

**METHIONINE OXIDATION BY MYELOPEROXIDASE ASSOCIATES  
WITH EARLY CYSTIC FIBROSIS AIRWAY DISEASE**

Joshua D. Chandler, Camilla Margaroli, Hamed Horati, Matthew B. Kilgore, Mieke Veltman, H. Ken Liu, Alexander J. Taurone, Limin Peng, Lokesh Guglani, Karan Uppal, Young-Mi Go, Harm A.W.M. Tiddens, Bob J. Scholte, Rabindra Tirouvanziam, Dean P. Jones, Hettie M. Janssens

**SUPPLEMENTARY INFORMATION**

- **List of Supplementary Data Files** (with description of contents)
- **Supplementary Methods**
- **Supplementary Figures 1-5**

## LIST OF SUPPLEMENTARY DATA FILES

*File accession:* Listed data files have been uploaded to the Dryad Digital Repository (datadryad.org) under digital object identifier (DOI) doi:10.5061/dryad.55m2m81. For direct access, use the following link: <https://doi.org/10.5061/dryad.55m2m81>

*SFile1\_RawFeatureTable.xlsx:* This is a table of the raw apLCMS-based peak intensity extraction of HRMS results, including blank (Water) and quality control (NIST, QSTD) samples. Total features detected, 11,188. “NIST” refers to the SRM 1950 reference standard. “QSTD” refers to an in-house prepared mixture of human plasma. All samples were injected in triplicate and the results have been median summarized from the triplicates. The first two columns pertain to the detected  $m/z$  value at a specific retention time, given in seconds. The remaining columns pertain to blanks, reference standards and samples. These columns are ordered left-to-right in the order of injection. For the samples, “\_A” or “\_B” designations pertain to shipment batches that took place between Erasmus and Emory. Three samples were ultimately excluded from analysis: one, 25, because of aberrant global sample composition, and two others, 09\_A and 15\_A, because aliquots were accidentally shipped and processed two independent times because the samples were blinded. For subsequent analyses, we arbitrarily kept 09\_B and 15\_B samples from the second batch and discarded their “\_A” counterparts, as the “\_B” versions were believed to have undergone fewer freeze-thaw cycles than “\_A” samples.

*SFile2\_HRMSResults.xlsx*: This is the results file of the untargeted analysis using an in-house R package (<https://github.com/kuppall2/xmsPANDA>) on raw HRMS data after removing features that did not meet data quality thresholds and dropping poor quality and redundant samples. QC materials were not analyzed in the untargeted test. Columns A-B pertain to observed  $m/z$  and retention time. Columns C-E pertain to accurate mass matches to the features (Annotation, Adduct, and ppm mass accuracy error; see Supplementary Methods for details). After accurate mass matching, spectral convolutions (i.e., isotopes and neutral losses) were designated by manual inspection of co-elution, intensity correlation and  $m/z$  shift. Retention times were taken into consideration for assignments (e.g., longer retention times generally correspond to hydrophilicity and spectral convolutions of adducts and isotopes should co-elute). Note, the [M+H]<sup>+</sup> ion of lidocaine (235.1804  $m/z$  by 127.6 sec; used as local anesthetic during bronchoscopy) held the maximum intensity value in each sample and was normalized to the same maximal value in each distribution, resulting in its removal during testing. Ppm error was calculated by METLIN except for spectral convolutions, which were calculated manually. Accurate mass annotations are only provided for those results that met significance criteria ( $p < 0.05$ ,  $q < 0.25$ ). Columns F-H indicate the raw  $p$ -value ("P.value"),  $q$ -value ("adjusted.p.value") and Pearson's  $r$  statistic ("Estimate\_var1"), respectively. Remaining columns show log<sub>2</sub>-transformed and quantile normalized HRMS intensities for each of the study samples. Missing values were imputed as "NA" to avoid skewing the correlation. The sample columns were automatically ordered from low-to-high PRAGMA-%Dis.

*SFile3\_ClinicalData.xlsx*: These data correspond to important clinical variables for the patient samples analyzed in this study.

*SFile4\_FollowUpStudiesData.xlsx*: Results used for follow-up studies with Spearman's correlations. MetO and methionine (Met) quantifications are based on reference calibration relative to the concentrations determined for SRM 1950 reference material. MPO measurements are based on the ELISA assay.

