SUPPORTING INFORMATION

Macrophage-Mediated Clofazimine Sequestration is Accompanied by a Shift in Host Energy Metabolism

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Running head: Clofazimine-induced changes in metabolism

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Confirmation of metabolite identification and quantification by nuclear magnetic resonance (NMR)

One advantage of NMR as an analytical platform for metabolomics, is that every compound has a distinct 1D-^{1}H-NMR spectrum and it is routinely quantitative with the addition of an internal standard (IS) to the sample.[1,2] Spectral analysis is optimized by using software platforms like Chenomx (chenomx.com) which aids in the analysis of a confounding mix of spectral peaks from biological samples and confirms metabolite identification (ID) using an established library of 338 compounds. It also permits reliable measurements of metabolite concentrations relative to the added IS. Chenomx-based spectral analysis is best when the software specified pulse sequence as reported in this manuscript is used. Nevertheless, because the spectrum of some compounds are complex, with multiple and/or numerous overlapping peaks, confirmation of metabolite identification may require more in depth analysis.

Following statistical analysis, the differentiating (FDR $\leq$ 15%) urine metabolites (µmole) of CFZ after 8 weeks of treatment are: 3-indoxylsulfate (HMDB00682), ascorbate (HMDB00044), glycine (HMDB00123), methionine (HMDB00696), taurine (HMDB00251), carnitine (HMDB00062), choline (HMB00097), N,N-dimethylglycine (HMDB00092), succinate (HMDB00254), cis-aconitate (HMDB00072), and 2-oxoglutarate (HMDB00208). Based on the 1D-^{1}H-NMR spectrum of each compound, they were grouped as follows:

Group 1 metabolites

No further NMR experiments were conducted for metabolites that have two or more indisputable peaks at distinct chemical shifts on the 1D-metnoesy spectrum. These compounds are: 2-oxoglutarate, 3-indoxylsulfate, ascorbate, cis-aconitate, and taurine. These metabolites are considered to be accurately identified by Chenomx software. The peak expansion figures for these distinct chemical shifts on representative 1D-^{1}H NMR spectra are shown in Fig. S1A-E.

Group 2 metabolites

For those differentiating metabolites for which confident and positive identification could not be readily achieved by Chenomx analysis, additional 1D (1H, 13C) and 2D (HSQC) NMR experiments were conducted. The metabolites for this analysis were: carnitine, choline, glycine, methionine, N,N-dimethylglycine and succinate. The HSQC high resolution experiments were used to compare the chemical shift correlation map of the directly bonded 1H-13C for each of these metabolites and these compounds from representative mice urine samples. The additional 13C dimension in the 2D-HSQC NMR experiment, provides additional resolution and secondary confirmation, towards a confident identification of the peaks for those metabolites. The peaks used for quantification in Chenomx from the urine samples, were cross-checked against the HSQC spectra of each pure metabolite in order to be confident in the peak assignment and quantification. The additional 1D and 2D NMR experiments were acquired at the University of Michigan´s Biochemical NMR Core Laboratory on an Agilent 500MHz spectrometer with a 5mm Agilent One-probe and a VNMRS console operated by host software VNMRJ 4.0 on two randomly selected urine samples from week 0 (cage 7) and week 8 (cage 12), which were technical replicates of the urine samples used for the

metnoesy acquisitions as reported in the main manuscript. For these NMR experiments, 13C-proton decoupled (18,000 scans) and 2D-HSQC (32 scans), the more sensitive HSQC pulse sequence, gc2hsqcse, was used and included in the experiment panel on VNMRJ 4.0; spectra were acquired at 295.45 ± 0.3 K and pH 7.0 ± 0.2. The data from 1H, 13C and gc2hsqcse experiments for these samples, as well as the raw free induction decay (FID) data for the individual metabolites, choline, glycine, N,N-dimethylglycine and succinate and the corresponding 1D and 2D-NMR spectra from the Human Metabolome Database (HMDB version 3.6; http://www.hmdb.ca/) were used. For carnitine, additional NMR experiments were conducted because no spectra were available in HMDB [3,4]. Spectra for all compounds were imported into MestreNova (10.0.2) Mestrelab Research S.L (Santiago de Compostela, Spain) and displayed in a stack-plot to identify each metabolite in the urine spectra (Fig. S2A-F).

Urine and Whole Blood Metabolite Datasets

The entire urine and whole blood metabolomics data sets can be found in the accompanying excel spreadsheet. The data and the free induction decay (FID) files for each sample are available on the NIH's Metabolomics Workbench website (http://www.metabolomicsworkbench.org/).

Figure Captions

Figure S1: The 1D-^{1}H-NMR distinct chemical shifts and associate peaks of (**A**) 2-oxoglutarate, with chemical shifts at 3.0ppm (left) and 2.44ppm (right); (**B**) indoxyl sulfate, with distinct chemical shifts at 7.68ppm (left) and 7.50ppm (right); (**C**) ascorbate,

with distinct chemical shifts at 4.52ppm (left) and 4.02ppm (right); (**D**) cis-aconitate, with distinct chemical shifts at 5.79ppm (left) and 3.14ppm (right); and (**E**) taurine*,* with distinct chemical shifts at 3.42ppm (left) and 3.26ppm (right).

Figure S2: The stack plot of the HSQC spectrum (top) from the HMDB data base (with the exception of carnitine) and the acquired urine HSQC spectrum (bottom) for: (**A**) carnitine, (**B**) choline, (**C**) glycine, (**D**) methionine (**E**) N, N-dimethylglycine, and (**F**) succinate. At the bottom, the 1D spectrum from Chenomx software showing the highlighted peak(s) used for metabolite quantification.

