Targeted NMR based serum metabolic profiling of serine, glycine and methionine in Acute-on-Chronic liver failure patients: Possible insights into mitochondrial dysfunction

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Annexure-I:

The assignments of NMR peaks in the 1D ^{1}H NMR spectrum corresponding to specific ^{1}H resonances of serine, glycine and methionine (as identified using profiler module of NMR suite in commercial software CHENOMX, shown here in **Figure S1A**) were also confirmed making composite use of two-dimensional (2D) ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra, ^{1}H-^{1}H total correlation spectroscopy (TOCSY) and J-resolved spectroscopy (JRES) acquired for normal control serum samples on 800 MHz NMR spectrometer operating at ^{1}H frequency of 800.21 MHz (See **Figure S1B, S1C** and **S1D**, respectively). Two-dimensional ^{1}H–^{1}H TOCSY spectra were acquired in the phase sensitive mode using time proportional phase incrementation (TPPI), and the DIPSI2 pulse sequence (pulse program name is DIPSI2ESGPPH) for the spin lock.[1,2] Each spectrum is acquired with 2048 data points along the direct ^{1}H (F_2) dimension with 16 transients per increment and 400 increments were acquired along the indirect ^{1}H (F1) with a spectral width of 12 ppm in both dimensions. The recycle delay between successive pulse cycles was 2 sec and the mixing time of the DIPSI2 spin lock was 100 ms. The FIDs were weighted using a sine–bell-squared function in both dimensions and zero filled to 2048 and 1024 data points, respectively, in the F_1 and F_2 dimensions prior to FT. The 2D ^{1}H–^{13}C HSQC spectra were measured using Bruker standard library pulse sequence (HSQCETGP; phase sensitive with Echo/Antiecho-TPPI gradient selection, trim pulses during double

inept transfer and ^{13}C decoupling during acquisition with inverse detection. A recycle delay of 2.2 s was used between pulses and a refocusing delay equal to $1/(4{*}^{1}J_{C\text{–}H}$ =1.75 ms) was employed. The transients were collected with 2048 data points and 96 scans per increment with spectral width of 12 ppm in the ^{1}H dimension. In the indirect ^{13}C dimension, total 328 increments were acquired with spectral width of 165 ppm. The FIDs were weighted using a sine–bell-squared function shifted by $\pi/2$ in both dimensions and zero filled to 2048 and 1024 data points, respectively, in the F_1 and F_2 dimensions prior to FT. Two-dimensional J-resolved (JRES) spectra were measured with solvent presaturation at 298 K.[3] The transients were collected into 8192 data points with a spectral width of 16 ppm, and the F1 (J-coupling) domain spectral width covered 78.125 Hz with 80 increments of t_1 and sixteen transients were collected for each t_1 increment. Prior to the double FT and magnitude calculation, the F2 and F1 data were zero-filled to 256 and 8192 points and apodized by means of a sinebell function in t2 and a sine-bell-squared function in t1. The spectra were tilted by 45° to provide orthogonality of the chemical shift and coupling constant axes and subsequently symmetrized about the F1 axis. Spectra were displayed both in the form of contour plots and as skyline F2 projections. The overall summary the ^{1}H and ^{13}C assignments obtained from this exercise are tabulated here as **Table S1**.

Table S1: The observed and non-observed peaks of serine, glycine and methionine in the 1D ^{1}H CPMG NMR spectra of serum samples (^{13}C chemical shifts are identified from the ^{1}H- ^{13}C HSQC spectrum). The HSQC peaks were calibrated w.r.t. methyl group of lactate: ^{1}H at 1.32 ppm and ^{13}C at 20.9 ppm.

Metabolite	$^{1}H/^{13}C$ Spins	$^{1}H/^{13}C$ Chemical shift in ppm	NMR Experiments
Serine	-NH_2 (amide protons)	Not observed	--
	-Cα-H	3.825/52.64	CPMG/^{13}C HSQC/TOCSY
	-Cβ-H_2	3.95/61.6	CPMG/^{13}C HSQC/TOCSY
Glycine	-NH_2 (amide protons)	Not observed	--
	-Cα-H_2	3.55/42.4	CPMG/^{13}C HSQC/JRES
Methionine	-NH_2 (amide protons)	Not observed	--
	-Cα-H	Not Observed	--
	-Cβ-H_2	Not Observed	--
	-Cγ-H_2	2.636/--- (triplet)	CPMG/JRES
	-S-CH_3	2.130/-- (Singlet)	CPMG/JRES

