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ORIGINAL ARTICLE

Comparative metabolome analysis reveals higher potential of haemoperfusion adsorption in providing favourable outcome in ACLF patients

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Abstract

Background and Aims: Acute-on-chronic liver failure (ACLF) is a serious illness associated with altered metabolome, organ failure and high mortality. Need for therapies to improve the metabolic milieu and support liver regeneration are urgently needed.

Methods: We investigated the ability of haemoperfusion adsorption (HA) and therapeutic plasma exchange (TPE) in improving the metabolic profile and survival in ACLF patients. Altogether, 45 ACLF patients were randomized into three groups: standard medical therapy (SMT), HA and TPE groups. Plasma metabolomics was performed at baseline, post-HA and TPE sessions on days 7 and 14 using high-resolution mass spectrometry.

Results: The baseline clinical/metabolic profiles of study groups were comparable. We identified 477 metabolites. Of these, 256 metabolites were significantly altered post 7 days of HA therapy ($p < .05$, FC > 1.5) and significantly reduced metabolites linked to purine (12 metabolites), tryptophan (7 metabolites), primary bile acid (6 metabolites) and arginine-proline metabolism (6 metabolites) and microbial metabolism respectively ($p < .05$). Metabolites linked to taurine-hypotaurine and histidine metabolism were reduced and temporal increase in metabolites linked to phenylalanine and tryptophan metabolism was observed post-TPE therapy ($p < .05$). Finally, weighted metabolite correlation network analysis (WMCNA) along with inter/intragroup analysis confirmed significant reduction in inflammatory (tryptophan, arachidonic acid and bile acid metabolism) and secondary energy metabolic pathways post-HA therapy compared to TPE and SMT ($p < .05$). Higher baseline plasma level of 11-deoxycorticosterone (C03205; AUROC > 0.90, HR > 3.2) correlated with severity ($r^2 > 0.5$, $p < .05$) and mortality (log-rank-$p < .05$). Notably, 51 of the 64 metabolite

Abbreviations: ACLF, acute on chronic liver failure; ALSS, artificial liver support systems; AUC, area under curve; DAMPs, damage associated molecular patterns; DEMs, differentially expressed metabolites; ELSs, extracorporeal liver support system; FFP, fresh frozen plasma; HA, haemoperfusion adsorption; HC, healthy control; PAMPs, Pathogen-Associated Molecular Patterns; PLS-DA, partial least square discriminant analysis; PV, plasma volume; SMT, standard medical therapy; TPE, therapeutic plasma exchange.

signatures (ACLF non-survivor) were reversed post-HA treatment compared to TPE and SMT($p < .05$).

Conclusion: HA more potentially (~80%) improves plasma milieu compared to TPE and SMT. High baseline plasma 11-deoxycorticosterone level correlates with early mortality in ACLF patients.

KEYWORDS
extracorporeal liver support system, haemoperfusion adsorption, metabolomic profile, safety and efficacy, therapeutic plasma exchange

1 | INTRODUCTION

Acute-on-chronic liver failure (ACLF) has high short-term mortality and is seen in up to 40% of patients with cirrhosis requiring hospitalization.[1,2] Systemic inflammation associated with hepatic insult is a key driver for ACLF progression.[3,4] For ACLF patients, liver transplant is the only definitive treatment; therefore, for the management of such patients, extracorporeal liver support systems (ELSs) have been an emerging area of interest.[5] During the last decades, many types of ELSs, such as haemoperfusion adsorption, continuous haemodiafiltration, molecular absorbents recirculating system (MARS) and fractionated plasma separation, absorption and dialysis system (FPSA), have been used for the management of liver failure.[6] These artificial liver support systems (ALSs) can serve as a bridge to liver transplant by temporary removal of several toxic metabolites making the internal milieu suitable for the diseased liver to recover and regenerate.[7]

In the current study, we compared the efficacy in terms of plasma purification and survival benefits or reversal in clinical indicators of liver failure in patients treated with either therapeutic plasma exchange (TPE) or haemadsorbtion therapy (HA). TPE is an extracorporeal procedure that involves the replacement of one or more volumes of patient's plasma with an equivalent volume of FFP and fluids.[7,8] This procedure is aimed to achieve metabolic homeostasis and improve deranged organ functions and hemodynamic parameters by removing inflammatory proteins, abnormal proteins and their breakdown products, DAMPs, soluble B7 (CD80/CD86) and others from the circulation.[9] In the case of HA therapy, patient's plasma is passed through the adsorbing membranes coated with resins and specific binding molecules for the removal of toxic metabolites and then re-circulated back into patient.[10] It neither requires large volumes of plasma nor bears the risk of plasma-associated allergic reaction or disease transmission.[2,6]

The metabolome of patients with ACLF is quite unique. Systemic inflammation is considered as key player in pathophysiology of the disease and is depicted by the accumulation of several toxic metabolites that derange the core energy generation and liver regeneration pathways.[3] Overall, the plasma milieu in ACLF patients is inflammatory and contains variable levels of DAMPs and PAMPs[11] which if removed could provide favourable environment for hepatic regeneration and survival of patients. Recently, Maiwall et al. showed that TPE improves systemic inflammation and lowers the development of MOF in patients with ACLF.[12] Another group had shown that the development of ACLF was associated with elevated amounts of chemicals of microbial origin (aromatics, secondary or sulphated bile acids, benzoate and oestrogen metabolites), as well as decreased levels of phospholipids.[13]

ACLF is characterized by the accumulation of metabotoxins produced by intense proteolysis and lipolysis, amino acid catabolism, depressed mitochondrial ATP-producing fatty acid β-oxidation; and extra-mitochondrial amino acid metabolism. However, it is not clear which metabotoxins are removed by these modalities and how long these changes persist. Therefore, our aim was to track the metabotoxins in ACLF post-SMT, HA and TPE.

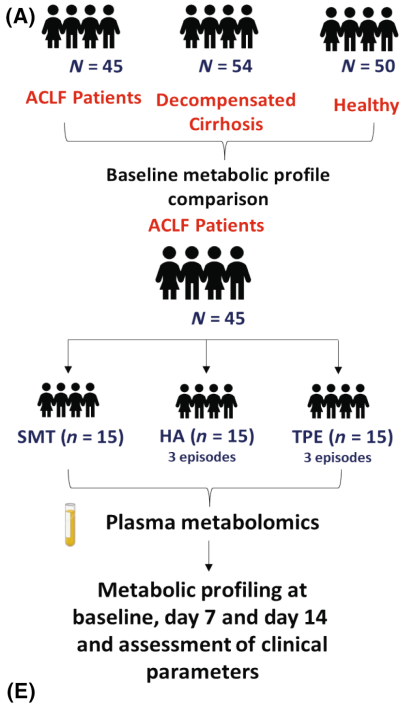
The present study was undertaken to investigate the metabolic profile of ACLF patients and the changes that follow after SMT, HA and TPE. Metabotype of patients was assessed serially; at baseline, day 7 and day 14. We also compared the metabotype of survivors and non-survivors to determine the potential metabolic signatures segregating patients likely to have worse outcomes.

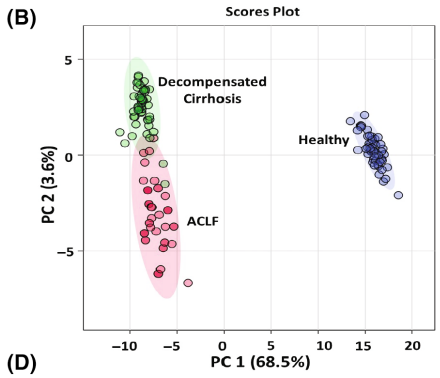
Key points

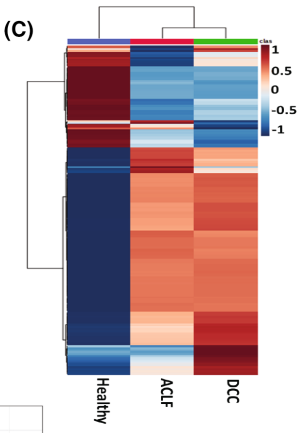
Acute-on-chronic liver failure (ACLF) has high short-term mortality and is characterized by intense systemic inflammation. Extracorporeal liver support systems help bridging patients to liver transplant by improving the plasma milieu, increasing liver recovery and regeneration. The use of ELSs as therapeutic option is expensive and thus evaluation of the efficacy of various ELS systems (haemoperfusion and therapeutic plasma exchange) over standard medical therapy in ACLF patients may benefit them. Based on the dynamic evaluation of metabotypes over time, we have shown that HA therapy is more efficient in reversing the pathological metabolic phenotype. This is also associated with improved survival. We also identified 11-deoxycorticosterone as an indicator of poor outcome in plasma of ACLF patients at baseline. The small sample size, however, remains the limitation.