Figure S3: Candidate metabolites of clofazimine (CFZ)-induced metabolic reprogramming that were detected in both urine (left) and whole blood (WB, right) after 8-weeks of treatment. Choline (**A**), an essential nutrient that is required for one carbon metabolism, was elevated in both urine ($p=0.002$, FDR=5.2%) and WB ($p<0.0001$, FDR=0.6%). Urine taurine (**B**), an endogenous sulfonic acid that is a major constituent of bile and is required for mitochondrial ATP production, was lower in the urine of CFZ-treated mice ($p<0.0001$, FDR=0.3%) but higher in WB ($p=0.001$, FDR=4%). Another amino acid, glycine (**C**), which is pivotal to one carbon metabolism via the folate cycle, was also lower in the urine ($p<0.0001$, FDR=0.3%) and higher in the blood ($p=0.005$, FDR=5.3%) of CFZ-treated mice. Carnitine (**D**), which is required for the transport of fatty acids into the mitochondria, was lower in the urine ($p=0.002$, FDR=1.1%) and higher in WB ($p=0.03$, FDR=11%). Methionine (**E**), a metabolite important to one carbon metabolism, was lower in the urine ($p<0.0001$, FDR=0.3%) and unchanged ($p=0.16$, FDR=31%) in the WB by CFZ-treatment. Clofazimine did not change ($p=0.46$, FDR=68%) urine betaine levels (**F**) but WB levels were increased ($p=0.03$, FDR=11%)

compared with sham controls. The crossbars represent the medians and the error bars, the interquartile ranges, of 6 urine samples/group and 6 and 7 WB samples from CFZ- and sham-treated mice, respectively. Urine p values are those that resulted from ANOVA post-hoc testing by a Sidak test and WB p values were generated by an unpaired Student's t-test of the normalized urine and WB metabolite concentration data, respectively.

Figure S4: Metabolite pathways (compound networks) of differentiating urine metabolites (red hexagons; false discovery rate (FDR) $\leq$ 15%) induced by CFZ at 8 weeks generated by Metscape (http://metscape.ncibi.org/). Pink hexagons indicate compounds associated with inputted metabolites. Networks were generated by inputting the Kyoto Encyclopedia of Genes and Genomes (KEGG) identification numbers of the differentiating urine metabolites (see Fig. 3E-N) into the Metscape pathway based analysis.

Figure S5: Metabolite pathways of differentiating whole blood (WB) metabolites (red hexagons; false discovery rate (FDR) $\leq$ 15%) induced by CFZ at 8 weeks generated by Metscape (http://metscape.ncibi.org/). Pink hexagons indicate compounds associated with inputted metabolites. Networks were generated by inputting the Kyoto Encyclopedia of Genes and Genomes (KEGG) identification numbers of the 20 differentiating WB metabolites (see Fig. 5) into the Metscape pathway based analysis.

