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Original Article

EVALUATION OF SERUM LEVELS OF CYP2D6, CYP3A4, GSH, AND MDA AS PREDICTIVE BIOMARKERS FOR METHAMPHETAMINE-INDUCED LIVER DYSFUNCTION USING ADVANCED STATISTICAL MODELING

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ABSTRACT

Objective: This study aimed to evaluate the predictive potential of serum protein levels of CYP2D6, CYP3A4, glutathione (GSH), and malondialdehyde (MDA) as early biomarkers of METH-induced liver dysfunction using multivariate statistical approaches, compared to standard LFTs.

Methods: This case–control study included 90 METH users and 45 healthy control individuals. Serum CYP2D6, CYP3A4, GSH, and MDA levels, and routine LFTs were determined. Analysis was performed using principal component analysis (PCA), receiver operating characteristic (ROC) curves, and decision tree modeling.

Results: METH users showed significantly decreased serum levels of CYP2D6, CYP3A4, and GSH and higher MDA levels ($p<0.0001$), suggestive of oxidative metabolic disequilibrium. While most routine LFTs were normal, other markers, including AST, AST/ALT ratio, and albumin-to-globulin ratio, increased significantly. PCA demonstrated that early injury components could be divided into separate groups according to the markers of their metabolic and oxidative components. CYP2D6 ≤ 1.98 ng/ml was the best discriminating predictor according to the decision tree with 93.3% accuracy (AUC = 94).

Conclusion: Serum protein levels of CYP2D6, CYP3A4, GSH, and MDA demonstrated high sensitivity for detecting subclinical liver injury in METH users before conventional LFTs became abnormal. Their integration into diagnostic models may facilitate early interventions in high-risk populations.

Keywords: Methamphetamine, Cytochrome P-450 CYP2D6, Cytochrome P-450 CYP3A4, Glutathione, Malondialdehyde, Principal component analysis

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INTRODUCTION

Cytochrome P450 enzymes (CYPs) are a group of heme-containing enzymes that play an important role in the metabolism of many drugs and other xenobiotics. CYP enzymes are located in the endoplasmic reticulum of cells throughout the body but are most abundant in the liver. CYP enzymes can catalyze an extensive range of reactions including oxidation, reduction, hydrolysis, and isomerization [1]. Of the 57 potentially functional human CYP enzymes, members of the CYP1, CYP2, and CYP3 families account for nearly 80% of clinically used drugs [2]. CYP-dependent metabolism not only transforms lipophilic compounds into water-soluble forms for easier elimination but also influences therapeutic outcomes by modulating drug efficacy, safety, bioavailability, and resistance, both in major metabolic organs and at local sites of drug action, with CYP3A4 and CYP2D6 contributing to over 50% of CYP-related drug metabolism [3, 4].

Among the CYP isoforms, CYP2D6 and CYP3A4 have received a great deal of clinical attention mainly because they are two important enzymatic members in phase I studies (75% drugs) with unique regulatory pathways [5]. 2D6 mediates the oxidative metabolism of 20-25% of drugs used in a clinical setting. Most individuals have highly variable enzymatic activity owing to genetic polymorphisms, which can lead to phenotypes of poor or ultrarapid metabolizers [6, 7]. In contrast, the most abundant hepatic isoform, CYP3A4, is involved in metabolism in more than 30% of clinically recommended drugs. In contrast to CYP2D6, factors other than genotype influence their activity more substantially [5, 8, 9].

Methamphetamine (METH), a highly addictive psychostimulant, was originally synthesized from its related compound amphetamine [10]. The metabolism of METH is primarily mediated by hepatic CYPs, particularly CYP2D6 and CYP3A4, which convert it to its main metabolites amphetamine and 4-hydroxyamphetamine [10]. This metabolic process, which is essential for detoxification, is also a source of cellular stress. Enzymatic conversion can lead to the production of reactive oxygen species (ROS), a process known as bioactivation. This contributes to a state of oxidative stress that, in conjunction with other pathways, such as the activation of intracellular nitric oxide (NO), can overwhelm cellular antioxidant defenses and lead to hepatocyte injury and apoptotic cell death [11].

