Supplementary Methods

Hepatic lipids quantification

The lipid content in the liver tissue were quantified using the Folch lipid extraction method. A 50 mg tissue sample was homogenized in chloroform/methanol in ratio of 2:1 (v/v) and centrifuged at 15,000 rpm for 15 minutes. The upper aqueous phase was discarded, and the lower organic phase was transferred to a pre-weighed, clean tube. The samples were evaporated to complete dryness, and the lipid fraction was weighed and normalized to the initial tissue sample weight.

Western Blot Analysis

Liver tissues were lysed with a lysis buffer (composed of 20 mM Tris-HCl (pH 7.4), 145 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM NaVO4, 5 mM NaF and 1% protease inhibitor cocktail (PIC, Sigma P8340). Lysates were centrifuged at 14,000 rpm for 15 minutes, and the protein concentration was determined by the Bradford method, using bovine serum albumin (BSA) as a standard. Equal amount of protein lysates was separated by SDS-PAGE, transferred to nitrocellulose membraned, blotted with primary antibodies: PCNA (BioLegend, PC10) overnight at 4 °C and Peroxidase-conjugated Affini-Pure goat anti-mouse (115-035-003, Jackson Immuno-Research Laboratories, West Grove, PA, USA). The band optical density was normalized to lane total content visualized with ponceau S solution (Sigma-Aldrich, USA) and analyzed on an Image lab system (Bio rad) and expressed as arbitrary units.

Caspase-3 activity

The liver caspase-3 enzyme activity was determined with the Caspase-3 Substrate II, Fluorogenic, Merck (Caspase-3 Substrate II, Fluorogenic, 235425, sigma-Aldrich). In brief, liver tissue (20 mg) was homogenized in 1 mL Lysis Buffer (provided in Supporting Table S3) and kept on ice for 30 minutes. The homogenate was centrifuged at 15,000 rpm for 15 minutes and supernatant was transferred to a new vial. Total protein concentration was determined

via Bradford assay and diluted with lysis buffer and 10 mM fresh DTT to get 2µg/µl protein. For the assay, 100 µL of each sample was added to a black 96-well plate. Then, 5 µL of caspase-3 substrate was added to each well (to get a final concentration of 20 µM), and the plate was incubated in the dark at 37 °C with shaking for 20 minutes. The total fluorescence was measured at Ex360/Em465 nm using an ELISA reader (Tecan, GENios) for 1.5 hours every 10 minutes.

Protein carbonylation assay

Proteins were extracted using lysis buffer (as mentioned in supplementary Table S3 with the addition of 0.1Mm PMSF, 0.2mM NaVO4, 5Mm and protease inhibitor). Protein concentration was determined by Bradford reagent and then diluted to 1 mg/mL using lysis buffer. For the DNPH staining, 0.4 mL of protein were transferred to a new 1.5 Eppendorf vial and mixed with 80µL of 10Mm DNPH. For the untreated sample, the same volume was mixed with 80µL 2N HCl (DNPH solvent). The mixtures were incubated for 60 minutes in the dark in room temperature with vortex-mixing every 10–15 min. then, a 1:1 mixture of the solution with 20% TCA was prepared sand incubated on ice for 15 minutes. Afterword samples were centrifuged at 10,000 × g for 5 minutes, at 4◦C and the supernatant was discarded, and the protein pellets were washed once with 1 ml of 20% TCA. The pellets were collected again after centrifuging. The supernatants were discarded and protein pellets were washed with 1 ml of 1:1 (v/v) ethanol:ethyl acetate in order to remove any free DNPH. The last step was repeated until supernatants were completely transparent. The pellets were let to dry in room temperature to allow complete solvent evaporation. Lastly, protein pellets resuspended in 0.3 mL 6M guanidine solution (dissolved in 20 mM sodium phosphate buffer, 0.5M sodium chloride and 50mM imidazole, Ph-7.4) and incubated at 37◦C for 30 minutes with vortex mixing. Once protein pellets were completely dissolved, results were read using Infinite M Plex Plate reader (Tecan Trading AG, Swizerland) at wavelength 366nm.