Figure S1A: The spectral regions derived from the 1D ^{1}H CPMG NMR spectrum of normal control serum sample opened in the profiler module of CHENOMX software and showing explicitly the NMR peaks used for concentration profiling the circulatory metabolites: serine, glycine and methionine. This is for the reference for future targeted NMR based metabolomics studies aiming to evaluate the changes in these metabolic profiles after the standard clinical treatment so that to monitor the treatment response or improvement in ACLF patients. To be mentioned here is that the NMR spectra on serum samples were recorded using coaxial insert (Wilmad, 50 mm stem length) containing 0.5 mM Trimethylsilylpropanoic acid (TMSP or TSP) solution prepared in 100% D_2O in order to avoid any metabolic addition to the serum samples. However, selecting TSP peak at 0.00 ppm as a calibration standard (which then serves as an external reference) making it difficult to achieve the adequate fitting of experimental peaks with metabolite peaks of CHENOMX 800 MHz library. Therefore, selecting formate as an internal calibration standard circumvented this issue and provided adequate fitting of experimental NMR peaks with metabolic patterns of 800 MHz compound library of CHENOMX NMR suite.

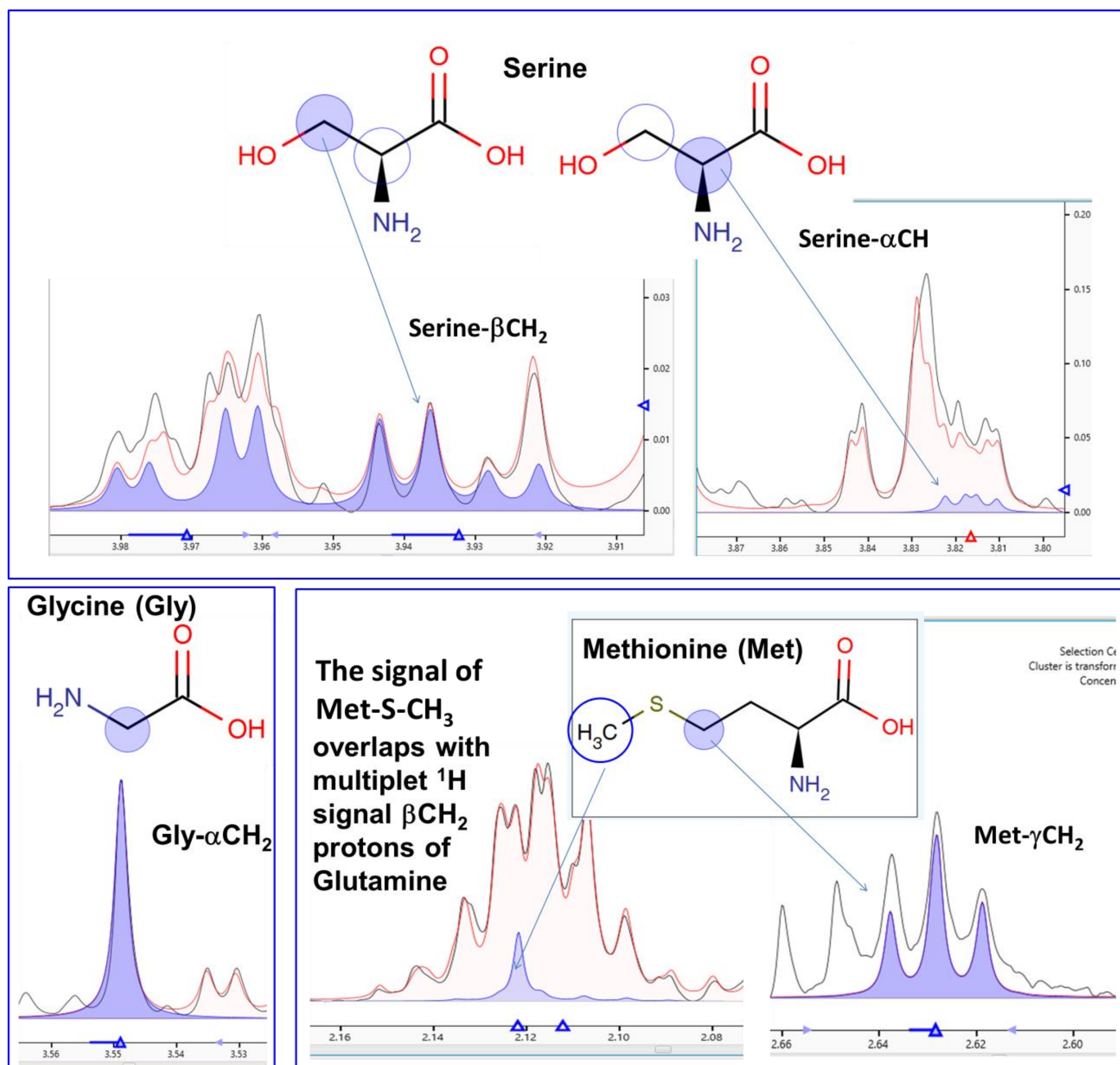


Figure S1B: (A) The molecular structure of serine with circles highlighting the ^{1}H resonances for which the signals are evident in the 1D ^{1}H CPMG NMR spectrum. **(B,C)** The overlay and stacking of 1D ^{1}H CPMG spectra recorded on the normal control serum sample (blue) with varying concentration of L-serine (as indicated in **(C)**). **(D)** The 2D ^{1}H-^{13}C HSQC spectrum of serum sample showing spectral region zoomed to highlight the CH cross peaks of serine (at 52.64/3.825 ppm for αCH and 61.6/3.95 for βCH_2) and glycine (at 42.4/3.55 ppm for αCH_2. For NMR spiking experiments, the amino-acid L-serine was purchased from MP Biomedicals and a stock solution of 200 mM was prepared in 100% D_2O for further titration experiments