Exposure to METH is associated with oxidative stress, characterized by increased malondialdehyde (MDA) levels, a marker of lipid peroxidation, and decreased glutathione (GSH) levels, a vital intracellular antioxidant [12, 13]. The imbalance between MDA and GSH levels reflects oxidative damage, which plays a central role in METH-induced hepatotoxicity pathogenesis [13].

Principal component analysis (PCA) is a powerful multivariate statistical technique that reduces data dimensionality and explores latent patterns within complex datasets. PCA is widely used, especially in medical informatics, to discover underlying pathological axes grouping related biomarkers, thus providing exposure points for critical biochemical pathways [14, 15]. Concurrently, decision tree models present a method of supervised machine learning that classifies samples according to the most discriminative variable splitting, thereby providing interpretable prediction criteria [14]. Combining both methods would result in a better conception and categorization of METH-induced hepatic changes by biochemical parameters [14-16].

Despite growing recognition of the impact of METH on liver function and CYPs regulation, integrative studies that simultaneously examine its biochemical, enzymatic, and toxicological consequences within a single experimental framework are lacking [17]. To the best of our knowledge, multivariate statistical approaches, such as PCA and decision tree modeling, have rarely been used to identify predictive biomarkers or classification patterns in METH-induced hepatic dysfunction [18, 19]. Therefore, this study aimed to evaluate the detection and classification potential of serum levels of the

metabolic enzymes CYP2D6 and CYP3A4, the oxidative stress markers GSH and MDA, and conventional liver function tests (LFTs) in METH-induced hepatic dysfunction using PCA and decision tree classification as advanced statistical tools.

MATERIALS AND METHODS

Subject

This case-control study was conducted between January and June 2025. A total of 135 participants were enrolled, comprising 90 patients diagnosed with METH, and 45 age-and BMI-matched healthy individuals who served as controls. The participants were aged between 18 and 40 y old.

The inclusion criteria for the METH group were a confirmed diagnosis of METH use disorder by a psychiatrist according to the DSM-5 criteria, a history of chronic METH use (≥3 times per week for at least one year), and a positive urine screening test for amphetamines at the time of recruitment. The route of administration (smoking, injection, or oral administration) was not systematically recorded for all the participants. Exclusion criteria for all participants included known pre-existing liver diseases (e. g., hepatitis B/C and cirrhosis), comorbid substance use disorders (except nicotine), HIV infection, current use of medications known to affect CYPs activity or liver function, and any chronic medical conditions (e. g., diabetes and cardiovascular disease).

Ethical approval and informed consent

The study protocol was reviewed and approved by the Ethical Committee of the Al-Qanat Center for Social Rehabilitation in Baghdad, Iraq. This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all the participants after the nature of the study procedure was fully explained. (IRB 1068 12/01/2025)

Blood sample collection and biochemical analysis

Venous peripheral blood samples (5 ml) were collected from all participants. The samples were allowed to clot at room temperature for 30 min before being centrifuged at 1000 × g for 20 min to separate the serum. The collected serum was promptly aliquoted into sterile Eppendorf tubes and stored at − 80 °C until analysis. This procedure was implemented to minimize the potential proteolytic degradation of enzyme proteins and prevent the oxidation of sensitive analytes, such as glutathione [20].

Biochemical assays

Liver function tests

LFTs, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, and total serum bilirubin (TSB), were performed on the same day of blood collection using a Beckman Coulter 480 AU Chemistry Analyzer (Beckman Coulter, Germany).

ELISA kit validation

To ensure the reliability of ELISA data, the performance of each kit was validated in our laboratory. The intra-and inter-assay CVs values were<8% and<10%, respectively, for all analytes. Spike-and-recovery experiments demonstrated recovery rates between 90 and 110%. The assay also showed excellent linearity over the tested concentration range (R^2>0.99).

