Evaluation of Hepatic Damage by Serum Levels of Cleaved Cytokeratin 18 Fragments M30 and M65 in Methamphetamine Abusers

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ABSTRACT

Methamphetamine (METH) abuse has been known to cause serious liver injury, but conventional liver function tests (LFTs) may not detect the damage early enough. This study investigated the utility of caspase-cleaved cytokeratin 18 (M30) and total cytokeratin 18 (M65) as sensitive biomarkers for METH-induced liver damage. To assess the serum concentrations of M30 and M65 in METH users and to determine their diagnostic efficacy for detecting subclinical liver injury. In this study, 30 chronic METH users and 30 healthy controls were enrolled. The serum levels of M30, M65, and conventional LFTs were measured. Results: METH users exhibited profoundly elevated serum levels of both M30 ($p < 0.001$) and M65 ($p < 0.001$) compared to the control group. Receiver Operating Characteristic Curve (ROC) analysis demonstrated diagnostic accuracy for M65 (AUC = 1.00) and M30 (AUC =0.97), significantly outperforming traditional LFTs, which remained within the normal ranges for all participants. In the multiple regression model, M65 was identified as the strongest predictor of METH group membership rather than of hepatic damage per se. A strong positive correlation was observed between M30 and M65 levels ($r = 0.972$). Serum M30 and M65 levels demonstrated high sensitivity and specificity for detecting subclinical liver injury in chronic METH abusers and showed better accuracy than conventional LFTs. Further longitudinal studies are required to evaluate the prognostic utility of M30 and M65 in this population.

Keywords: Methamphetamine; Drug-Induced Liver Damage; Apoptosis; Cytokeratin-18; Alanine Transaminase.

INTRODUCTION

Methamphetamine is an addictive psychostimulant that has emerged as one of the most significant global public health challenges and a growing epidemic. According to the 2023 United Nations World Drug Report, amphetamine-type stimulants are the third most prevalent substance class used globally[1], with an estimated number of 36 million users. There are an estimated 1.6 million people living with an METH use disorder in the United States alone, exemplifying how far-reaching this epidemic truly is[2]. Chronic use of METH leads to deleterious multisystemic pathologies, as it causes tremendous damage to these systems, especially the cardiovascular, neurological and renal systems. The major site of xenobiotics metabolism and a highly sensitive organ for METH-induced toxicity is the liver[3]. In fact, the spectrum of hepatic injury includes asymptomatic increases in liver enzymes to more serious pathologies such as steatosis, fibrosis, fulminant hepatic failure and cirrhosis[4].

The mechanisms of action by which METH affects the liver are complex and multifactorial, involving a cascade of detrimental cellular events. Recent mechanistic insight from preclinical and clinical data identifies oxidative stress as a predominant pathogenic driver[5,6]. METH metabolism generates a large amount of reactive oxygen species (ROS), which exceeds the antioxidant defense capacity in liver and initiates an avalanche of lipid peroxidation and cell death[7]. Wang et al. also showed that METH directly kills hepatocytes by apoptosis (programmed cell death) and necrosis (uncontrolled cell death) where it enhances greatly the organ inflammation of the liver[8]. These include drug-induced hyperthermia, mitochondrial toxicity and bile acid homeostasis disorder[9,10].

At present, the clinical assessment of hepatotoxicity in METH users can be extrapolated from classic LFTs. Despite being indicators of hepatocellular damage, liver enzymes have many limitations[11]. They are therefore not specific as to the mechanism of cell death and may be insufficiently sensitive to pick up subclinical, or early-stage liver damage, most of which is asymptomatic. This limitation emphasizes the urgent need for more specific and sensitive serum biomarkers, as they allow noninvasive diagnosis of different modes of hepatocyte death and early characterization of METH-induced liver injury prior to becoming clinically apparent[12].

Fragments of cytokeratin 18 (CK18) have emerged as promising biomarkers for the CK18 is cleaved by caspases during apoptosis to yield a specific fragment that can be quantified using M30 immunoassay. In contrast, cell death (apoptosis and necrosis) entails the release of both cleaved and full-length CK18, which can be measured using the M65 immunoassay[13]. The ratio of M30 to M65 may serve as a more detailed readout of the mode of cell death. M30 and M65 have been demonstrated to be useful in several hepato-diseases, such as MAFLD and viral hepatitis, but their use in the case of METH-induced hepatotoxicity has not been widely studied[13,14].

