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# Bacteroides intestinalis AM-1 rewires glutathione and bile acid metabolism to restore mitochondrial homeostasis and ameliorate necro-apoptosis in acute liver failure

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# Abstract

Acute liver failure (ALF) is characterized by rapid hepatocellular injury with profound metabolic and microbial dysregulation. Plasma metabolomics and metaproteomics performed in ALF patients (n = 270) identified a distinct non-survivor signature marked by elevated primary bile acids and tryptophan metabolites associated with inflammation, mitochondrial dysfunction, and cell death ($p < 0.01$, FDR < 0.01, FC > 1.5). ALF non-survivors also showed altered alpha/beta diversity ($p < 0.05$) with depletion of bacterial species linked to bile acid metabolism and redox homeostasis. Among cytotoxic metabolites, chenodeoxycholic acid (CDCA; logFC > 10) strongly predicted early mortality (HR > 1.7, $p < 0.05$). In an acetaminophen-induced ALF mouse model, Bacteroides intestinalis AM-1 ($10^9$ CFU/ml), administered prophylactically and therapeutically, significantly reduced toxic metabolites, enhanced glutathione detoxification via L-histidine amino ligase and GSH synthetase (FC > 1.5, $p < 0.05$), activated FXR-FGF15 signaling, suppressed IL-1β/TLR4 pathways, reduced oxidative stress and caspase-3–mediated necroptosis ($p < 0.05$), and ameliorated liver injury by ~ 70%, supporting its therapeutic potential in ALF.


# INTRODUCTION

Acute liver failure (ALF) is a rapidly progressive, life-threatening condition marked by sudden liver dysfunction in individuals without pre-existing liver disease(1). It is most often triggered by acute viral hepatitis or drug toxicity, especially acetaminophen overdose(1). Histologically, ALF involves massive hepatocellular necrosis and mitochondrial dysfunction(2)

Despite advances in the management, ALF is still a condition with high mortality(3). Interventions such as plasma exchange, renal replacement therapy, and artificial liver support systems provide temporary support and serve as a bridge to transplantation(4). However, the success of these therapies or liver transplant depends on the timely identification of patients who are likely to benefit(4). Further identification of therapeutic agents which could be used for improving the prognosis of ALF patients is critical.

This underscores the urgent need for reliable prognostic indicators to stratify ALF patients at high-risk of early mortality. Early identification of such patients could prompt the use of aggressive medical therapies and guide transplant decisions. One promising approach is to analyse the circulating plasma of ALF patients(2). Plasma is easily accessible and reflects systemic changes occurring due to liver failure, including critical changes in the metabolome and microbiome both of which are crucial components of ALF pathogenesis(2).

Recent research emphasizes that the interplay between metabolic disturbances and gut-derived microbial products contributes to the hyper-inflammatory state(5). Changes in the plasma metabolome such as elevated bile acids, amino acids, and oxidative stress markers and circulating bacterial peptides can potentially serve as biomarkers for disease severity and outcome(5).

An additional approach could be use of next generation probiotics particularly directed towards defined metabolic functions, in our study neutralization of toxic metabolites and enhancing antioxidant response followed by reduction of endotoxemia and systemic inflammation. Importantly, the toxic metabolite educated next generation probiotics can reshape the gut metaproteome, leading to improved metabolic homeostasis. Therefore, next-generation probiotic therapy represents a novel precision-microbiome approach for mitigating inflammation and improving outcomes in liver-associated diseases.

Our investigation focused on baseline plasma Meta-proteome (bacterial peptides) and metabolome alterations to identify markers predicting early mortality in ALF patients. Multiomics profile of ALF plasma showed hyper-inflammation and significant disruption in bile acid haemostasis (accumulation of primary bile acid; chenodeoxycholic acid) and a prominent decrease in linked circulating microbiome; validated in 270 ALF patients using high-resolution mass spectrometry (HRMS) and machine learning. These observations were used to curate a toxic metabolite educated next generation probiotic therapy which was validated in a preclinical mouse model of APAP-induced acute liver failure followed by therapeutic intervention study using Bacteroides intestinalis AM-1 ($10^9$ cfu/ml), human intestinal isolate(6) directed towards modulation of the identified prognostic marker (chenodeoxycholic acid). The therapeutic effect of Bacteroides intestinalis-AM1 in ameliorating liver injury and hepatocyte death and restorative alterations in bile acid composition, inflammatory plasma proteomics, and metabolomics was evaluated in detail.

## RESULTS

# Plasma metabolome and meta-proteome diversity could stratify ALF non-survivor

Our study was explicitly framed as hypothesis-generating and subsequent validation for which the Acute liver failure patients enrolled (Fig. 1A and 1B, Supplementary-Table-1) were subjected to baseline plasma metabolomics(2) (Supplementary-Figures 1 and 2). Discovery cohort; ALF survivors (n = 8), non-survivors (n = 32), and healthy controls (n = 5) showed 9,814 features with 548 annotations across positive/negative ionization modes. ALF patients showed 321 differentially expressed metabolites (DEMs; 153 upregulated, 168 downregulated; FC > 1.5; $p < 0.05$; FDR < 0.01, Supplementary-Table-2) when compared to controls. Additionally, 233 DEMs (114-up, 119-downregulated) distinguished ALF non-survivors (ALF-NS) from survivors (ALF-S; Supplementary-Figures 3 and 4). ALF-NS were distinct (PLS-DA, Fig. 2A, Supplementary-Figures 3, 4 and 5) with significant increase in tryptophan and primary bile acid metabolic pathways (Fig. 2B). Notably, plasma levels of chenodeoxycholic acid and L-tyrosine were highest in ALF-NS ($p < 0.05$).

Plasma meta-proteome showed > 15 phyla and > 25 genera dysregulated in ALF-NS, with increased Firmicutes, Tenericutes and others ($p < 0.05$; Fig. 2C, Supplementary-Figures 6–8, Supplementary-Table-3). Alpha and beta diversity significantly distinguished ALF-NS (Fig. 2C). Inflammation-associated taxa, including Enterobacteriaceae, Salmonella, and Actinobacteria, exhibited significant increases and are

associated with xenobiotic degradation, amino acid metabolism in ALF-NS. Conversely, bacterial populations involved in bile acid metabolism or primary contributors to antioxidant responses were substantially diminished in ALF-NS (Supplementary Figs. 6–8).

Bacterial peptides from non-survivors, including Actinobacteria and Cyanobacteria, were directly correlated with circulating metabolites and liver function and severity indices (Fig. 2D and 2E).

The probability of detecting non-survival using the top five metabolites or bacterial peptides exceeded 85%, with AUROC > 0.85 ($p < 0.05$; Fig. 2F), indicating that baseline plasma metabolites and bacterial peptides effectively distinguished ALF-NS. These results suggest that gut dysbiosis in ALF-NS correlates to the change in circulating metabolome and clinical phenotype seen in ALF patients. Pre-admission antibiotic exposure among ALF patients could not be ascertained therefore, the results are interpreted as being independent of potential confounders such as rifaximin. Consequently, the plasma microbiome profile seen is considered reflective of the intrinsic host response rather than an effect of prior antimicrobial therapy

**Increase in pathogenic bacterial peptide is linked to metabolic pathway and ALF severity.**

Integration of plasma metabolome and meta-proteome using Weighted Metabolite/Meta-protein Correlation Network Analyses WMCNA/WMpCNA(7) showed ALF-NS specific clusters. WMCNA of 548 metabolites showed eight modules, with red, yellow, and turquoise modules enriched in ALF-NS (soft threshold > 9, scale-free topology fit index > 0.85; $p < 0.05$; Fig. 3A). WMpCNA of 579 bacterial peptides showed five modules, with Mp_Grey, Mp_Turquoise, and Mp_Yellow linked to dysregulated lipid and amino acid metabolism (soft threshold > 9, scale-free topology fit index > 0.85; $p < 0.05$; Fig. 3A and Supplementary Figure-9). Cross-correlation (MMCN) demonstrated strong associations ($p < 0.05$, $R^2 > 0.5$) between ALF-NS specific metabolite and metaproteome modules, highlighting inflammation-driven metabolic pathways ($p < 0.05$; $R^2 > 0.5$; Fig. 3B, Supplementary-Figure-10 and 11 and Supplementary-Table-4).

Metabolome modules were associated with AKI and HE, while functional modules correlated with infection at baseline, sepsis, necrosis, and multiorgan failure (Fig. 3C, Supplementary-Figure-12). Interestingly metabolites and bacterial peptide used for POD calculation were linked to increase in severity and liver parameters (Fig. 3D). Suggesting that increase in the pathogenic bacteria and linked metabolic pathway are direct indicators of ALF severity. POD metabolome superseded POD meta-proteome for predicting non survival (AUC = 0.986; Fig. 3E).

Notably, there were no significant differences in top 5 plasma signatures (metabolome and meta-proteome) across different etiologies of ALF, indicating that these biomolecular signatures are pan ALF and can be used to segregate ALF-NS with highest robustness and specificity (Supplementary-Figures-13, 14 and 15).

