doi.org/10.36721/PJPS.2026.39.10.285.1

Serum zinc protoporphyrin and protoporphyrin IX levels as potential biomarkers for anti-tuberculosis drug-induced hepatotoxicity

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Abstract: **Background**: Anti-tuberculosis drug-induced hepatotoxicity (ATDH) remains a significant clinical challenge, highlighting the critical demand for predictive biomarkers. **Objectives**: This study aimed to investigate serum zinc protoporphyrin (ZnPP) and protoporphyrin IX (PPIX) as potential ATDH predictors. **Methods**: Blood samples were collected at baseline and two subsequent liver function tests in 58 cases and 191 controls from four hospitals. **Results**: The ZnPP levels of controls at baseline and two subsequent time points (147.9, 152.0 and 147.5 pg/ml, respectively) were significantly higher than cases (113.1, 134.3 and 119.0 pg/ml), while the PPIX levels were significantly higher in ATDH cases (8.6, 9.4 and 9.1 ng/ml, respectively) than controls (7.0, 7.6 and 7.1 ng/ml) ($P<0.05$). Multivariate analysis identified liver disease history, baseline serum ZnPP and PPIX as independent ATDH risk factors. A predictive model combining the three variables achieved an area under curve (AUC) of 0.714 (95% CI, 0.639~0.790), with improved AUC in moderate-severe cases (AUC, 0.782; 95% CI, 0.688~0.877). **Conclusion**: The present study provides the first clinical evidence linking baseline serum ZnPP/PPIX levels with ATDH risk, suggesting their potential as predictive biomarkers, especially for moderate-severe cases.

Keywords: Anti-tuberculosis drug-induced hepatotoxicity; Case-control study; Protoporphyrin IX; Zinc protoporphyrin

Submitted on 16-05-2025 – Revised on 10-06-2025 – Accepted on 09-12-2025

INTRODUCTION

Tuberculosis (TB) is a chronic *Mycobacterium tuberculosis* infection that continues to be a serious public health challenge. It was reported by the World Health Organization (WHO) that approximately 8.2 million newly diagnosed TB cases were identified in 2023, with 1.25 million deaths globally(World Health Organization, 2024). WHO-recommended TB therapy involves a multidrug regimen of isoniazid (INH), rifampicin (RIF), ethambutol and pyrazinamide. The treatment cycle typically includes a 2-month intensive period and a 4-month consolidation period (World Health Organization, 2017).

However, prolonged use of multiple medications can trigger several adverse reactions, leading to drug interruption, increasing the risk of multidrug resistant TB, which ultimately results in therapeutic failure, TB recurrence or fatal outcomes (El Hamdouni *et al.*, 2020). Anti-TB drug-induced hepatotoxicity (ATDH) is a frequent adverse effect, typically arising within the initial 3 months of TB treatment. The presentation ranges from asymptomatic or mild liver transaminase elevation to fatal hepatic failure (Choi *et al.*, 2022). A worldwide meta-analysis revealed an 11.50% prevalence of ATDH and demonstrated an increasing trend from 1999-2020 (Zhang *et al.*, 2016). Another prospective cohort study from China reported that 9.8-12.9% of patients with TB developed ATDH during treatment, which notably decreased the rate of sputum conversion and lung cavity closure, leading to treatment failure (Zhang *et al.*, 2016; Sun *et al.*, 2016). Therefore, ATDH remains a formidable challenge for TB treatment and control.

Existing evidence revealed that INH induces hepatotoxicity through acetylisoniazid accumulation catalyzed by N-acetyltransferase and its subsequent excretion through glutathione S-transferase enzyme (Ulanova *et al.*, 2024). Moreover, RIF can cause liver injury through direct toxic effects on hepatocytes and the development of cholestasis (Ezhilarasan, 2023). A previous animal and cellular experiment demonstrated that INH-RIF coadministration induces hepatic protoporphyrin IX (PPIX) accumulation via pregnane X receptor (PXR)-regulated heme biosynthesis dysregulation, suggesting a novel ATDH mechanism (He *et al.*, 2017; Brewer *et al.*, 2019; Wang *et al.*, 2020). PPIX is a pro-oxidant and the accumulation of PPIX could increase protein oxidation and mitochondrial oxidative stress and lead to disruptions of mitochondrial function and alter the balance of biliary bile acids, phospholipids and cholesterol, finally elevating hepatotoxicity risk (Martínez *et al.*, 2023; He *et al.*, 2017; Lyoumi *et al.*, 2011; Wang *et al.*, 2020). INH/RIF treatment significantly reduced ferrochelatase (FECH) expression in mRNA and protein levels in both mouse and