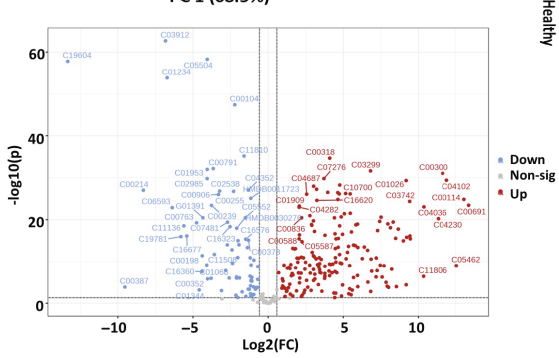
TABLE 1 Demographic profile of ACLF patients and laboratory parameters at baseline, day 7 and day 14.

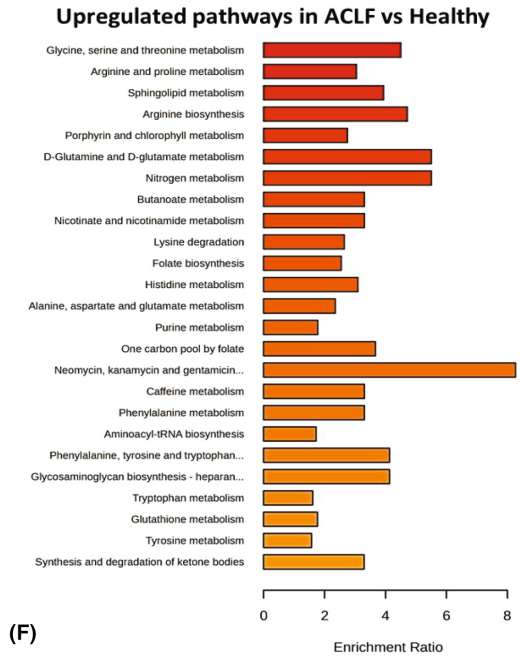
		Baseline				Day 7			Day 14		
Variable	Total ($n=45$)	Standard therapy (SMT) ($n=15$)	HA + SMT ($n=15$)	TPE + SMT ($n=15$)	p value	Standard therapy (SMT)	HA + SMT	TPE + SMT	Standard therapy (SMT)	HA + SMT	TPE + SMT
Alcohol related (n%)	20 44%)	7 (46%)	6 (40%)	7 (46%)	0.32	—	—	—	—	—	—
Viral (n (%))	10 22%)	2 (14%)	5 (34%)	3 (20%)	0.28	—	—	—	—	—	—
Others (n (%))	15 33%)	6 (40%)	4 (26%)	5 (34%)	0.78	—	—	—	—	—	—
Age (years)	47.5 ± 11.7	50.20 ± 11.5	44.1 ± 12.3	47 ± 12.02	0.59	—	—	—	—	—	—
Haemoglobin (g/dL)	9.94 ± 1.518	10.06 ± 1.594	9.58 ± 1.362	9. 9 ± 1.112	<0.01	9.28	8.95	8.1	9.08	8.26	6.34
TLC (×10 *3/cumm)	10.7 ± 1.1	10.93 ± 1.1	0.558 ± 1.2	0.9 ± 1.3	0.45	9.28	8.95-	8.1-	9.08-	8.26-	6.34-
Platelet (×10*6 / cumm)	121 ± 1.16	106 ± 59	103 ± 53	116 ± 1.5	0.7	9.28	8.95	8.1	9.08	8.26	6.34
INR	2.52 ± 0.64	2.35 ± 0.49	2.22 ± 0.46	2.95 ± 0.75	0.8	2.6375	2.064667	2.417857	2.38	2.007	1.44
Serum Creatinine (mg/dL)	0.78 ± 0.9	1.2 ± 0.7	0.46 ± 0.14	0.54 ± 0.21	0.62	1.046429	0.754375	1.025714	0.96286	1.665	0.457
Serum bilirubin (mg/dL)	25.4 ± 9.7	21.7 ± 12	26.9 ± 6.1	27.6 ± 8.81	0.5	14.018571	15.9125	18.40714	11.2614	13.651	11.04
Total protein (g/dL)	5.66 ± 1	5.4 ± 0.7	5.38 ± 1.38	5.9 ± 0.6	0.39	5.999286	5.528125	4.965	5.955	5.65	4.29
Serum Albumin (g/dl)	2.5 ± 0.59	2.48 ± 0.71	2.55 ± 0.55	2.47 ± 0.55	0.96	2.675714	2.43375	2.587143	2.72786	2.65	2.203
Total Bile acids (mg/dL)	136.6 ± 21.3	131.43 ± 17.3	126.8 ± 11	138 .14 ± 10.8	0.78	107.285714	161.68	142.5	32.5714	135.79	82.76
CTP	11.19 ± 1.29	10.7 ± 1.56	11.56 ± 1.01	11.4 ± 1.134	0.31	9.7 ± 2.54	9.56 ± 2.00	10.4 ± 2.11	11.7 ± 3.44	9.56 ± 2.00	12.4 ± 2.25
MELD	29 ± 3.9	29 ± 4.2	28 ± 3.5	31.4 ± 3.4	0.182	27 ± 3.1	23 ± 2.1	27.4 ± 4.3	27 ± 3.1	24 ± 2.1	28.4 ± 3.9
AARC	9.5 ± 1.39	9.5 ± 1.78	8.71 ± 0.95	10.1 ± 0.92	0.138	8.5 ± 1.78	6.71 ± 1.01	9.1 ± 1.36	9.5 ± 2.36	7.71 ± 1.26	10.1 ± 2.01

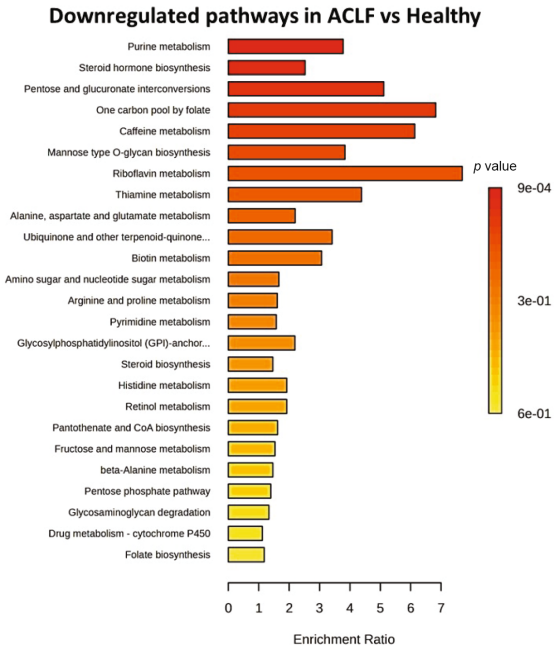












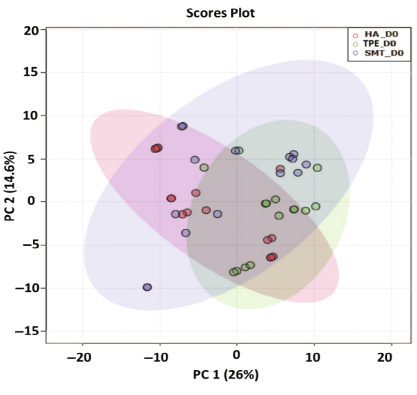


FIGURE 1 Baseline Metabolic Profile of ACLF patients. (A) The figure depicts the study design. Plasma metabolic profile of 45 ACLF patients was compared with 54 decompensated cirrhotic patients and 50 healthy subjects. Forty-five ACLF patients were randomized into three distinct groups: SMT, HA and TPE (15 in each). Patients were followed up till day 28 and the plasma samples were collected before the start of therapy, that is, day 0 and at days 7 and 14 post-HA and TPE sessions. (B) Principal component analysis (PCA) plot shows variability among the plasma metabolic profile of ACLF, DCC and healthy subjects at baseline. Red dots depict ACLF patients, green dots depict DCC patients and blue represents healthy subjects. (C) Hierarchical clustering heatmap displays that ACLF and DCC have similar metabolic profiles and large number of metabolites have higher expression when compared to healthy. (D): Volcano plot shows the differentially expressed metabolites between ACLF and healthy controls. *X*-axis represents fold change and *y*-axis represents significance (*p*-value). Metabolites highlighted by blue colour were downregulated in ACLF whereas red colour metabolites were upregulated. (E) The bar plot represents the pathways up-regulated and down-regulated in ACLF patients compared to HC respectively ($p < .05$; FC > 1.5). The *x*-axis represents the enrichment ratio and *y*-axis represents the pathways. (F) Partial least square-discriminant analysis (PLS-DA) represents the overlapping metabolic profile of ACLF patients segregated into different groups: SMT, HA and TPE. Red dots depict patients in the HA group, green dots depict the TPE group and purple dots depict patients in the SMT group. Day 0 = D0.