## Circulating chenodeoxycholic acid correlate with severity and early mortality in ALF

Pan ALF specific metabolic signature panel was significantly elevated in ALF-NS as compared to ALF-S and disease control (SAH; 70) patients when validated in three distinct cohorts (Test-Cohort-1; plasma and one drop blood, of 160 ALF (ALF-S; 53, ALF-NS; 107) samples, Test-Cohort-2; External Validation cohort; 70 ALF samples (ALF-S; 25, ALF-NS; 45) and Test-Cohort-3; Disease cohort; 70 SAH samples; Fig. 4A and 4B and Supplementary-Figures 16, 17 and 18).

Interestingly the ALF metabolite indicator panel showed highest AUROC > 0.95, $p < 0.05$ when benchmarked against ALF clinical scores (Fig. 4C) and showed highest sensitivity, specificity, accuracy for prediction of early mortality using machine learning in 270 ALF patients (Supplementary-Figures 17 and 18).

Of the ALF metabolite indicator panel higher plasma levels (> 10 folds) of chenodeoxycholic acid (C02528) showed highest AUROC and hazard ratio (HR = 1.7; $p < 0.05$) for prediction of early mortality (log-rank < 0.05, Fig. 4D, Supplementary-Figure 19) and ALF patients with chenodeoxycholic acid > 10 folds showed higher severity and mortality (Supplementary-Table-5). Interestingly, primary bile acids were increased with significant reduction of microbiome taxa responsible for converting primary to secondary bile acids, such as *Bacteroides* and *Clostridia and others* (Fig. 4E). This pattern shows that primary bile acid (Chenodeoxycholic acid) accumulates across all etiologies (viral, DILI, and others), likely driven by microbiome insufficiency and underlying hepatic inflammation.

## Chenodeoxycholic Acid Accumulation Correlates with Severity in a Preclinical Acute Liver Failure Model

Increased plasma levels of chenodeoxycholic acid correlates to higher severity and early mortality in ALF patients. In-addition change in bile acid homeostasis and dysregulation of the FXR axis in bile acid accumulation has been reported in various liver diseases(8, 9) Fig. 5A.

Furthermore, the level of chenodeoxycholic acid was significantly elevated in non-survivors across all acute liver failure (ALF) etiologies. To validate the association between chenodeoxycholic acid accumulation, bile acid dysregulation, inflammation, and necro-apoptosis, an acetaminophen (APAP)-induced ALF model (250 mg/kg and 500 mg/kg at 24 and 72 hours) was established as a prototype. Liver and plasma metabolome, proteome, and metaproteome analyses were subsequently conducted (Fig. 5B). A significant difference in necrosis, AST, and ALT levels was seen between the control and APAP-treated mice (250 mg/kg and 500 mg/kg, Fig. 5C). Consistent with ALF patients, the preclinical model of ALF showed marked increases in liver and plasma levels of chenodeoxycholic and muricholic acids, showing severe bile acid dysregulation and inflammation-associated tryptophan metabolism (Fig. 5D, Fig. 5E). Interestingly the expression of CDCA-3β-glucuronide(10) (deactivation of FXR indicating impaired bile acid transport) was significantly higher in preclinical model of ALF (Fig. 5F). In-

addition, key bile acid transporters (FXR, FGF15, SLC10A1, and others.) were downregulated in liver and intestine, showing impaired transport and primary bile acid accumulation in ALF model (Fig. 5F, Supplementary-Table-6). Overall, our results show that the preclinical ALF model mirrors ALF patient pathology, confirming its suitability for testing therapeutic interventions.

**Toxic metabolites directed use of Bacteroides Intestinalis AM-1 therapy restructured the microbiome and ameliorates liver injury, necrosis, and inflammation in preclinical model of ALF**

To establish the potential of gut modulation in improving liver failure and evaluate the therapeutic utility of Bacteroides Intestinalis AM-1 (known for metabolism of primary bile(6)and regulation of FXR axis (11). *Bacteroides intestinalis* AM-1 was cultured in an anaerobic medium and a suspension containing $1\times10^9$ CFU/mL colonies was administered to the preclinical model of ALF (Supplementary-Figure 20). In brief, *Bacteroides intestinalis* AM-1 was given following gut wash as detailed

1. Before APAP injection (7 days- 2 dose per day for gut colonization)
2. After APAP injection (3 days-2 dose per day) to mitigate ALF (Fig. 6A) and both the groups were compared for alleviation of liver injury, necrosis, and inflammation Supplementary-Figure-21

Stool analysis confirmed gut colonization of Bacteroides intestinalis AM-1 (Fig. 6A), which also promoted the growth of other bile acid metabolising taxa, including Bacteroides, Clostridiaceae, Lactobacillaceae and others (Fig. 6A). In the ALF model, Bacteroides intestinalis AM-1 pre-treatment improved outcomes, reducing primary bile acid accumulation, liver injury, necrosis, and inflammation in both the treatment groups (Fig. 6B). We also observed significant reduction of AST, ALT, and other liver function markers (Fig. 6C and Supplementary-Figure-22).

Interestingly, *Bacteroides intestinalis* AM-1 showed significant reduction in chenodeoxycholic acid levels in the plasma, liver, and intestine of ALF model (Figs. 6D and 6E). Bacteroides intestinalis AM-1 treatment upregulated key bile acid homeostasis genes (FXR, FGF15, SLC10A1, etc.) in liver and intestine, showing recalibrated bile acid metabolism, reduced primary bile acid accumulation, and improved liver functions (Fig. 6F, Supplementary-Figure 23). These findings highlight its potential to improve bile acid dysregulation and inflammation in the preclinical ALF model

**Bacteroides intestinalis AM-1 colonization modulates molecular pathways linked to liver injury and inflammation in pre-clinical model of ALF.**

Bacteroides intestinalis AM-1 treatment reduced necro-apoptosis by up to 70% in the ALF model. To assess microbiome-driven molecular changes, plasma and liver metabolome and proteome were analyzed across all mice groups.

Plasma and liver metabolome analysis showed that Gut microbiota modulation with Bacteroides intestinalis AM-1 in the ALF model led to reduced inflammation and oxidative stress, elevated glutathione metabolism, and improved antioxidant defence. This intervention decreased primary bile

acid accumulation and hepatic stress, showing protective effects on the liver (Fig. 7A and 7B and Supplementary Figures-24, 25, 26, 27, 28 and 29).

Bacteroides intestinalis AM-1 treatment suppressed liver NF-κB, MAPK, TLR4 signaling, and mitochondrial β-oxidation, further validating reduced inflammation, apoptosis, oxidative stress, and cellular damage (Fig. 7C). Glutathione-mediated detoxification was increased, enhancing oxidative stress neutralization and hepatic repair (Fig. 7C and Supplementary-Figure- 30, 31 and 32). These findings highlight the potential of gut microbiota modulation to influence key signaling and metabolic pathways, enhancing detoxification, limiting harmful mitochondrial activity, and protecting the liver and reducing severity.

**Dissecting the molecular mechanism linked to reduction of inflammation and Necro-apoptosis post Bacteroides intestinalis AM-1 treatment in preclinical model of ALF.**

In order to dissect the molecular mechanism linked to decrease in inflammation and necro-apoptosis seen post Bacteroides intestinalis AM-1 treatment in preclinical model of ALF. Liver proteomics and metabolomics were analysed. Liver expression of cytochrome P450 enzymes, particularly CYP2E1 and CYP3A41 was markedly increased which enhanced the conversion of APAP into toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) depleting glutathione and raising ROS levels. The resulting oxidative stress drives mitochondrial dysfunction, inflammation, bile acid accumulation, and widespread necrosis, accelerating liver damage (Fig. 8A).

Interestingly, treatment with Bacteroides intestinalis AM-1 in preclinical model of ALF not only significantly reduces NAPQI liver levels ($p < 0.05$) but also increased the expression of key enzymes such as L-histidine amino ligase, urocanate hydrates, Imidazolonepropionase, Glutamate cysteine ligase and GSH synthetase in the gut (> 1.5 folds, $p < 0.05$, known for the conversion of histidine to glutathione) we also observed significant increase in mitochondrial GSH transporters in the liver (SLC25A39, SLC25A40; $p < 0.05$, Fig. 8B, Supplementary Figure-31) which ascertains that gut bacteria driven glutathione reaches mitochondria and help in countering ROS and oxidative stress. Our results also suggest that colonization of Bacteroides intestinalis AM-1 in the gut not only modulates bile acid homeostasis but also provides a redox protection thereby increasing the systemic glutathione levels and mitigating mitochondrial stress and liver injury though our study provides Supportive mechanistic evidence, which warrants definitive proof of clinical human efficacy.

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Bacterial treatment significantly upregulated proteins across mitochondrial complexes I to V ($p < 0.05$), including NDUFS1/2/3/7/8, SDH subunits, UQCR proteins, COX4I1/5A/6A1, and ATP5F1A/B/C. This elevation shows improved electron transport, ATP synthesis, and hepatocyte bioenergetics (FC > 1.5; $p < 0.05$) (Fig. 8C and Supplementary-Figure-33).