Metabolomics Profiling LC-MS analysis

For polar metabolites detection, LC-MS metabolomics analysis was performed as described previously (PMID: 26358905) with some slight changes. ThermoFisher Scientific Vanquish ultra high-performance liquid chromatography (UHPLC) system coupled to Exploris 240 Orbitrap Mass Spectrometer

(ThermoFisher Scientific) was used with a resolution of 120,000 at 200 mass/charge ratio (m/z), electrospray ionization, and polarity switching mode to enable both positive and negative ions across a mass range of 67 to 1000 m/z. HPLC setup consisted of ZIC pHILIC column (SeQuant; 150 mm x 2.1 mm, 5µm). 5 µL of biological extracts were injected and the compounds were separated using mobile phase gradient of 15 min, starting at 20% aqueous (20 mM ammonium carbonate (ThermoFisher Scientific, 10785511) adjusted to pH = 9.2, with 0.1% of 25% ammonium hydroxide (ThermoFisher Scientific, 15547049) and 80% acetonitrile. The gradient was terminated with 20% acetonitrile. Flow rate and column temperature were maintained at 0.2 mL/min and 45 ºC, respectively, for a total run time of 27 minutes. All metabolites were detected using a mass accuracy below 5 ppm. Xcalibur (ThermoFisher Scientific) was used for data acquisition.

Metabolomics data analysis

TraceFinder 5.0 (ThermoFisher Scientific) was used for analysis. Peak areas of metabolites were determined using the exact mass of singly charged ions. The retention time of metabolites was predetermined on the pHILIC column by analyzing an in-house mass spectrometry metabolite library consisting of commercially available standards. Raw data files were processed with Compound Discoverer 3.3 to obtain total peak areas for each sample. Each metabolite's peak area value was normalized to total measurable ions in the sample as well as to the tissue weight. Metabolite AutoPlotter 2.6 (PMID: 32670572) and Metaboanalyst (PMID: 34019663) were used for data visualization.

Dimethyl fumarate/Monomethyl fumarate/fumarate -thiol containing metabolites conjugates

The conjugates of interest were synthesized as described previously (PMID 17049250). Shortly, to a solution of 0.16 mmol of thiol containing metabolite (GSH/Cysteine, N-Acetylcysteine, etc..) in 5 mL phosphate buffer, pH 7.4, 0.16 mmol of eighter DMF/MMF/Fumarate, dissolved in 2 mL acetone was slowly added under stirring. After stirring for 1 h, the reaction crude extracts were injected to the LC-MS system described herein under the same chromatographic conditions to record the MS-MS spectra, retention time, adduct and ionization mode and add those into the in-house metabolites library for further analysis in the liver samples.

Supplementary Tables

Table S1. Normal diet and HFDCA composition

Ingredients	**Normal diet (ND)**		**HFDCA**	
	gr	**kcal**	**gr**	**kcal**
casein	200.000	800	265.000	1060
L-Methionine	3.000	12	4.000	16
Corn strach	397.500	1590	0.000	0
maltodextrin	132.000	528	160.000	640
sucrose	100.000	400	90.000	360
cellulose	50.000	0	65.500	0
soybean oil	30.000	270	30.000	270
lard	40.000	360	310.000	2790
mineral mix	35.000	0	51.400	0
vitamin mix	10.000	0	21.000	0
cholic acid	0.000	0	5.000	0
choline chloride	2.500	0	3.000	0
BHT	0.014	0	0.014	0
total	1000.0	3960	1005	5136
Protein (%)		20.5		21.0
Carbohydrate (%)		63.6		19.5
Fat (%)		15.9		59.6