ELISA-based measurements

The serum protein concentrations of CYP2D6 (catalog no. EH0783; Fine Test, China), and CYP3A4 (Catalog No. EH0777; Fine Test, China) were measured using commercial sandwich ELISA kits. The serum levels of reduced glutathione (GSH) (catalog no. EH3524; Fine Test, China) and malondialdehyde (MDA) (Catalog No. ELK1135; ELK Biotechnology, China) was determined using a competitive inhibition ELISA kit. The ELISA measurements were conducted using a BioTek ELISA microplate reader (BioTek Instruments, USA). All assays were performed strictly in accordance with the manufacturer's protocols. To ensure the reliability of the ELISA data, the performance of each kit was validated using pooled serum samples. Intra-assay precision was determined by running 10 replicates of the same sample in a single assay, while inter-assay precision was determined by running the same sample in duplicate across five independent assay runs. The coefficients of variation (CVs) were within acceptable limits for all biomarkers.

Statistical analysis

The data were subjected to statistical analysis using the IBM SPSS Statistics software (version 27, IBM Corp., Armonk, NY, USA). Data are expressed as mean±SD. Comparisons between patients and normal controls were carried out by t-test, and p<0.001 and<0.05 were considered to be highly significant or significant differences, respectively. The Pearson's coefficient (r) was used to express the correlation between the studied parameters. The sensitivity and specificity of the tests were evaluated, and the cut-off value was established using receiver operating characteristic (ROC) curve analysis (performed using the ROC Curve module in SPSS). The interrelationships among biochemical variables were estimated using principal component analysis (PCA) with the Factor Analysis module in SPSS, and the potential interaction patterns among them were determined by a decision tree using the Chi-squared Automatic Interaction Detection (CHAID) method (performed using the Decision Tree module in SPSS) [21]. All advanced statistical analyses (ROC, PCA, and CHAID) were performed using SPSS.

RESULTS

There was no statistically significant difference in age and body mass index (BMI) between the METH use disorder and control groups (table 1). Patients had significantly lower serum protein levels of CYP2D6 and CYP3A4 and lower serum GSH levels than controls (p<0.0001). They also had significantly higher levels of MDA, AST/ALT, albumin, globulin, and A/G ratio.

Table 1: Comparison of demographic, biochemical and enzymatic markers between METH users and healthy controls

Parameters	Control group (n=45) mean±SD	METH patients (n=90) mean±SD	P value
Age (years)	27.75±7.49	30.25±6.15	0.096
BMI (kg/m^2)	22.64±2.30	23.44±2.47	0.064
CYP2D6 (ng/ml)	4.17±0.92	1.145±0.69	<0.0001**
CYP3A4 (pg/ml)	1539±448.65	483.43±180.5	<0.0001**
MDA (ng/ml)	21.63±1.511	27.70±2.80	<0.0001**
GSH (ng/ml)	101.43±1.74	59.01±9.34	<0.0001**
ALT (U/l)	28.06±8.57	31.2±14.82	0.123
AST (U/l)	21.95±8.00	26.5±13.20	0.01*
AST/ALT	0.810±0.26	1.008±0.61	0.009*
ALP (U/l)	82.53±14.30	88.34±31.41	0.142
TSB (mg/dl)	0.86±0.193	0.84±0.378	0.807
A/G	1.35±0.08	1.54±0.466	0.00022*

Data are presented as mean±SD. *P<0.05 is considered significant. **P<0.001 is considered highly significant. Abbreviations: BMI, body mass index; CYP2D6, cytochrome P450 2D6; CYP3A4, cytochrome P450 3A4; MDA, malondialdehyde; GSH, glutathione; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TSB, total serum bilirubin; A/G, albumin-to-globulin ratio.

CYP2D6 demonstrated high diagnostic performance (AUC = 0.995, sensitivity and specificity = 95.6%). MDA had excellent predictive ability, with an AUC of 0.967, sensitivity of 95.6%, and moderate specificity (77.8%). Notably, CYP3A4 and GSH showed perfect classification performance (AUC = 1.000) in the training dataset. Although these results are promising, perfect classifiers are rare in clinical practice and may reflect the homogeneity of our study cohorts or potential overfitting. All AUCs were highly significant (p<0.001), as shown in table 2.