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This is a major research requirement. Although the use of METH is common and its hepatotoxicity is known, only a few studies have introduced specific types of hepatocyte death using these advanced biomarkers[8,11]. Understanding whether METH predominantly induces apoptosis or necrosis is crucial for elucidating precise pathogenic mechanisms and developing targeted therapeutic interventions. Therefore, the aim of this study was to evaluate the serum M30 and M65 in a cohort of chronic METH users compared to healthy controls.

MATERIALS AND METHODS

Study Design: This case-control study was conducted between mid-January and November 2025. The recruited 60 participants included 30 patients with methamphetamine use disorder (MUD), diagnosed by a qualified psychiatrist according to DSM-5 criteria, and 30 age-matched healthy controls. Participants were 18–40 years old.

Inclusion Criteria: Eligible METH users had a confirmed clinical diagnosis of methamphetamine use disorder and a urine toxicology screen positive for amphetamines at the time of admission to the rehabilitation center. Chronic methamphetamine use was defined as continuous use for at least one year. Route of administration was not systematically documented and is therefore not reported as an enrollment characteristic; this limitation is addressed in the corresponding section. Blood sampling was performed at admission, immediately after the positive urine screen, without a standardized abstinence interval. A subset of participants were occasional tobacco smokers; none met the threshold for heavy smoking (≥ 10 cigarettes per day), and smoking was retained as a documented covariate rather than an exclusion criterion to preserve sample size.

Exclusion Criteria: Current or past alcohol use disorder, hepatitis B or C infection, cirrhosis, or any other documented chronic liver disease; HIV infection; treatment with potentially hepatotoxic medications within the six months preceding enrollment; active malignancy; or major comorbidities including diabetes mellitus and overt cardiovascular disease.

Healthy Controls Recruitment: Healthy controls were recruited from hospital staff and from university faculty and students known to the investigators. All controls underwent a urine toxicology screen, and only individuals with a negative result were enrolled. None of the controls had a history of substance use or chronic illness based on self-report and routine clinical evaluation.

Biochemical Analysis and Blood Sample Collection: A peripheral venous blood (5 mL) sample was obtained from all subjects. After sampling, the serum was separated, allowed to clot at room temperature for 30 min, and centrifuged at 1000 × g for 20 min. Serum was collected and then aliquoted into sterile Eppendorf tubes and stored (− 80°C) until analyzed[15].

Biochemical Assays

Liver Function Tests: At the time of blood collection, ALT, AST, ALP and GGT were performed using a Beckman Coulter AU480 Chemistry Analyzer (Beckman Coulter GmbH; Germany). Clinical reference intervals for ALT, AST, ALP and GGT were those supplied by the manufacturer of the AU480 system and are consistent with internationally recognized adult reference ranges.

ELISA Assay: Serum M30 and M65 concentrations were measured using commercial sandwich ELISA kits (Human Cytokeratin 18-M30 GENLISA™, KBH1610; and Human Cytokeratin 18-M65 GENLISA™, KBH1666; KRISHGEN BioSystems, India). According to the manufacturer, the calibration range for M30 was 1–16 ng/mL with a limit of quantification (LOQ) of 0.5 ng/mL, whereas the calibration range for M65 was 200–3200 pg/mL with an LOQ of 195 pg/mL. The reported intra-assay and inter-assay coefficients of variation were < 15 % and < 18 %, respectively, for both assays. Standard curves demonstrated acceptable linearity ($r^2 > 0.99$). Absorbance was measured at 450 nm using a BioTek microplate reader (BioTek Instruments, USA), and unknown sample concentrations were interpolated from the standard curve, with appropriate dilution factors applied where required.