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human hepatocytes (He *et al.*, 2017). FECH is known to be the rate-limiting enzyme that catalyzes PPIX and Fe^{2+} to synthesize heme and is regulated by PXR (Li *et al.*, 2013; Zhang *et al.*, 2022). Additionally, long-term INH treatment results in hepatic PPIX accumulation through PXR-mediated FECH inhibition and aminolevulinate synthase 1 (ALAS1) upregulation, the key regulatory enzyme of porphyrin synthesis (Sachar *et al.*, 2016; Zhang *et al.*, 2022). Therefore, INH and RIF disrupt PXR-mediated heme biosynthesis pathway by upregulating ALAS1 and suppressing FECH, ultimately leading to pathological PPIX accumulation and hepatotoxicity (Sachar *et al.*, 2016). Zinc protoporphyrin (ZnPP) forms through the binding of zinc ions to PPIX, constituting a normal metabolite produced in small amounts during heme biosynthesis (Schliemann *et al.*, 2023). Typically, iron is the preferred metal to chelate with PPIX in heme synthesis (Obi *et al.*, 2022); however, in conditions such as iron deficiency or impaired iron utilization, excess free PPIX may accumulate and zinc can substitute as an alternative substrate, enhancing ZnPP biosynthesis and accumulating in reticulocytes (Labbe *et al.*, 1999). Therefore, the ZnPP level in peripheral blood may be associated with the PPIX level and consequently correlate with liver injury risk.

ZnPP has commonly been used for the clinical diagnosis of heme synthesis disorders such as lead poisoning and anemia (Leventi *et al.*, 2021; Słota *et al.*, 2021). A previous study suggested that ZnPP induced apoptosis and exacerbated liver ischemia/reperfusion injury *in-vitro* (Wang *et al.*, 2014). Another study using human blood samples reported a significant association between blood PPIX levels after anti-TB treatment and the occurrence of ATDH ($P<0.01$) (Zhang, 2020). However, the relationship between baseline ZnPP and PPIX levels, as well as and their dynamic changes during therapy with ATDH risk remains unclear. This study was conducted to investigated serum ZnPP and PPIX associations with ATDH risk, describe the changes of ZnPP and PPIX levels during anti-TB treatment and evaluate the discriminatory ability of ZnPP and PPIX levels as potential biomarkers for ATDH, thereby facilitating personalized treatment for patients with TB.

MATERIALS AND METHODS

Study population

Participants on anti-TB regimens were enrolled from four hospitals in Jiangsu Province, China (The People's Hospital of Taixing, The People's Hospital of Jurong, The Second People's Hospital of Changshu and The Third People's Hospital of Zhenjiang) from June 2017 to June 2021. The study protocol was approved by Nanjing Medical University's Ethics Committee (Nanjing, China) and in comply with the Helsinki Declaration principles, with all participants providing documented informed consent before study enrollment.

Participants met the following inclusion criteria: i) Newly-diagnosed TB and current administration of treatment regimens based on first-line anti-TB drugs recommended by WHO (World Health Organization, 2017); ii) during the first 2 months of treatment, performance of regular liver function tests, with available blood samples at ≥3 different time points (initial anti-TB treatment and two subsequent routine liver function tests); and iii) obtained written informed consent. Furthermore, participants meeting these criteria were excluded: i) Inability to complete the related epidemiological investigation due to mental illness; ii) diagnosis of critical medical conditions, such as neoplasms and survival time of <6 months; iii) abnormal levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBil) and/or alkaline phosphatase (ALP) prior to starting anti-TB treatment; iv) administration of other hepatotoxic drugs such as acetaminophen, carbamazepine and amoxicillin clavulanic acid during anti-TB treatment; v) coinfection with other infectious diseases, including acquired immune deficiency syndrome or syphilis; and vi) diagnosis of anemia.

After initiating the anti-TB treatment, all patients were followed up until completion of therapy unless they withdrew from the study.

Definitions

The definition of ATDH followed the Tuberculosis Branch of the Chinese Medical Association criteria: ALT ≥3 times the upper limit of normal (ULN) and/or TBil ≥2 ULN, or when AST, ALP and TBil are elevated simultaneously, with ≥1 being ≥2 ULN (Chinese Society of Tuberculosis, 2019); The updated Roussel Uclaf Causality Assessment Method (RUCAM) results were possible (3-5 score), probable (6-8 score) or highly probable (>8 score) (Danan *et al.*, 2015). Patients meeting the ATDH criteria were assigned to the case group, while participants maintaining normal liver function during anti-TB treatment served as controls. Liver injury severity followed WHO classifications: mild (ALT <5ULN), moderate (5 ULN ≤ ALT ≤10 ULN) or severe (ALT >10 ULN) (Tostmann *et al.*, 2008). Hepatoprotectant use including silibinin, bicyclol, compound glycyrrhizin and reduced glutathione and so on.