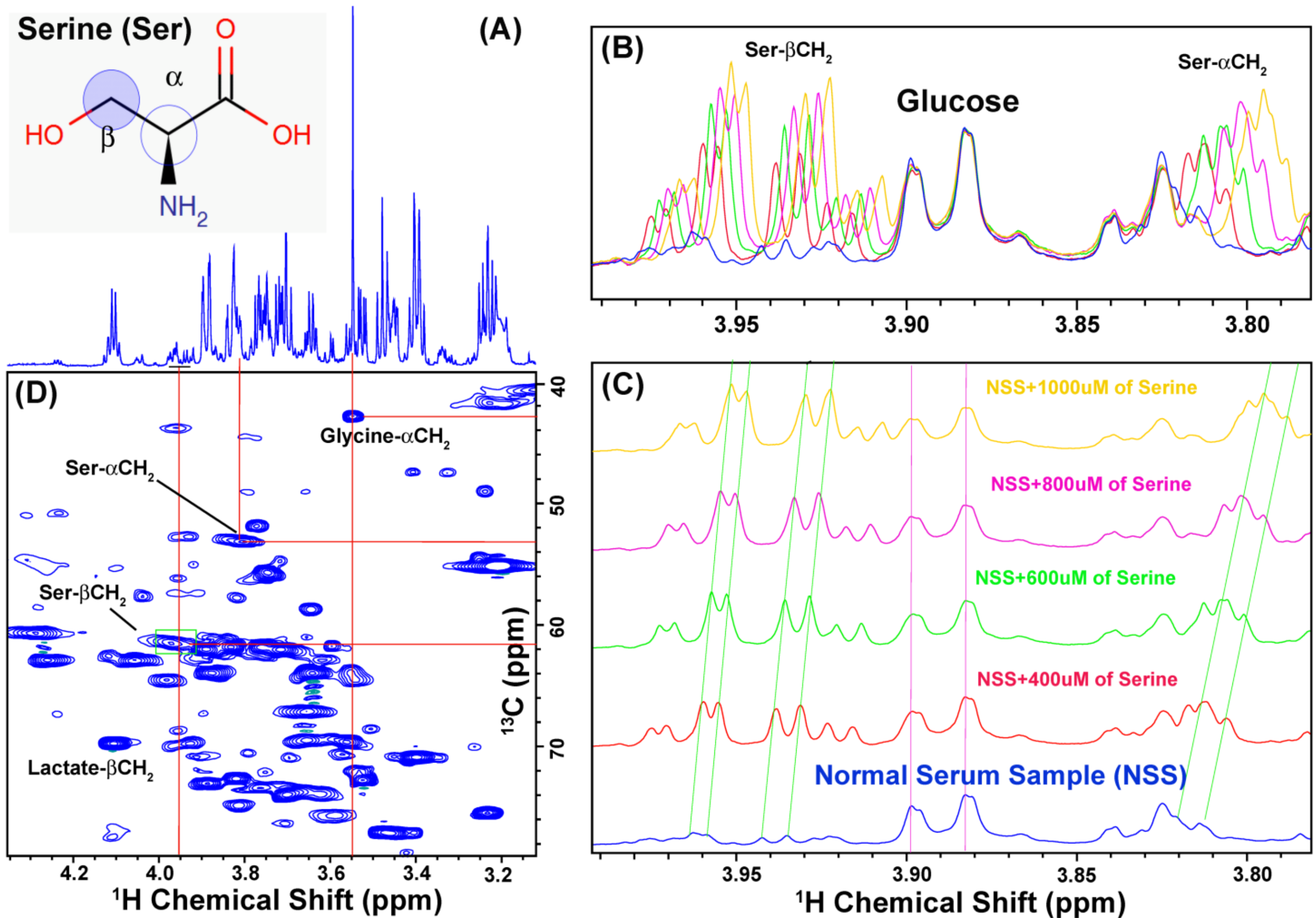
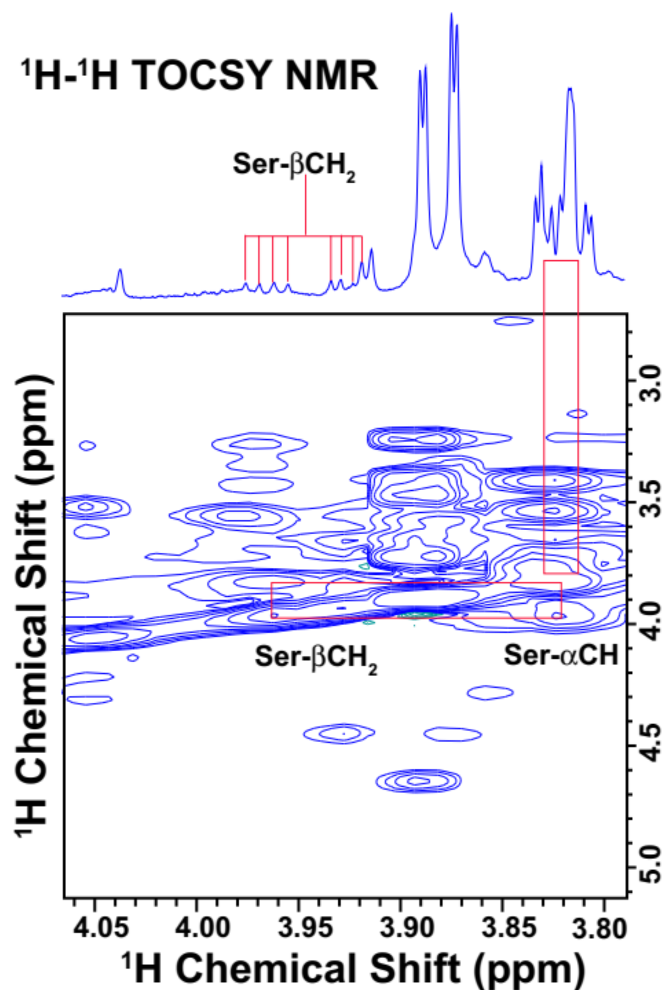


Figure S1C: A zoomed spectral region of the 800 MHz 1H-1H 2D TOCSY NMR spectrum of control human serum sample measured at 298 K, showing the correlation peaks between the diagonal signals of alpha (a) and beta 1H-spins of serine (i.e. Ser-βCH2 and Ser-αCH); thus confirming the assignment of peaks for serine metabolite.