Table 2: Diagnostic performance of biomarkers for differentiating METH users from controls

Marker	AUC	Cut-off	Sensitivity (%)	Specificity (%)	Accuracy (%)	P-Value	95% CL
CYP2D6	0.995	2.6100	95.6%	95.6%	95.6%	<0.001	0.989-1.000
CYP3A4	1.000	849.37	100%	100%	100%	<0.001	1.000-1.000
GSH	1.000	88.145	100%	100%	100%	<0.001	1.000-1.000
MDA	0.967	23.85	95.6%	77.8%	89.6%	<0.001	0.941-0.994

Abbreviations: AUC, area under the curve; CL, confidence limit; CYP2D6, cytochrome P450 2D6; CYP3A4, cytochrome P450 3A4; GSH, glutathione; MDA, malondialdehyde. PCA was conducted on ten biochemical variables, including metabolic enzymes, oxidative stress indicators, and liver function markers (table 3). The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was 0.591, indicating mediocre suitability for factor analysis.

Table 3: Suitability of data for principal component analysis (KMO and bartlett's test)

Kaiser-meyer-olkin measure of sampling adequacy		.591
Bartlett's test of sphericity	Approx. Chi-Square	815.514
	Df	45
	Sig.	.000

Abbreviations: KMO, Kaiser-Meyer-Olkin; Df, degrees of freedom; Sig., significance. Given the median KMO value (0.591), three factors with eigenvalues greater than 1 were retained, contributing to 71.305% of the total variance (table 4). The first, second, and third components explained 33.965%, 23.107%, and 14.233% of the variance, respectively.

Table 4: Total variance explained by the extracted principal components

Component	Initial eigenvalues			Extraction sums of squared loadings		
	Total	% of variance	Cumulative %	Total	% of variance	Cumulative %
1	3.396	33.965	33.965	3.396	33.965	33.965
2	2.311	23.107	57.072	2.311	23.107	57.072
3	1.423	14.233	71.305	1.423	14.233	71.305
4	.917	9.165	80.470			
5	.598	5.983	86.453			
6	.522	5.219	91.672			
7	.459	4.589	96.261			
8	.198	1.979	98.240			
9	.124	1.240	99.480			
10	.052	.520	100.000			

Extraction method: principal component analysis.

The rotated component matrix (varimax rotation) is listed in table 5. While the KMO value suggests only mediocre sampling adequacy, the variables were grouped as follows for exploratory purposes: component 1 (33.97%) was associated with oxidative stress and serum cytochrome protein levels, with strong positive loadings for CYP2D6 (0.917), CYP3A4 (0.913), and GSH (0.900), and a strong negative loading for MDA (-0.768). Component 2 (23.11%): Traditional automatic liver function parameters such as ALT (0.732), AST (0.782), ALP (0.762), and TSB (0.766). Component 3 (14.23%) was primarily driven by the AST/ALT ratio (0.923) and ALT (0.544), whereas it was enriched with AST (0.391) and A/G ratio (0.436)}.

Table 5: Rotated component matrix showing factor loadings of biochemical variables

Biochemical variables	Component		
	1	2	3
ALT (U/l)	-.080	.732	-.544
AST (U/l)	-.186	.782	.391
AST/ALT	-.216	.007	.923
ALP (U/l)	-.077	.762	-.024
TSB (mg/dl)	.073	.766	-.175
MDA (ng/ml)	-.768	.139	.008
GSH (ng/ml)	.900	-.004	.030
CYP2D6 (ng/ml)	.917	-.086	-.051
CYP3A4 (pg/ml)	.913	.034	.048
A/G Ratio	-.281	.089	-.436

Rotation Method: Varimax with Kaiser Normalization. a. Rotation converged in 5 iterations.

To visualize the distinction between METH users and healthy controls, a two-dimensional PCA plot was generated using the first two principal components, which collectively explained the largest proportion of total variance. Although the KMO value indicated only mediocre data

suitability for PCA, as depicted in fig. 1, the score plot of the first two PCs revealed a distinct separation between the two groups. The METH user cohort and the control group formed separate clusters that were primarily distinguished along the axis of first principal component 1.