ZnPP and PPIX quantification

Blood draws were performed by nurses between 7:30 and 9:30 a.m., following an 8-hour overnight fast. Samples were then centrifuged by trained technicians and stored at -80˚C pending testing. Serum samples selected for subsequent testing were taken at baseline (the time of initial anti-TB treatment), at the first abnormal liver function time point and peak liver function time point (for ATDH cases) and at two subsequent routine liver function tests (for controls). ZnPP and PPIX levels were quantified using human-specific enzyme-linked immunosorbent

assay (ELISA) kits (Shanghai Enzyme-linked Immunoassay Biotechnology Co., Ltd) (Tabatabaei *et al.*, 2022). The detection ranges were 5-180 pg/mL for ZnPP and 0.2-9 ng/mL for PPIX (samples were 5-fold dilution prior to analysis). The optical density values were measured at 450 nm and ZnPP and PPIX levels were quantified using standard curves established per assay (Figs. S1 and S2). To ensure data quality, 5% of samples covering high, medium and low concentration ranges were selected for repeat testing, which demonstrated ≤15% absolute bias and the coefficient of variation ranging from 5.6%-7.9% (Tiwari *et al.*, 2010).

Statistical analysis

Continuous data are expressed as either mean ± standard deviation or median (interquartile range), with intergroup comparisons analyzed by t-tests or Wilcoxon tests. Categorical variables are described as counts (n) and differences were compared using unconditional logistic regression analysis. The treatment regimens were compared between the two groups using χ^2 test. Receiver operating characteristic (ROC) analysis was employed to assess biomarkers' discrimination capacity. The optimal ZnPP and PPIX thresholds were determined by maximizing Youden index (sensitivity + specificity - 1), with area under the curve (AUC) and 95% confidence intervals (CIs) were obtained. Univariate and multivariate logistic regression were employed to identify ATDH risk factors. Multicollinearity was assessed using tolerance (TOL) and variance inflation factor (VIF) (Wei, 2019). Factors that did not exhibit collinearity ($0.9 < TOL < 1$; $VIF < 10$) were incorporated into the multivariate model, with outcomes reported as odds ratios (ORs) with 95% CIs. For subgroup analysis, patients were stratified by hepatotoxicity severity (mild vs. moderate-severe) and ROC curves were generated to evaluate the predictive ability for ATDH in each group as previously described. Statistical analysis was performed in R 4.3.1 (The R Foundation). Graphical outputs were generated with R v4.3.1 and GraphPad Prism 9.1.0.221 (Dotmatics). All analyses used two-tailed testing with $P<0.05$ significance threshold.

RESULTS

Characteristics of patients undergoing anti-TB treatment

The present study included 249 patients, comprising 58 ATDH cases and 191 controls. There were 199 patients who received the 2HREZ/4HR regimen, 17 patients received the 3HREZ/9HR regimen and 33 patients received other regimens. The case and control groups showed comparable treatment regimens ($P>0.05$).

Among the 58 ATDH cases, 20 cases (34.4%) were classified as possible, 28 cases (48.3%) were classified as probable and 10 cases (17.2%) as highly probable according to RUCAM criteria. The number of mild liver injury cases and moderate-severe cases was evenly distributed. The two groups showed comparable demographic and clinical characteristics, including age, sex, hepatoprotectant use or smoking and drinking history ($P>0.05$, Table 1). However, liver disease history was more prevalent in ATDH than controls (24.1 vs. 11.5%; P=0.017). Moreover, the median baseline AST level of ATDH cases was significantly elevated compared to controls (22.0 vs. 19.0 U/l; P=0.005). ATDH cases exhibited significantly elevated peak liver function values compared to controls ($P<0.001$, Table 1).

Distribution of ZnPP and PPIX levels

ATDH cases and controls showed comparable testing time intervals (P=0.645 and 0.761, respectively; Table 2). Furthermore, ATDH patients demonstrated significantly lower baseline ZnPP levels than controls (113.1 vs. 147.9 pg/ml; P=0.002; Fig. 1A) and the baseline PPIX levels were significantly elevated in the ATDH group (8.6 vs. 7.0 ng/ml; P=0.003; Fig. 1B). ATDH cases showed significantly lower ZnPP levels than controls at the first abnormal liver function time point (134.3 vs. 152.0 pg/ml; P=0.031) and peak liver function time point (119.0 vs. 147.5 pg/ml; P=0.010) (Fig. 1A), while the PPIX levels were reversed (first abnormal liver function time point, 9.4 vs. 7.6 ng/ml; P=0.002 and peak liver function time point, 9.1 vs. 7.1 ng/ml; P=0.001; Fig. 1B and Table 2). Although absolute ZnPP levels remained lower in the ATDH group, the changes from baseline were not significantly different between groups (P=0.493 and P=0.886, respectively), while the PPIX changes from baseline at both follow-up timepoints were significantly larger in the ATDH group (P=0.004 and $P<0.001$, respectively) (Table 2).