Improved mitochondrial stress reduced cytochrome c release and apoptosome formation (Fig. 8C), thereby lowering BAX, CASP6, and CASP3 activation and suppressing apoptosis and necrosis (FC > 1.5; $p < 0.05$). Through modulation of glutathione metabolism, Bacteroides intestinalis AM-1 enhanced antioxidant defense, improved mitochondrial function, and inhibited cell death pathways, collectively supporting liver protection in preclinical model (Fig. 8D, Supplementary-Figure 32). Together, these results provide a mechanistic basis for reduced necrosis and redox stress modulation by AM-1 treatment in a preclinical ALF model

# DISCUSSION

The study investigated the plasma metabolome and bacterial peptides in ALF patients to identify putative indicator capable of segregation of ALF patients predisposed to early mortality. Identification and validation of the marker chenodeoxycholic acid in ALF patients led us to the hypothesis that Bacteroides intestinalis AM-1 treatment could benefit ALF patients and its therapeutic efficacy was evaluated in a preclinical mice model of ALF.

ALF plasma metaproteome and metabolome analysis showed marked dysbiosis with increased inflammation-associated taxa, increased inflammation metabolites and reduced bile acid metabolizing, antioxidant-linked bacteria, suggesting that microbial imbalance drives metabolome alterations and clinical phenotype in ALF-NS patients.

ALF plasma analysis revealed metabolite and microbial peptide panels, with metabolites showing superior accuracy for predicting early mortality. Chenodeoxycholic acid (> 10-fold increase) showed the highest hazard ratio and strongest discrimination. Similar elevations, including toxic tryptophan metabolites, were confirmed in APAP-induced ALF mice, accompanied by reduced bile acid–metabolizing taxa (Bacteroides, Clostridia). These observations suggest that the preclinical model of ALF recapitulates the molecular pathology observed in ALF patients.

In the preclinical ALF model, B. intestinalis AM-1 treatment significantly reduced necro-apoptosis and inflammation by restoring bile acid homeostasis, lowering toxic metabolites (NAPQI, chenodeoxycholic acid), and suppressing hepatic caspase-3/6 activity. It also improved ROS detoxification via microbial glutathione biosynthesis, enhanced antioxidant defense, and strengthened mitochondrial function, promoting liver recovery.

Systemic inflammation and stress predispose ALF patients to early mortality, with plasma metabolites and metaproteome amplifying oxidative stress inflammation loops that accelerate disease progression(2). Analysis of the plasma metaproteome as the functional correlate (reflecting functional host-microbe interactions and microbial translocation) of the gut microbiome in ALF-NS showed substantial gut dysbiosis, marked by inflammation and impaired detoxification linked to reduced conversion of primary to secondary bile acids due to loss of taxa such as Bacteroides and Clostridia(12).

Plasma analysis of ALF non-survivors revealed elevated chenodeoxycholic and cholic acids, reduced secondary bile acids, and depletion of associated bacteria. Disrupted amino acid metabolism was evident, with decreased branched-chain and increased aromatic amino acids indicating oxidative stress and impaired protein synthesis (13). Elevated tryptophan and kynurenine pathway activation correlated with immune dysfunction(14, 15), while excessive chenodeoxycholic acid was linked to early mortality through microbiota imbalance, impaired detoxification, and mitochondrial dysfunction(16–18). Increased carnosine suggested a neuroprotective response to ammonia-induced stress(19, 20). Non-survivors also showed higher microbial diversity and circulating bacterial peptides, indicating gut dysbiosis and compromised intestinal integrity(5, 21). Collectively, these metabolomic and microbial signatures provide prognostic value and mechanistic insights into ALF progression.

A strong correlation was seen between plasma metabolome and meta-proteome in ALF non-survivors, as revealed by MMCC network analysis. Module-trait relationships identified key metabolic pathways tryptophan, bile acid, and tyrosine metabolism linked to specific microbial modules, especially the turquoise module, highlighting microbial influence in ALF pathogenesis(22). Tryptophan metabolism, modulated by gut microbiota, were notably associated with inflammation and immune responses(22). The turquoise meta-proteome module, enriched in Actinobacteria, Proteobacteria, and Firmicutes, corresponded to elevated aromatic amino acids, indicative of disease severity. These microbial shifts reflect gut dysbiosis and increased intestinal permeability, promoting systemic inflammation via altered bile acid metabolism(23, 24). The meta-proteome diversity predicted infection and multi-organ failure, while metabolome changes associated with AKI and hepatic encephalopathy.

CDCA and related metabolites/meta-proteins were significantly elevated versus SAH controls, across ALF etiologies, and between survivors and non-survivors/transplanted patients (supplementary figure-15, 16 and 19), indicating disease-independent microbial-metabolic signatures with universal prognostic potential.

Cross validation of five plasma metabolites, including chenodeoxycholic acid, in three distinct validation cohorts, with random forest models achieving high predictive accuracy for early mortality. This pilot study proposes a robust, mass spectrometry-based plasma metabolite panel for early ALF risk stratification, offering significant clinical potential.

Our study confirms that the accumulation of primary bile acids, particularly chenodeoxycholic acid (CDCA) and cholic acid/ muricholic acid, contributing to inflammation and hepatocellular death in preclinical mouse model of acute liver failure (ALF). The dose-dependent elevation of serum ALT and AST levels, along with histological evidence of necrosis, confirmed liver injury in APAP-treated groups.

Plasma and liver metabolome in the ALF model showed elevated primary bile acids and altered tryptophan metabolism, reflecting inflammation and systemic dysregulation consistent with ALF patients.

A critical finding was the marked increase in CDCA and its metabolite CDCA-3β-glucuronide, which is known to inhibit farnesoid X receptor (FXR) signaling(10). FXR is a key nuclear receptor that regulates bile acid homeostasis and protects against bile acid-mediated liver injury(8). Its deactivation likely exacerbates bile acid toxicity, contributing to hepatocellular damage and inflammation. This was further supported by significant downregulation of FXR and other bile acid transport genes (e.g., FGF15, SLC10A1) in both hepatic and intestinal tissues of preclinical mice model, indicating impaired enterohepatic circulation and bile acid clearance(8). These results suggest that bile acid accumulation drive inflammation, oxidative stress as a central pathogenesis of APAP-induced liver injury.

Bacterial gut modulation has been studied in patients with alcoholic hepatitis, COPD, and the other organ system(11).

Gut microbiome modulation and targeting bile acid metabolism represent promising strategies to mitigate ALF progression (9). This was achieved by using *Bacteroides intestinalis AM-1*, a bacterium involved in primary bile acid metabolism, and observed a significant reversal of liver damage in preclinical model of APAP-induced acute liver failure (ALF)(6). Metabolomic profiling of plasma and liver supported the beneficial effect of *Bacteroides intestinalis* AM-1 and showed reduced primary bile acid, inflammatory metabolites, and oxidative stress. Importantly, upregulation of bile acid transport and regulatory genes (FXR, FGF15, SLC10A1) in both liver and intestine suggests restoration of bile acid signalling and improved enterohepatic circulation(11). These findings highlight the therapeutic potential of modulating gut microbiota to influence host metabolic and immune pathways, offering a promising strategy for mitigating liver injury and promoting recovery in preclinical model of ALF.

Interestingly, *Bacteroides intestinalis* AM-1 colonization modulate the microbiome to enhanced glutathione biosynthesis via increasing key enzymes (L-histidine amino ligase, urocanate hydrates, Imidazolonepropionase, Glutamate cysteine ligase and GSH synthetase) in the gut supporting enhanced oxidative detoxification. This was corroborated with significant reduction of hepatotoxic metabolites (NAPQI, chenodeoxycholic acid), significantly reducing liver stress. These findings suggest that gut microbial modulation is associated with changes in metabolic pathways that may influence liver injury outcomes

Proteomic analysis confirmed the protective effects of Bacteroides intestinalis AM-1, showing reduced activation of pro-inflammatory NF-κB and MAPK signaling pathways, along with suppression of TLR4-mediated immune activation(25). These changes indicate a dampened inflammatory response and reduced apoptosis in the liver. Additionally, the increase in glutathione metabolism, causing decrease in mitochondrial dysfunction thus decreasing oxidative stress and further supporting hepatoprotection(26–28).

Hepatic proteomics revealed a marked upregulation of mitochondrial GSH transporters (SLC25A39, SLC25A40), confirming efficient delivery of gut bacteria derived glutathione to mitochondria, where it neutralizes ROS and mitigates oxidative stress(29). This adaptation enhanced electron transport, ATP production, and overall hepatocyte bioenergetics. Consequently, mitochondrial stress was alleviated,

reducing cytochrome c release and apoptosome formation, which in turn suppressed activation of BAX, CASP6, and CASP3, limiting apoptosis and necrosis. Through modulation of glutathione metabolism, B. intestinalis AM-1 strengthened antioxidant defense, improved mitochondrial performance, and blocked cell death pathways, thereby supporting liver protection and recovery in the preclinical model. Collectively, these data provide a mechanistic explanation for how Bacteroides intestinalis AM-1reduces necrosis and regulates redox stress in preclinical ALF. The results of our study strongly show that treatment with Bacteroides intestinalis AM-1could mitigate liver injury and could be evaluated in ALF patients (30, 31)

A limitation of this study is the reliance on a preclinical APAP-induced ALF model, which may not fully capture the complexity and heterogeneity of human ALF, although key metabolome and metaproteome alteration were similar to those observed in ALF patients. Safety studies would be required in patients with HAV and HEV induced ALF, as bacteria can induce strong immune reactions(32). Additionally, validation of our panel in larger, multicenter patient cohorts is necessary. The translational efficacy of Bacteroides intestinalis AM-1 in humans remains to be established, and long-term safety and microbiome dynamics require further investigation. Furthermore, antibiotic exposure details for every patient were not available however consistent increase of the markers in ALF-NS suggests that observed signals are not solely antibiotic-driven but reflect underlying disease biology.