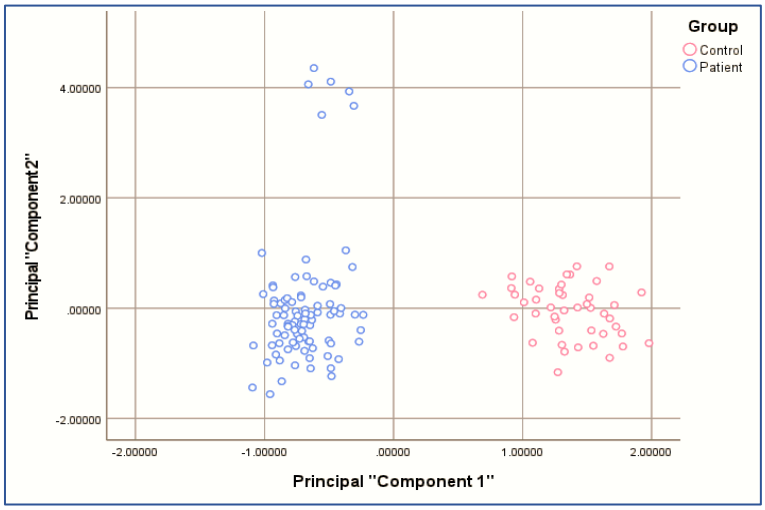

Fig. 1: PCA score plot of the first two principal components. the plot shows a clear separation between METH users (red) and healthy controls (blue), with the distinction occurring primarily along the first principal component (PC1), which represents an axis of oxidative stress and cytochrome P450 protein levels

A decision tree classification model was constructed using the Chi-squared Automatic Interaction Detection (CHAID) algorithm to identify the most significant predictors for differentiating METH users from healthy individuals. The algorithm identified CYP2D6 as the most statistically significant predictor (χ^2 = 101.25, df=1, p<0.001), with an optimal cutoff value of 1.980 ng/ml (fig. 2).

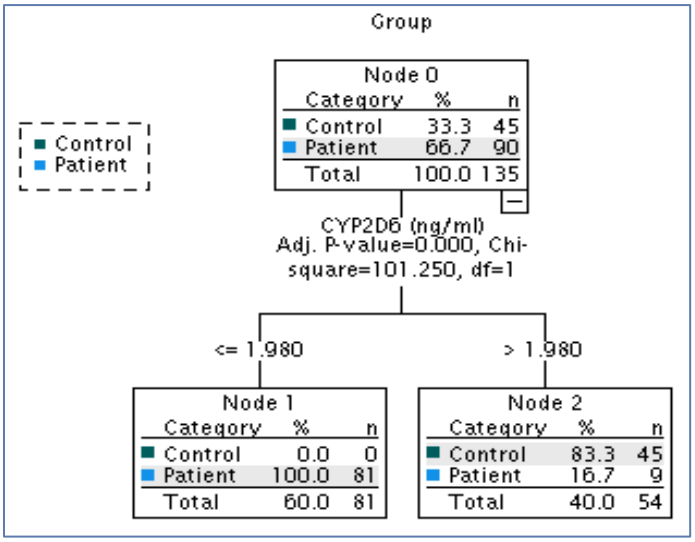

Fig. 2: CHAID decision tree model for classifying participants. The model identifies a single, optimal split point based on the serum level of CYP2D6 (≤ 1.980 ng/ml) to differentiate METH users from healthy controls

As shown in table 7, the model achieved a sensitivity of 90% and specificity of 100% on the training data, with an overall classification accuracy of 93.3%. It is important to note that these performance metrics represent the training set results without an independent test set validation or cross-validation. This suggests that CYP2D6, using a threshold of ≤1.980 ng/ml, may serve as a discriminating biomarker for differentiating METH users from controls within our study population, although external validation is required to confirm its generalizability.