The ROC analysis demonstrated that the AUC of ZnPP and PPIX were 0.638 and 0.629 respectively (Table 3), which exhibit superior predictive performance for ATDH compared to conventional liver function biomarkers such as ALT (AUC=0.575), AST (AUC=0.621), TBil (AUC=0.574), DBil (AUC=0.549) and ALP (AUC=0.538) (Table S1).

Logistic regression and logistic ROC curve analysis

In the univariate analysis, liver disease history (OR =2.444; 95% CI, 1.157~5.162; P=0.019), baseline ZnPP (OR =0.996; 95% CI, 0.993~1.000; P=0.049) and PPIX (OR =1.074; 95% CI, 1.025~1.126; P=0.003) were significantly associated with ATDH (Table 3). After removing the variables with multicollinearity, the multivariate model incorporated eight variables, identifying liver disease history (OR =2.422; 95% CI, 1.076~5.451; P=0.033), baseline ZnPP (OR =0.995; 95% CI, 0.991~0.999; P=0.013) and PPIX (OR =1.099; 95% CI, 1.041~1.161; P=0.001) as independent risk factors of ATDH (Table 3). After including the aforementioned three variables in logistic regression model, ROC analysis achieved an AUC of 0.714 (95% CI, 0.639~0.790; $P<0.001$; Fig. 2 and table 4).

Table 1 Characteristics of ATDH cases and controls.

Variables	ATDH cases (n=58)	Controls (n=191)	P value
Age (years)[a]	46.6±16.9	48.9±17.2	0.409[c]
Sex (male/female)	40/18	149/42	0.158[d]
Hepatoprotectant (use/not use)	22/36	67/124	0.691[d]
Liver disease history (yes/no)	14/44	22/169	0.017[d]
Smoke history (yes/no)	5/53	14/177	0.746[d]
Drink history (yes/no)	2/56	6/185	0.908[d]
Treatment regimen			0.669
2HREZ/4HR[*]	44	155	
3HREZ/9HR[*]	5	12	
Other[#]	9	24	
Baseline value			
ALT (U/L)[b]	16.0 (9.5~16.8)	12.0 (8.8~17.0)	0.083[e]
AST (U/L)[b]	22.0 (18.8~24.4)	19.0 (16.0~22.1)	0.005[e]
TBil (μmol/L)[b]	10.5 (8.8~12.1)	9.8 (7.0~12.6)	0.089[e]
DBil (μmol/L)[b]	3.5 (2.1~4.0)	3.3 (2.3~4.3)	0.259[e]
ALP (U/L)[b]	83.0 (68.6~91.2)	82.5 (66.0~94.0)	0.376[e]
During treatment (peak value)			
ALT (U/L)[b]	195.5 (149.3~317.5)	22.1 (14.8~33.4)	<0.001[e]
AST (U/L)[b]	125.4 (80.0~264.5)	27.5 (22.8~34.9)	<0.001[e]
TBil (μmol/L)[b]	19.4 (15.0~30.3)	13.8 (10.3~19.1)	<0.001[e]
DBil (μmol/L)[b]	7.7 (6.0~13.2)	5.1 (3.4~7.1)	<0.001[e]
ALP (U/L)[b]	123.5 (89.8~176.3)	89.0 (74.0~112.6)	<0.001[e]

ATDH, anti-tuberculosis drug-induced hepatotoxicity; ALT, alanine transaminase; AST, aspartate transaminase; TBil, total bilirubin; DBil, direct bilirubin; ALP, alkaline phosphatase. Normal range: ALT < 40U/L; AST < 40U/L; TBil < 21μmol/L; DBil < 6.9μmol/; ALP < 130U/L. [a] Values are presented as mean ± standard deviation (range); [b] Values are presented as median (interquartile range); [c]Independent-samples t-test; [d] Unconditional logistic regression; [e] Wilcoxon rank-sum test. [*]H: isoniazid, R: ifampin, E: ethambutol, Z: pyrazinamide. [#]Modified first-line regimen.