To conclude our study generates the hypothesis and validates that circulating level of chenodeoxycholic acid could segregate ALF patient predisposed to early mortality. We also evaluated the therapeutic benefit of Bacteroides intestinalis AM-1 treatment to mitigate liver failure in preclinical model of ALF (integrating clinical outcomes with mechanistic biology ) providing a rationale for evaluating this as a strategy for treatment of patients with liver failures (Graphical abstract).

# METHODS

## Patient Cohorts

A total of 270 ALF patients and 25 healthy controls were prospectively enrolled at admission between December 2019 and April 2023 at ILBS, New Delhi, India. Baseline samples were obtained before treatment initiation. ALF was diagnosed using standard criteria: acute jaundice with encephalopathy within four weeks, INR >1.5, and absence of chronic liver disease(1).

Patients with cirrhosis-related features (splenomegaly, ascites, HBV/HCV infection, or chronic alcohol use) were excluded. ALF etiologies included DILI (training 42.8%, test 38.58%), viral causes (training 48.2%, test 51.8%), and others such as Wilson disease and autoimmune hepatitis (training 9%, test 9.62%). All patients underwent baseline CT brain to exclude cerebral edema before L-ICU transfer. Management included ventilation when needed, sedation (fentanyl/propofol), hemodynamic-guided fluids, and vasopressors (norepinephrine ± vasopressin)(3). All patients received intravenous N-acetylcysteine for 5 days, and antimicrobials per protocol. CRRT was initiated in cases with suspected

cerebral edema(33). Liver transplantation was offered when feasible according to the King's College Criteria (subject to donor and resource availability(34) . Survivor patients were clinically followed for 90 days and survivors were classified as ALF-S. Non-survivors (ALF-NS) included those who died from progressive liver failure, cerebral edema, sepsis, or underwent transplant (within 30 day) (Death= 146; Eligible for Liver Transplant=32). All Human studies were ethical approval (IEC/2019/70/NA06) and conducted in accordance with the Declaration of Helsinki.

Ethical approval (IEC/2019/70/NA06) and written informed consent were obtained further detailed in (Supplementary methods and Figure 1A).

Untargeted Metabolomics and Meta-proteomics analysis:

Untargeted plasmaMetabolomics and meta-proteomics analysis of plasma samples were performed using ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS). The detailed protocol is provided in the Supplementary method and our recent publication(2)

Weighted co-expression analysis (WM/MpCNA) was constructed for metabolites and meta-proteins using bi-weight mid-correlation (soft threshold>9, power>12, scale-free topology (0.85). Additionally, Probability of Death (POD) wascalculated followed by machine learning (ML) detailed in supplementary method.

Bacterial and Cell Culture: Bacteroides intestinalis AM-1 (DSM 17393; DSMZ, Germany) was cultured anaerobically on Columbia agar supplemented with 5% sheep blood at 37°C (details in Supplementary Methods).

Preclinical ALF Model: Male germ-free C57BL/6 mice (6 weeks) were bred in-house, and all procedures were approved by the ILBS Animal Ethics Committee (IAEC/ILBS/23/1). and the study is reported in accordance with ARRIVE guidelines. Acute liver failure was induced by intraperitoneal acetaminophen (APAP; 250 or 500 mg/kg) in warm saline in non-fasted mice. For bacterial intervention, mice received gut cleansing with polyethylene glycol, followed by B. intestinalis AM-1 ($10^9$ CFU/200 µL, intragastric). detailed in Supplementary method.

Histology and Biochemistry and molecular analysis:FFPE liver sections underwent H&E staining for injury assessment; serum ALT/AST were measured. Liver and intestinal RNA was analyzed by qPCR using SYBR Green, GAPDH normalization, and bacterial gene detection was performed.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 6; GraphPad Software Inc., La Jolla, CA, USA), and SPSS (version 20; IBM Co., Armonk, NY, USA). A *P*-value <0.05, adjusted using the Benjamini-Hochberg correction, was considered statistically significant. Group comparisons were done by using t-test, Mann–Whitney U, ANOVA, or Kruskal–Wallis tests; correlations applied Spearman ($r^2$>0.5, p<0.05). Missing values were imputed, data log-normalized, and Pareto-scaled via MetaboAnalyst 5.0 for

PCA, PLS-DA, heatmaps, and random-forest analysis. Top metabolites underwent univariate and multivariate AUROC, hazard ratio, and Kaplan–Meier survival analysis and Machine learning further detailed in supplementary method.

## Abbreviations

AKT/PKB Protein kinase B

ALF Acute Liver Failure

ALP Alkaline Phosphatase

ALS Artificial Liver Support

ALT Alanine transaminases

AST Aspartate transaminase

AUROC Area under the Receiver Operating Characteristics

CART Classification and Regression Tree

CDCA chenodeoxycholic acid

CRRTContinuous Renal Replacement Therapy

CT Computed Tomography Scan

CTPChild-Turcotte-Pugh

DEM Differentially Expressed Metabolite

DEMP Differentially Expressed Meta

DILI Drug Induced Liver Injury

ERK Extracellular signal

GGTGamma-glutamyl Transferase

HAV Hepatitis A virus

HC Healthy Control

HEV Hepatitis E virus

HR Hazard Ratio

HRMSHigh Resolution mass spectorometery

IL1β- Interleukin-1-beta

JNKJun N-terminal kinase

KCH King's College Criteria

KNNk-nearest neighbours

LDA Linear discriminant analysis

MELDModel for End-Stage Liver Disease

ML Machine Learning

NLDB National Liver disease Biobank

NLRP3Nucleotide-binding domain, leucine-rich–containing family, pyrin domain containing-3

NS non- survivors

PLSDAPartial least squares-discriminant analysis

POD Probability of detection

RF Random Forest

ROS Reactive Oxygen Species

S Survivors

SAH Severe Alcoholic Hepatitis

SLEDSustained low-efficiency dialysis

SVM Support Vector Machine

TC Test Cohort

TRAILTNF-related apoptosis-inducing ligand (TRAIL)

# Declarations

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**Ethical approval Statement- All Human studies were ethical approval (IEC/2019/70/NA06) and conducted in accordance with the Declaration of Helsinki and Animal Ethical approval Institutional Animal Ethics Committee (Approval No: IAEC/ILBS/23/1), he study is reported in accordance with ARRIVE guidelines.**

Authors' contribution: JSM conceptualized the work. SP, and NS helped in sample enrolment, processing, and experimental work and were helped by NS, SB, VS, MK, AK, YM, AG, BM, GT, VB, SY, MY, AK, SS, CB, AK, RM and SKS provided the samples. Data analysis was performed by SP and NS under the guidance of JSM. The manuscript was drafted by SP, NS, and JSM. RM and SKS read the manuscript and provided expert advice. This manuscript has been seen approved by all authors.

DATA availability statement: The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials further the data can also be found on https://doi.org/10.6084/m9.figshare.31323484 further the raw data of the work is available on request to the corresponding author.