Statistical Methods: For statistical analyses GraphPad Prism 9 was used. Continuous variables are shown as mean ± SD. The Shapiro–Wilk test was used to assess normality. Between-group comparisons were performed using independent t-tests (with Cohen's d) or Mann–Whitney U tests, mean differences with 95% CIs. The Benjamini–Hochberg FDR procedure was used to adjust for multiple comparisons. Pearson's correlation assessed associations of biomarkers with liver enzymes. Diagnostic performance of the candidate biomarkers was evaluated by Receiver Operating Characteristic (ROC) analysis, with the Youden index used to identify optimal cut-off values; 95 % CIs were reported for the area under the curve (AUC), sensitivity, and specificity. Predictors of group status (METH user vs. healthy control) were determined by a stepwise multiple linear regression using LFTs and CK18 fragments. Assumptions of the regression models (multicollinearity, homoscedasticity and residual normality) were assessed with VIF, Breusch–Pagan and Shapiro–Wilk tests. Statistical significance was defined as two-sided $p < 0.05$[16,17].

RESULTS

Demographic, biochemical and enzyme markers were compared between each of the METH user groups and healthy control group. METH patients and control subjects did not differ significantly in age (26.60 ± 5.86 years vs. 27.10 ± 5.71 years, $p = 0.722$). METH users had higher serum ALT, AST and GGT levels than controls (all $p < 0.001$). ALP was likewise elevated in the METH group ($p = 0.0407$). In contrast, the AST/ALT ratio did not differ between groups ($p = 0.19$). Both apoptosis and total cell death markers, M30 ($p < 0.001$) and M65 ($p < 0.001$), were significantly higher in METH users than controls. Very large Cohen's d effect sizes for M65 ($d = 5.161$) and M30 ($d = 4.246$) indicate the magnitude of these differences. Mean differences between the two groups, together with their 95 % confidence intervals, are also provided in Table 1, and the principal biomarker comparisons remained statistically significant after Benjamini–Hochberg correction for the false discovery rate, Table 1.

Data are presented as Mean ± SD. Independent t-test; 95 % CIs were computed from the pooled-variance formula. The principal biomarker comparisons remained statistically significant after Benjamini–Hochberg FDR adjustment.

The proportion of abnormal liver enzyme values in relation to the clinical reference limits is summarized in Table 2. High GGT levels (>60 U/L) were found in 5/30 (16.7 %) of the METH group compared to 0/30 (0.0 %) among the controls. ALP levels outside the reference range (30–120 U/L) were found in 12/30 (40.0 %) of the METH group versus 0/30 (0.0 %) in the control group. There were no instances of ALT or AST levels of >40 U/L in either group. AST/ALT ≥ 1.0 was 8/30 (26.7 %) in the METH group and 14/30 (46.7 %) in the control group.

Normality was assessed using the Shapiro–Wilk test. Most biomarkers departed from normality in the control group, and several variables

(AST, GGT, ALP) also departed from normality in the METH group; this prompted the parallel Mann–Whitney U sensitivity analyses described in the Methods, which fully reproduced the parametric pattern of significance. Detailed p-values are reported in Table 3.

Pearson's correlation coefficient was calculated between M30 and M65 values with liver enzymes. There was no significant association between M30 or M65 and serum ALT, AST, GGT or ALP levels in the METH group (all $p > 0.05$). Additionally, the AST/ALT ratio was not significantly correlated with either apoptosis marker. Notably, Figure 1 indicates a very high positive and significant correlation between M30 and M65; ($r = 0.972$, $p < 0.001$).

ROC analysis was used to test M30 and M65 for distinguishing between METH users and controls. M65 had AUC of 1.00 (95% CI: 0.94-1.00; cut-off ≥1737·18 pg/mL; sensitivity and specificity both were 100%). M30 had AUC 0.97 (95% CI: 0.90-0.99; cut off ≥8.153 ng/mL; sensitivity, 100%, specificity 90%). GGT had the best performance among liver enzymes (AUC=0.893; cut-off ≥31.5 U/L; 86.7% sensitivity, 93.3% specificity), followed by ALT (AUC=0.767), AST (AUC=0.761) and ALP (AUC=0.628), Figure 2.