Table 7: Classification performance of the CHAID decision tree model

Actual \ Predicted	Predicted		
	Predicted control	Predicted patient	Percent correct
Actual Control	45 (TN)	0(FP)	100.0%
Actual Patient	9(FN)	81(TP)	90.0%
Overall Percentage	40.0%	60.0%	93.3%

Abbreviations: TN, true negative; FP, false positive; FN, false negative; TP, true positive.

DISCUSSION

The present study introduced a significant advancement in monitoring METH-induced hepatotoxicity by demonstrating that serum levels of CYP2D6 and CYP3A4, in conjunction with markers of oxidative stress, serve as highly sensitive and early predictors of subclinical liver injury. Our central and most novel finding is the profound decrease in circulating concentrations of these key metabolic enzymes in METH users, a change that occurs before conventional LFTs become abnormal. By employing advanced statistical models, we not only confirmed the predictive power of these biomarkers but also elucidated a hierarchical pattern of injury, positioning metabolic and oxidative dysregulation as primary events in METH-induced hepatic pathology.

The significant reduction in serum CYP2D6 and CYP3A4 protein levels was the cornerstone of our findings. Historically, a major limitation in using CYPs as biomarkers is that they are primarily intracellular membrane-bound proteins within the liver, making their serum concentration an unconventional measure of hepatic function. However, our results are strongly supported by an emerging body of evidence demonstrating that CYP enzymes are packaged into extracellular vesicles (EVs) or exosomes and released into circulation [22, 23]. Groundbreaking work has shown that circulating EV-containing CYPs are metabolically active, and their levels can change in response to liver damage [24]. For instance, studies have shown that circulating CYP activity increases following drug-induced liver injury (DILI), suggesting that the release of these enzymes reflects the underlying hepatic stress [25, 26].

Therefore, decreased serum CYP levels observed in METH users may represent a novel pathophysiological mechanism. This could be attributed to several factors, including METH-induced transcriptional downregulation of hepatic synthesis, altered packaging of CYPs into exosomes, or accelerated clearance of circulating vesicles [27, 28]. While the precise mechanism requires further investigation, our study provides compelling evidence that measuring circulating CYP protein levels is a viable and sensitive noninvasive strategy for detecting early hepatic dysregulation, aligning with research that calls for the use of CYP activity to evaluate acute hepatic dysfunction [25, 29, 30].

The present findings of decreased GSH and increased MDA levels corroborate the well-established role of oxidative stress in METH-induced toxicity [31]. Chronic METH exposure generates ROS, which depletes cellular antioxidants such as GSH and causes lipid peroxidation, as indicated by elevated MDA [32]. Turan *et al.* (2023) investigated oxidative stress biomarkers in 50 METH users versus 36 controls and demonstrated significantly elevated serum thiol/disulfide ratios and ischemia-modified albumin levels [33]. Their research supports the present findings by establishing that multiple oxidative stress parameters are simultaneously altered in METH users, reinforcing the concept of a unified metabolic-redox injury axis. According to Raspopović *et al.* (FAN, 2024), GSH is considered a sensitive marker of oxidative stress with the ability to reflect initial cellular damage before cell injury becomes readily apparent in organs [34]. Similarly, Cherian *et al.* [2019] found malondialdehyde to be a good marker of lipid peroxidation under inflammatory conditions, indicating that the strong negative correlation between these two variables in the heatmap indicates that METH-induced oxidative damage involves concurrent antioxidant exhaustion and membrane injury [35]. This bimodal pattern may indicate a more realistic picture of early drug-induced liver injury.

As a secondary observation, we noted modest but statistically significant increases in the AST/ALT and albumin-to-globulin (A/G) ratios, despite most conventional LFTs remaining within the normal range. While an AST/ALT ratio greater than 1 may suggest mitochondrial injury [36, 37] and an elevated A/G ratio is atypical, the clinical significance of these findings in the context of METH use remains unclear and requires further investigation [38, 39]. Given their limited diagnostic relevance compared to primary biomarkers (CYP2D6, CYP3A4, GSH, and MDA), these observations should be considered exploratory [36].