2 | PATIENTS AND METHODS

In this pilot study, metabotype of ACLF patients defined as per APASL ACLF Research Consortium (AARC)[14] criteria and admitted to the Department of Hepatology were screened. The primary aim of the study was to compare metabotype of ACLF with DCC and healthy controls (HC) and ACLF patients undergoing SMT, HA and TPE at baseline, day 3 and day 7. Of the 101 screened patients, 45 patients were enrolled based on inclusion and exclusion criteria (Supplementary Methods), 54 DCC and 50 HC were enrolled in the study. These 45 patients underwent block randomization and were divided into three groups: SMT, HA and TPE groups. A complete history of the cause of acute and chronic insult with clinical and physical examination, demographic profile, standard of care biochemical investigations and a detailed record of organ dysfunction/failure were recorded. Three sessions of HA and TPE were performed on alternate days (details in Supplementary Methods). Written informed consent was obtained from the patients or relatives. The institutional ethics committee (ILBS ethical committee (IEC/2020/70/NA6) approved the study).

2.1 | Untargeted plasma metabolomics

Untargeted metabolomics of the plasma was performed as per previous publication[15] and is further detailed in the Supplementary Methods. In brief, metabolites were isolated from 100-μL plasma using organic phase separation method and were analysed using LC-MS. Identified metabolites were annotated using compound discoverer.

2.2 | Statistical analyses

Standard statistical analysis was performed including unpaired (two-tailed) Student's *t*-test, Mann-Whitney *U* test and ANOVA-Kruskal–Wallis test respectively. Spearman correlation ($R^2 > 0.5$, $p < .05$) was used. Annotated metabolites were subjected to WMCNA as detailed in Supplementary Methods. Correlation, AUROC and Kaplan-Meier (KM) curve analysis for the significant metabolites were performed using SPSS 20 (USA). For the KM curve, log rank $p < .05$ was considered significant.

3 | RESULTS

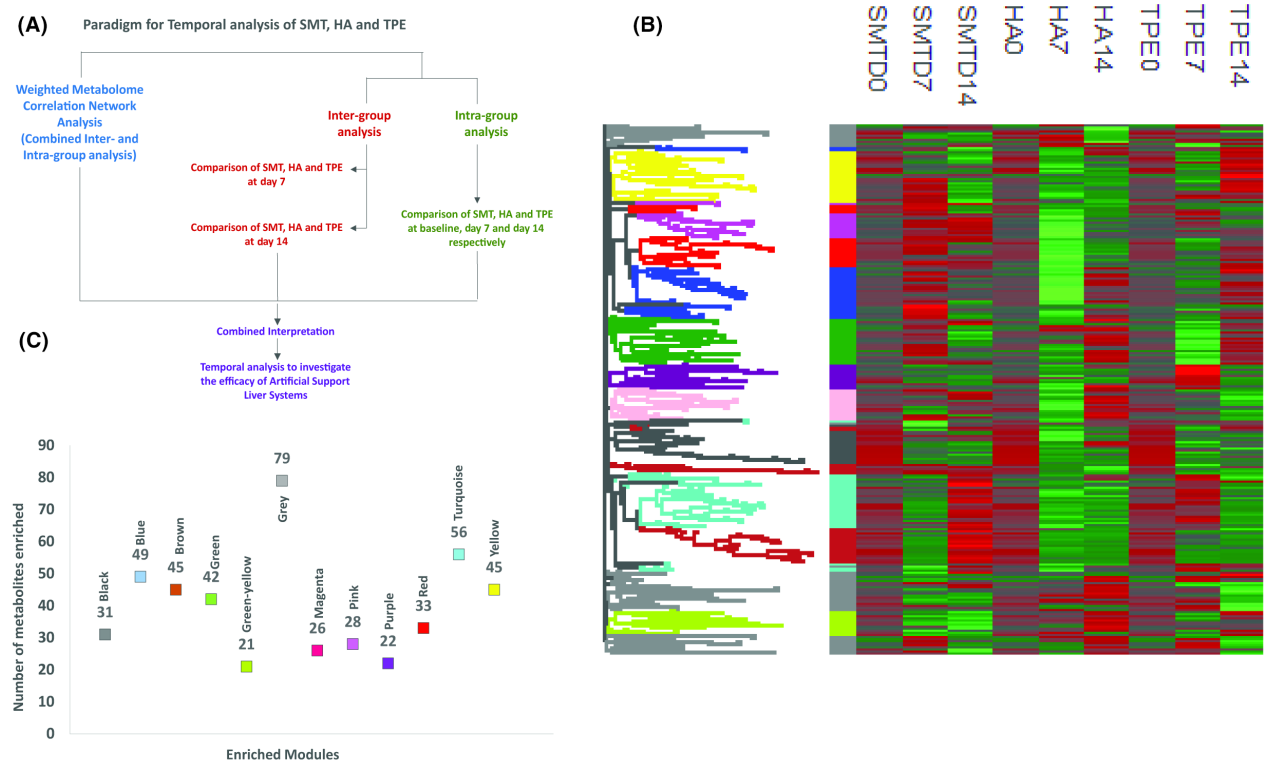
3.1 | Demographic profile

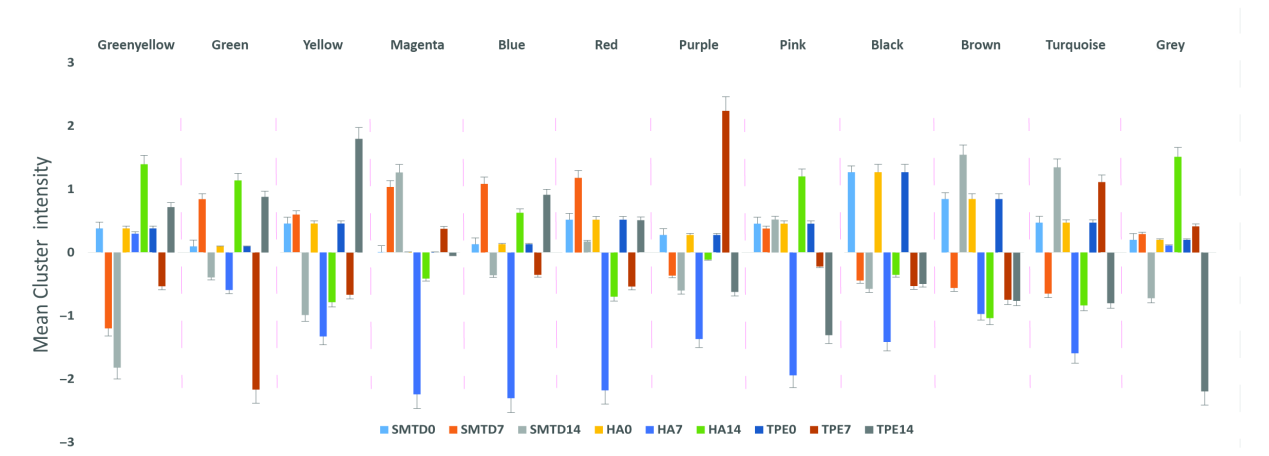
In this pilot study, a total of 45 patients were randomized in either SMT, HA + SMT or TPE + SMT groups respectively (15 in each). The baseline clinical profile of ACLF patients in three groups was comparable (Table 1).

3.2 | Baseline metabolic profile of ACLF patients

Baseline plasma samples of ACLF patients, decompensated cirrhotic patients and healthy individuals were subjected to metabolomic analysis and clinical characterization (Figure 1A). Based on the chemical structure, metabolites were segregated into super-classes, main classes and sub-classes. Annotated 477 metabolites (Table S1) in our data belonged to organic acids, nucleic acids, carbohydrates and other super-classes. These metabolites were linked to amino acids, hippuric acid, indoles, indole-3 acetic acid derivatives and other sub-classes as detailed in Figure S1 (Table S5).