BHT, butylated hydroxytoluene

Table S2. Primers sequences

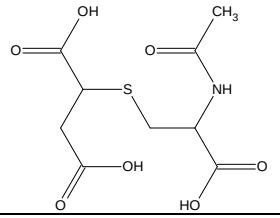
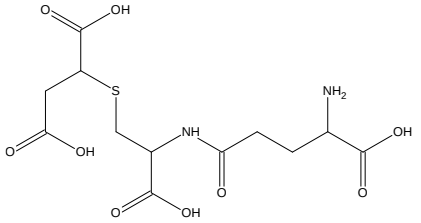
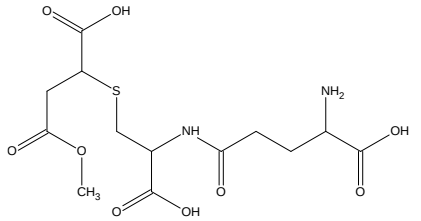
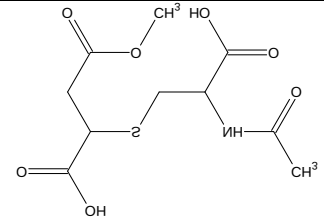
Name	Reverse	Forward
18s	5′-CCTCAGTTCCGAAAACCAAC-3′	5′-ACCGCAGCTAGGAATAATGG-3′
HO-1	5′-CTTCCAGGGCCGTGTAGAT-3′	5′-CAGAAGGGTCAGGTGTCCA-3′
GSTA1	5′-TGCAGCTTCACTGAATCTTGAAAG-3′	5′-CCCCTTTCCCTCTGCTGAAG-3′
NQO1	5′-CCTTTCAGAATGGCTGGCA-3′	5′-GGAAGCTGCAGACCTGGTGA-3′
GCLC	5′-TCGCCTCCATTCAGTAACAA-3′	5′-CGAGGTGGAGTACATGTTGG-3′
CYP7A1	5′-TTGTTCAAGACCGCACATAAAGCC-3′	5′-CGTAGACGGATCAGTTCAGAGCC-3
FXR	5'-TCACTGCACATCCCAGATCTC-3'	5'-TCCGGACATTCAACCATCAC-3'
SHP	5'-AGGATCGTGCCCTTCAGGTA-3'	5'-CAGCGCTGCCTGGAGTCT-3'

18S – 18S ribosomal RNA; HO-1 – Hemeoxygenase-1; GSTA1 – Glutathione S-transferase A1; NQO1 – NAD(P)H Quinone Dehydrogenase 1; GCLC – Glutamate-Cysteine Ligase Catalytic Subunit; CYP7A1 – Cholesterol 7 alpha-hydroxylase; FXR – farnesoid X receptor; SHP – Small heterodimer partner

Table S3. The composition of Lysis Buffer used for the Caspase-3 activity assay.

Ingredient	Final Concentration
Trizma Base (pH 7.4)	20 mM
NaCl	145 mM
Glycerol	10%
EDTA	5 mM
Triton X-100	1%

Table S4. Compounds as mentioned in the paper and their IUPAC names

Compound names as mentioned in paper	IUPAC name	Molecular formula	Formula weight (gr/mol)	Chemical structure
Fumarate-cysteine conjugate	2-[(2R)-2-acetamido-2-carboxyethyl]sulfanylbutanedioic acid	$C_9H_{13}NO_7S$	279.263	
Fumarate-N-Acetyl cysteine conjugate	2-[(2R)-2-[[(4S)-4-amino-4-carboxybutanoyl]amino]-2-carboxyethyl]sulfanylbutanedioic acid	$C_{12}H_{18}N_2O_9S$	366.341	
MMF-N-Acetylcysteine conjugate	2-amino-5-[[1-carboxy-2-(1-carboxy-3-methoxy-3-oxopropyl)sulfanylethyl]amino]-5-oxopentanoic acid	$C_{13}H_{20}N_2O_9S$	380.368	
MMF-Cysteine conjugate	2-[(2R)-2-acetamido-2-carboxyethyl]sulfanyl-4-methoxy-4-oxobutanoic acid	$C_{10}H_{15}NO_7S$	293.29	