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# Figures

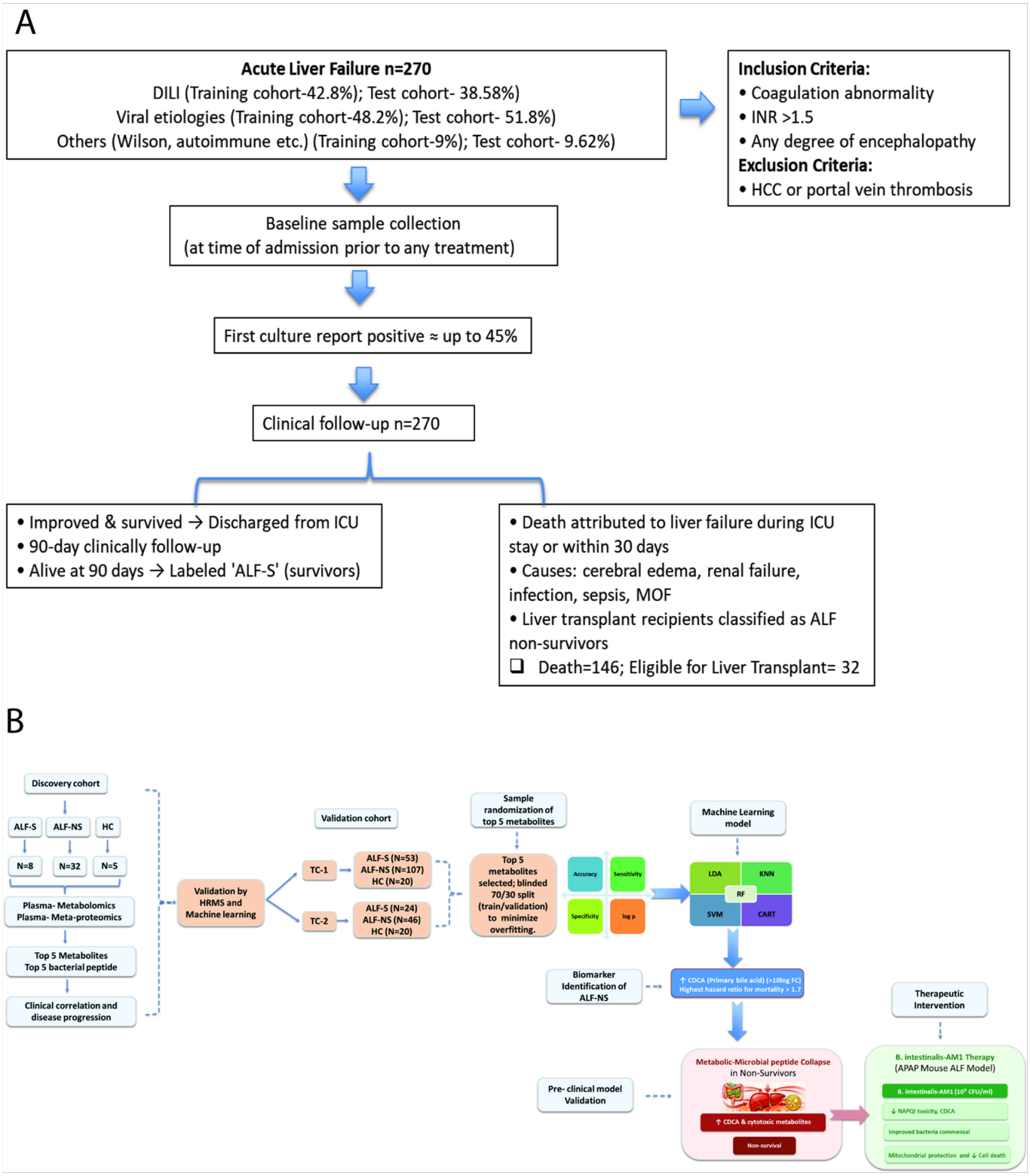
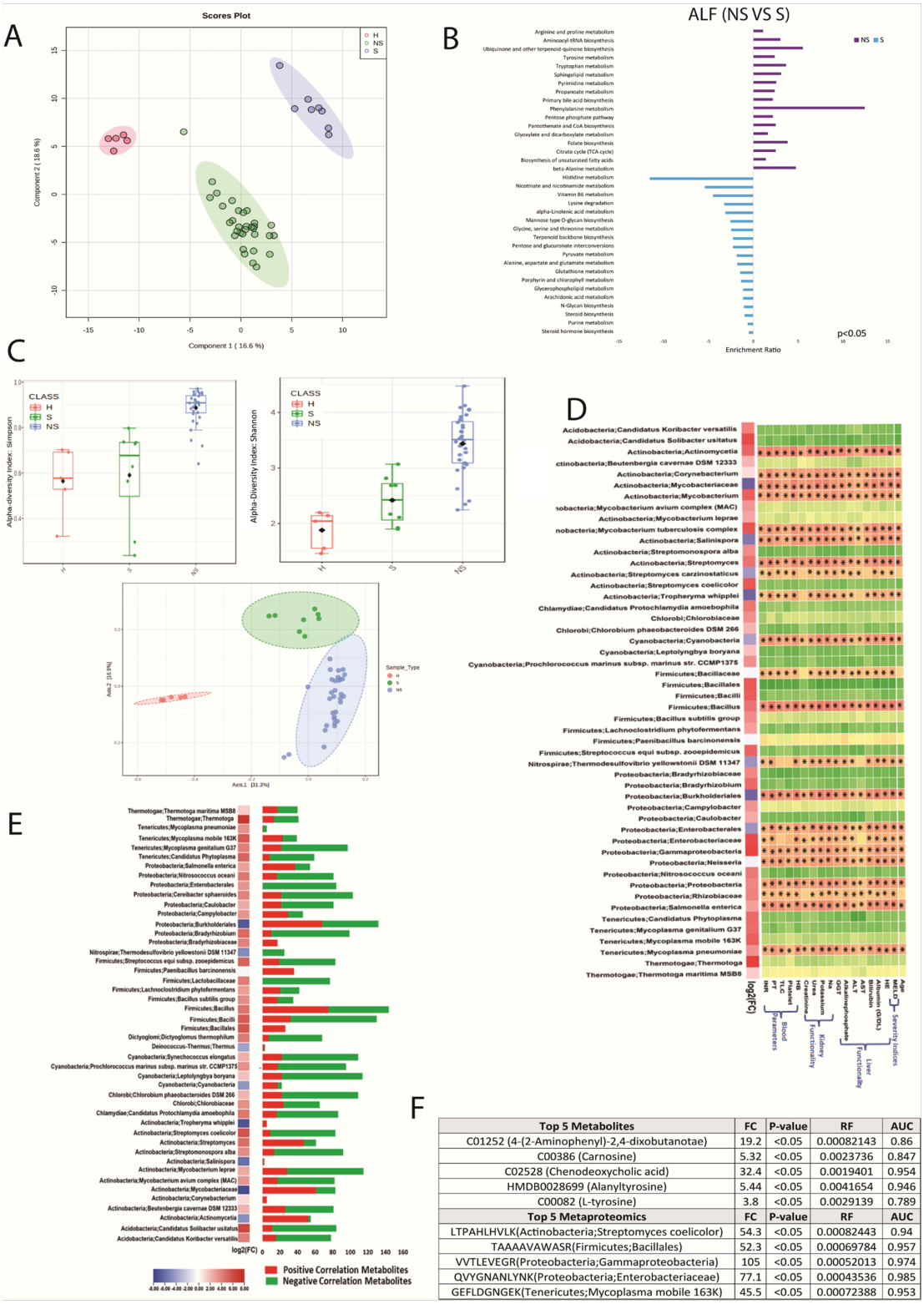



**Figure 1**

1A- Overview of patient enrolment and selection criteria.