A stepwise multiple linear regression model was constructed with group status as the dependent variable (METH user = 1, healthy control = 0) and the conventional LFTs and CK18 fragments as candidate predictors. The final model retained four independent variables — M65, GGT, ALT and ALP — and explained 90.4 % of the variance in group status ($R^2 = 0.904$, adjusted $R^2 = 0.897$; $F(4,55) = 129.3$, $p < 0.0001$). M65 emerged as the strongest predictor of METH group membership ($\beta = 0.00050$, 95 % CI 0.00043 to 0.00056, $p < 0.0001$), followed by GGT ($\beta = 0.00339$, 95 % CI 0.00078 to 0.00600, $p = 0.0118$), ALT ($\beta = 0.00668$, 95 % CI 0.00055 to 0.01281, $p = 0.0334$) and ALP ($\beta = 0.00061$, 95 % CI 0.00001 to 0.00120, $p = 0.0455$). AST, the AST/ALT ratio and M30 did not contribute significantly once M65 had entered the model and were therefore excluded. Multicollinearity was not detected (all VIF values < 2). The Breusch–Pagan test showed no evidence of heteroscedasticity ($p = 0.465$), while residuals showed only mild deviation from normality on the Shapiro–Wilk test ($p = 0.043$); the regression estimates are therefore reported with appropriate caution. Although M30 and M65 were strongly correlated ($r = 0.972$), only M65 was retained in the final stepwise model, and its VIF (1.64) confirmed that this correlation did not destabilise the regression solution (Table 4).

Table 1. Demographic, biochemical and enzymatic markers of the comparison between METH users and healthy controls

Variable	METH	Control	Mean difference (95 % CI)	P-value	Cohen's d
Age (years)	26.60 ± 5.86	27.10 ± 5.71	—	0.722	−0.087
ALT (U/L)	18.6 ± 7.88	11.8 ± 5.3	6.87 (3.37 to 10.36)	<0.001	0.729
AST (U/L)	15.3 ± 6.34	9.56 ± 2.45	5.57 (2.96 to 8.17)	<0.001	1.118
AST/ALT	0.84 ± 0.26	0.95 ± 0.38	−0.11 (−0.28 to 0.06)	0.19	−0.335
GGT (U/L)	48.87 ± 19.1	23.97 ± 5.85	24.89 (16.72 to 33.07)	<0.001	1.599
ALP (U/L)	131.21 ± 68.55	93.11 ± 15.07	38.10 (0.95 to 75.26)	0.0407	0.541
M30 (ng/mL)	11.83 ± 1.96	6.1 ± 1.82	6.51 (5.71 to 7.30)	<0.001	4.246
M65 (pg/mL)	2275.10 ± 339.17	767.47 ± 235.84	1507.63 (1356.27 to 1659.00)	<0.001	5.161

Table 2. Prevalence of Elevated Liver Enzymes.

Parameter	METH (n=30)	Control (n=30)
ALT > 40 U/L	0/30 (0.0 %)	0/30 (0.0 %)
AST > 40 U/L	0/30 (0.0 %)	0/30 (0.0 %)
GGT > 60 U/L	5/30 (16.7 %)	0/30 (0.0 %)
ALP outside 30–120 U/L	12/30 (40.0 %)	0/30 (0.0 %)
AST/ALT ≥ 1.0	8/30 (26.7 %)	14/30 (46.7 %)

Table 3. Shapiro–Wilk normality results for biochemical markers in METH users and healthy controls.

Variable	METH (p-value)	Control (p-value)
ALT	0.076	0.0004
AST	0.015	<0.0001
AST/ALT	0.102	0.213
GGT	0.017	0.0018
ALP	<0.0001	0.0076
M30	0.481	0.0011
M65	0.158	<0.0001

Table 4. Stepwise regression coefficients with 95 % confidence intervals and collinearity statistics.

Variable	β (coefficient)	95 % CI	p-value	VIF
ALT	0.00668	0.00055 to 0.01281	0.0334*	1.194
ALP	0.00061	0.00001 to 0.00120	0.0455*	1.045
GGT	0.00339	0.00078 to 0.00600	0.0118*	1.511
M65	0.00050	0.00043 to 0.00056	<0.0001**	1.640

*$p < 0.05$; **$p < 0.0001$. Dependent variable = group status (METH = 1, control = 0). Model: $F(4,55) = 129.3$, $p < 0.0001$; $R^2 = 0.904$; adjusted $R^2 = 0.897$. AST, AST/ALT ratio and M30 were excluded from the final stepwise model. VIF = variance inflation factor.