The integrated application of PCA and decision tree modeling provided a deeper interpretation of the biomarker data. PCA stratified the injury process into distinct stages: PC1 represented the initial metabolic-oxidative shift, and PC2 (loaded with ALT, AST, etc.) represented subsequent hepatocellular injury. PC3, which was primarily driven by the AST/ALT ratio, may reflect additional injury patterns, although its interpretation remains exploratory, given the limited diagnostic relevance of this ratio in our cohort.

The decision tree model is more useful for the diagnosis of biomarkers of liver disease. In the present study, CHAID decision tree analysis identified CYP2D6 as the best discriminating variable between METH users and controls in our training dataset, with an optimal cutoff value for prediction of ≤1.98 ng/ml. This enzymatic function as a sensitive marker is also supported by clinical case reports, for example, a syndrome of persistent paroxetine toxicity associated with a phenoconversion event that profoundly decreased CYP2D6 metabolism [40]. This clinical observation is supported by experimental models showing that transcriptional downregulation of CYP2D6 is an early event in DILI [41]. The versatility of tree-based modeling has also been demonstrated in oncological applications, where ensemble models have efficiently identified diverse biomarkers for hepatocellular carcinoma with high accuracy AUC values (up to 0.997) across a number of datasets [42]. This joint evidence further emphasizes the potential of multivariate statistical methodologies that integrate feature optimization and ensemble learning for biomarker mining in hepatology. However, rigorous external validation is essential for clinical translation.

Limitations and future research directions

This study has several limitations that guide future research. The primary limitation is the measurement of serum CYP protein concentrations rather than direct hepatic enzymatic activity. However, by contextualizing our findings within the latest research on circulating exosomal CYPs [22, 23, 43, 44], we reframe this not as a simple surrogate measure but as a novel and potentially more specific indicator of hepatic stress. Future studies should aim to correlate serum protein levels with the direct characterization of hepatic exosomes to validate this mechanism.

Statistically, the perfect AUCs of 1.00 for CYP3A4 and GSH suggest potential overfitting, and the median KMO value (0.591) for our PCA indicates that the results should be interpreted with caution. Furthermore, a decision tree model was developed and evaluated on the same training dataset without independent test set validation or cross-validation procedures. While this model is highly interpretable, its reliance on a single predictor and lack of external validation limit conclusions about its generalizability and predictive performance in independent populations. Future studies should employ robust cross-validation techniques and explore more complex ensemble models, such as Random Forests, to build a more resilient predictive signature for METH-induced hepatotoxicity.

CONCLUSION

In conclusion, this study marks a shift in the understanding of METH-induced hepatic effects, revealing a subtle pattern of subclinical liver dysfunction that cannot be detected by conventional screening. The simultaneous decrease in the serum protein levels of CYP2D6 and CYP3A4, along with changes in oxidative stress markers, revealed an early biological injury occurring at the molecular level before clinical and biochemical abnormalities became apparent. It is important to note that this study measured serum protein levels rather than direct hepatic enzymatic activity. Advanced statistical models, specifically PCA and decision tree modeling, marked a turning point in data interpretation, identifying CYP2D6 as an early pivotal marker to better differentiate abusers from healthy controls. Beyond improving the classification accuracy, these models provide new ways to understand the hidden patterns of liver injury. Accordingly, integrating oxidative stress markers and cytochrome enzymes with predictive analytics tools is a necessary step toward developing early screening strategies capable of intervening before damage progresses to irreversible stages.

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ETHICAL APPROVAL

The study protocol was reviewed and approved by the Ethical Committee of the Al-Qanat Center for Social Rehabilitation in Baghdad, Iraq. This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all the participants after the nature of the study procedure was fully explained. (IRB 1068 12/01/2025).

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AUTHORS CONTRIBUTIONS

Conceptualization, AFS and GHA; methodology, AFS, GHA, and MTH; validation, AFS, GHA, and MTH; formal analysis, MTH; investigation, AFS, GHA, and MTH; resources, AFS; data curation, MTH; writing – original draft preparation, AFS and GHA; writing – review and editing, all authors; visualization, MTH; supervision, AFS; project administration, AFS; funding acquisition, AFS, and GHA. All the authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTERESTS

The authors have nothing to disclose

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