ACLF metabotype was compared with decompensated cirrhosis (DC) and healthy subjects. PCA plot showed variability among the three subsets of patients (Figure 1B). Hierarchical clustering heatmap showed that the metabolic profile of ACLF is similar to DC (Figure 1C). ACLF and DC have metabolic load as the majority of metabolites in their plasma are up-regulated compared to healthy (Figure 1C). ACLF showed 93 differentially up-regulated metabolites when compared to DC and were linked to amino acid metabolism, butanoate metabolism and others shown in Figure S2. A large number of metabolites (184 metabolites) were significantly up-regulated ($p < .05$, FC > 1.5, Figure S1D, Table S7) and were linked to tryptophan metabolism, arginine biosynthesis, histidine metabolism, aromatic amino acid synthesis, nitrogen metabolism, butyrate metabolism and others (Figure 1E) whereas 74 metabolites were down-regulated (*p*-value < .05, FC > 1.5,





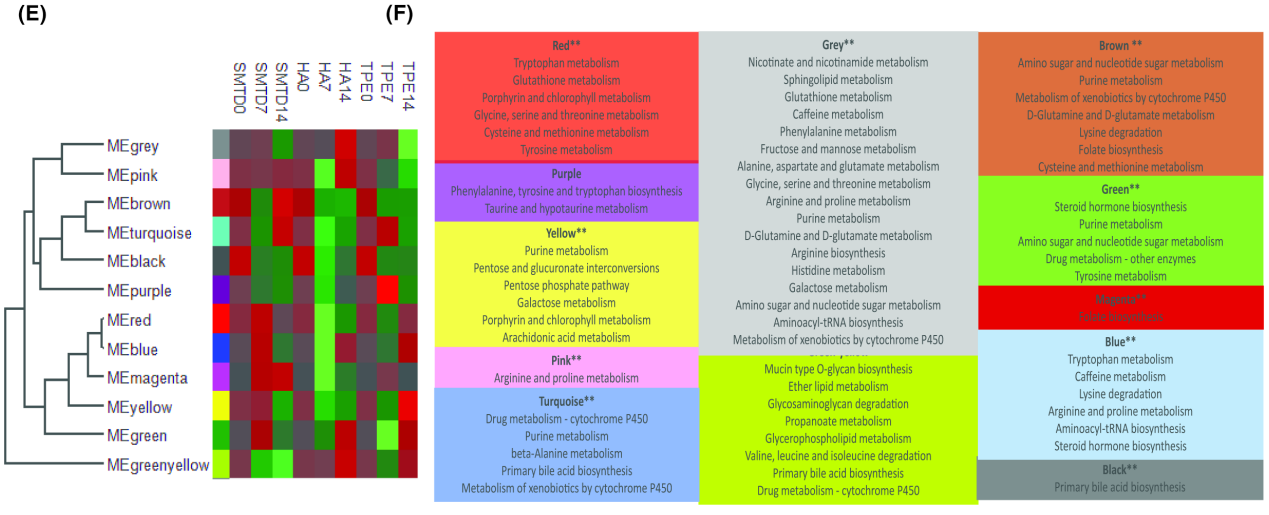


FIGURE 2 Temporal change in the metabotype of ACLF patients post SMT, HA and TPE. (A) Paradigm for temporal analysis of SMT, HA and TPE therapy. Inter-group analysis among SMT, HA and TPE was performed at baseline, day 7 and day 14. Intra-group analysis, that is, the temporal change in metabolic profile within the group pre- and post-therapy was also studied. (B) Weighted metabolome correlation network analysis heatmap showing 12 identified modules (cluster of metabolites). Each module is represented by a distinct colour. (C) Dot plot showing number of metabolites present in each module. *X*-axis represents the identified modules and *y*-axis represents the number of metabolites clustered together in each module. Colour of the dot represents the corresponding module. (D) Bar plot representing mean cluster intensity of modules in study groups at different time points. (E) Heatmap showing module trait (HA, TPE and SMT different time points) relationship. (F) Table represents the metabolic pathways associated with metabolites present in each module. (** implies metabolites enrichment =/>2).

Table S7) and were linked to one carbon pool of folate, folate, porphyrin and other metabolic pathways in ACLF compared to HC (Figure 1E). Metabotype of randomized ACLF patients into HA, TPE and SMT groups was similar at baseline as depicted by the PLS-DA analysis (Figure 1F).

These results suggest that ACLF plasma has a radical increase in metabolites linked to inflammation and immune activation such as tryptophan and aromatic amino acid and decrease in vitamin metabolism and energy metabolism. Enhanced tryptophan metabolism in the blood of ACLF patients is due to impaired activation of Kynurenine pathway which could result in perturbed peripheral organ functions.[16]

Hypothalamic-pituitary-adrenal axis and sympathetic nervous system arms are activated in ACLF due to systemic inflammation which results in intense activation of glycogenolysis, proteolysis and lipolysis which fuels the immune cells. This metabolic reprogramming further contributes to organ dysfunction and failure.[17]

3.3 | Temporal change in the metabotype of ACLF patients post-SMT, HA and TPE treatments

Weighted metabolome correlation network analysis (WMCNA: global analysis) and inter-intragroup investigation were performed to determine the changes in the metabotype of ACLF patients pre- and post-HA and TPE treatments as detailed in Figure 2A.

Weighted (metabolome) co-expression analysis (WMCNA) performs unsupervised hierarchical clustering of metabolites and groups metabolites with similar expression patterns into modules (clusters of metabolites) which are represented by different colour labels.

WMCNA: A total of 477 metabolites were grouped into 12 modules (soft-threshold >10, scale-free topology fit index >0.85; $p < .05$, Figure 2B). The number of metabolites enriched in each module (represented by a specific colour) is shown in Figure 2C. Analysis of the mean cluster intensity (Figure 2D) and the module trait relationship heatmap (Figure 2E) showed that HA treatment significantly reduced the expression of 'Red, Yellow, Pink, Turquoise, Brown, Magenta, Blue and Black' module in ACLF patients by day 7. These changes persisted till day 14 (shift from red to green in Figure 2E).

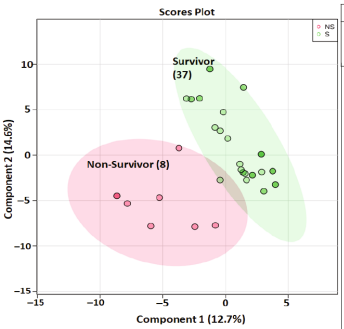
These modules were linked to inflammatory pathways (tryptophan, cysteine and methionine metabolism, arachidonic acid metabolism and others), secondary energy metabolism (pentose phosphate pathways, purine metabolism) and others (Figure 2F). We also observed significant reduction in primary bile acid biosynthesis post 7 days of HA therapy compared to other therapies. Together these results highlight a beneficial change in the plasma metabotype post-HA treatment which was not so evident when ACLF patients were treated with TPE or SMT.

3.4 | Inter-group HA versus TPE metabotype comparison on day 7

Inter-group analysis of post 7 days of treatment showed that ACLF patients treated with different therapies responded differently and had distinct metabotype by day 7 (Figure S3). Differentially expressed metabolites with significant fold change in HA versus SMT and TPE versus SMT at day 7 are mentioned in Table S3. We found HA-specific clusters (clusters 'i' and 'iii') consisting of metabolites associated with glutamine, glutamate, thiamine and tryptophan metabolism decreased post-HA at day 7 (Figure S4). On the other hand, TPE-specific cluster (Cluster-'iv') consisting of metabolites of taurine and hypo-taurine metabolism decreased post-TPE at day 7 (Figure S4).