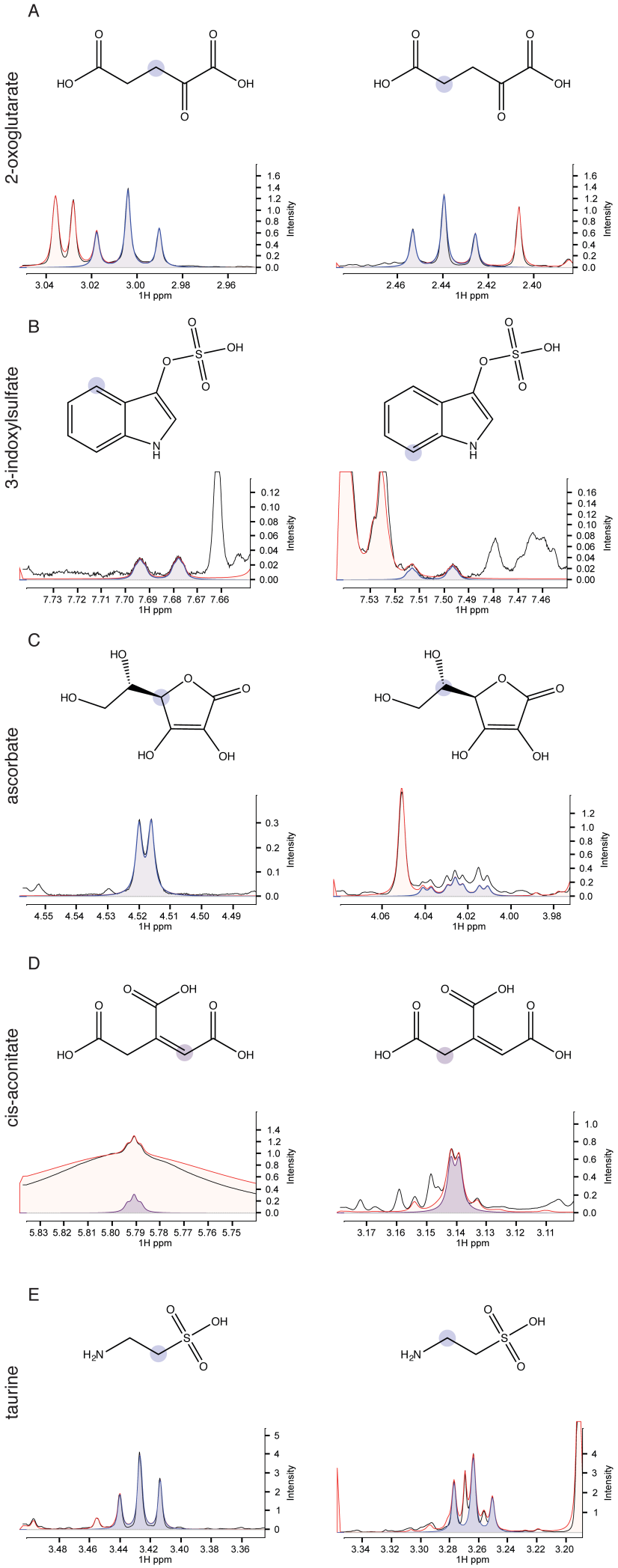
References

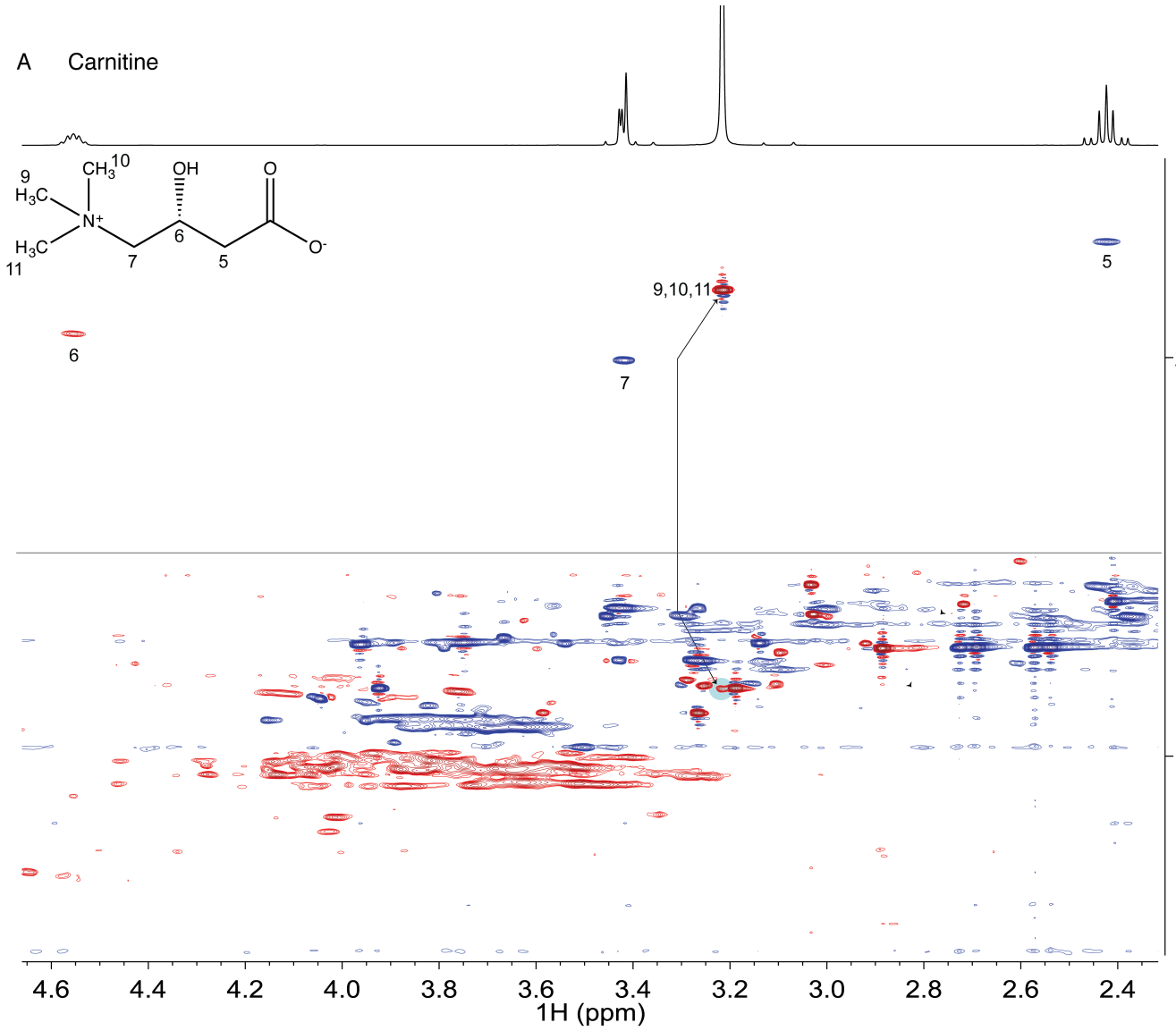
1. Serkova NJ, Brown MS 2012. Quantitative analysis in magnetic resonance spectroscopy: from metabolic profiling to in vivo biomarkers. Bioanalysis 4(3):321-341.

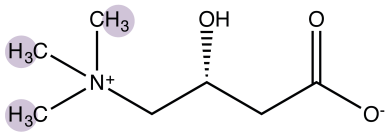
2. Serkova NJ, Standiford TJ, Stringer KA 2011. The emerging field of quantitative blood metabolomics for biomarker discovery in critical illnesses. Am J Respir Crit Care Med 184(6):647-655.

3. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai L, Lewis M, Coutouly MA, Forsythe I, Tang P, Shrivastava S, Jeroncic K, Stothard P, Amegbey G, Block D, Hau DD, Wagner J, Miniaci J, Clements M, Gebremedhin M, Guo N, Zhang Y, Duggan GE, Macinnis GD, Weljie AM, Dowlatabadi R, Bamforth F, Clive D, Greiner R, Li L, Marrie T, Sykes BD, Vogel HJ, Querengesser L 2007. HMDB: the Human Metabolome Database. Nucleic Acids Res 35(Database issue):D521-526.

4. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, Djoumbou Y, Mandal R, Aziat F, Dong E, Bouatra S, Sinelnikov I, Arndt D, Xia J, Liu P, Yallou F, Bjorndahl T, Perez-Pineiro R, Eisner R, Allen F, Neveu V, Greiner R, Scalbert A 2013. HMDB 3.0--The Human Metabolome Database in 2013. Nucleic Acids Res 41(Database issue):D801-807.


A
2-oxoglutarate
B
3-indoxylsulfate
C
ascorbate
D
cis-aconitate
E
taurine
Intensity
1H ppm