Supplementary Figures

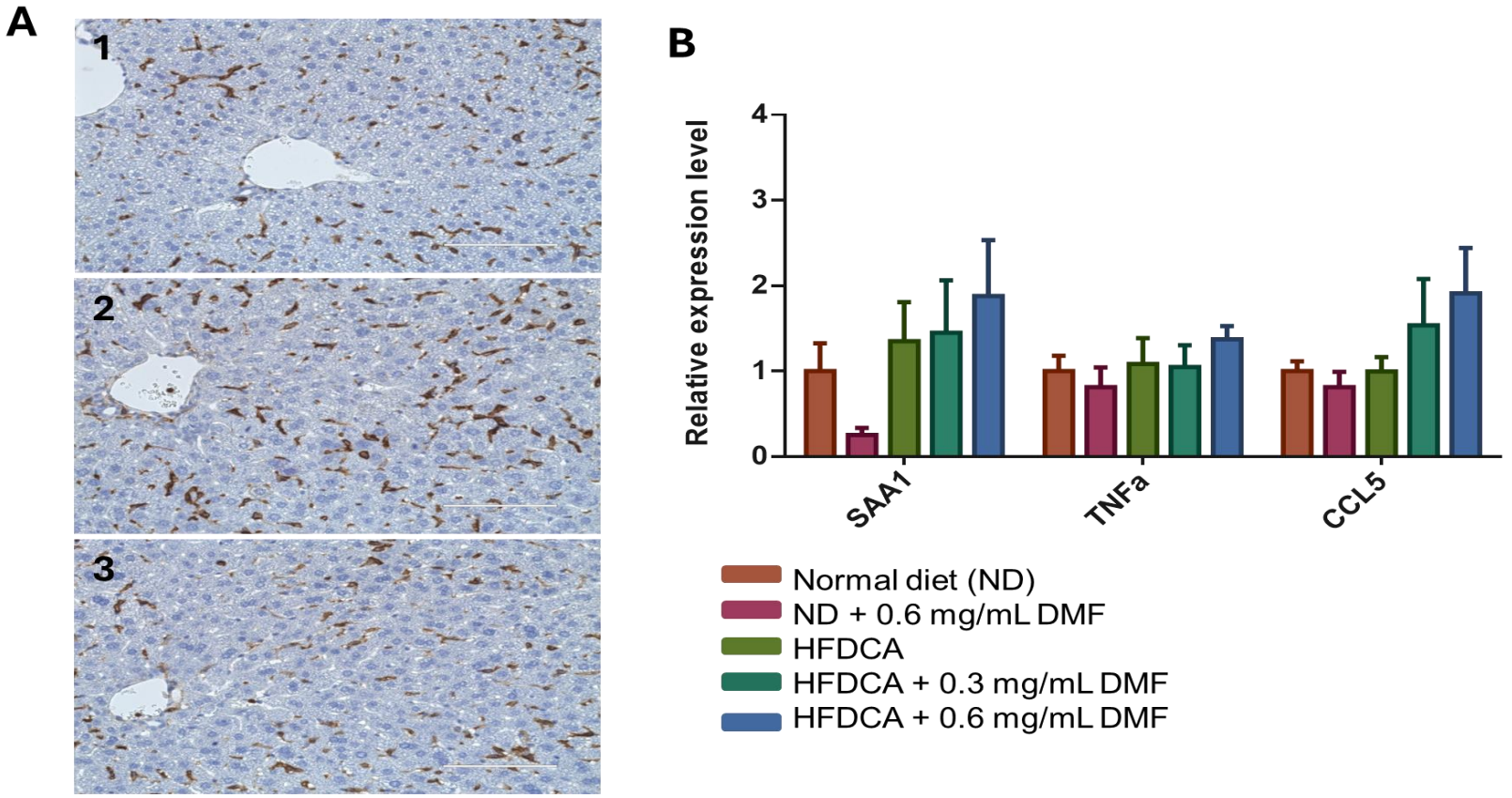


Figure S1. Inflammatory response in liver tissue. (A) IHC staining from liver tissue of (1) ND, (2) HFDCA, and (3) HFDCA + 0.6 mg/mL DMF group. IHC staining performed with an antimacrophage marker F4/80 using digital microscope EVOS M5000 imaging system. Scale bar = 100 µm. **(B)** Hepatic expression levels of Serum amyloid A1 (*SAA1*), Tumor necrosis factor alpha (*TNFa*) and Chemokine (C-C motif) ligand 5 (*CCL5*). All values are expressed as mean ± SEM (n = 6).