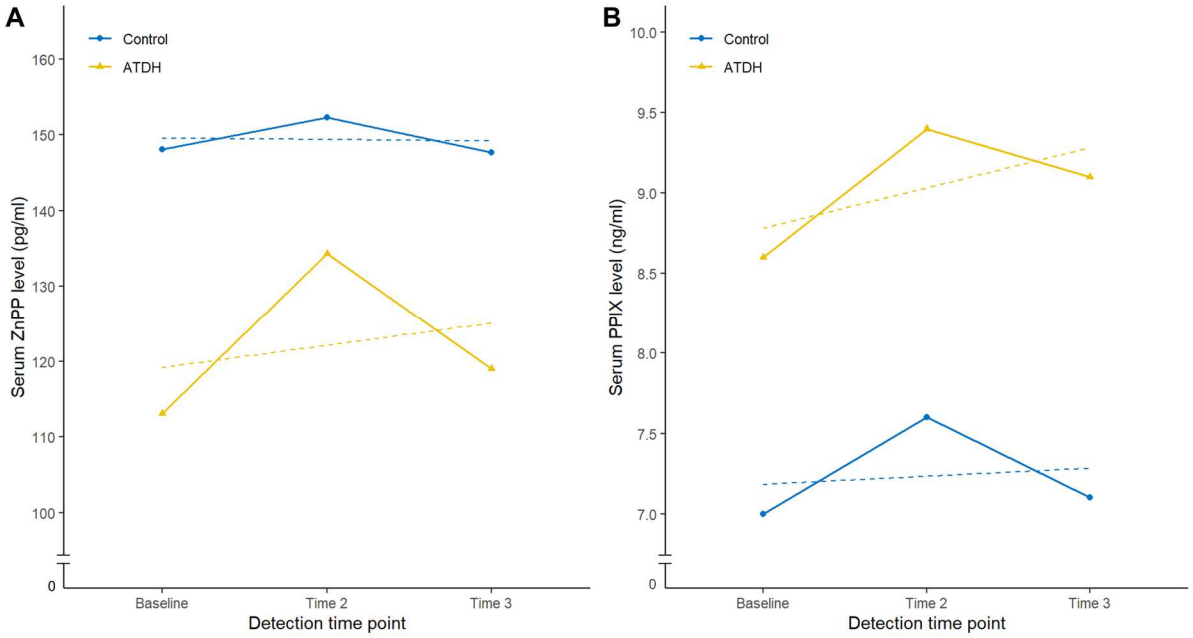

Fig. 1: Serum ZnPP (pg/mL) and PPIX (ng/mL) levels at three detection time points.
(The line plots show the temporal trends of ZnPP (A) and PPIX (B) levels across three time points in the ATDH and control groups. In the ATDH group, time 2 and time 3 refer to the first abnormal liver function test and the peak liver function test time points, and in control group refers to two subsequent liver function tests after baseline. The two time points represents approximately 24-25 days (Time 2) and 61-63 days (Time 3) from baseline respectively) (ATDH, anti-tuberculosis drug-induced hepatotoxicity; ZnPP, zinc protoporphyrin; PPIX, protoporphyrin IX)

Table 2: Comparison of serum indexes between ATDH cases and controls.

Variables	Time points	ATDH cases (n=58)	Controls (n=191)	Median difference (95% CI)	P value*
Days from baseline to detection time (days)	Baseline	-	-		-
	Time 2[b]	25.0 (12.0~49.0)	24.0 (10.0~40.0)	-1.0 (-6.0~4.0)	0.645
	Time 3[c]	63.0 (33.0~119.25)	61.0 (33.0~105.0)	-2.0 (-15.0~11.0)	0.761
ZnPP (pg/ml)[a]	Baseline	113.1 (73.4~187.4)	147.9 (118.1~187.5)	33.8 (13.7~51.7)	0.002
	Time 2[b]	134.3 (83.4~206.8)	152.0 (124.1~191.5)	25.4 (2.8~45.9)	0.031
	Time 3[c]	119.0 (80.7~188.3)	147.5 (120.5~198.8)	28.9 (7.5~48.3)	0.010
	ΔT1[d]	8.5 (-14.0~30.2)	5.7 (-21.5~31.8)	-4.7 (-19.0~8.4)	0.493
	ΔT2[e]	1.0 (-19.4~39.9)	7.1 (-36.4~35.4)	-1.3 (-18.7~14.7)	0.886
PPIX (ng/ml)[a]	Baseline	8.6 (5.7~17.6)	7.0 (5.2~8.5)	-1.7 (-3.1~-0.6)	0.003
	Time 2[b]	9.4 (5.9~26.2)	7.6 (5.8~9.9)	-2.3 (-4.4~-0.8)	0.002
	Time 3[c]	9.1 (6.4~37.5)	7.1 (5.5~9.6)	-2.7 (-6.7~-1.0)	0.001
	ΔT1[d]	1.9 (-0.8~10.4)	0.41 (-0.44~1.89)	-1.5 (-2.8~-0.5)	0.004
	ΔT2[e]	2.9 (-0.3~24.4)	0.37 (-0.74~1.51)	-3.2 (-7.2~-1.3)	<0.001

ATDH, anti-tuberculosis drug-induced hepatotoxicity; ZnPP, zinc protoporphyrin; PPIX, protoporphyrin IX. [a] Values are presented as median (interquartile range). [b] The time 2 in the case group refer to the first abnormal liver function detection time point. [c] The time 3 in the case group refer to the peak liver function detection time point. [d] The change at time 2 relative to the baseline. [e] The change at time 3 relative to the baseline. * Wilcoxon rank-sum test.