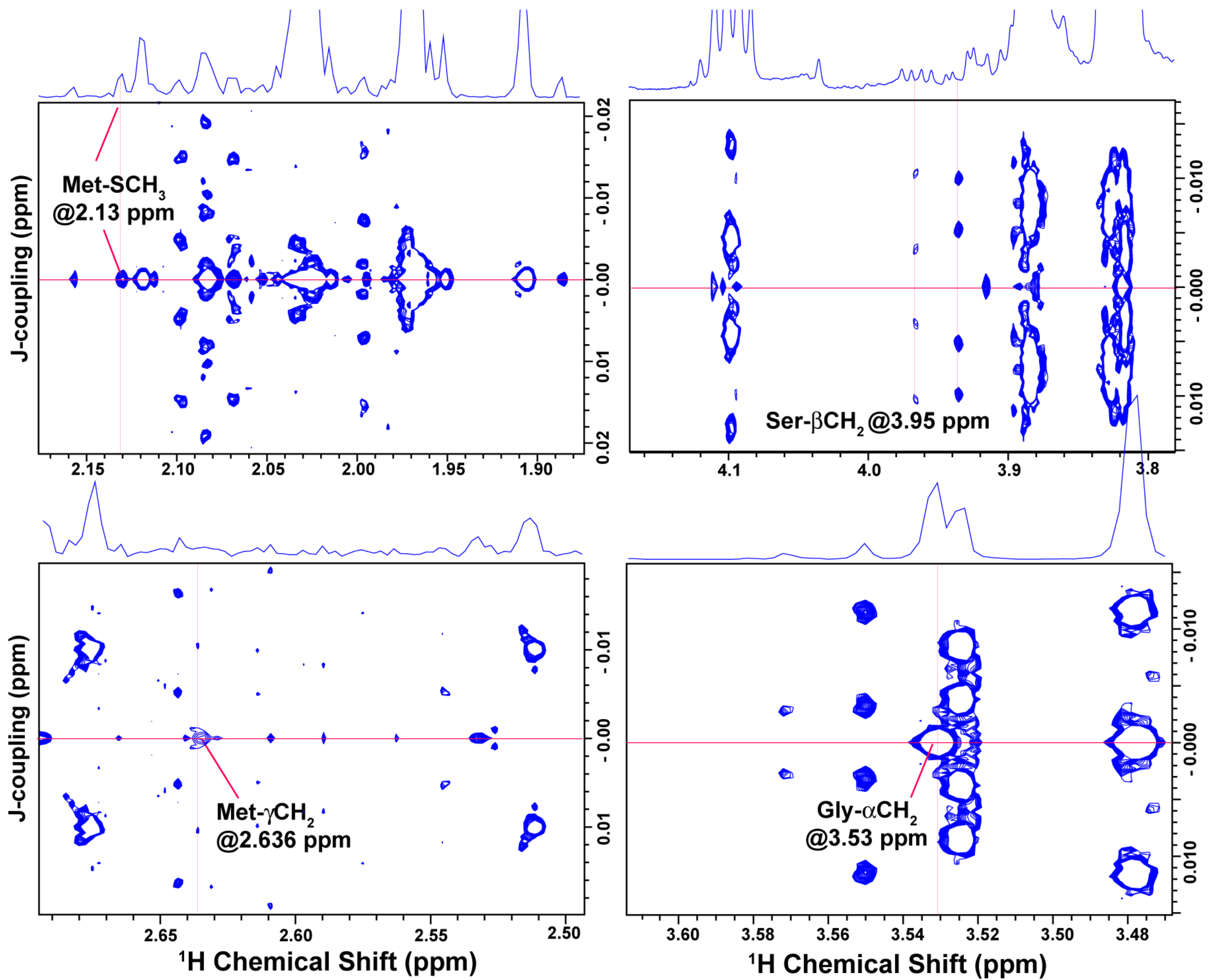