A Carnitine
9
CH3 10
OH
O
H3C
N+
H3C
11
7
6
5
O-
6
7
9,10,11
5
Carnitine UM-hsqc
Urine UM-hsqc
2
1
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
1H (ppm)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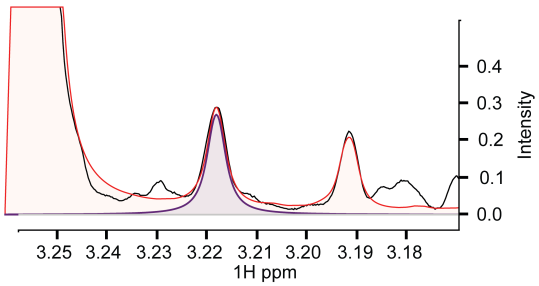
OH
O
H3C
N+
H3C
O-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0.3
0.2
0.1
0.0
Intensity
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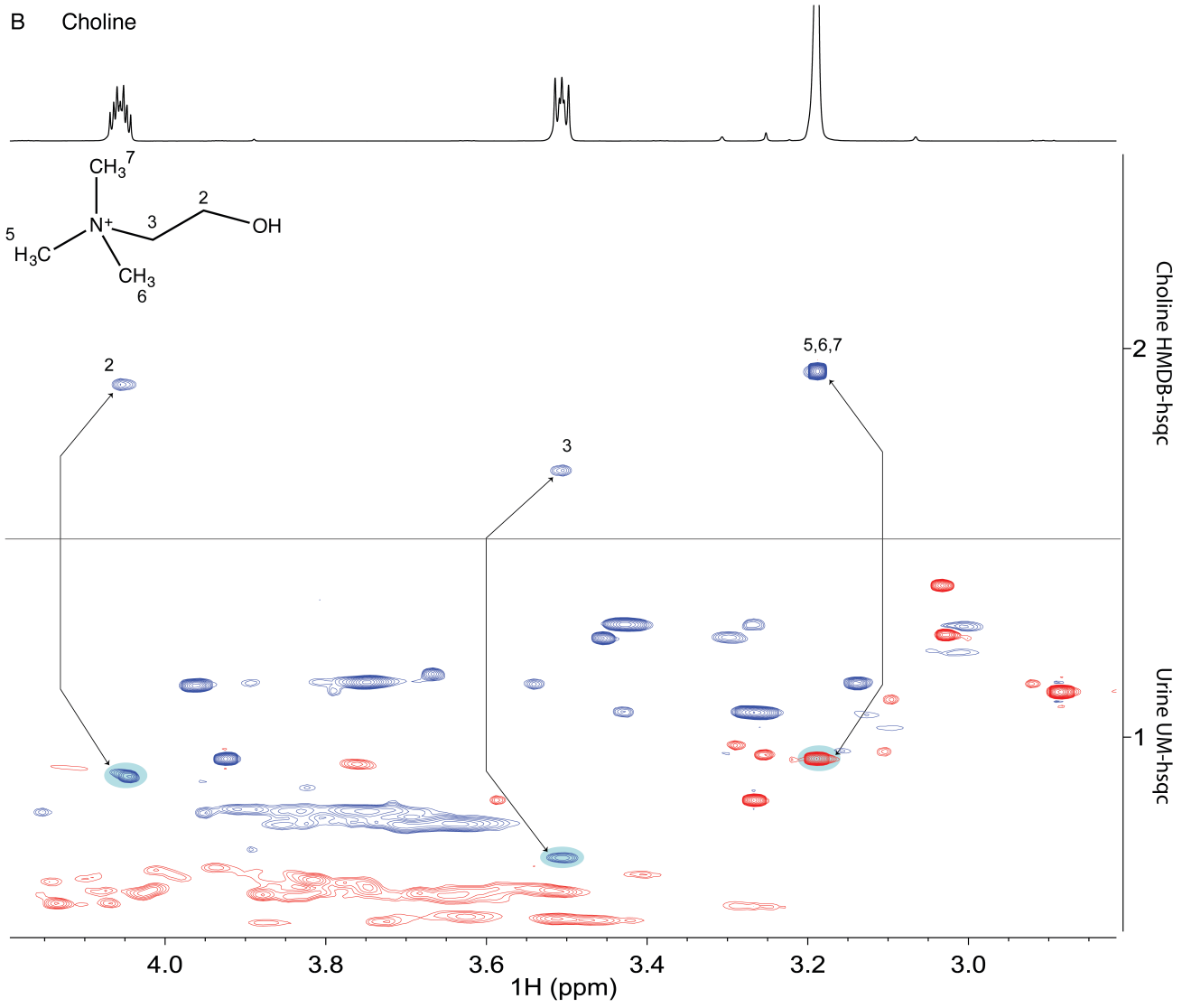
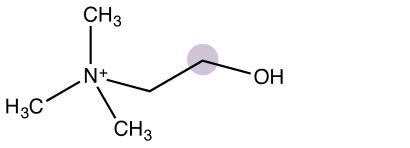
3.23
3.22
3.21
3.20
3.19
3.18
1H ppm