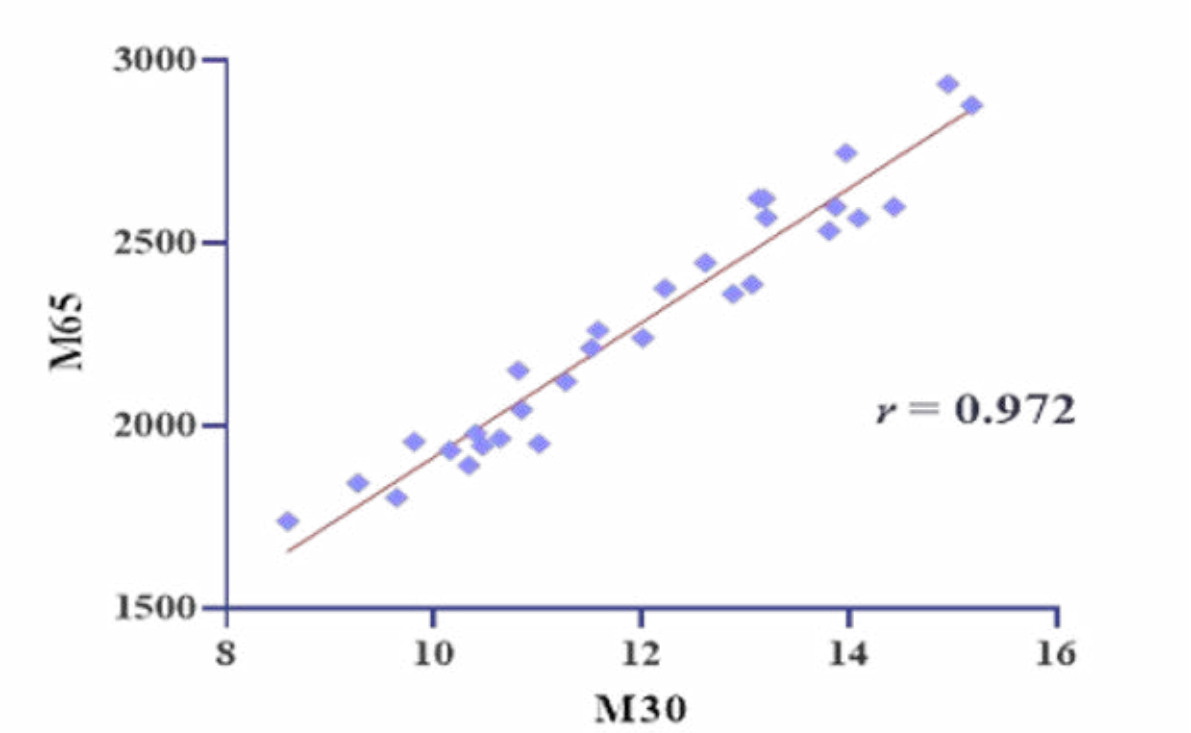

Figure 1. Correlation of cytokeratin 18 fragments M30 and M65. Scatter plot showing the positive correlation between M30 and M65.