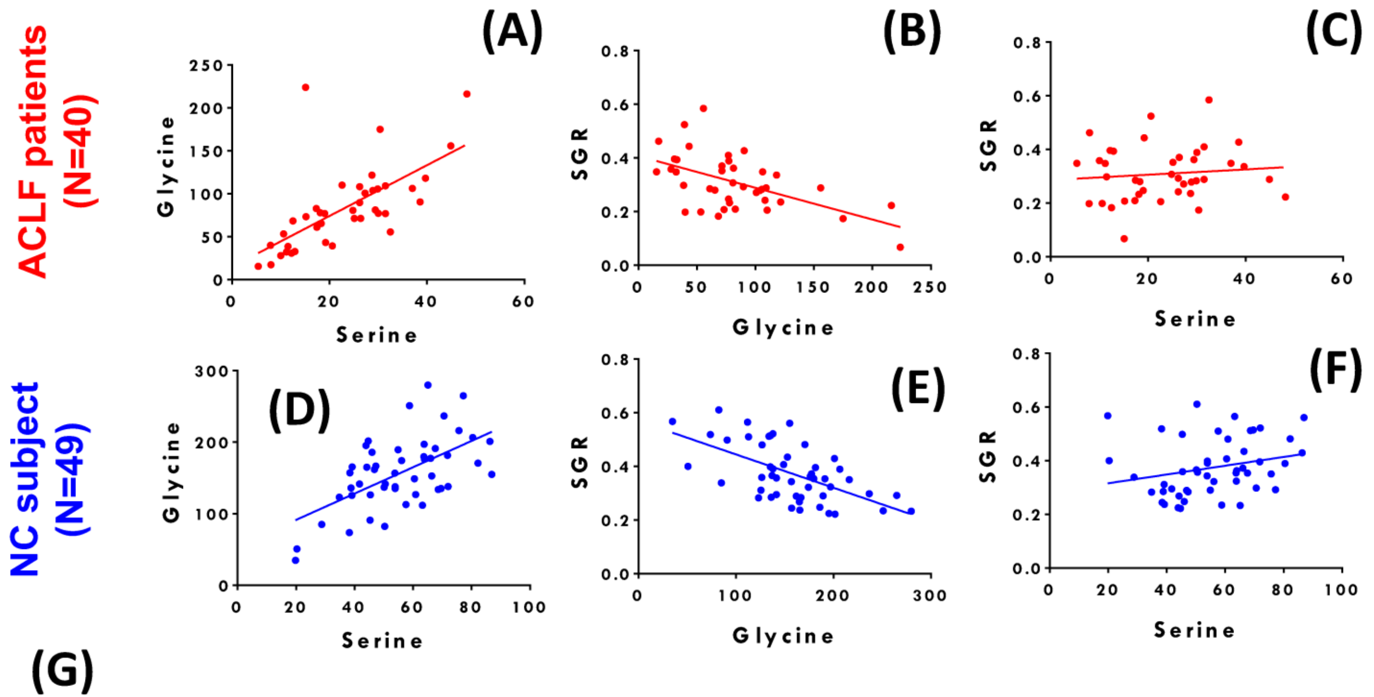


