Nuclear magnetic resonance measurements:

Beforehand, lyophilized serum samples (0.6 ml volume in each case) were thawed, dissolved in 100% deuterium oxide (D_2O) solution (0.6 ml volume in each case at pH 7.2) and centrifuged at 10, 000rpm for 5min to remove precipitates, if any. The NMR experiments were performed on 800 MHz NMR spectrometer equipped with a CryoProbe and an actively shielded gradient unit with a maximum gradient strength output of 53 G/cm and a proton frequency of 800.21 MHz. The metabolic profiles of serum samples (each group sample size $\geq$ 9) were measured using 1D ^{1}H transverse relaxation-edited radiofrequency (RF) pulse seqeunce named CPMG (i.e. Carr–Purcell–Meiboom–Gill) RF sequence (the acquisition parameters as per previous NMR based metabolomics study (1).. The CPMG spectra were acquired with pre-saturation of the water peak by exciting it continuously throughout the recycling delay of 5 seconds. Each CPMG spectra took around 30 minutes and consisted of an accumulation of 256 scans. The chemical shifts were referred to TSP methyl protons (applied externally) at 0.0 ppm after Fourier Transform and calibrated with lactate at 1.33 ppm. The recorded CPMG NMR spectra were manually phased followed by automatic baseline correction using the Whittaker smoother algorithm and appropriate manual multipoint baseline correction the PROCESSOR module of commercial software CHENOMX (NMR Suite, v8.2, Chenomx Inc., Edmonton, Canada). All spectra were calibrated w.r.t. formate δ (8.4442) ppm (used here as an internal reference) and the concentration of formate was set to 30 micromolar as per this study reference (2). The phase and baseline corrected one-dimensional proton (^{1}H) CPMG NMR spectra were imported into the PROFILER-Module of CHENOMX for concentration profiling of circulatory metabolites.

Multivariate Statistical analysis of Metabolites data matrix:

Multivariate analysis is used to transform the complex multivariate data into easily understandable graphical representations and identifying discriminatory variables. The data matrix (containing concentrations of all samples) was grouped according to their respective class information and subjected to multivariate and univariate statistical analysis using different modules of MetaboAnalyst (v4.0, a freely available, user-friendly, web-based analytical platform for metabolomics data analysis from the University of Alberta, Canada:

www.metaboanalyst.ca) to identify activity-specific metabolic disturbances(3). First, the original data table was row wise normalized with formate, square root transformation and pareto scaling for the analysis. The statistical strategy adopted for the analysis of the samples involved a preliminary unsupervised Principal Component Analysis (PCA), followed by a supervised PLS-DA analysis based on predictive and descriptive modelling with group separation. The correlation value of Asparagine, Uridine and Malonate with potential metabolite were measured using Spearman correlation coefficient. The models robustness was assessed by, 5-fold cross-validation algorithm based on the permutation tests (n = 100) using top 5 latent variables and further cross-validated using CV-ANOVA (analysis of variance testing of cross-validated predictive residuals). The marker metabolites were identified using the loading plots (for PLS-DA) and the scores of variable importance on projection (VIPs) and average values in heatmap clustering. The correlation value of Asparagine, Uridine and Malonate with potential metabolite were measured using Spearman correlation coefficient.

Univariate Statistical Analysis

Further hierarchical cluster analysis of data was conducted with considering Euclidean distance. measure and ward.D linkage clustering algorithm using the hclust function in package stat of MetaboAnalyst and represented as heatmap. Additional, univariate analysis was performed by applying the independent samples T-test with p-value <0.05 and non-parametric Wilcoxon Mann Whitney test. to metabolites of interest (identified by the multivariate analysis). The differences in the levels of significantly altered metabolites were presented in table 2. The pathways interpretation based on these metabolites were prepared using Biorender software.

References

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