technologies might be integrated into confirmation study to diminish the heterogeneity concern and untangle the complexity of gene regulation-related to ATDILI [59]. Idiosyncratic nature of ATDILI unavoidably contributed to low number of case samples and probably affect the reliability of statistical analysis. Lastly, as CYP2E1 is easily induced by various external exposures such as concomitant drugs, alcohol and carcinogen in their daily life, controlling these confounding factors is not straightforward. To address this aspect of limitations, the further study with more independent participant, which could be gained by expanding to additional clinical sites with high TB and/or ATDILI incidence is essential in order to affirm these recent findings.

To recapitulate, DNA methylation on *CYP2D6* and *CYP2E1* genes promoter may predispose patients to ATDILI and also related to ATDILI-associated parameters. Utilizing these markers as supportive tools for ATDILI diagnosis could refine clinical oversight and enhance patient recovery. However, due to the fluid nature of epigenetic changes, these results must be confirmed in larger, separate study groups before they can be integrated into medical practice.

## Acknowledgements

This study was granted by the 60<sup>th</sup> Year Supreme Reign of His Majesty King Bhumibol Adulyadej Scholarship, Faculty of Graduate Studies, Mahidol university. Primary data/Phenotypic data was collected and managed under the project “Pharmacogenetics of drug-induced liver injury 2024”, conducted by Assoc. Prof. Dr. Jiraphun Jittikoon. This research was made possible through access to biological materials, the data and findings generated by Genomics Thailand, Health Systems Research Institute, Department of Medical Sciences and National Science and Technology Development Agency, supported by the Ministry of Public Health and the Ministry of Higher Education Science Research and Innovation.

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## Beyond Bacterial Load: Impact of Gender and Body Mass Index on Treatment Outcomes Among Hospitalized Tuberculosis Patients in Central India

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Tuberculosis (TB) outcomes are influenced by nutritional status and gender, but their combined impact in hospitalized patients remains unclear. A retrospective analysis of hospitalized TB patients at IGGMC, Nagpur, from January 2024 to June 2025 was conducted. Outcomes were classified as discharge after improvement or death. Associations with body mass index (BMI), gender, cartridge-based nucleic acid amplification test (CBNAAT) semi-quantitative load, comorbidities, and alcohol use were analyzed using chi-square tests. Among 210 patients, 31 deaths occurred (overall mortality 14.8%). Severe undernutrition (BMI <15 kg/m<sup>2</sup>) was associated with the highest mortality. A significant association between BMI and outcomes was observed in females ( $p = 0.004$ ) and a borderline association in males ( $p = 0.068$ ). Mortality was higher in patients with comorbidities (23.5% vs 14.0%; OR 1.89). CBNAAT load and alcohol use were not significantly associated with outcomes. In conclusion, BMI and gender are significantly associated with TB outcomes in hospitalized patients. Undernourished females represent a particularly high-risk group. CBNAAT bacterial load alone may not reliably predict clinical outcomes.

**Keywords:** Tuberculosis; Body Mass Index; Gender Differences; Mortality

### Introduction

Tuberculosis (TB) remains a major public health challenge globally and in India, contributing significantly to morbidity and mortality. Despite advances in diagnostic modalities such as cartridge-based nucleic acid amplification test (CBNAAT), predicting clinical outcomes in hospitalized TB patients remains complex.

Nutritional status plays a crucial role in determining disease severity and outcomes in TB. Undernutrition impairs host immunity and has been consistently associated with increased mortality. Body mass index (BMI) serves as a simple and practical marker for assessing nutritional status in clinical settings.

Gender-based differences in TB outcomes have also been reported, influenced by biological, behavioral, and socioeconomic factors. However, the interaction between gender and BMI in determining inpatient outcomes is not well established.

Furthermore, while the CBNAAT provides semi-quantitative bacterial load estimation, its prognostic value in hospitalized patients remains uncertain.

This study aimed to evaluate the impact of body mass index (BMI), gender-wise differences, CBNAAT bacterial load, comorbidities, and alcohol use on in-hospital clinical outcomes.

### Material and Methods

#### Study Design and Setting

This retrospective observational study was conducted from January 2024 to June 2025 in the Department of Respiratory Medicine at Indira Gandhi Government Medical College (IGGMC), Nagpur, a tertiary care center in Central India.

#### Ethical Approval

This study was approved by the Institutional Ethics Committee of IGGMC, Nagpur (Approval No: 269/2024). As this was a retrospective study using anonymized data, the requirement for informed consent was waived.

## Study Population

All adult patients diagnosed with tuberculosis and admitted during the study period were included. Patients with incomplete BMI data were excluded.

## Diagnostic Criteria

TB diagnosis was based on microbiological confirmation (CBNAAT/smear/culture) and/or clinico-radiological findings as per national guidelines.