Venn analysis of the clusters which got reduced post-HA (clusters i, ii and iii) and TPE (clusters 'ii' and 'iv') (SMT normalized) showed that 256 metabolites linked to purine metabolism (12 metabolites), steroid hormone biosynthesis (8 metabolites), tryptophan metabolism (7 metabolites; L-tryptophan, 5-hydroxy indole acetic acid, hydroxyl L tryptophan, kynurenine, indole pyruvate, indole acetic acid), primary bile acid biosynthesis (6 metabolites; 7a, 12a-dihydroxy-5b-cholestan-3-one, chenodeoxycholic acid, cholic acid, taurocholic acid and others), arginine and proline metabolism (6 metabolites) and arachidonic acid metabolism (5 metabolites; eicosatrienoic acid, arachidonic acid, prostaglandin, leukotriene and THETA) specifically reduced post day 7 of HA (Figure S5). Further, only 21 metabolites linked to taurine and hypotaurine, phenylalanine and others were reduced post 7 days of TPE (Figure S5). This reduction in metabolites associated with tryptophan metabolism, and arachidonic acid metabolism post 7 days of HA therapy suggests that HA more efficiently remove metabolites associated with inflammation and bile acids and helps in establishing normal metabolic plasma milieu. Taurine and hypotaurine metabolites are known to exhibit anti-oxidative properties. A reduction in this pathway was observed post 7 days of TPE indicating suppressed anti-oxidative system in TPE patients.

(A)



(B)

Area Under the Curve

Test Result Variable(s)	Area	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval Lower Bound	Upper Bound
1,2-Dihydroxy-3-keto-5-methylthiopentene	0.135	0.001	0.021	0.250
1-Methylnicotinamide	0.875	0.001	0.690	1.000
Deoxycorticosterone	0.909	0.000	0.801	1.000
Deoxyadenosine monophosphate	0.084	0.000	0.000	0.189
Tranexamic Acid	0.818	0.005	0.684	0.951
S-Allylcysteine	0.216	0.013	0.057	0.376
L-Fucose	0.750	0.028	0.576	0.924
Cysteic acid	0.206	0.010	0.036	0.376
Pyridoxal 5'-phosphate	0.223	0.015	0.093	0.353
4-(3-Methylbut-2-enyl)-L-tryptophan	0.247	0.026	0.093	0.401
Phosphoribosyl formamidocarboxamide	0.206	.010	0.015	0.397
Vitamin K2	0.777	0.015	0.537	1.000
Inosine triphosphate	0.182	0.005	0.000	0.373
PC(16:0/16:0)	0.750	0.028	0.492	1.000
NADP	0.814	0.006	0.634	0.995

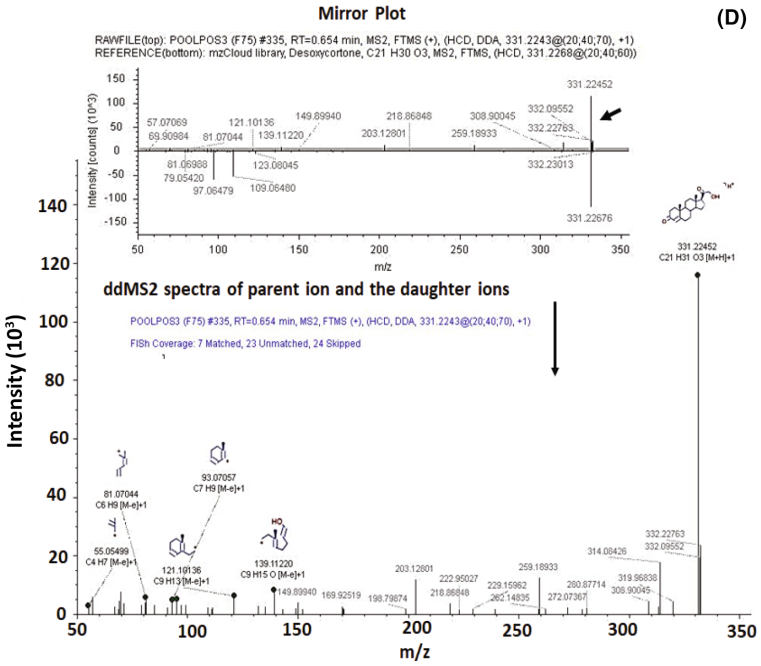
a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

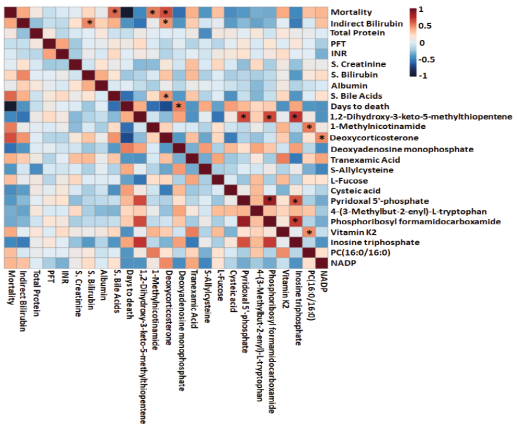
Univariate Analysis

Srno		B	SE	Wald	Sig.	Exp(B)	95.0% CI for Lower	Upper
1	1,2-Dihydroxy-3-keto-5-methylthiopentene	-1.828	0.646	8.002	0.005	0.161	0.045	0.570
2	1-Methylnicotinamide	1.656	0.436	14.427	0.000	5.237	2.229	12.308
3	Deoxycorticosterone	1.182	0.281	17.758	0.000	3.261	1.882	5.652
4	Deoxyadenosine monophosphate	-2.212	0.657	11.331	0.001	0.110	0.030	0.397
5	Tranexamic Acid	0.661	0.261	6.427	0.011	1.936	1.162	3.227
6	S-Allylcysteine	-1.370	0.551	6.180	0.013	0.254	0.086	0.748
7	L-Fucose	0.929	0.421	4.873	0.027	2.532	1.110	5.778
8	Cysteic acid	-1.200	0.407	8.685	0.003	0.301	0.135	0.669
9	Pyridoxal 5'-phosphate	-0.905	0.411	4.847	0.028	0.404	0.181	0.905
10	4-(3-Methylbut-2-enyl)-L-tryptophan	-0.530	0.282	3.534	0.060	0.589	0.339	1.023
11	Phosphoribosyl formamidocarboxamide	-0.892	0.480	3.449	0.063	0.410	0.160	1.051
12	Vitamin K2	1.207	0.412	8.584	0.003	3.344	1.491	7.499
13	Inosine triphosphate	-0.872	0.276	9.982	0.002	0.418	0.243	0.718
14	PC(16:0/16:0)	2.158	0.749	8.303	0.004	8.653	1.994	37.556
15	NADP	1.245	0.555	5.043	0.025	3.474	1.172	10.300
16	s.bildir0	0.096	0.035	7.414	0.006	1.101	1.027	1.179
17	Tpro0	0.309	0.420	0.542	0.462	1.362	0.598	3.101
18	PFT0	0.000	0.000	0.063	0.801	1.000	0.999	1.001
19	INR0	-0.009	0.089	0.010	0.920	0.991	0.832	1.180
20	Screat0	0.124	0.724	0.029	0.864	1.132	0.274	4.676
21	Sbilirub0	0.074	0.045	2.733	0.098	1.077	0.986	1.176
22	alb0	0.159	0.591	0.072	0.788	1.172	0.368	3.730
23	BA0	0.015	0.005	10.733	0.001	1.015	1.006	1.024
	Multivariate analysis							
Step 1	Deoxycorticosterone	1.182	0.281	17.758	0.000	3.261	1.882	5.652
Step 2	Deoxycorticosterone	1.030	0.334	9.500	0.002	2.801	1.455	5.393
	BA0	0.012	0.005	6.800	0.009	1.012	1.003	1.022
Step 3	Deoxycorticosterone	1.167	0.369	10.030	0.002	3.214	1.560	6.619
	PC(16:0/16:0)	1.479	0.569	6.748	0.009	4.390	1.438	13.402
	BA0	0.018	0.006	8.130	0.004	1.019	1.006	1.031

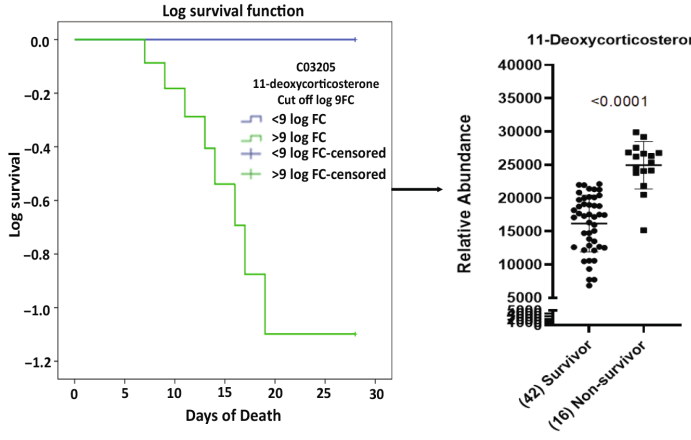
(C)



(D)



(E)