Table 3: Univariate and multivariate logistic regression analysis of ATDH influencing factors.

Inclusion indicators*	Univariate		Multivariate	
	OR (95%CI)	P value	OR (95% CI)	P value
Age (years)	0.992 (0.976~1.010)	0.384	-	-
Sex (male/female)	1.596 (0.831~3.068)	0.160	-	-
Liver disease history (yes/no)	2.444 (1.157~5.162)	0.019	2.422 (1.076~5.451)	0.033
Smoking history (yes/no)	1.193 (0.411~3.464)	0.746	-	-
Hepatoprotectant (use/not use)	1.131 (0.616~2.077)	0.691	-	-
Baseline TBil (μmol/L)	1.069 (0.997~1.145)	0.061	-	-
Baseline AST (U/L)	1.039 (0.992~1.088)	0.105	-	-
Baseline ALP (U/L)	1.007 (0.993~1.021)	0.347	-	-
Baseline PPIX (ng/ml)	1.074 (1.025~1.126)	0.003	1.099 (1.041~1.161)	0.001
Baseline ZnPP (pg/ml)	0.996 (0.993~1.000)	0.049	0.995 (0.991~0.999)	0.013

ATDH, anti-tuberculosis drug-induced hepatotoxicity; TBil, total bilirubin; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; PPIX, protoporphyrin IX; ZnPP, zinc protoporphyrin; SE, standard error; OR, odds ratio; 95%CI, 95% confidence interval; TOL, tolerance; VIF, variance inflation factor. *Collinearity diagnostic: 0.9 < TOL < 1, VIF < 10.

Table 4: ROC curve parameters for discrimination of ATDH.

Group	Variables	Cut-off value	AUC (95% CI)	P value
All ATDH	ZnPP (pg/mL)	94.59 pg/ml	0.638 (0.543~0.732)	0.002
	PPIX (ng/mL)	8.86 ng/ml	0.629 (0.541~0.718)	0.003
	ZnPP+PPIX	-	0.695 (0.615~0.776)	<0.001
	ZnPP+PPIX+liver disease history	-	0.714 (0.639~0.790)	<0.001
Moderate-severe ATDH	ZnPP+PPIX+liver disease history	-	0.782 (0.688~0.877)	<0.001
Mild ATDH	ZnPP+PPIX+liver disease history	-	0.672 (0.569~0.775)	0.003

Subgroup analysis

ROC analysis demonstrated that ZnPP, PPIX and liver disease history had an improved discriminatory ability in moderate-severe cases (AUC =0.782; 95% CI, 0.688~0.877; P<0.001) compared with in mild cases (AUC =0.672; 95% CI, 0.569~0.775; P=0.003) (Fig. 2 and Table 4). Furthermore, when comparing differences between the subgroups, no significant baseline differences were observed, such as age, sex, hepatoprotectant use, liver disease history, smoking and drinking, or liver biochemical parameters (ALT, AST, TBil, DBil and ALP) between the moderate-severe and mild ATDH subgroups (P>0.05; Table S2). The moderate-severe group exhibited significantly elevated ALT, AST and TBil levels at peak liver function time point compared to the mild group (P<0.05; Table S2). However, ZnPP and PPIX levels showed no significant difference between groups across three time points, nor their changes (P>0.05; Table S3).