B
Choline
CH3 7
N+
3
2
OH
5
H3C
CH3
6
2
3
5,6,7
Choline HMDB-hsqc
Urine UM-hsqc
2
1
4.0
3.8
3.6
3.4
3.2
3.0
1H (ppm)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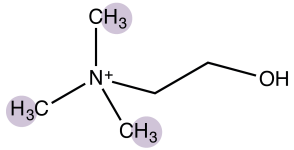
N+
OH
H3C
CH3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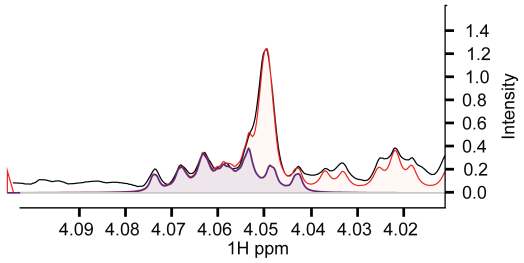
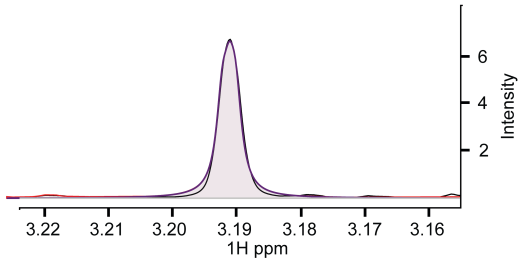
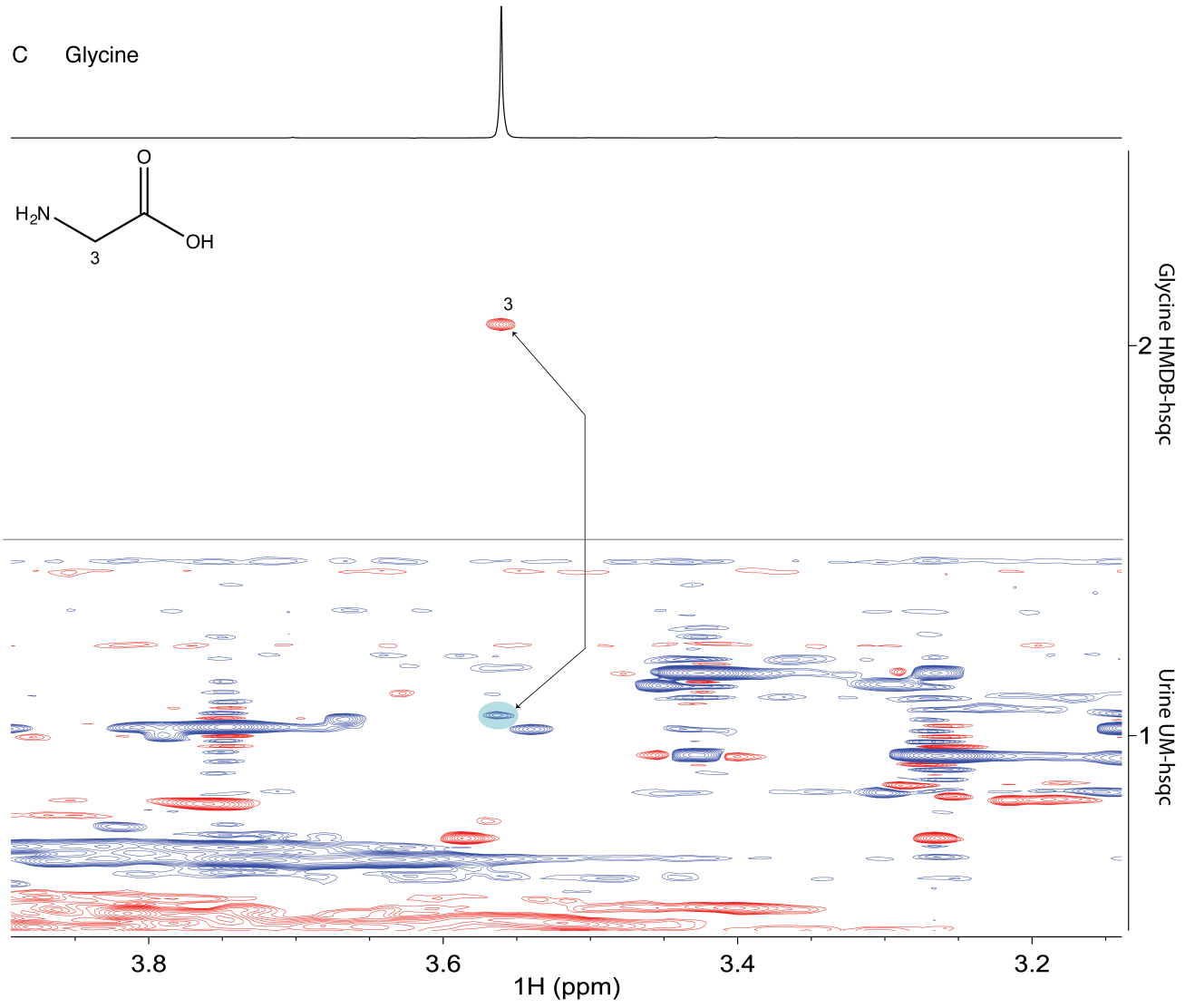
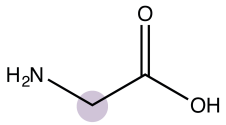
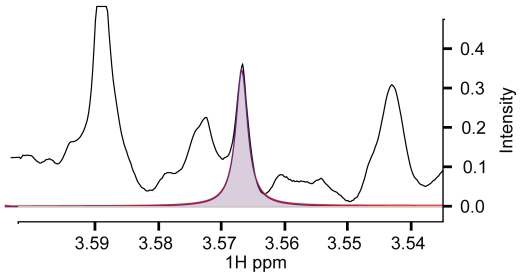
N+
OH
H3C
CH3


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Intensity
4.09 4.08 4.07 4.06 4.05 4.04 4.03 4.02
1H ppm


6
4
2
Intensity
3.22 3.21 3.20 3.19 3.18 3.17 3.16
1H ppm


C Glycine
O
H2N
OH
3
3
Glycine HMDB-hsqc
2
Urine UM-hsqc
1
3.8
3.6
3.4
3.2
1H (ppm)