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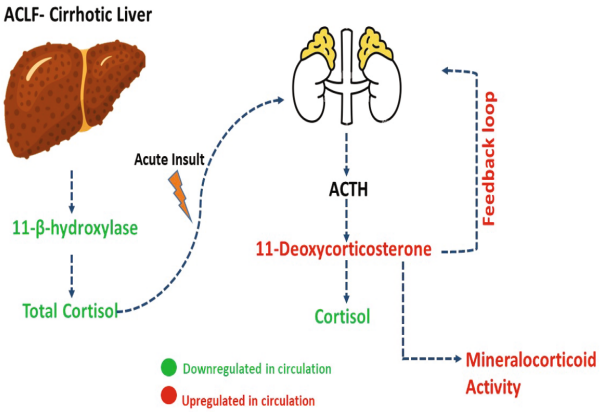

FIGURE 3 Baseline plasma metabolic profile can identify non-survivors: (A) PLS-DA showing distinct metabotype of non-survivor and survivor at baseline. Red dots represent non-survivors and green dots represent survivors. Heatmap also shows that the expression of metabolites in non-survivors was different compared to survivors. Red bar represents profile of non-survivors and green bar represents metabolic profile of survivors. (B) Area under the curve (AUC) of top 15 putative metabolite indicators of poor outcome in ACLF patients identified based on the significant differential expression. Univariate and multivariate analysis; 11-deoxycorticosterone (C03205) augmented highest AUC value (0.909) with a hazard ratio of 3.26. (C) Mirror spectral image of 11-deoxycorticosterone identified in the pool and annotated by mzCloud library and the ddMS2 spectra of the parent ion and all the daughter ion of 11-deoxycorticosterone identified in the pool. (D) Correlation of putatively identified indicators of poor outcome in ACLF patients with clinical parameters such as PT, INR, creatinine, bilirubin and others at baseline. 11-deoxycorticosterone (C03205) showed significant correlation with mortality. (E) Kaplan-Meier curve showing clear distinction between patients with C03205 levels >9 log-FC (non-survivors) as compared to patients with C03205 levels <log 9-FC (survivors). Whisker dot plot showing relative expression of 11-deoxycorticosterone in the validation group (Survivor-42 and non-survivor-16) ($p < .0001$). (F) Mechanism depicting mechanism by which 11-deoxycorticosterone level increases in the circulation.

3.5 | Inter-group HA versus TPE metabotype comparison on day 14

PLS-DA followed by hierarchal clustering analysis showed clear segregation of the study groups by day 14 (Figure S6). Differentially expressed metabolites with significant fold change in HA versus SMT and TPE versus SMT at day 14 are mentioned in Table S4. K-means clustering identified five clusters. HA-specific clusters: cluster 'iv' (linked with tryptophan, cysteine and methionine and histidine metabolism) reduced whereas cluster 'v' (linked to caffeine, nicotinate and nicotinamide metabolism) increased (Figure S7). We observed metabolites linked to cysteine and methionine, histidine metabolism and tyrosine metabolism pathways were specifically increased in TPE groups versus others ($p < .05$, Figure S8). A significant decrease in glutamate and glutamine metabolism, arginine biosynthesis and others was observed post day14 of HA ($p < .05$, Figure S9) whereas caffeine, nicotinate and nicotinamide, phenylalanine metabolism and other pathways decreased post-TPE ($p < .05$, Figure S9). These results suggest that even after 14 days of HA, the internal environment of HA patients has less concentration of metabolites associated with inflammatory signalling (tryptophan pathway) and oxidative radicles (cysteine, methionine and histidine), whereas in the TPE group, concentration of aromatic amino acids and non-aromatic amino acid strikingly increased, suggesting an increase in proteolysis and catabolic processes known to fuel immune system.

3.6 | Intra-group (SMT, HA and TPE) comparison; from baseline to day 14

To document the temporal association of metabolites in ACLF patients, a comparative analysis of baseline to day-14 metabotype was performed in SMT, HA and TPE groups (Figure S10). Temporal analysis showed that plasma metabotype changed distinctly in the SMT, HA and TPE groups over time (Figure S11). Metabolites linked to phenylalanine, glutamine and glutamate metabolism, alanine and aspartate and others decreased in the SMT group (Figure S13). We observed a significant increase in caffeine metabolism, folate biosynthesis and others in the SMT group and purine metabolism, folate biosynthesis and others in the HA group respectively (Figure S12). Interestingly, metabolites linked to inflammatory signalling and microbial metabolism such as phenylalanine, tyrosine, tryptophan biosynthesis, tryptophan metabolism and others were specifically increased post-TPE therapy (Figure S12). Metabolites linked to glutamine and glutamate metabolism, phenylalanine metabolism, histidine and others decreased in the HA group, and metabolites linked to drug metabolism, glycosaminoglycan, arginine and proline metabolism decreased over time (baseline to day 14) in the TPE group (Figure S13). These results suggest that HA treatment more prominently eliminates pro-inflammatory metabolites produced by intestinal microbes, which influences overall outcome in ACLF patients, thus improving 30-day survival in HA-treated patients.

3.7 | Baseline plasma metabolic profile can identify non-survivors

Eight of the 45 patients died (3/15 in SMT, 2/15 in HA and 3/15 in TPE groups) by 28 days. Early mortality was observed in SMT and TPE groups (two in each group by days 14 and 0 in HA). PLS-DA showed distinct profile of survivors and non-survivors at baseline (Figure 3A, Figure S14). The differentially expressed metabolites between non-survivors and survivors are highlighted in volcano plot (Figure S15).

Based on significant fold change (>±1.5) and AUC-value, a panel of 15 putative indicators capable of segregating non-survivors from survivors was identified (Figure 3B). On performing univariate and multivariate analysis, 11-deoxycorticosterone (C03205) showed highest diagnostic efficiency (AUC = 0.909) with hazard ratio of 3.26 (Figure 3B). The mirror plot in Figure 3C shows the peak of identified biomarker, that is, 11-deoxycorticosterone in the pooled samples (quality control). ddMS2 spectra were observed for 11-deoxycorticosterone and the parent and daughter ions of captured as shown in Figure 3C. 11-deoxycorticosterone (C03205) also showed strong positive correlation with bilirubin levels, bile acids and mortality in non-survivor patients (Figure 3D). Based on the AUROC, a cut-off of log9FC of 11-deoxycorticosterone (C03205) identified patients to have high mortality (log rank <0.05, Figure 3E).

Taken together, these results identified 11-deoxycorticosterone (C03205) as an independent predictor of early mortality in ACLF patients. C03205 was then validated in an external cohort of 58 ACLF patients (42 survivors and 16 non-survivors) (Figure 3E).