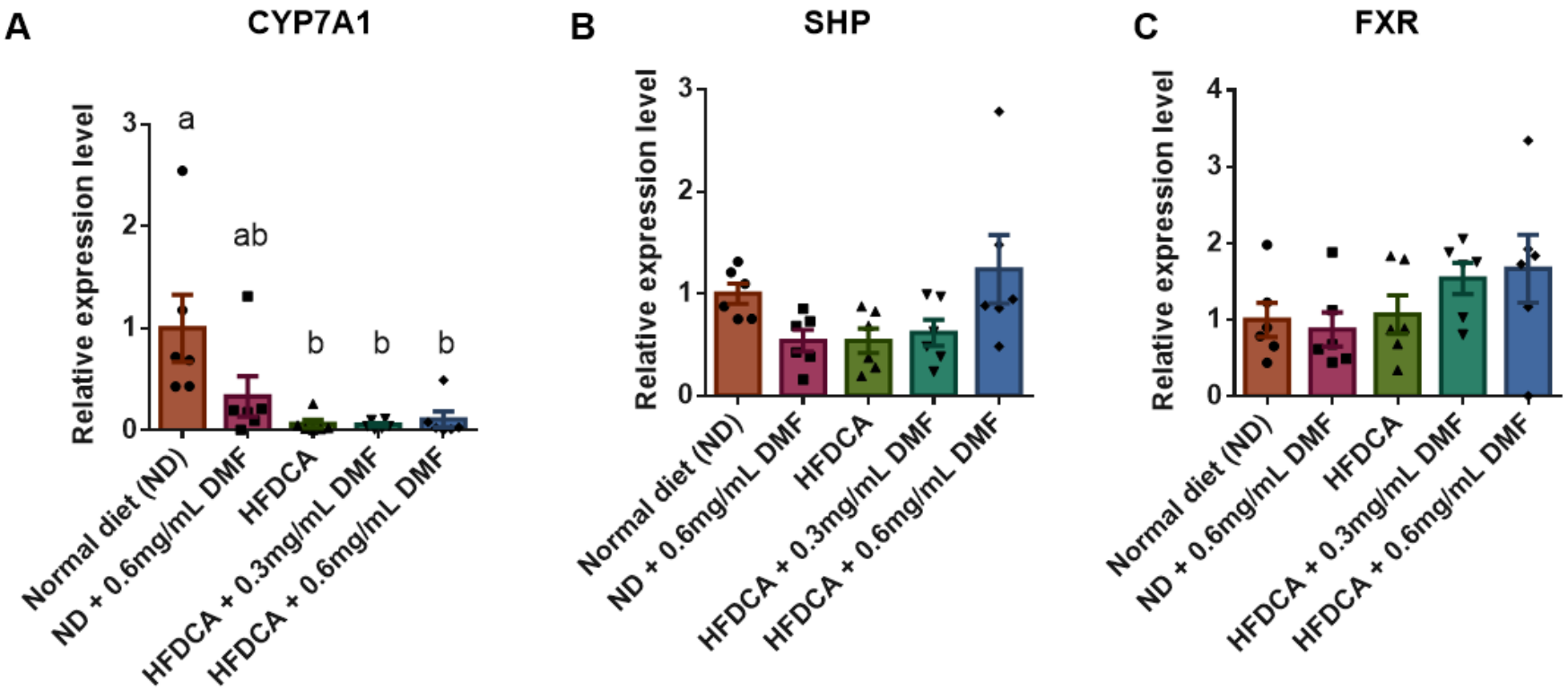


Figure S2. Hepatic cholesterol-related genes expression. Hepatic expression levels of **(A)** Cholesterol 7 alpha-hydroxylase (*CYP7A1*), **(B)** small heterodimer partner (*SHP*) and **(C)** Farnesoid X receptor (*FXR*). All values are expressed as mean ± SEM (n = 6) and each individual measurement represented by point. Columns marked with different letters (a, b) are significantly different ($p < 0.05$) in the Tukey-Kramer post hoc test.