Figure S1D: 800 MHz J-Resolved 2D 'H NMR spectrum of a normal control serum sample measured at 298 K with skyline F_2 projections. The various spectral regions are zoomed and shown in different panels to highlight the assignments of serine, glycine and methionine peaks in the 1D 1H CPMG NMR spectrum.



Pearson r (95% CI)	p-value (significance)	Pearson r (95% CI)	p-value (significance)
Serine vs Glycine for ACLF patients		**Serine vs Glycine for NC patients**	
0.66 (0.43 to 0.80)	< 0.0001 (Yes****)	0.59 (0.37 to 0.75)	< 0.0001 (Yes****)
Serine vs SGR for ACLF patients		**Serine vs SGR for NC patients**	
0.10 (-0.22 to 0.40)	0.5302 (No)	0.28 (-0.04 to 0.50)	0.086 (No)
Glycine vs SGR for ACLF patients		**Glycine vs SGR for NC patients**	
-0.55 (-0.74 to -0.29)	0.0002 (Yes***)	-0.59 (-0.75 to -0.37)	0.0001 (Yes****)

Figure S2: The serum metabolic profiles of serine, glycine and SGR subjected to Pearson r based correlation analysis. The plot sets **A-C** and **D-F** represent the correlations in ACLF patients and NC subjects, respectively. The statistical relevance of the correlation plots can be assessed by p-value nearly close to statistical significance (i.e. p-value <0.05).