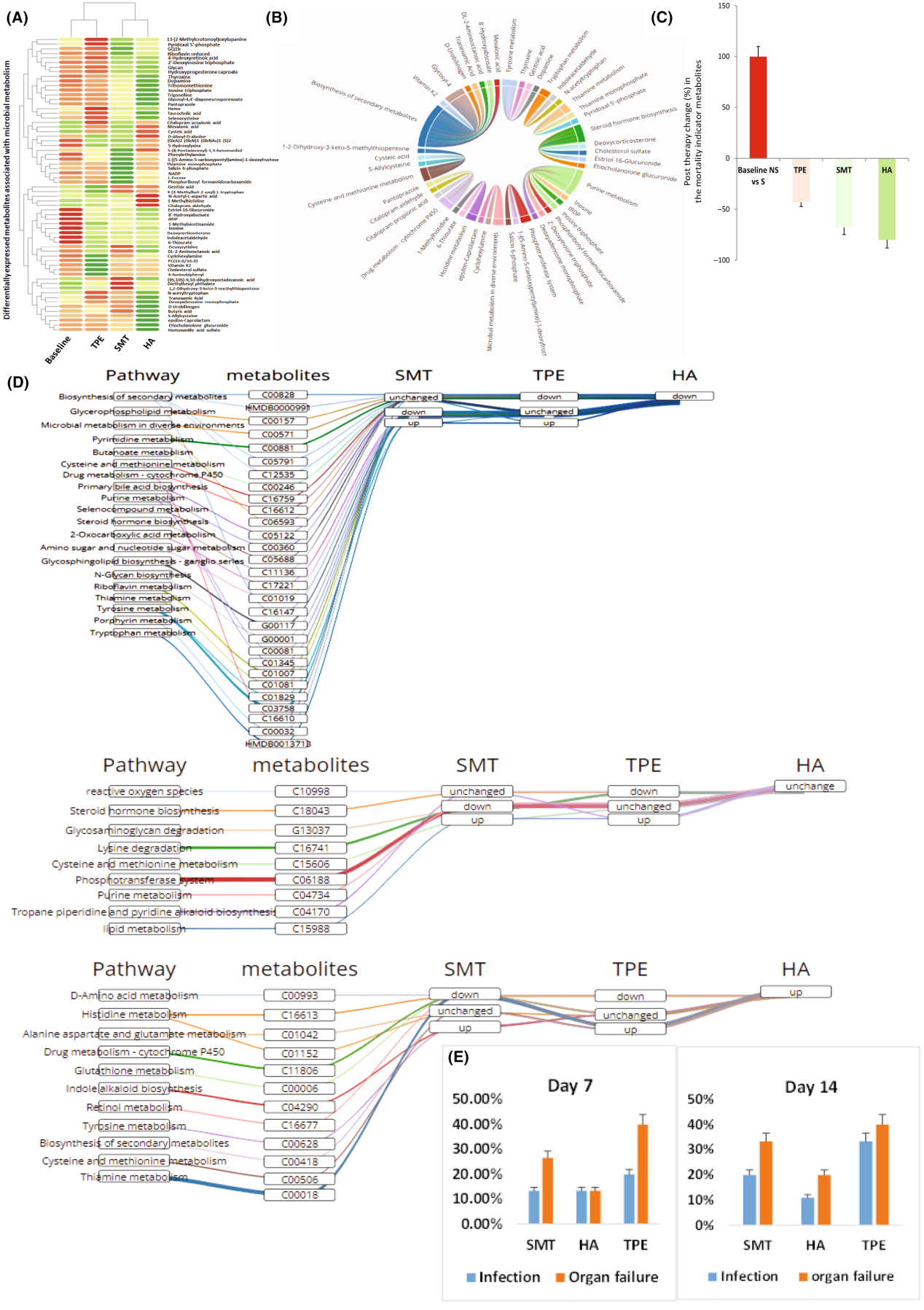

FIGURE 4 Effectiveness of HA, TPE over SMT: (A): Heatmap showing relative expression (green to red = low to high) of DEMs segregating survivors and non-survivors at baseline and post day 7 of TPE, SMT and HA. (B) Circos showing altered metabolic pathways associated with DEMs signature between non-survivors and survivors at baseline. (C) Bar plot showing % change in expression of metabolite-based signatures of poor outcome post 7 days of SMT (~60%), TPE (~48%) and HA (~80%) respectively. (D) Alluvial plot showing metabolite-based signatures and associated pathways which got down-regulated (panel a), unchanged (panel b) and up-regulated (panel c) post 7 days of HA treatment compared to SMT and TPE. (E) Bar plot showing percentage of infection and organ failure occurrence after HA and TPE therapy post 7 days and 14 days respectively.

Total cortisol level is known to be lower in ACLF patients,[18] which results in persistent stimulation of ACTH, thus increasing 11-deoxycorticosterone levels in these patients. Cirrhotic patients also have deficiency of 11-β-hydroxylase enzyme responsible for converting 11-deoxycorticosterone/11-deoxycortisol to cortisol. Thus, again leading to accumulation of 11-deoxycorticosterone in the plasma (Figure 3F, Figure S16).

3.8 | Effectiveness of HA and TPE in improving metabolic milieu

To evaluate the efficacy and effectiveness of HA and TPE over SMT, the 64 differentially expressed metabolites between non-survivors and survivors at baseline were compared with their expression post 7 days of therapy (Figure 4A). These signature metabolites belonged to biosynthesis of secondary metabolites, cysteine and methionine metabolism, drug metabolism, steroid hormone biosynthesis, tryptophan metabolism, etc. (Figure 4B). Modulation in metabolite expression was found to be lowest (43%) post-TPE, 60% post-SMT and highest (80%) post-HA (Figure 4C).

Detailed analysis showed that HA therapy reduces the metabolites associated with microbial metabolism (biosynthesis of secondary metabolites; urobilinogen and five other metabolites, glycerophospholipid and butanoate metabolism), inflammatory metabolites (tryptophan metabolism, tyrosine metabolism, cysteine and methionine and primary bile acid biosynthesis) and steroid biosynthesis linked metabolites. In addition, metabolites associated with amino acid, histidine metabolism, drug metabolism and glutathione metabolism were higher post 7 days in the HA therapy group (Figure 4D). Also, the percentage of occurrence of infection and organ failure was low post-HA therapy as depicted in Figure 4E. Additionally, HA and TPE therapy improved bilirubin and INR, whereas MELD, CTP and AARC scores were reduced in the HA group as shown in Figure S17. These results portray beneficial metabolic changes and outline the efficacy of HA therapy over SMT or TPE therapy in ACLF patients.

4 | DISCUSSION

In this pilot study, we assessed the potency of HA and TPE over SMT in improving transplant-free survival in Asian-ACLF patients by examining the metabotype using high-throughput untargeted metabolomics. The aim of our study was to assess the temporal change in ACLF metabolome post 7 and 14 days of HA and TPE. Further, we evaluated the potency of HA and TPE over SMT in removing toxic metabolites and improving ACLF milieu. Finally, we identified metabolic signatures predicting poor outcome in ACLF patients.

ACLF metabolic profile showed significant increase in concentration of ~60% metabolites linked to inflammation and fatty acid metabolism such as tryptophan metabolism and aromatic amino acid metabolism similar to the results shown by other groups.[13,16,19] We observed a decrease in energy production in the diseased state similar to earlier observations.[20]

Global analysis (WMCNA) showed that HA treatment significantly reduced the expression of more than 90% of the modules after 7 days of treatment and the changes persisted till 14 days. These modules were specifically linked to canonical inflammatory pathways (tryptophan, cysteine and methionine metabolism, arachidonic acid metabolism and others[16]) and secondary energy metabolism (pentose phosphate pathways, purine metabolism), primary bile acid biosynthesis and others. Inter- and intra-group analysis also revealed similar results and explicated that after 7 days of HA treatment, the concentration of seven metabolites of tryptophan pathway was reduced. Treatment with HA specifically reduces arachidonic acid (unsaturated fatty acid) pathway and associated metabolites known to be pro-inflammatory in nature and associated with downstream activation of various ROS species and systemic inflammation.[21] Remarkable reduction in concentration of six primary bile acids post-HA therapy validates improvement in hepatocytes overall health and 28-day mortality.[22] These results were concordant with reduction in CTP, MELD and the AARC score in ACLF patients post-HA treatment (Table 1). On the contrary, a down-regulation of taurine and hypo-taurine pathway involved in providing antioxidant defence mechanism was observed post 7 days of TPE treatment reflecting reduced anti-oxidative environment in the TPE group. Post 14 days of TPE, a significant increase in circulating amino acids such as cysteine, methionine,[23] histidine and tyrosine[23] was observed indicating high proteolysis which is known to be linked with systemic inflammation.[24] Further elevated methionine and homocysteine levels are associated with liver injury progression.[25] Similar results were documented upon temporal analysis. These results indicate that by day 14, the toxic metabolites linked to liver injury remained accumulated in the plasma of ACLF patients and could be a potential reason for higher severity, organ failure and infection observed in the TPE group. The 14-day profile of the TPE group summons for requirement of additional sessions of TPE therapy which could be lifesaving in such patients.

In order to evaluate the potency of therapies, along with the reduction in the clinical severity scores such as MELD and AARC;

DEMs in non-survivors at baseline were compared with their expression post 7 days of therapies. HA therapy effectively reversed the expression of ~51 metabolites (~80%) compared to 60% in SMT and 40% in TPE treatment of 64 DEMs. A favourable response was observed post-HA, as it reduced the concentration of metabolites associated with biosynthesis of secondary metabolites (urobilinogen), glycerophospholipid, butanoate, aromatic amino acid metabolism and steroid biosynthesis. Increase in the secondary metabolic product and butanoate metabolism clearly suggests that HA therapy could neutralize gut-derived bacterial by-products which are considered to be inflammatory and harmful.[26,27] It also improved CTP, MELD and AARC scores.