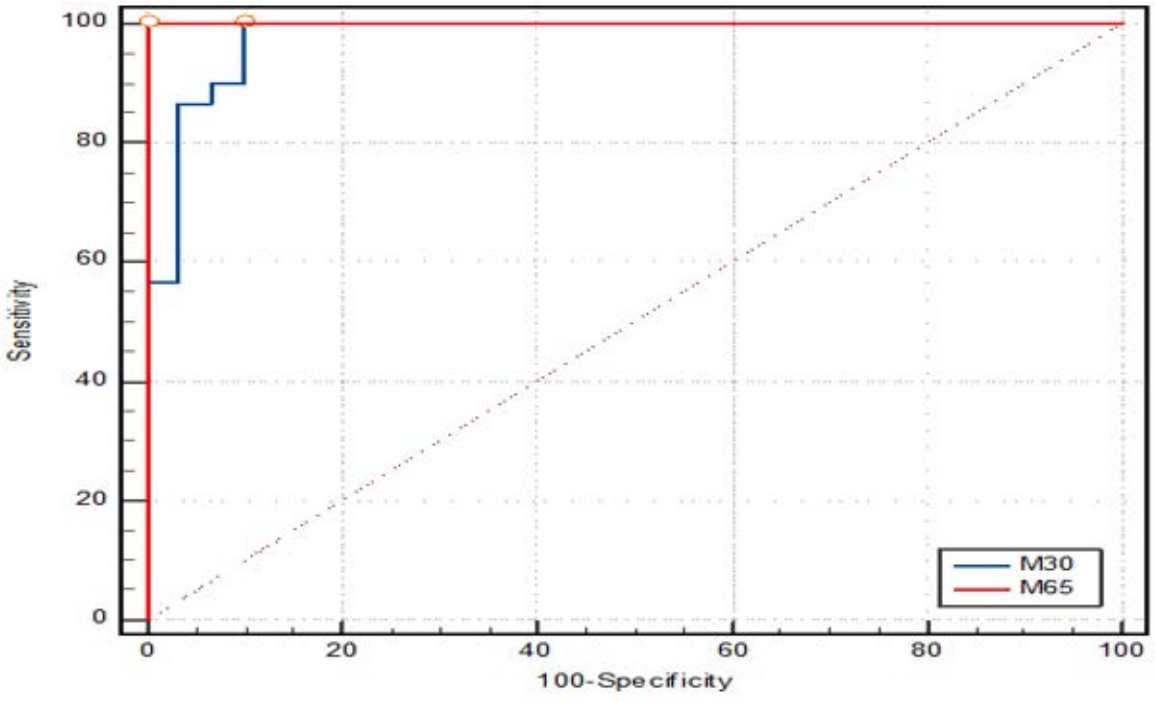

Figure 2. ROC Curves for All Biomarkers. This figure illustrates the diagnostic performance of each biomarker.

DISCUSSION

This study was the first to investigate M30 and M65 as possible biomarkers of liver injury in chronic METH abusers. The most important result is that METH users have strikingly increased serum levels of M30 and M65 compared to healthy controls, suggesting significant hepatocyte death. These markers were notably better than standard LFTs at diagnostic accuracy and showed promise as improved testing strategies for the subclinical detection of liver injury in this high-risk group.

A critical result was the remarkable capacity of M30 and M65 to differentiate between METH users and non-drug abusers. Both markers achieved high AUC values, indicating very good separation between the two groups in our sample. This is in sharp contrast to the only moderate diagnostic value for other chronic liver diseases, as recently

demonstrated in large meta-analyses. Chen et al. 2025 including more than 2,200 patients demonstrated an AUC of 0.81 for significant fibrosis and an AUC of 0.79 for significant inflammation in NAFLD patients using CK18[18]. Similarly, a more extensive 2025 systematic review by Ye et al., encompassing 63 studies and over 9,000 patients, reported AUROC values for M30 and M65 ranging from 0.70 to 0.82 for various stages of fibrosis and inflammation in viral hepatitis and steatotic liver disease[13]. These findings should be interpreted cautiously because of the relatively small sample size and the absence of external validation.

This study also indicated that traditional LFTs have significant limitations in the early or mild stages of liver injury and disease. Although serum ALT, AST, and GGT levels were significantly elevated in the METH group, none of the participants had values that exceeded the threshold limits used clinically (e.g., >40 U/L for either ALT or AST), which would generally guide further clinical evaluation. However, the large increases in M30 and M65 in these patients clearly indicated continued hepatocellular damage. This is in accordance with increasing literature pointing to the need for more sensitive biomarkers for detecting subclinical organ damage[19]. As Humphries and Dear noted, standard markers such as ALT perform well for diagnosing established drug-induced liver injury (DILI) but often lack sensitivity for early detection and are not prognostic[20]. The observed elevations in M30 and M65 should therefore be interpreted as indirect evidence of hepatocyte injury rather than as direct histological confirmation.