O
H2N
OH


0.4
0.3
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0.1
0.0
Intensity
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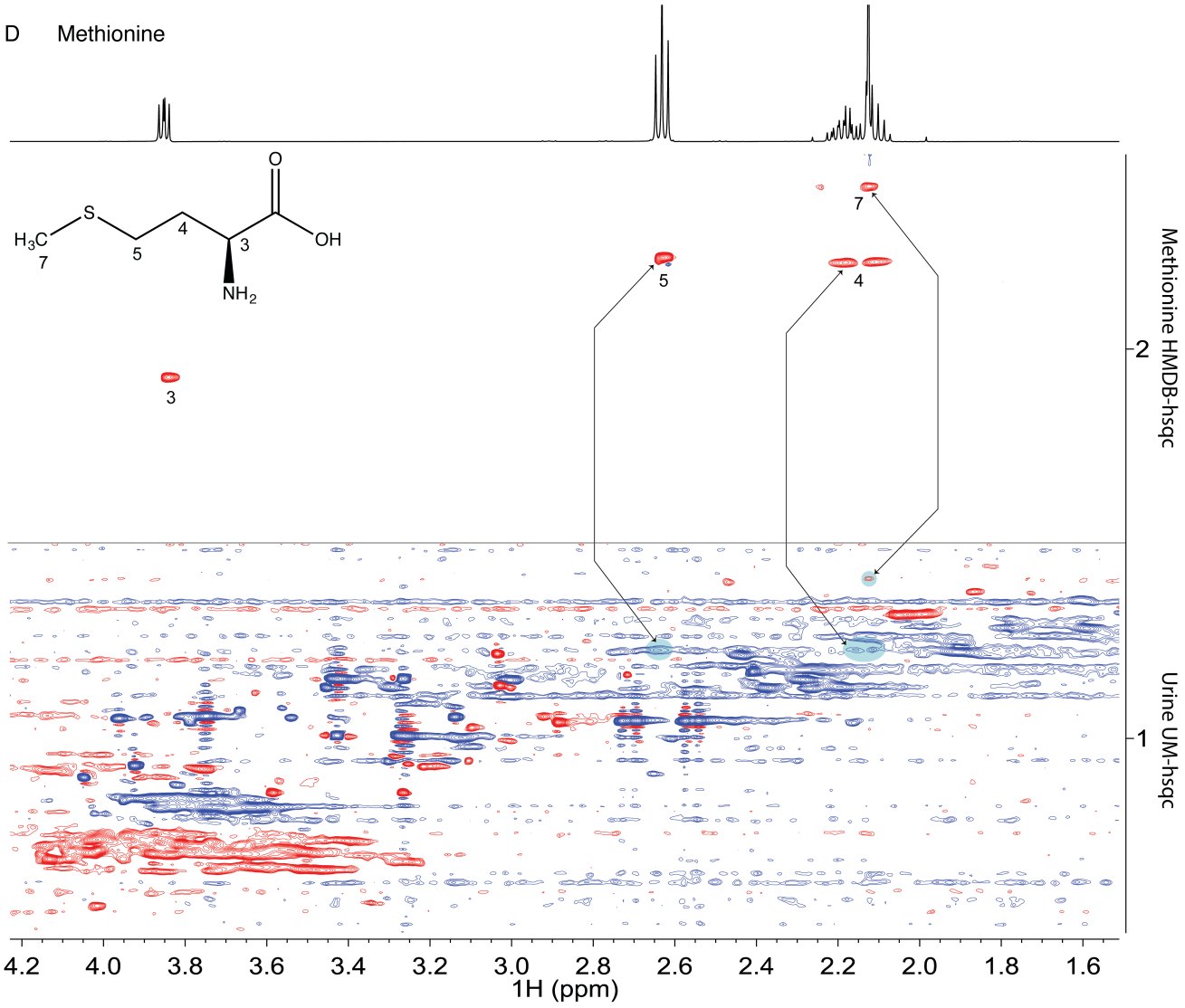
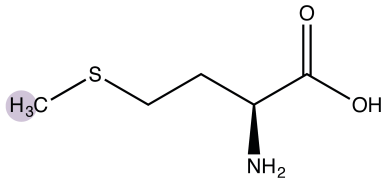
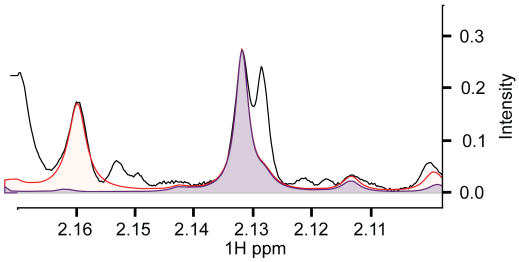
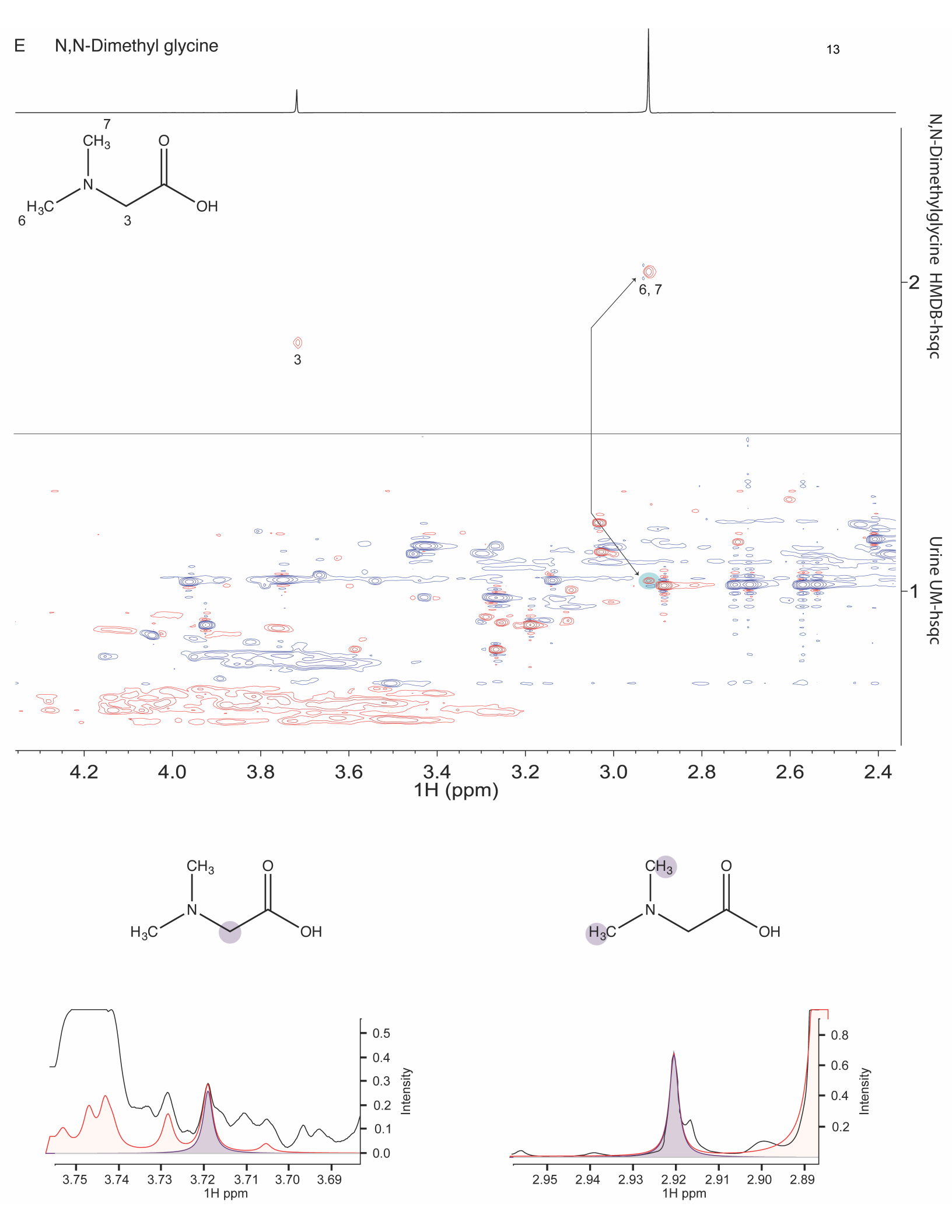
3.58
3.57
3.56
3.55
3.54
1H ppm