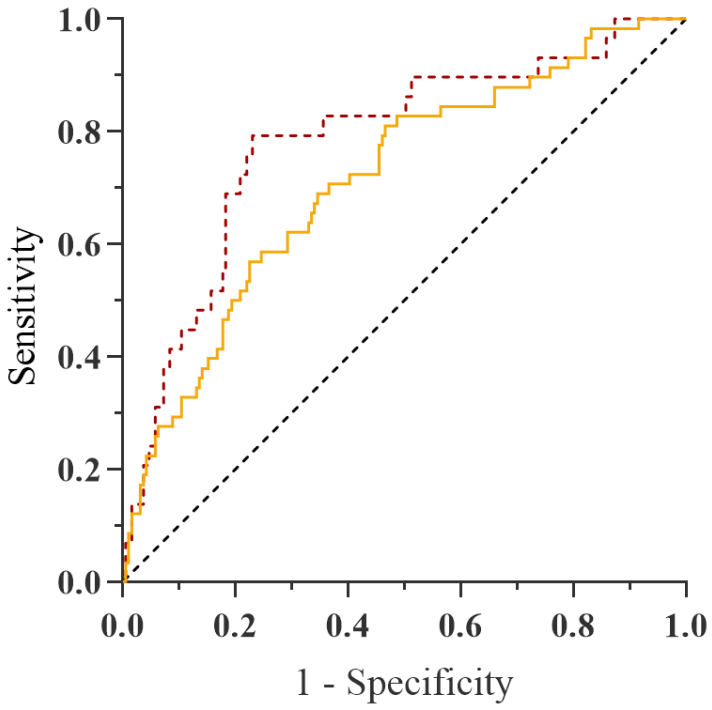

Fig. 2: ROC curves of ZnPP (pg/mL) + PPIX (ng/mL) + liver disease history for distinguishing ATDH and moderate-severe ATDH cases.
(The yellow solid line represented ROC curve for all ATDH cases, and red dashed line represented for moderate-severe ATDH cases)
(ATDH, anti-tuberculosis drug-induced hepatotoxicity; ZnPP, zinc protoporphyrin; PPIX, protoporphyrin IX; ROC, receiver operating characteristic curve; AUC, area under the curve)

DISCUSSION

PPIX is considered an endogenous hepatotoxic substance metabolized by the liver (Sachar *et al.*, 2016). Previous studies have reported a significant downregulation of FECH and an upregulation of ALAS1 protein expression in mouse liver after treatment with INH and RIF, with a significant increase of PPIX level (P<0.01) (He *et al.*, 2017; Wang *et al.*, 2020). A randomized control trial reported that RIF increased the mean uroporphyrin concentration 3.7-fold in comparison with the placebo (1.80±0.6 vs. 6.73±4.4 nmol/l; P=0.003) (Tolonen *et al.*, 2023). During the catalysis by FECH, excessive PPIX combines with zinc ions to generate an increased amount of ZnPP. Accumulated PPIX in liver induces endoplasmic reticulum stress, proteasome inhibition and disruption of mitochondrial function, resulting in hepatocellular damage (He *et al.*, 2017; Wang *et al.*, 2020). As PPIX is secreted from the liver into the biliary system, excess PPIX can accumulate and obstruct in the bile duct, which may lead to cholestatic liver injury (Levy *et al.*, 2025). A previous study using PXR-humanized mice showed RIF and INH coadministration significantly elevated ALT (P<0.01) and ALP (P<0.05) activity, with bile plugs in the liver observed (Li *et al.*, 2013). Additionally, research has reported that blood PPIX levels in patients who received anti-TB treatment were significantly higher compared to untreated patients (P<0.01) and blood PPIX levels after anti-TB treatment showed a significant association with ATDH risk (P<0.01) (Zhang, 2020). Aligning with prior reports, the present study revealed that PPIX levels were consistently elevated in the ATDH group compared to controls across all three time points, with significantly greater changes observed in the case group. The serological test results of these patients undergoing anti-TB treatment also further demonstrate the role of abnormal PPIX synthesis pathway in the occurrence of ATDH.

In addition to the direct association between PPIX and ATDH, the present study also demonstrated that ZnPP has a certain ability to identify hepatotoxicity. The ATDH group exhibited consistently lower ZnPP levels than controls across all three detection time points. Under physiological conditions, zinc serves as an alternative substrate of FECH to form ZnPP in trace amounts. The reduced baseline ZnPP levels in the case group may reflect lower FECH activity and slow metabolic clearance of PPIX, potentially predisposing to more PPIX accumulation in subsequent anti-tuberculosis therapy (Paul *et al.*, 2021; He *et al.*, 2017). As a byproduct of heme synthesis, ZnPP is widely used as a biomarker for heme synthesis disorders, including lead poisoning and anemia (Leventi *et al.*, 2021; Słota *et al.*, 2021). Furthermore, ZnPP competitively inhibits heme oxygenase-1 (HO-1), an enzyme against

oxidative stress in cells (Wang *et al.*, 2015). In neonatal rats, intraperitoneal injection of ZnPP significantly inhibited hepatic heme oxygenase (HO) activity (P<0.01) (Fujioka *et al.*, 2016), suggesting its potential as a treatment for neonatal jaundice. HO-1 expression is upregulated in cancerous tissues, making ZnPP a valuable tool in cancer therapies (Cheng *et al.*, 2016; Li *et al.*, 2023). Additionally, previous study has suggested that ZnPP induces apoptosis and exacerbates hepatic ischemia/reperfusion injury *in vitro* through HO-1 inhibition (Wang *et al.*, 2014). However, these studies were usually performed at higher concentrations of ZnPP, which may not be consistent with the low levels of endogenous ZnPP *in-vivo* (Paul *et al.*, 2021). Moreover, previous research reported that patients hepatic comorbidities such as non-alcoholic fatty liver (P<0.05) (Liu *et al.*, 2021), hepatitis C [hazard ratio (HR) =3.425; 95% CI, 1.752~6.697; P<0.001] and hepatocellular carcinoma (HR =3.361; 95% CI, 1.212~9.322; P=0.02) (Kim *et al.*, 2016) may have an significantly increased risk of liver injury. Multivariate logistic regression indicated that liver disease history and baseline ZnPP and PPIX were independent risk factors of ATDH. The ROC analysis of ZnPP + PPIX + liver disease history for discriminating ATDH achieved an AUC of 0.714 and subgroup analysis further revealed that these three variables had an improved discriminatory ability for moderate-severe liver injury (AUC =0.782) compared with overall cases. These findings indicate that ZnPP + PPIX + liver disease history has a certain ability to distinguish liver injury cases, especially for moderate-severe cases. Baseline ZnPP/PPIX tests could help identified high-risk patients (Baseline PPIX >8.86 ng/ml and ZnPP <94.59 pg/ml), enable individualized treatment and timely interventions, potentially reducing the incidence of liver injury. In addition, the return of ZnPP and PPIX levels to baseline at peak liver function time point suggests that these biomarkers may primarily reflect early-stage liver injury. The monitoring of ZnPP/PPIX can be performed simultaneously with routine liver function tests (WHO recommends once a week during the first two months), enabling earlier detection of liver injury before clinical symptoms or biochemical abnormalities arise and could help guide adjustments to anti-tuberculosis drug regimens and hepatoprotectant use.