The profound increase in both M30 and M65, which were highly correlated, supports the idea that METH provokes a mixed pattern of hepatocyte death, where either apoptosis or necrosis plays an important role. This is in line with preclinical data, clearly supporting that a cascade of upstream cellular processes, mainly based on oxidative stress and mitochondrial damage, plays key roles in METH-triggered hepatotoxicity[21–23]. In the liver, METH is extensively metabolized by the cytochrome P450 system to produce an abundance of reactive oxygen species (ROS), which supersede its antioxidant capacity[24]. This oxidative insult causes lipid peroxidation, causes direct damage to cellular macromolecules, and compromises mitochondrial integrity. MPT pore opening is induced by METH, which results in the release of pro-apoptotic factors such as cytochrome c (M30 triggers the caspase cascade) and an ATP collapse that ultimately leads to necrotic cell death (measured by M65)[25]. Our findings provide clinical support for this dual-mechanism model, positioning M30 and M65 as integrated biomarkers that reflect the downstream consequences of METH-induced oxidative injury. However, it should be emphasised that elevated CK18 fragments have also been reported in NAFLD, viral hepatitis, alcoholic liver disease, and drug-induced liver injury, and the marker is therefore not biologically specific to methamphetamine; the strict exclusion of these competing etiologies in our cohort is central to the interpretation of the present findings.

Furthermore, the prognostic implications of these findings warrant further consideration. A meta-analysis by Zhang *et al.* on patients with advanced alcohol-related liver disease (ALD) established that elevated M30 and M65 levels were powerful predictors of adverse outcomes, with an approximately doubled risk of death or liver transplantation[26]. Although the current study design does not allow direct prognostic implications, similarities between METH-induced and alcohol-induced liver injury (both exacerbated by substance toxicity and oxidative stress) imply that the elevated M30 and M65 levels in our METH cohort may indicate a high risk of disease worsening. Because the current case-control study cannot establish temporal or causal relationships, these observations remain hypothesis-generating; further longitudinal studies are needed to assess the predictive utility of M30 and M65 before any role in clinical decision-making can be considered.

Clinical and therapeutic implications of these findings should be framed cautiously. The high sensitivity of M30 and M65 for the detection of subclinical injury translates into wider clinical applications. The critical window of opportunity is the ability to recognize liver damage prior to clinically significant levels of ALT/AST. Moreover, these results provide new opportunities to investigate selective therapeutic strategies. Antioxidants are biologically plausible candidates, since oxidative stress is the central phenomenon. As an example, N-acetylcysteine (NAC), a strong antioxidant and precursor of glutathione, is the standard of care for acetaminophen-induced liver injury[27] and has also been used in several non-acetaminophen DILI contexts. The potential therapeutic role of N-acetylcysteine in methamphetamine-induced liver injury remains hypothetical and requires prospective clinical evaluation.

Strengths and Limitations

The small sample size limits the generalizability of the results and may have contributed to an over-fitting of the regression models and the ROC curve. Data on liver histological examination and assessment of fibrosis using imaging were unavailable; therefore, conclusions regarding apoptosis and necrosis are based on circulating biomarkers rather than direct morphological evidence. Information on methamphetamine dosage, cumulative exposure, route of administration, and precise abstinence period prior to sampling was lacking. Finally, the observational case-control study design indicates association rather than causation and does not allow for the assessment of temporal, causal, or predictive value relationships for M30 and M65.

CONCLUSIONS

This study provides the first clinical evidence that serum M30 and M65 are promising biomarkers of hepatocellular injury in chronic methamphetamine users and demonstrated greater sensitivity than traditional liver function tests for detecting subclinical liver damage. In this cohort, both M30 and M65 showed better discriminatory performance than traditional liver function tests. In summary, methamphetamine use was associated with elevated biomarkers of hepatocyte injury (M30 and M65) in the present cohort. Further longitudinal studies are required to evaluate the prognostic utility of M30 and M65 and to confirm their value as early indicators of methamphetamine-related hepatocellular injury before any routine clinical implementation can be considered.

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Competing Interest: None

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