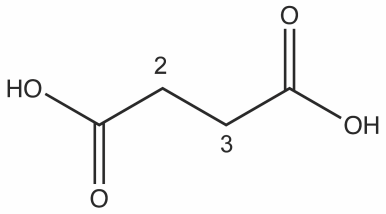

D Methionine
O
S
4
3
OH
H_3C
7
5
NH_2
7
5
4
3
2
1
Methionine HMDB-hsqc
Urine UM-hsqc
4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6
1H (ppm)


O
S
OH
H_3C
NH_2

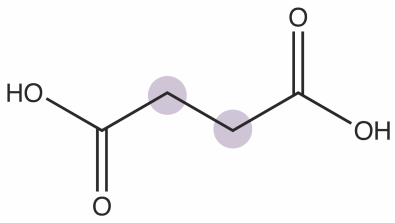

0.3
0.2
0.1
0.0
Intensity
2.16 2.15 2.14 2.13 2.12 2.11
1H ppm

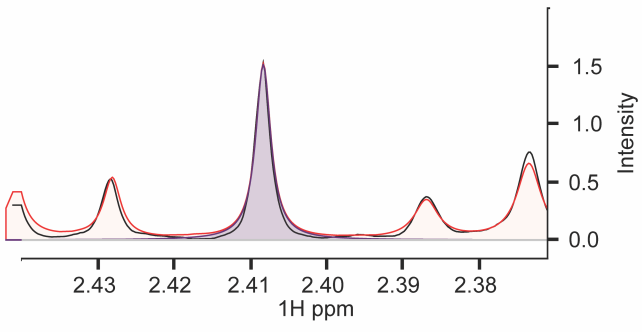

E
N,N-Dimethyl glycine
13
7
CH3
O
N
H3C
OH
6
3
6, 7
3
N,N-Dimethylglycine HMDB-hsqc
Urine UM-hsqc
2
1
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
1H (ppm)
CH3
O
N
H3C
OH
CH3
O
N
H3C
OH
0.5
0.4
0.3
0.2
0.1
0.0
Intensity
3.75
3.74
3.73
3.72
3.71
3.70
3.69
1H ppm
0.8
0.6
0.4
0.2
Intensity
2.95
2.94
2.93
2.92
2.91
2.90
2.89
1H ppm

F Succinate


HO
O
2
3
OH
O

2, 3
Succinate HMDB-hsqc
Urine UM-hsqc
2
1
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1H (ppm)


HO
O
OH
O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0.0
Intensity
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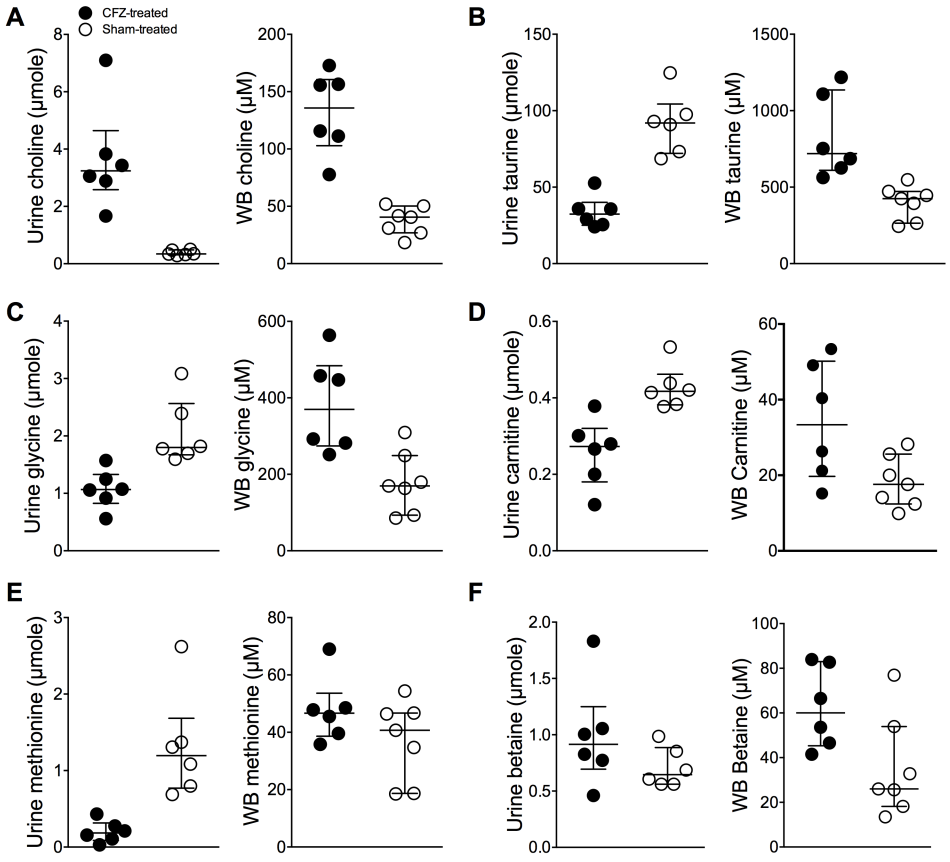
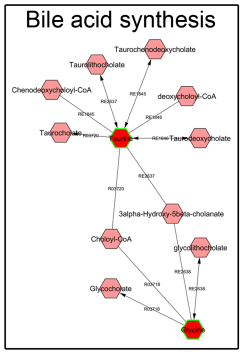
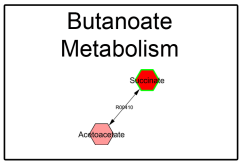
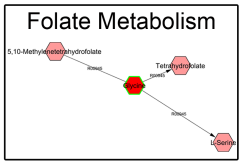
2.42
2.41
2.40
2.39
2.38
1H ppm