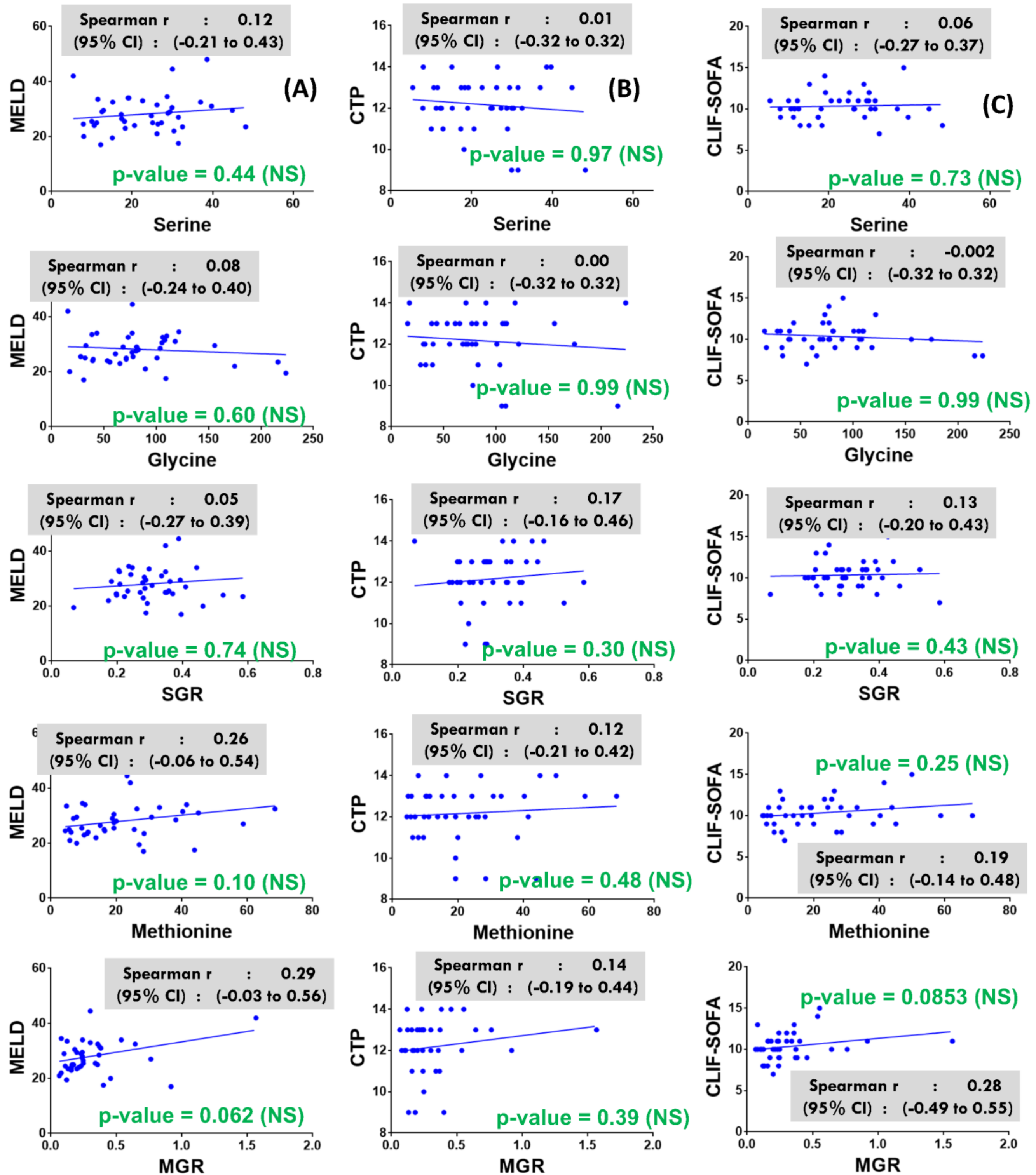


Figure S3: The metabolic profiles subjected to Spearson r based correlation analysis. The plots in columns **A, B** and **C** represent the correlations of MELD, CTP and CLIF-SOFA with circulatory levels of serine, glycine, SGR, methionine, and MGR, respectively. The statistical relevance of the correlation plots can be assessed by p-value nearly close to statistical significance (i.e. p-value <0.05).

References:

(1) Cavanagh, J.; Rance, M. Sensitivity improvement in isotropic mixing (TOCSY) experiments. *Journal of Magnetic Resonance (1969)* **1990**, 88, 72-85.

(2) Cavanagh, J.; Rance, M. Suppression of cross-relaxation effects in TOCSY spectra via a modified DIPSI-2 mixing sequence. *Journal of Magnetic Resonance (1969)* **1992**, 96, 670-678.

(3) Nicholson, J. K.; Foxall, P. J.; Spraul, M.; Farrant, R. D.; Lindon, J. C. 750 MHz 1H and 1H-13C NMR spectroscopy of human blood plasma. *Analytical chemistry* **1995**, 67, 793-811.