Figure 1B- Study Design

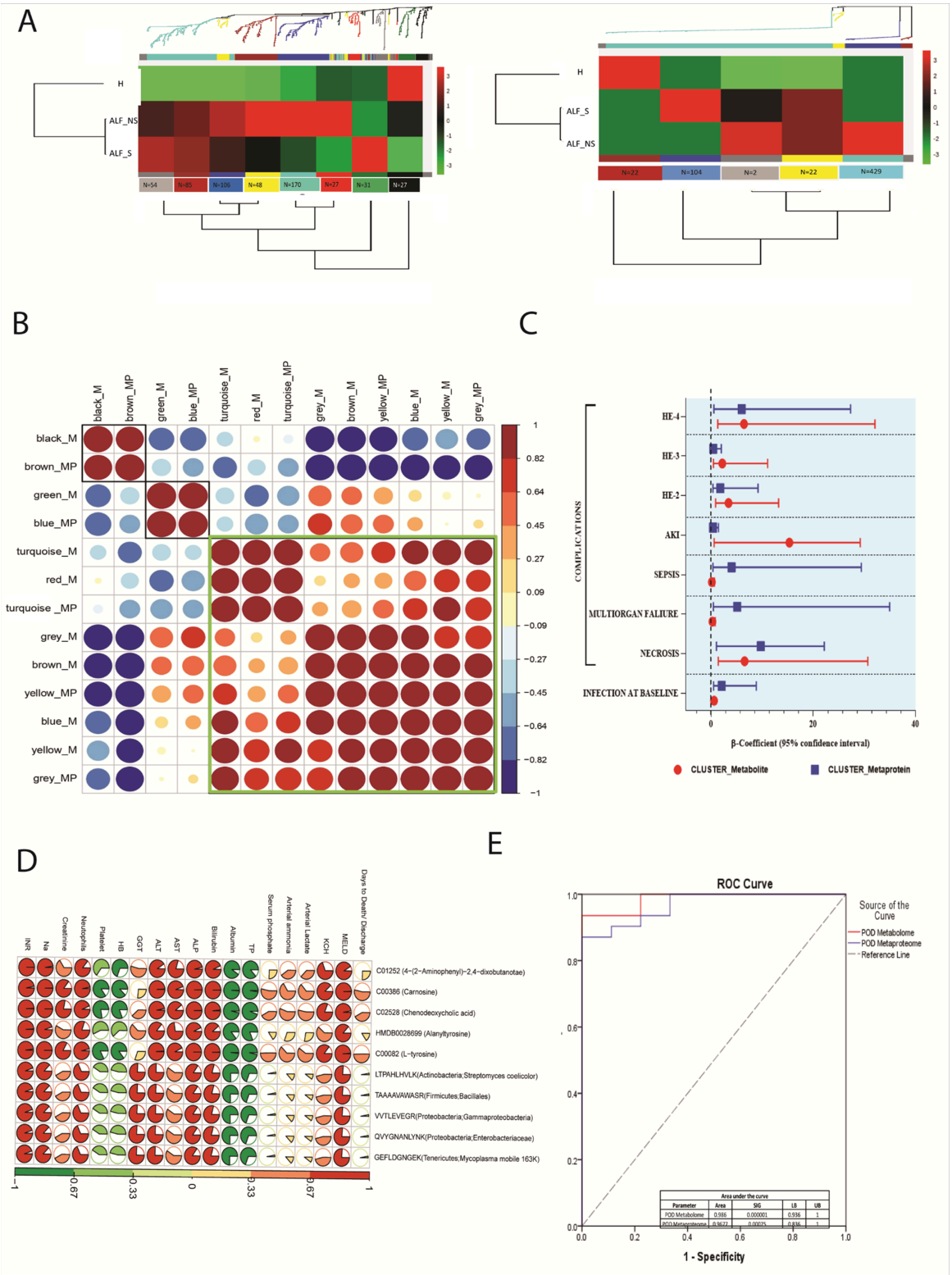



| Top 5 Metabolites | FC | P-value | RF | AUC |
|---|---|---|---|---|
| C01252 (4-(2-Aminophenyl)-2,4-dixobutanotae) | 19.2 | <0.05 | 0.00082143 | 0.86 |
| C00386 (Carnosine) | 5.32 | <0.05 | 0.0023736 | 0.847 |
| C02528 (Chenodeoxycholic acid) | 32.4 | <0.05 | 0.0019401 | 0.954 |
| HMDB0028699 (Alanyltyrosine) | 5.44 | <0.05 | 0.0041654 | 0.946 |
| C00082 (L-tyrosine) | 3.8 | <0.05 | 0.0029139 | 0.789 |
| **Top 5 Metaproteomics** | **FC** | **P-value** | **RF** | **AUC** |
| LTPAHLHVLK(Actinobacteria;Streptomyces coelicolor) | 54.3 | <0.05 | 0.00082443 | 0.94 |
| TAAAAVAWASR(Firmicutes;Bacillales) | 52.3 | <0.05 | 0.00069784 | 0.957 |
| VVTLEVEGR(Proteobacteria;Gammaproteobacteria) | 105 | <0.05 | 0.00052013 | 0.974 |
| QVYGNANLYNK(Proteobacteria;Enterobacteriaceae) | 77.1 | <0.05 | 0.00043536 | 0.985 |
| GEFLDGNGEK(Tenericutes;Mycoplasma mobile 163K) | 45.5 | <0.05 | 0.00072388 | 0.953 |

## Figure 2

**(A)** Partial least squares discriminant analysis (PLS-DA) demonstrating clear metabolic segregation of healthy controls (red), ALF survivors (blue), and ALF non-survivors (green).
**(B)** Pathway enrichment analysis comparing ALF non-survivors versus survivors, showing significantly upregulated (purple) and downregulated (blue) pathways ($p<0.05$).
**(C)** Alpha-diversity (Shannon and Simpson indices) and beta-diversity (PCoA) analyses of

metaproteomic profiles at the class level across study groups ($p<0.05$).
**(D)** Correlation of clinical parameters with metaproteomic phyla, indicating differential expression patterns (red, upregulated; blue, downregulated).
**(E)** Bacterial peptides associated with positively and negatively correlated metabolites, with corresponding metaproteomic expression levels (red, positive correlation; green, negative correlation; $R^2<0.5$, $p<0.05$).
**(F)** Top five discriminatory metabolites and bacterial peptides identified based on fold change, p-value, random forest importance, and AUC, highlighting links between pathogenic bacterial peptides, metabolic pathways, and ALF severity.




## Figure 3

**(A)** Weighted metabolome and metaproteome correlation network analysis heatmaps illustrating module-trait relationships across groups ($R^2$>0.5, p<0.05).

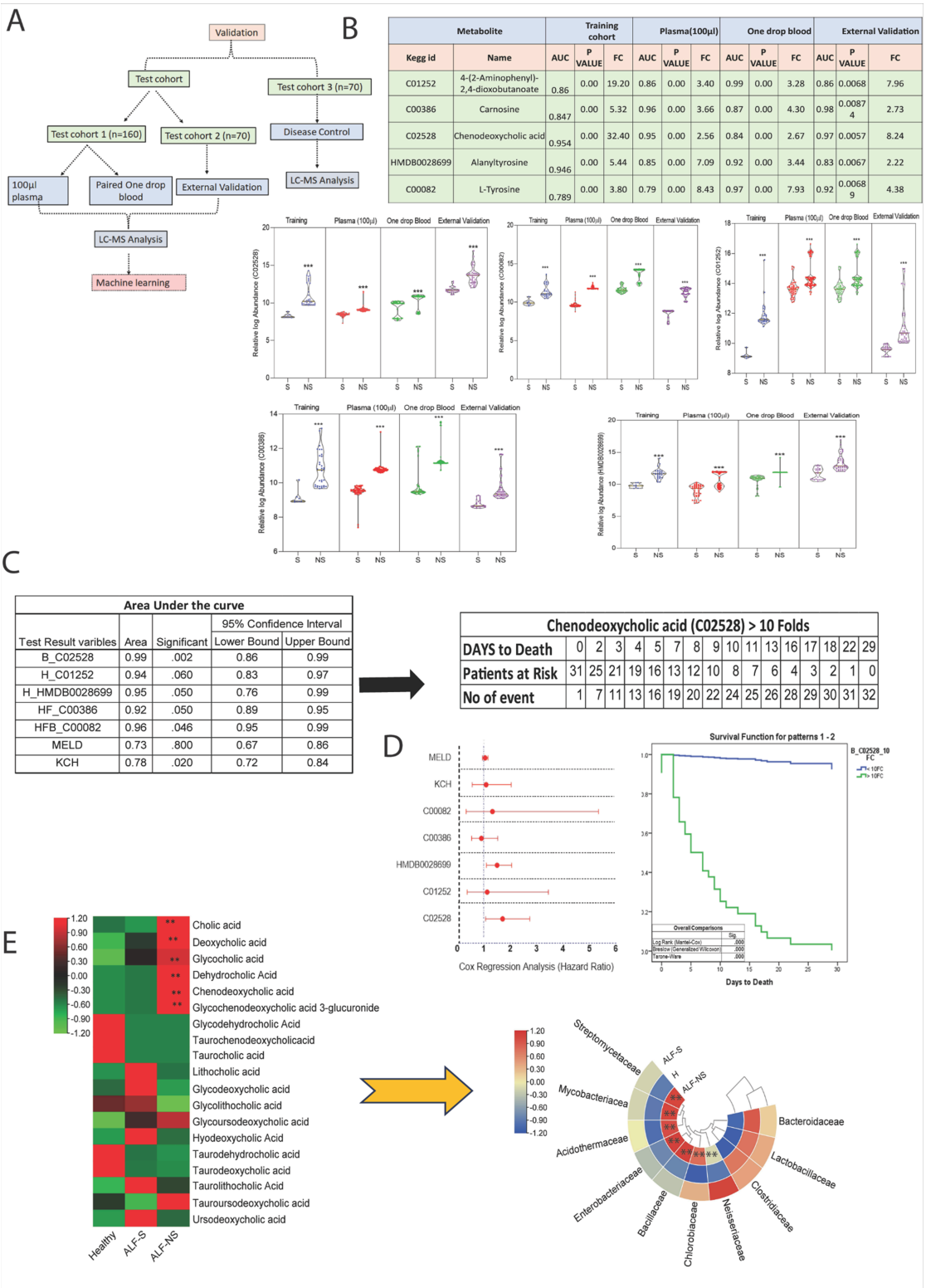
**(B)** Multi-modular correlation cluster (MMCC) plot showing significant interconnections between metabolomic and metaproteomic modules ($R^2$>0.4, p<0.05).

**(C)** Linear regression analysis linking metabolome-metaproteome module clusters with ALF-associated

clinical complications.

**(D)** Correlation of clinical parameters with the top five metabolomic and metaproteomic features (red, upregulated; green, downregulated).