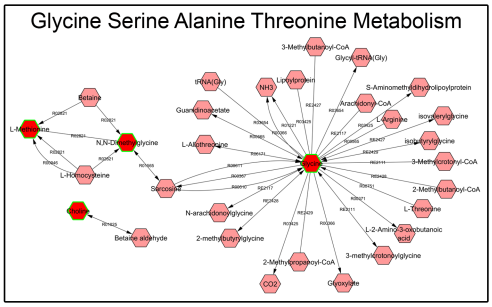

A
CFZ-treated
Sham-treated
Urine choline (µmole)
WB choline (µM)
B
Urine taurine (µmole)
WB taurine (µM)
C
Urine glycine (µmole)
WB glycine (µM)
D
Urine carnitine (µmole)
WB Carnitine (µM)
E
Urine methionine (µmole)
WB methionine (µM)
F
Urine betaine (µmole)
WB Betaine (µM)

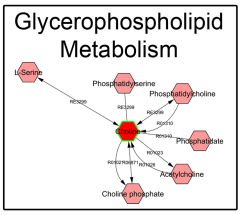
Urine Metabolites: CFZ-induced Pathways at 8 weeks

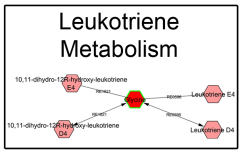


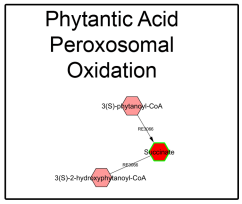


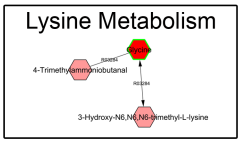


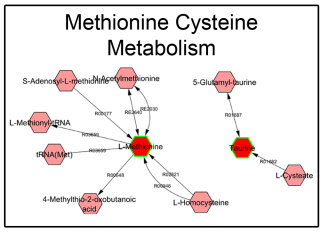


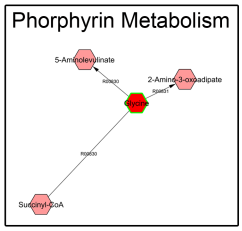


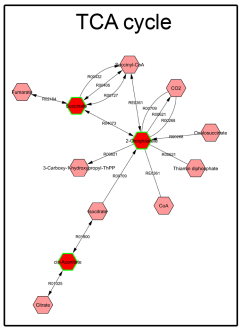


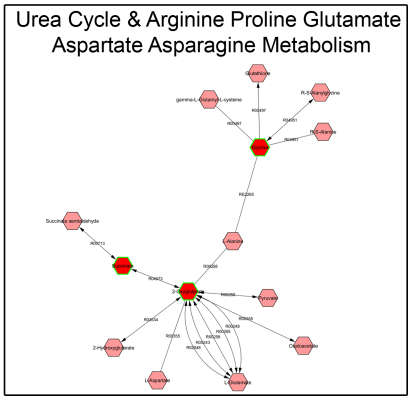


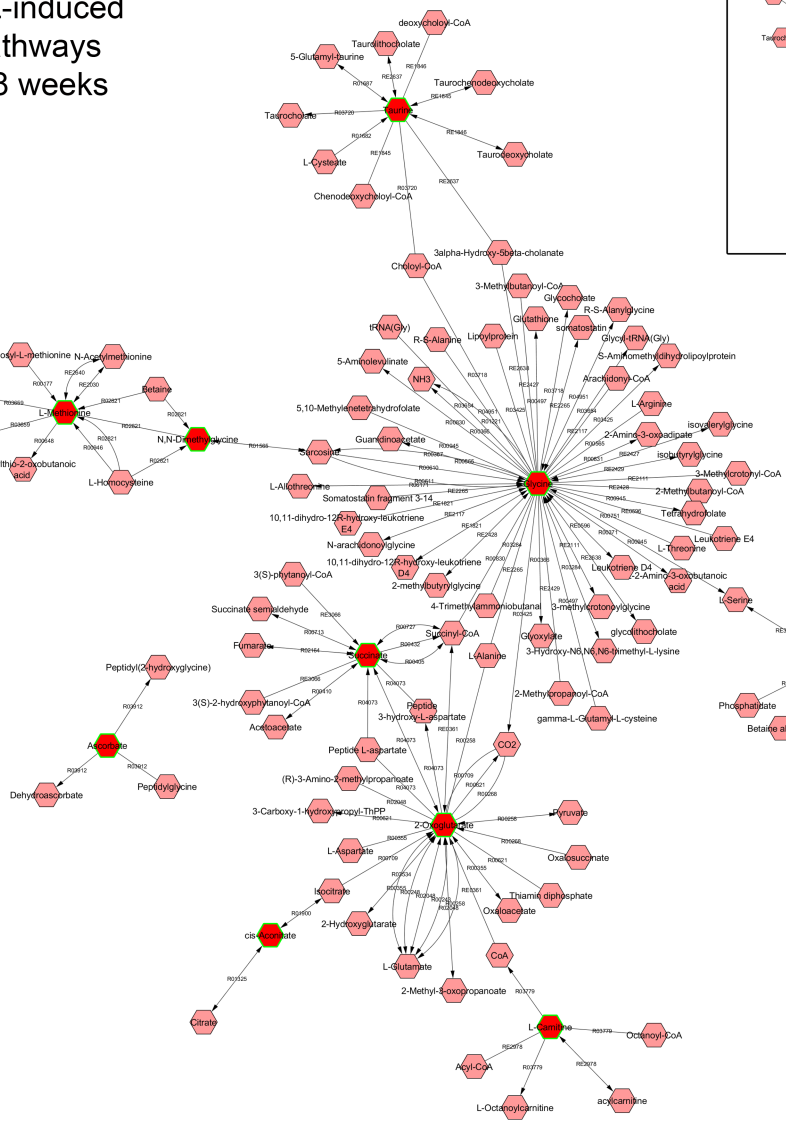












Whole Blood Metabolites: CFZ-induced Pathways at 8 weeks