Methods

Collection Summary:
Liver tissue was harvested on post-natal day 240 after challenge with Western-style diet.Tissue was snap-frozen in liquid nitrogen.

Treatment Summary:
Neonatal rats were treated with vehicle (sesame oil) or bisphenol A (BPA; 50 µg/kg dissolved in sesame oil) orally via pipette tip on post-natal days 1, 3, and 5. Littermates were randomly assigned to the treatment groups. BPA was obtained from the National Institute of Environmental Health Sciences (NIEHS). The dose and route of administration recapitulates human exposure to BPA. At day 180, adult rats in both treatment groups were fed a diet high in fat (40% kcal), fructose (20% kcal) and cholesterol (2%) (Western-style diet) for 60 days (D09100301, Research Diets, Inc). Rats were fasted overnight prior to tissue collection.

Sample Preparation Summary:
Metabolites were extracted from crushed liver samples and a mouse liver pool was used for quality control. Twenty-five mg of crushed liver was used for the metabolic extraction. The extraction step started with the addition of 750 µL ice-cold methanol:water (4:1) containing 20 µL spiked internal standards to each tissue sample. Ice-cold chloroform and water were added in a 3:1 ratio for a final proportion of 1:4:3:1 water:methanol:chloroform:water. The organic (methanol and chloroform) and aqueous layers were mixed, dried and resuspended with 50:50 methanol: water. The extract was deproteinized using a 3kDa molecular filter (Amicon ultracel-3K Membrane; Millipore Corporation, Billerica, MA) and the filtrate was dried under vacuum (Genevac EZ-2plus; Gardiner, Stone Ridge, NY). Prior to mass spectrometry, the dried extracts were re-suspended in identical volumes of injection solvent composed of 1:1 water: methanol and were subjected to liquid chromatography-mass spectrometry. Fifty µl of sample was used for preparation. Internal standards were spiked into the samples. Then it was processed through a 3 kDa filter. After that, 50 µl of sample was diluted with 450 µl solvent (methanol: water = 50:50 v/v) and subjected to LC/MS analysis. The injection volume was 10 µl. For internal standards, high-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, and water were procured from Burdick & Jackson (Morristown, NJ). Mass spectrometry-grade formic acid was purchased from Sigma-Aldrich (St Louis, MO). Calibration solution containing multiple calibrants in a solution of acetonitrile, trifluroacetic acid, and water was purchased from Agilent Technologies (Santa Clara, CA). Metabolites and internal standards, including N-acetyl Aspartic acid-d3, Tryptophan-15N2, Sarcosine-d3, Glutamic acid-d5, Thymine-d4, Gibberellic acid, Trans-Zeatine, Jasmonic acid, 15N Anthranilic acid, and Testosterone-d3, were purchased from Sigma-Aldrich (St. Louis, MO). Three LC- MS methods were used to separate metabolites. Method A: In ESI positive mode the HPLC column was waters X-bridge amide 3.5 µm, 4.6 x 100 mm (Waters, Milford, MA). Mobile phase A and B were 0.1% formic acid in water and acetonitrile, respectively. Gradient flow: 0-3 min 85% B; 3-12 min 30% B, 12-15 min 2% B, 16 min 95%B, followed by re-equilibration till the end of the gradient 23 min to the initial starting condition of 85% B. Flow rate of the solvents used for the analysis is 0.3 ml/min. Injection volume was 10 µL. Method B: In ESI negative mode the HPLC column

was waters X-bridge amide 3.5 µm, 4.6 x 100 mm (Waters, Milford, MA). Mobile phase A and B were 20 mM ammonium acetate in water with pH 9.0 and 100% acetonitrile, respectively. Gradient flow: 0-3 min 85% B, 3-12 min 30% B, 12-15 min 2% B, 15-16 min 85% B followed by re-equilibration till the end of the gradient 23 min to the initial starting condition of 85% B. Flow rate of the solvents used for analysis is 0.3 ml/min. Injection volume was 10 µL. Method C: In ESI positive mode the HPLC column was Luna 3 µM NH2 100 A0 Chromatography column (Phenomenex, Torrance, CA). Mobile phase A and B were 20 mM ammonium acetate in water with pH 9.0 and 100% acetonitrile, respectively. Gradient flow: 0-3 min 85% B, 3-12 min 30% B, 12-15 min 2% B, 15-16 min 85% B followed by re-equilibration till the end of the gradient 23 min to the initial starting condition of 85% B. Flow rate of the solvents used for analysis is 0.3 ml/min. Injection volume was 10 µL. For data acquisition through LC/MS analysis, 10 µL of suspended samples were injected and analyzed using a 6495 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) coupled to a HPLC system (Agilent Technologies, Santa Clara, CA) via Multiple reaction monitoring (MRM). Source parameters were as follows: Gas temperature- 250°C; Gas flow- 14 l/min; Nebulizer - 20psi; Sheath gas temperature - 350°C; Sheath gas flow- 12 l/min; Capillary - 3000 V positive and 3000 V negative; Nozzle voltage- 1500 V positive and 1500 V negative. Approximately 8–11 data points were acquired per detected metabolite. The data acquired using Agilent mass hunter software and data was analyzed using mass hunter quantitative analysis software.

MS acquisition and Data Processing Comments:

For data acquisition through LC/MS analysis, 10 µL of suspended samples were injected and analyzed using a 6495 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) coupled to a HPLC system (Agilent Technologies, Santa Clara, CA) via Multiple reaction monitoring (MRM). Source parameters were as follows: Gas temperature- 250°C; Gas flow- 14 l/min; Nebulizer - 20psi; Sheath gas temperature - 350°C; Sheath gas flow- 12 l/min; Capillary - 3000 V positive and 3000 V negative; Nozzle voltage- 1500 V positive and 1500 V negative. Approximately 8–11 data points were acquired per detected metabolite. The data acquired using Agilent mass hunter software and data was analyzed using mass hunter quantitative analysis software.