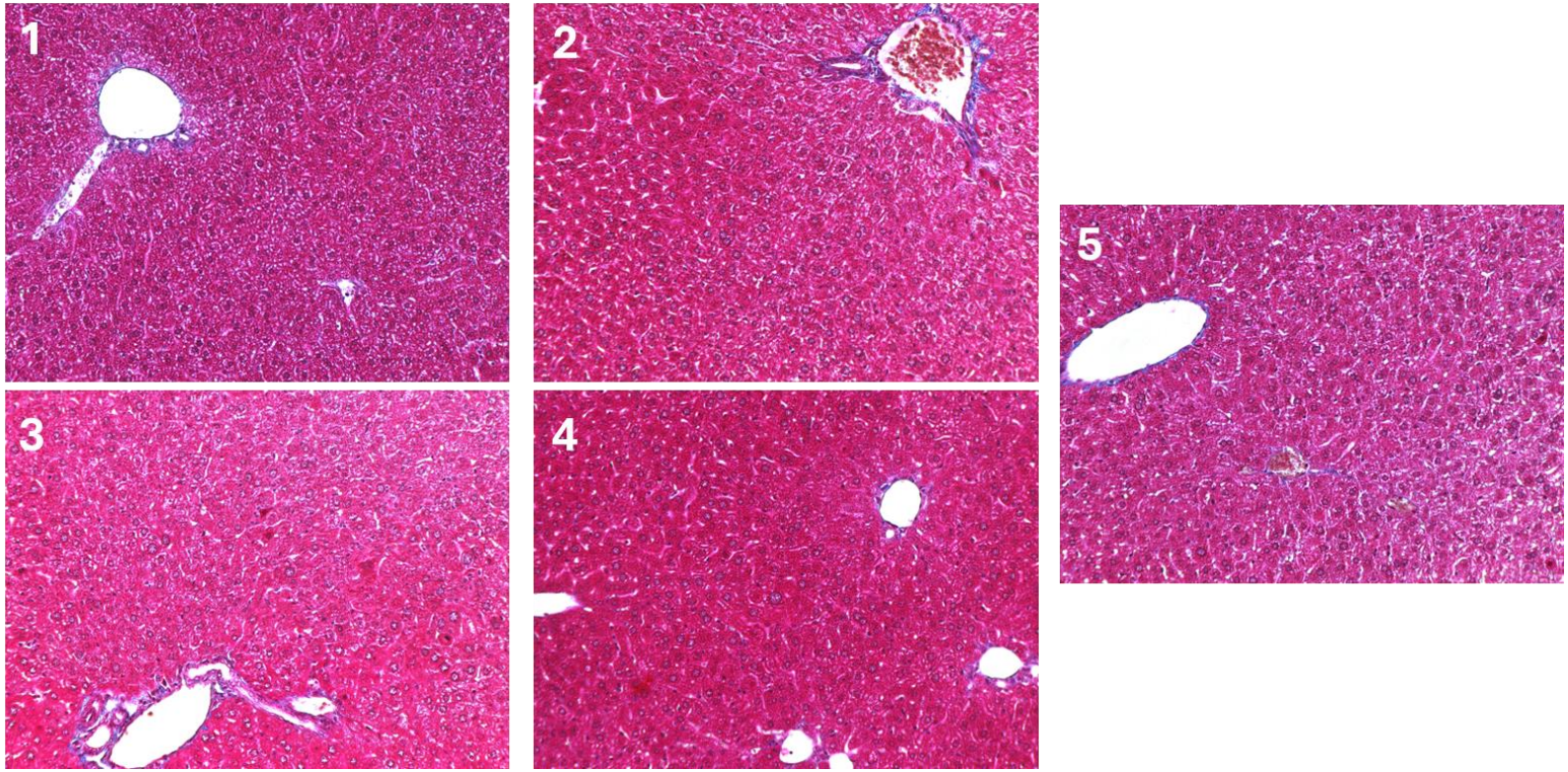


Figure S3. Hepatic fibrosis. Masson's Trichrome staining of collagen within the liver tissue of (1) ND, (2) ND + 0.6 mg/mL DMF, (3) HFDCA, (4) HFDCA + 0.3 mg/mL DMF and (5) HFDCA + 0.6 mg/mL DMF group. Representative liver Masson's trichrome staining under ×20 magnification using digital microscope EVOS M5000 imaging system.