Comparison of survivors and non-survivors resulted in identification of panel of signature metabolites that could predict poor outcomes in ACLF patients at baseline. Of these, 11-deoxycorticosterone (C03205) documented maximum diagnostic efficacy and hazards for the poor outcome prediction. Increased level of 11-deoxycorticosterone in plasma results in activation of mineralocorticoid signalling and mineralocorticoid-based hypertension.[27] Mineralocorticoids are known to activate pro-inflammatory immune system, involved in production of oxidative radicles and fibrosis.[28] Increased 11-deoxycorticosterone further increases basal steroid level inducing feedback inhibition loop (particularly seen in steroid non-responders). We, therefore, hypothesizes that increase in 11-deoxycorticosterone could be one of the plausible reasons for higher inflammatory state and high basal steroid level seen particularly in non-responders/non-survivors SAH patients. Thus, the hypothesized feedback inhibition due to baseline increase in steroid warrants further elucidation in SAH patients.

Our study provided robust results and showed that HA is more potent in removing toxic metabolite, improves plasma milieu over SMT and TPE and reduces the risk of infection and organ failure in ACLF. However, a similar study with a larger sample size is needed. In this pilot and first of its kind study, the metabotype of ACLF patient's pre- and post-therapeutic interventions are analysed using metabolomics. Since HA and TPE are very expensive treatments, investigating the effects of these therapies would be highly beneficial.

Our study has a few limitations. Since this was a pilot study to determine the effects of TPE and HA on the plasma metabolome, the sample size was small and hence the adequacy of therapies to improve clinical outcomes could not be assessed. This would require a larger patient cohort. Secondly, 11-deoxycorticosterone as a marker of poor prognosis in ACLF needs to be validated in a larger cohort before recommendation as a reliable biomarker.

In conclusion, our study presents the potential of ELSs in ACLF patients. Our data provide a metabolomic overview that HA therapy is more efficient than therapeutic plasma exchange and should be the therapy of choice in sick ACLF patients. The circulating level of 11-deoxycorticosterone (C03205) in plasma could be used to predict mortality in ACLF patients at baseline and for evaluating effectiveness of ELSs.

AUTHOR CONTRIBUTIONS

JSM and SKS conceptualized the work. MY and GT RM were responsible for sample processing and experimental work and were helped by BM, NS and VB. Data analysis was performed by MY under the guidance of JSM. The manuscript was drafted by MY and JSM. Professor Sarin proofread the manuscript and provided critical inputs. This manuscript has been approved by all authors.

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CONFLICT OF INTEREST STATEMENT

All authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data for metabolomics performed in the study are available on request from the corresponding author. The analysed data are presented in Supplementary Tables.

ETHICS STATEMENT

Written informed consent was obtained from the patients or relatives. The institutional ethics committee (ILBS ethical committee (IEC/2020/70/NA6)) approved the study.

PATIENT CONSENT STATEMENT

Written consent was taken from all the patients enrolled in the study.

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REFERENCES

1. Kulkarni S, Sharma M, Rao PN, Gupta R, Reddy DN. Acute on chronic liver failure-in-hospital predictors of mortality in ICU. *Journal of Clinical and Experimental Hepatology*. 2018;8(2):144-155.
2. Arroyo V, Moreau R, Jalan R, Ginès P, EASL-CLIF Consortium CANONIC Study. Acute-on-chronic liver failure: a new syndrome that will re-classify cirrhosis. *Journal of Hepatology*. 2015;62(1 Suppl):S131-S143.
3. Laleman W, Claria J, Van der Merwe S, Moreau R, Trebicka J. Systemic inflammation and acute-on-chronic liver failure: too much, not enough. *Canadian Journal of Gastroenterology & Hepatology*. 2018;2018:1027152.
4. Kumar R, Mehta G, Jalan R. Acute-on-chronic liver failure. *Clinical Medicine (London, England)*. 2020;20(5):501-504.
5. Ichai P, Samuel D. Extracorporeal liver support with MARS in liver failure: has it a role in the treatment of severe alcoholic hepatitis? *Journal of Hepatology*. 2003;38(1):104-106.
6. Heemann U, Treichel U, Loock J, et al. Albumin dialysis in cirrhosis with superimposed acute liver injury: a prospective, controlled study. *Hepatology*. 2002;36(4 Pt 1):949-958.
7. Rademacher S, Oppert M, Jorres A. Artificial extracorporeal liver support therapy in patients with severe liver failure. *Expert Review of Gastroenterology & Hepatology*. 2011;5(5):591-599.

8. Rassi AB, d'Amico EA, Tripodi A, et al. Fresh frozen plasma transfusion in patients with cirrhosis and coagulopathy: effect on conventional coagulation tests and thrombomodulin-modified thrombin generation. *Journal of Hepatology*. 2020;72(1):85-94.
9. Maiwall R, Moreau R. Plasma exchange for acute on chronic liver failure: is there a light at the end of the tunnel? *Hepatology International*. 2016;10(3):387-389.
10. Claria J, Stauber RE, Coenraad MJ, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. *Hepatology*. 2016;64(4):1249-1264.
11. Moreau R. The pathogenesis of ACLF: the inflammatory response and immune function. *Seminars in Liver Disease*. 2016;36(2):133-140.
12. Maiwall R, Bajpai M, Choudhury AK, et al. Therapeutic plasma-exchange improves systemic inflammation and survival in acute-on-chronic liver failure: a propensity-score matched study from AARC. *Liver International*. 2021;41(5):1083-1096.
13. Bajaj JS, Reddy KR, O'Leary JG, et al. Serum levels of metabolites produced by intestinal microbes and lipid moieties independently associated with acute-on-chronic liver failure and death in patients with cirrhosis. *Gastroenterology*. 2020;159(5):1715-1730 e12.
14. Sarin SK, Choudhury A, Sharma MK, et al. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific association for the study of the liver (APASL): an update. *Hepatology International*. 2019;13(4):353-390.
15. Sharma N, Bhat SH, Tripathi G, et al. Global metabolome profiling of COVID-19 respiratory specimen using high-resolution mass spectrometry (HRMS). *STAR Protocols*. 2022;3(1):101051.
16. Zaccherini G, Aguilar F, Caraceni P, et al. Assessing the role of amino acids in systemic inflammation and organ failure in patients with ACLF. *Journal of Hepatology*. 2021;74(5):1117-1131.
17. Liu G, Wang X, Fan X, Luo X. Metabolomics profiles in acute-on-chronic liver failure: unveiling pathogenesis and predicting progression. *Frontiers in Pharmacology*. 2022;13:953297.
18. Hartl L, Simbrunner B, Jachs M, et al. An impaired pituitary-adrenal signaling axis in stable cirrhosis is linked to worse prognosis. *JHEP Rep*. 2023;5:100789.
19. Claria J, Moreau R, Fenaille F, et al. Orchestration of tryptophan-kynurenine pathway, acute decompensation, and acute-on-chronic liver failure in cirrhosis. *Hepatology*. 2019;69(4):1686-1701.
20. Ganeshan K, Nikkanen J, Man K, et al. Energetic trade-offs and hypometabolic states promote disease tolerance. *Cell*. 2019;177(2):399-413.e12.
21. Tallima H, El Ridi R. Arachidonic acid: physiological roles and potential health benefits—a review. *Journal of Advanced Research*. 2018;11:33-41.
22. Stadlbauer V, Krisper P, Beuers U, et al. Removal of bile acids by two different extracorporeal liver support systems in acute-on-chronic liver failure. *ASAIO Journal*. 2007;53(2):187-193.
23. McPhail MJW, Shawcross DL, Lewis MR, et al. Multivariate metabotyping of plasma predicts survival in patients with decompensated cirrhosis. *Journal of Hepatology*. 2016;64(5):1058-1067.
24. Moreau R, Clària J, Aguilar F, et al. Blood metabolomics uncovers inflammation-associated mitochondrial dysfunction as a potential mechanism underlying ACLF. *Journal of Hepatology*. 2020;72(4):688-701.
25. Li Z, Wang F, Liang B, et al. Methionine metabolism in chronic liver diseases: an update on molecular mechanism and therapeutic implication. *Signal Transduction and Targeted Therapy*. 2020;5(1):280.
26. Strasser B, Sperner-Unterweger B, Fuchs D, Gostner JM. Mechanisms of inflammation-associated depression: immune influences on tryptophan and phenylalanine metabolisms. *Current Topics in Behavioral Neurosciences*. 2017;31:95-115.
27. Gupta V. Mineralocorticoid hypertension. *Indian J Endocrinol Metab*. 2011;15(Suppl 4):S298-S312.
28. Duque Ede A, Munhoz CD. The pro-inflammatory effects of glucocorticoids in the brain. *Front Endocrinol (Lausanne)*. 2016;7:78.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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