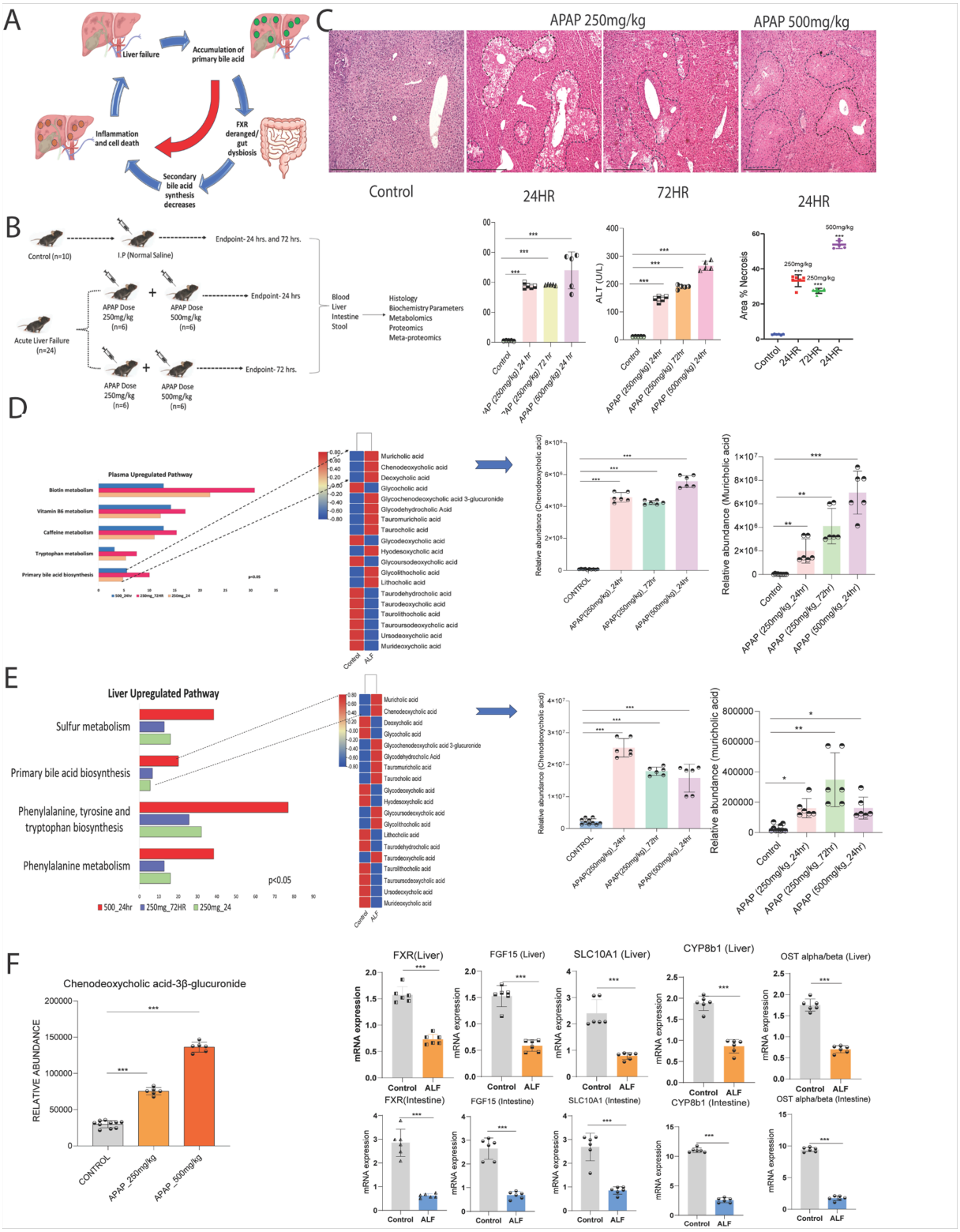
**(E)** Comparative performance of post-onset day (POD) metabolome and metaproteome, demonstrating superior AUROC for metabolomics (>0.95, p<0.05).




| Metabolite | | Training cohort | | | Plasma(100µl) | | | One drop blood | | | External Validation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kegg id | Name | AUC | P VALUE | FC | AUC | P VALUE | FC | AUC | P VALUE | FC | AUC | P VALUE | FC |
| C01252 | 4-(2-Aminophenyl)-2,4-dioxobutanoate | 0.86 | 0.00 | 19.20 | 0.86 | 0.00 | 3.40 | 0.99 | 0.00 | 3.28 | 0.86 | 0.0068 | 7.96 |
| C00386 | Carnosine | 0.847 | 0.00 | 5.32 | 0.96 | 0.00 | 3.66 | 0.87 | 0.00 | 4.30 | 0.98 | 0.00874 | 2.73 |
| C02528 | Chenodeoxycholic acid | 0.954 | 0.00 | 32.40 | 0.95 | 0.00 | 2.56 | 0.84 | 0.00 | 2.67 | 0.97 | 0.0057 | 8.24 |
| HMDB0028699 | Alanyltyrosine | 0.946 | 0.00 | 5.44 | 0.85 | 0.00 | 7.09 | 0.92 | 0.00 | 3.44 | 0.83 | 0.0067 | 2.22 |
| C00082 | L-Tyrosine | 0.789 | 0.00 | 3.80 | 0.79 | 0.00 | 8.43 | 0.97 | 0.00 | 7.93 | 0.92 | 0.00689 | 4.38 |

**Figure 4**

**(A)** Schematic overview of the overall study design.
**(B)** Panel of five top-ranked metabolites selected using minimal depth analysis, fold change, and significance, compared across study groups and cohorts.
**(C)** AUROC comparison of MELD score, KCH criteria, and metabolic indicators (C02528, HMDB0028699, C00386, C00082, C01252).
**(D)** Hazard ratio and survival analysis stratified by elevated chenodeoxycholic acid (C02528; >log10 fold change).
**(E)** Heatmap of bile acid profiles in ALF non-survivors, survivors, and healthy controls, highlighting primary bile acid accumulation and reduced primary to secondary bile acid converting bacteria.




## Figure 5

**(A)** Working hypothesis linking primary bile acid accumulation, gut dysbiosis, FXR disruption, reduced secondary bile acids, inflammation, and hepatocyte death in ALF.

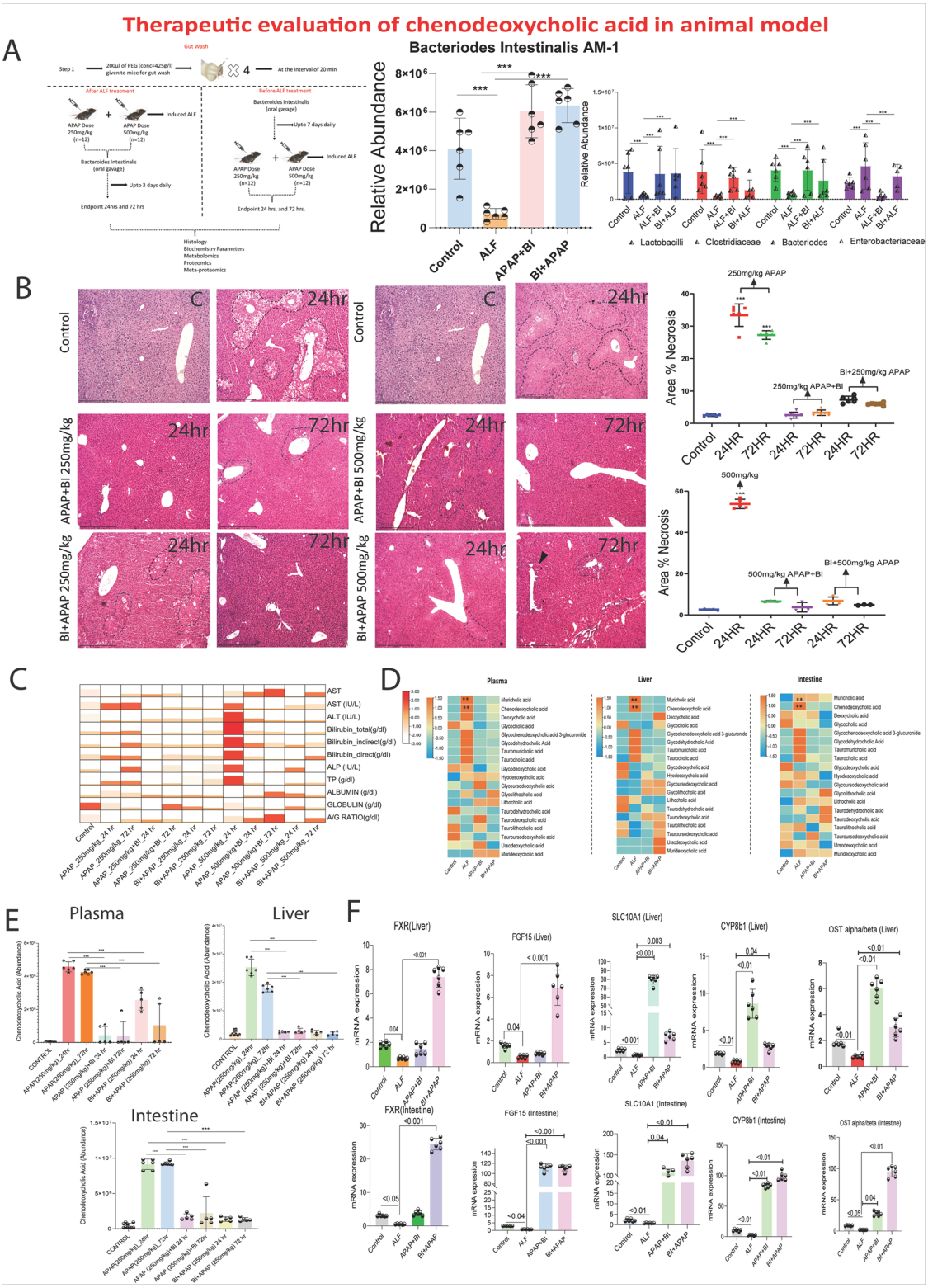
**(B)** Experimental design of APAP-induced liver injury using 250 mg/kg and 500 mg/kg doses in male C57BL/6 mice.

**(C)** H&E staining showing progressive necrosis at 24 and 72 hours post-APAP administration (scale bar

500 μm, 10×), accompanied by increased serum AST and ALT.
**(D)** Elevated plasma primary bile acids, including chenodeoxycholic and cholic acid, in APAP-treated mice ($p<0.05$).

**(E)** Elevated liver primary bile acids, including chenodeoxycholic and cholic acid, in APAP-treated mice ($p<0.05$).

**(F)** Increased chenodeoxycholic acid β-glucuronide levels (left) and altered bile transporter gene expression (right) in APAP-induced ALF ($p<0.05$).




## Figure 6

**(A)**Schematic of Bacteroides intestinalis AM-1 therapeutic intervention, stool colonization levels, and bile acid-converting bacterial expression across groups ($p<0.05$).