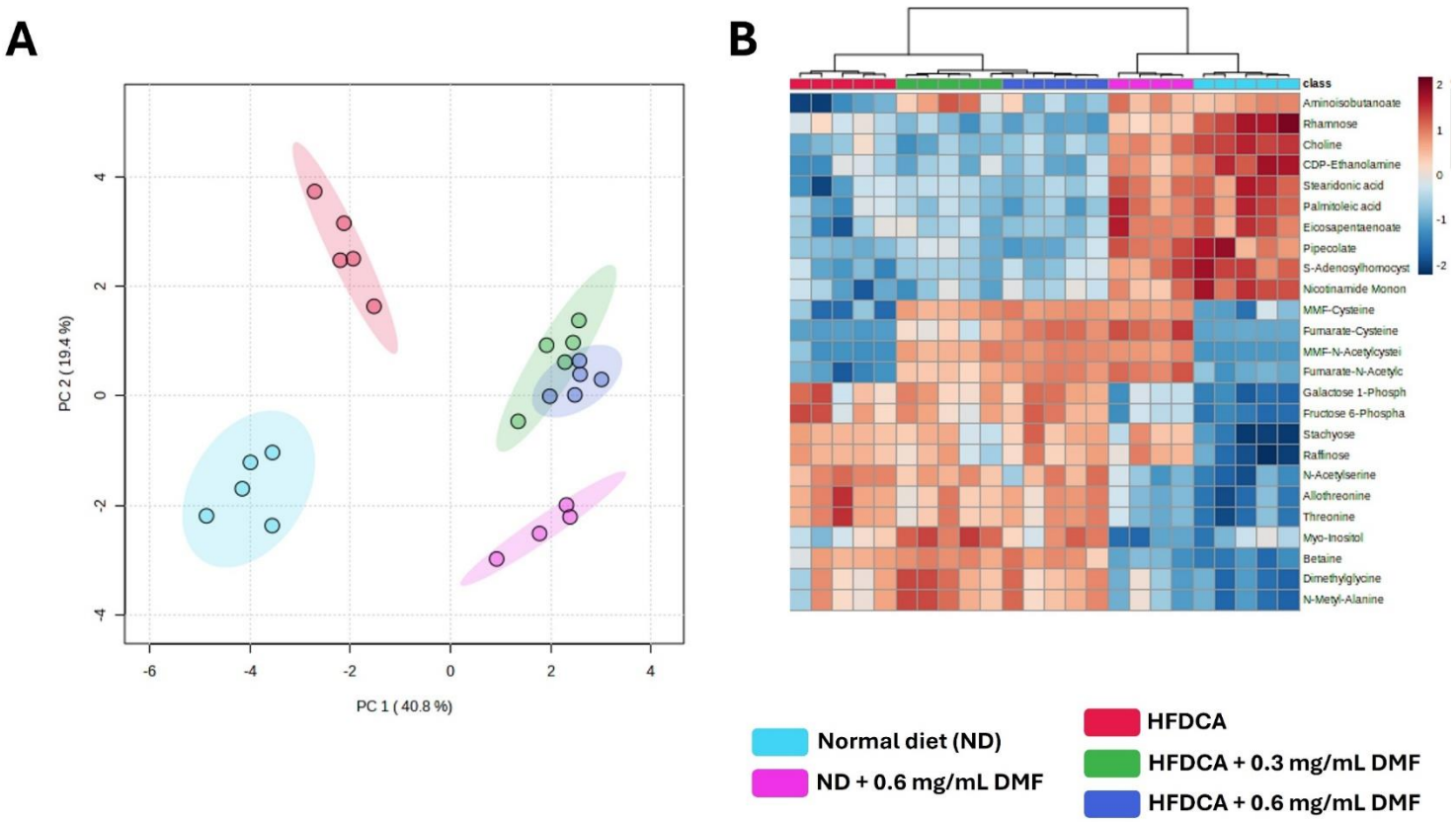


Figure S4. Liver metabolic analysis of ND and HFDCA fed mice supplemented/or not with DMF. (A) Principal Component Analysis (PCA) of 241 chromatographic feature intensities in the liver (n=4 ND + 0.6 mg/mL DMF, n=5 rest of the mice) and **(B)** heatmap of 25 compounds out of 241. (1) ND group, (2) ND + 0.6 mg/mL DMF group, (3) HFDCA group, (4) HFDCA + 0.3 mg/mL DMF group and (5) HFDCA + 0.6 mg/mL DMF group.