## Data Collection

Data extracted from hospital records including demographic characteristics (age and gender), admission body mass index (BMI), clinical and behavioral factors (comorbidities including HIV, and alcohol use), diagnostic parameters including semi-quantitative CBNAAT results (very low, low, medium, or high), length of hospital stay, and final treatment outcomes (discharge versus death).

## Definitions

BMI categories were defined according to WHO criteria: underweight (<18.5 kg/m<sup>2</sup>), normal (18.5–24.9), overweight (25–29.9), and obese (≥30 kg/m<sup>2</sup>). Additionally, severe undernutrition (BMI <15 kg/m<sup>2</sup>) was analyzed separately.

Discharge was defined as clinical improvement with stabilization of vital parameters and physician decision for continuation of treatment on an outpatient basis.

Alcohol use was defined as self-reported regular consumption of alcohol.

## Statistical Analysis

Categorical variables were analyzed using the chi-square test. Odds ratios (OR) with 95% confidence intervals were calculated where appropriate.

## Results

### Baseline Characteristics

A total of 210 patients were analyzed, including 142 males (67.6%) and 68 females (32.4%).

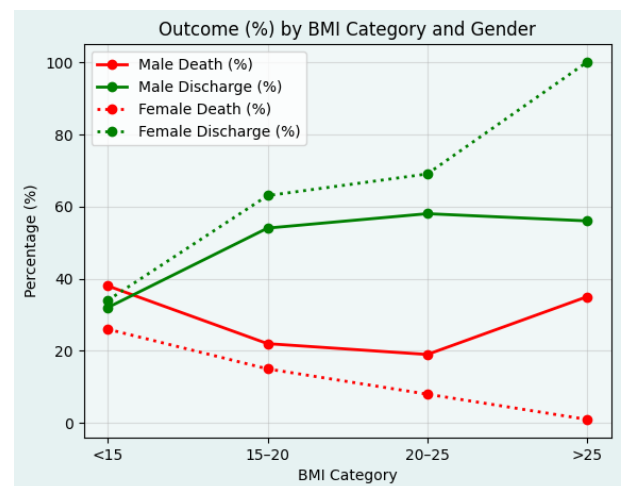
**Table 1:** Baseline Characteristics of Study Population

Variable	Value (%)
<b>Total patients</b>	210
<b>Male</b>	142 (67.6%)
<b>Female</b>	68 (32.4%)
<b>Deaths</b>	31 (14.8%)

### BMI and Treatment Outcomes

Severe undernutrition (BMI <15 kg/m<sup>2</sup>) was associated with the highest mortality in both genders. Mortality decreased progressively with increasing BMI, demonstrating a dose-response relationship in female patients; however, the same effect was not observed in male patients as shown in the Figure. This may be explained by a higher burden of comorbidities among male patients, including HIV, diabetes mellitus, alcohol use, and chronic liver disease.

A significant association between BMI and outcomes was observed in females ( $\chi^2 = 18.90$ ,  $p = 0.004$ ) and a borderline association in males ( $\chi^2 = 11.73$ ,  $p = 0.068$ ).



### Comorbidities and Outcomes

Mortality was higher among patients with comorbidities:

- With comorbidities: 23.5%
- Without comorbidities: 14.0%

Odds ratio (OR) for mortality in patients with comorbidities was 1.89.

### CBNAAT and Outcomes

No statistically significant association was observed between CBNAAT semi-quantitative bacterial load and outcomes ( $\chi^2 = 6.72, p = 0.347$ ).

### Alcohol Use and Outcomes

Alcohol use was not significantly associated with in-hospital mortality ( $p = 0.58$ ).

### Discussion

This study highlights the significant impact of nutritional status and gender on TB outcomes among hospitalized patients.

Severe undernutrition emerged as a strong predictor of mortality, consistent with existing literature linking malnutrition to impaired immune response and worse clinical outcomes. The observed dose-response relationship between BMI and outcomes further reinforces the importance of nutritional status in TB management.

The association between BMI and outcomes was more pronounced in females, suggesting that gender-related biological and social factors may amplify the effects of undernutrition.

Comorbidities were associated with increased mortality, nearly doubling the risk, highlighting the importance of comprehensive patient assessment and management.

CBNAAT bacterial load did not correlate with clinical outcomes, suggesting limited prognostic utility in hospitalized patients.

### Limitations

This study has several limitations. Being retrospective, it is subject to information bias and missing data. As a single-center study, generalizability may be limited. The sample size in certain subgroups was small, reducing statistical power. Additionally, long-term outcomes beyond hospitalization were not assessed.

### Conclusion

BMI and gender are significantly associated with in-hospital outcomes among TB patients. Severe undernutrition, particularly among females, is associated with higher mortality. Nutritional assessment and targeted interventions should be integral to TB management in hospitalized settings.

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