Inhaled glucocorticoid-induced metabolome changes in asthma – Supplementary Materials

Short title: Inhaled glucocorticoids and altered metabolome

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SUPPLEMENTARY METHODS

Metabolomic profiling was performed as described previously (Ford et al., 2020). An overview of the process is provided below.

Sample Preparation

Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes. Samples (100 μL) were extracted with methanol (500 μL) under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) to precipitate the protein and dissociate small molecules bound to the protein or trapped in the precipitated protein matrix, followed by centrifugation (10 minutes at 680g) to recover chemically diverse metabolites. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods using positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS using negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS using negative ion mode ESI, and one reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

Quality Assurance/Quality Control

The following mitigations were used to ensure quality assurance (QA) and quality control (QC) between samples: 1) technical replicate samples derived from a pool of well-characterized human plasma (MTRX) or, alternatively, generated by combining a small portion of each (non-plasma) experimental sample (CMTRX), were spaced evenly among experimental samples; 2) extracted water samples (process blanks) and solvent blanks; and 3) a cocktail of QC standards, were carefully chosen so as not to interfere with the measurement of endogenous compounds, and were spiked into every analyzed sample, allowing instrument performance monitoring and aiding with chromatographic alignment.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible with each of the four methods. Each reconstitution solvent contained a series of standards (isotopically labeled or halogenated compounds; see Supplementary Table 1) at fixed concentrations to ensure injection and chromatographic consistency, and for alignment during data processing. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm) using a gradient consisting of water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). A second aliquot was also analyzed using acidic positive ion conditions, and was chromatographically optimized for more hydrophobic compounds. In this method, the extract was eluted over a gradient of methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA, and was operated using an overall higher organic content. A third aliquot was analyzed using negative ionization optimized conditions using a separate dedicated C18 column. The basic extracts were eluted using a gradient of methanol and water with 6.5 mM ammonium bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a
HILIC column (Waters Acquity UPLC BEH Amide 2.1 x 150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, overall pH 10.8 with ammonium hydroxide. The MS analysis alternated between MS and data-dependent MS scans using dynamic exclusion. The scan range varies slightly between methods, but covers approximately 70-1000 m/z. Raw data files are archived and extracted as described below.

**Reference**

Ford L, Kennedy AD, Goodman KD, Pappan KL, Evans AM, Miller LAD, Wulff JE, Wiggs BR, Lennon JJ, Elsea S, Toal DR. Precision of a Clinical Metabolomics Profiling Platform for Use in the Identification of Inborn Errors of Metabolism. *J Appl Lab Med.* **2020** Mar 1;5(2):342-356.

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**Supplemental Table 1. Chromatography Conditions**

| Chromatographic Conditions                                      | LC/MS Positive (positive ionization chromatography optimized for hydrophilic compounds) | LC/MS Positive (positive ionization chromatography optimized for hydrophobic compounds) | LC/MS Negative (negative ionization optimized conditions) | LC/MS Negative (negative ionization with HILIC chromatography) |
|----------------------------------------------------------------|--------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|----------------------------------------------------------|------------------------------------------------------------|
| Column                                                         | Waters BEH C18 1.7µm 2.1 x 100mm                                                      | Waters BEH C18 1.7µm 2.1 x 100mm                                                      | Waters BEH C18 1.7µm 2.1 x 100mm                          | Waters BEH C18 1.7µm 2.1 x 150mm                           |
| Mobile Phase A                                                | 0.1% formic acid and 0.05% PFPA in water, pH ~2.5                                   | 0.1% formic acid and 0.05% PFPA in water, pH ~2.5                                    | 6.5 mM ammonium bicarbonate in water, pH 8               | 10 mM ammonium formate in 15% water/5% methanol/80% acetonitrile (effective pH 10.16 with NH₄OH) |
| Mobile Phase B                                                | 0.1% formic acid and 0.05% PFPA in methanol, pH ~2.5                                | 0.1% formic acid and 0.05% PFPA in 50% methanol/50% acetonitrile, pH ~2.5           | 6.5 mM ammonium bicarbonate in 95% methanol/5% water    | 10 mM ammonium formate in 50% water/50% acetonitrile (effective pH 10.60 with NH₄OH) |
| Flow Rate                                                     | 0.35 mL/min                                                                          | 0.60 mL/min                                                                            | 0.35 mL/min                                              | 0.50 mL/min                                               |
| Gradient Elution                                              | Linear gradient from 5% B to 80% B over 3.35 minutes                                 | Linear gradient from 40% B to 99.5% B over 1.0 minute, hold 99.5% B for 2.4 minutes. | Linear gradient from 0.5 to 70% B over 4.0 minutes, then rapid gradient to 99% B in 0.5 minutes. | Linear gradient from 5% B to 50% B in 3.5 minutes, then linear gradient from 50% B to 95% B in 2 minutes. |
| Instrument Performance Standards                              | d7-glucose                                                                           | Br-phenylalanine                                                                      | d7-glucose                                               | d35-octadecanoic acid                                     |
|                                                                | d5-glutamine                                                                         | d5-androstene                                                                         | d3-methionine                                            | d5-indole acetate                                         |
|                                                                | d2-threonine                                                                         | d9-progesterone                                                                       | d3-leucine                                               | Br-phenylalanine                                          |
|                                                                | d5-hippuric acid                                                                     | d4-dioctyphthalate                                                                    | d8-phenylalanine                                         | d5-tryptophan                                             |

**Notes:**
- PFPA: Perfluoro-1-butyl-acetic acid
- HILIC: Hydrophilic Interaction LC/MS
| d3-methionine | d5-tryptophan | d4-tyrosine |
|---------------|--------------|------------|
| d3-leucine    | Br-phenylalanine | d3-serine |
| Br-phenylalanine | d15-octanoic acid | d3-aspartic acid |
|               | d19-decanoic acid | d7-ornithine |
|               | d27-tetradecanoic acid | d4-lysine |
|               | d35-octadecanoic acid |         |
|               | d2-eicosanoic acid |         |

**Process Assessment Standards**
- fluorophenylglycine
- d6-cholesterol
- tridecanoic acid
- fluorophenylglycine
- chlorophenylalanine
- chlorophenylalanine
- chlorophenylalanine
- chlorophenylalanine

BEH, ethylene bridged hybrid; LC, liquid chromatography; MS, mass spectrometry; PFPA, perfluoropentanoic acid