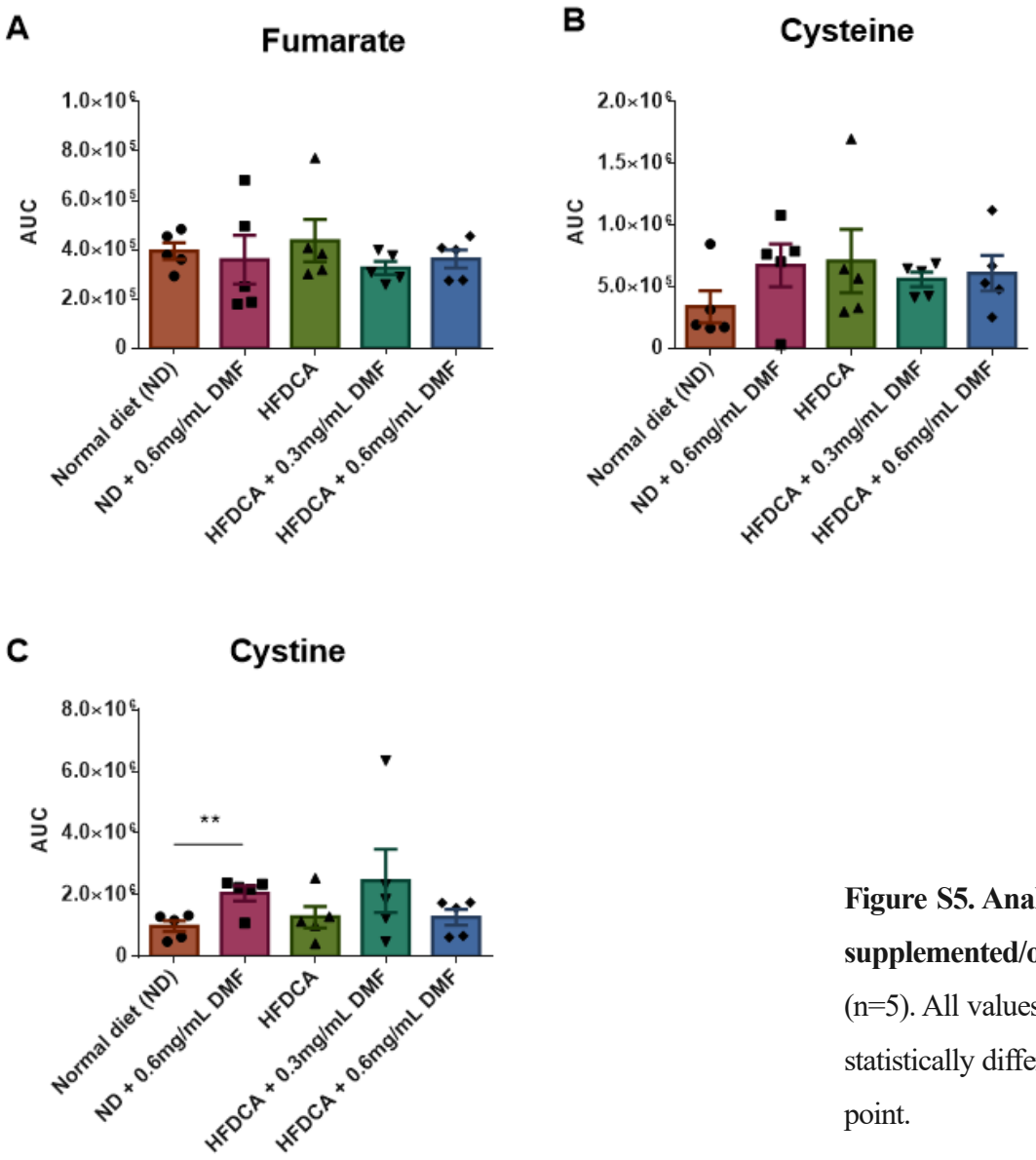


Figure S5. Analysis of DMF metabolites in liver of ND and HFDCA fed mice supplemented/or not with DMF. (A) Fumarate, (B) Cysteine and (C) Cystine levels. (n=5). All values are expressed as mean ± SEM Columns marked with asterisks are statistically different (**, $p < 0.01$) and each individual measurement represented by point.