Although the present study firstly evaluated the correlations between serum ZnPP/PPIX levels and ATDH risk during anti-TB treatment, a few limitations warrant mention. First, the present study focused exclusively on serum ZnPP and PPIX levels, without concurrently assessing their concentrations in urine and red blood cells. While measuring these samples could provide a more comprehensive understanding, urine concentrations are highly influenced by hydration status and measuring metabolites in red blood cells is usually complex, making them unsuitable for clinical application (Weaver *et al.*, 2016). This strategic focus on serum was designed to develop clinically relevant biomarkers that could be readily implemented in anti-TB treatment monitoring (Fernández Ventureira *et al.*, 2021). Second, while several mechanisms are well understood, including the accumulation of acetylisoniazid and RIF induced cholestasis (Ulanova *et al.*, 2024; Ezhilarasan, 2023), the present study focused solely on the novel mechanism of bilirubin synthesis pathway to identify new biomarkers. This design allowed for the exploration of new mechanisms, potentially leading to innovative biomarker discovery. Future studies should integrate other established mechanisms with current findings to further develop a more comprehensive predictive model. By ensuring that all included patients received a first-line anti-TB regimen recommended by WHO, the present study minimized treatment heterogeneity, thereby enhancing the comparability of results. However, the limitation in treatment variability may restrict the generalizability to different clinical settings. Further studies should expand to multiple drug resistant TB cohorts receiving second-line drugs to assess regimen-specific effects. Finally, the present data were derived from individuals of the Han ethnic population in the eastern part of China; a single genetic background and diagnostic criteria may limit the generalizability of the findings (Cheng *et al.*, 2025; Chinese Society of Tuberculosis, 2019). Further studies in larger anti-TB treatment cohorts encompassing diverse racial backgrounds are needed to further validate the potential predictive ability of ZnPP and PPIX for ATDH.

CONCLUSION

This study provides the first evidence linking serum ZnPP/PPIX levels to ATDH risk in Chinese anti-TB treated patients. The present case-control study revealed that ATDH cases had significantly elevated PPIX levels than controls, while the opposite was demonstrated for ZnPP. These findings indicate that baseline ZnPP and PPIX levels combined with a history of liver disease may be potential predictive factors for ATDH, especially for moderate-severe cases. However, further investigation across expanded demographic groups is needed.

Acknowledgments

We would like to thank The People's Hospital of Taixing, The People's Hospital of Jurong, The Second People's Hospital of Changshu and The Third People's Hospital of Zhenjiang in Jiangsu province, China, for their support of this study. We also extend our gratitude to all the individuals who provided valuable assistance throughout the research process.

Authors' contributions

All authors contributed to the conception and design of this study. MLZ., LZL and XYX: Performed material preparation; MZ, FW, LZL, YW, RNC and JRC: Conducted experiments; MZ and FW: Performed data collection; MZ and FW: Performed data analysis; MZ and

FW: Wrote the first draft of the manuscript; SWT: Critically revised the manuscript and approved the final version of the manuscript. All authors have read and approved the final manuscript.

Funding
This study was funded by the National Natural Science Foundation of China (82073614), the Medical Research project of Jiangsu Provincial Health Commission (Z2022089), the Jiangsu University General project of Medical Education Collaborative Innovation Fund (JDYY2023039), and the Changshu Science and Technology Development Plan Project (CS202230).

Data availability statement
The data generated and analyzed in the study are available from the corresponding author upon reasonable request.

Ethical approval
The study was approved by the Ethics Committee of Nanjing Medical University (No. (2018)579) and was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from every participant before enrolment.

Conflict of interest
The authors declare no conflict of interest.

Supplementary data
https://www.pjps.pk/uploads/2026/07/SUP1783416592.pdf

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