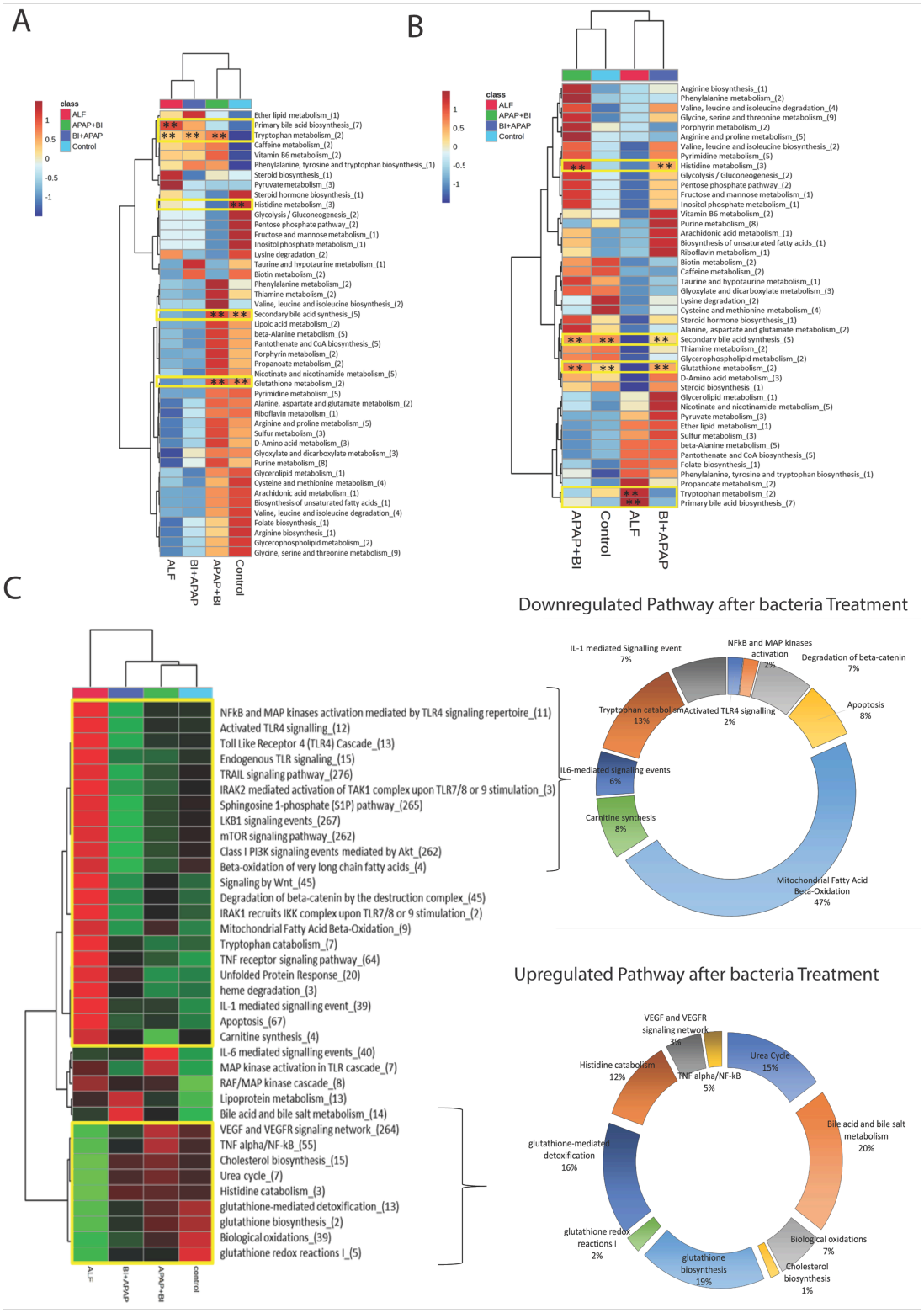
**(B)** Reduced necrotic progression in BI-treated mice by H&E staining.

**(C)** Decreased serum AST and ALT indicating liver recovery following BI treatment.

**(D)** Heatmaps showing bile acid redistribution in plasma, liver and intestine in different groups

**(E)**Reduced chenodeoxycholic acid levels in treated groups (p<0.05).

**(F)** Differential bile transporter mRNA expression among control, APAP, and BI-treated mice (p<0.05).




## Figure 7

(A) Unsupervised hierarchical clustering of plasma metabolome pathways separated treated, ALF (250_24h), and control mice groups.

(B) Unsupervised hierarchical clustering of liver metabolome pathways separated treated, ALF (250_24h), and control mice groups.

(C) Unsupervised hierarchical clustering with different group showing liver proteome pathway segregate Treatment and ALF (250_24hrs) and control group in mice model.

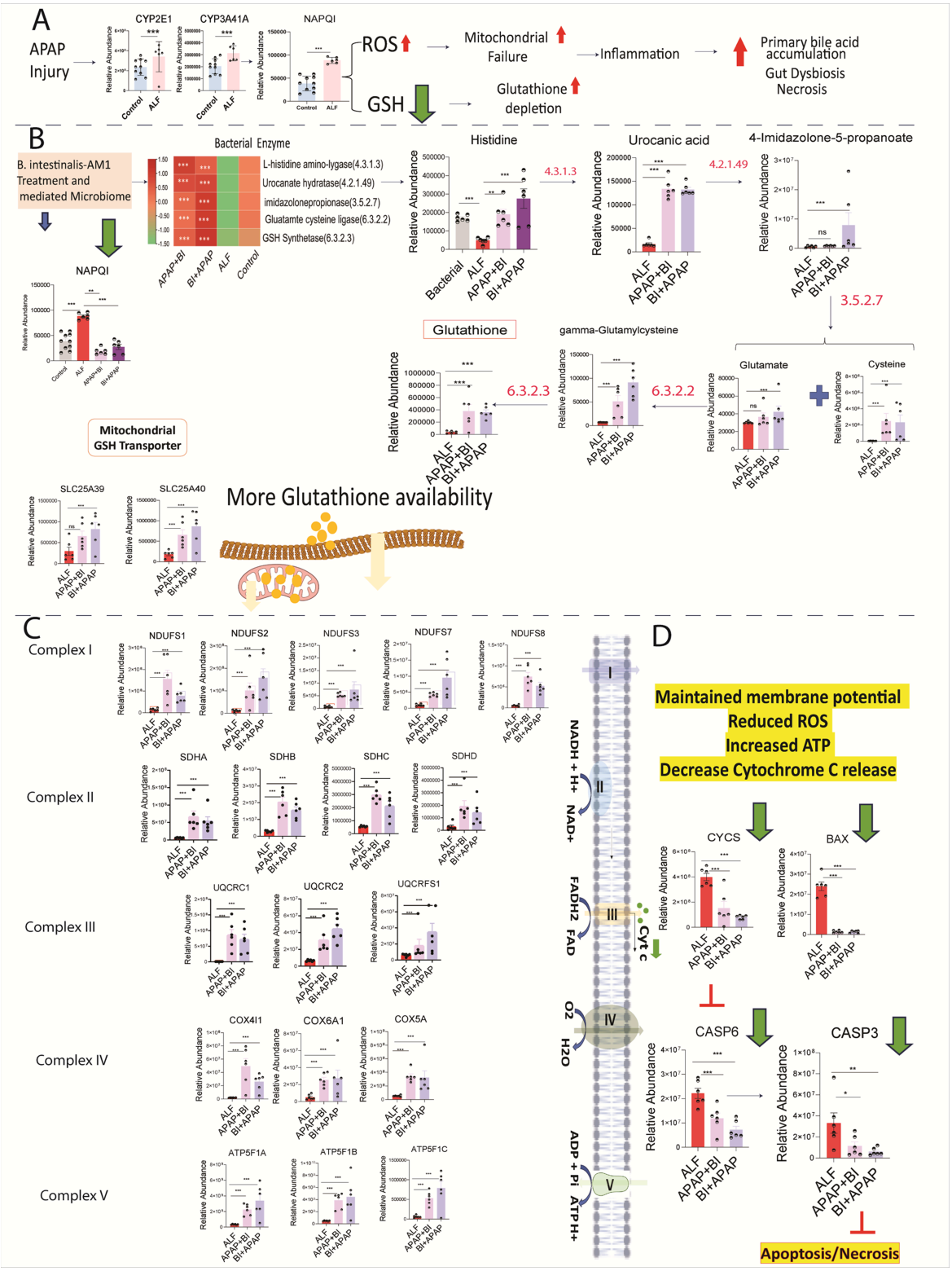



**Figure 8**

**(A)** APAP-induced acute liver injury, CYP2E1/CYP3A4 generate toxic NAPQI, depleting GSH and raising ROS. Resulting mitochondrial dysfunction and inflammation drive oxidative stress, hepatocyte death, and bile acid accumulation.

**(B)** Bacteroides intestinalis AM-1 modulated gut microbiota, promoting primary-to-secondary bile acid conversion and glutathione synthesis; metaproteomics showed histidine-to-glutathione pathways (FC>1.5; p<0.05).

(**C)** Bacterial treatment significantly upregulated proteins across mitochondrial complexes I to V (FC>1.5; p<0.05)

**(D)** Bacteroides intestinalis AM-1 boosted antioxidant defense, improved mitochondrial function, and reduced cell death, protecting liver in preclinical models (FC>1.5; p<0.05).

## Supplementary Files

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