## SUPPLEMENTARY METHODS

### *Metabolomics*

Metabolites were extracted from BALF by 1:2 mixture with acetonitrile (ACN) plus a mixture of internal standards. Internal standards from Cambridge Isotope Labs were added to all samples at the following final concentrations: [ $^{13}\text{C}_6$ ]-D-glucose (5 mM), [ $^{15}\text{N}$ ]-indole (10  $\mu\text{M}$ ), [ $^{13}\text{C}_5$ ,  $^{15}\text{N}$ ]-L-methionine (25  $\mu\text{M}$ ), [2- $^{15}\text{N}$ ]-L-lysine dihydrochloride (150  $\mu\text{M}$ ), [ $^{15}\text{N}$ ]-choline chloride (8  $\mu\text{M}$ ), [ $^{13}\text{C}_5$ ]-L-glutamic acid (25  $\mu\text{M}$ ), [ $^{13}\text{C}_7$ ]-benzoic acid (10  $\mu\text{M}$ ), [ $^{15}\text{N}$ ]-L-tyrosine (50  $\mu\text{M}$ ), [ $^{15}\text{N}_2$ ]-uracil (0.1  $\mu\text{M}$ ), [3,3- $^{13}\text{C}_2$ ]-cystine (60  $\mu\text{M}$ ), and [trimethyl- $^{13}\text{C}_3$ ]-caffeine (10  $\mu\text{M}$ ). The extract supernatant was isolated after centrifugation at 16,100  $g \times 10$  min at 4 °C and transferred to autosampler vials. Reference standards extracted and analyzed in parallel were National Institute of Standards and Technology (NIST) standard reference material (SRM) 1950 plasma and a separate pooled plasma sample prepared for batch QC purposes. Vials of extract supernatant were maintained at 4 °C on the autosampler tray while awaiting injection. Ten  $\mu\text{l}$  of supernatant were injected into an Accucore HILIC 100 x 2.1 mm column (2.6  $\mu\text{m}$  particle size, 80 Å pore size) with a 10 x 2.1 mm matching pre-column, held at 30 °C with mobile phases of 100% water, 100% ACN and 2% aqueous formic acid. Starting conditions were 20% water/80% ACN held for 1.5 min at a flow rate of 350  $\mu\text{l}/\text{min}$ , followed by a linear gradient to 90% water/10% ACN over 4.5 min and a simultaneous flow rate gradient to 500  $\mu\text{l}/\text{min}$ . These conditions were held for 9 min prior to 5 min of re-equilibration at 20% water/80% ACN. Formic acid was maintained at 0.2% throughout. Column eluate was introduced to a HESI source held at +2.5 kV. Thermo Q Exactive High Field quadrupole scanning was set at 85-1275  $m/z$  and Orbitrap

resolution was set to 120,000 full width at half maximum. After feature alignment by apLCMS and data quality assessment by xMSanalyzer, a list of 11,188 *m/z*-by-retention time features was extracted. These were further selected for analysis based on thresholds for mean sample intensity ( $<10^6$ ), mean sample intensity relative to water blanks ( $<1.5$ -fold that of water), missing values ( $>20\%$  among samples), median technical replicate coefficient of variation ( $>20\%$ ) and retention time ( $<30$  seconds, corresponding to void volume). Two redundant samples and one with aberrant missing values were dropped (see '*SFile1\_RawFeatureTable.xlsx*'). The final list contained 1,798 *m/z*-by-retention time features for analysis. After completion of Pearson's correlation of each feature with PRAGMA-%Dis (see '*SFile2\_HRMSResults.xlsx*'), significant ( $p < 0.05$ ,  $q < 0.25$ ) features were annotated using METLIN (metlin.scripps.edu) with a  $\text{ppm} \leq 3$  mass accuracy threshold and searching for [M+H], [M+Na], [M+H-H<sub>2</sub>O], [M+K], [M+2H] and [M+2Na-H] adducts. Reference calibration of BALF samples by SRM 1950 to estimate absolute concentrations of MetO and Met was carried out by multiplying the ratio of sample intensities to the average SRM 1950 intensity by the observed concentrations in SRM 1950. I.e.,  $(\text{Intensity}_{\text{sample}} / \text{mean Intensity}_{\text{SRM 1950}}) * (\text{C}_{\text{SRM 1950}})$ .

For validation and quantification of methionine sulfoxide (MetO), an isotopically enriched standard of  $^{13}\text{C}_5, ^{15}\text{N}$ -L-MetO and  $^{13}\text{C}_5, ^{15}\text{N}$ -L-methionine (Met) was made by adding sodium hypochlorite dropwise to  $^{13}\text{C}_5, ^{15}\text{N}$ -L-Met in PBS pH 7.4 to a final ratio of 1:10. Resulting  $^{13}\text{C}_5, ^{15}\text{N}$ -L-MetO and  $^{13}\text{C}_5, ^{15}\text{N}$ -L-Met were measured as 0.294 and 1.613 mM (respectively) by calibrating with pure reference standards (Sigma-Aldrich). The

heavy-labeled standard was used to confirm co-elution and MS/MS in SRM 1950 and pooled BALF, and to quantify the compounds in SRM 1950 for reference calibration.

Heatmaps and chromatograms were visualized using R 3.3.3 ([www.R-project.org](http://www.R-project.org)) and chemical structures were generated in ChemDraw 16.0 (Perkin Elmer).

### *MPO Assay*

Samples were assayed at dilutions of 200-, 100- and 20-fold. Unless stated otherwise, dilutions were in assay buffer containing 1% bovine serum albumin and 0.025% Tween-20 in phosphate-buffered saline, pH 7.4 (PBS); wash steps were 3 x 10 minutes of 200 µl PBS with 0.05% Tween-20; reaction volumes were 50 µl; and temperature ambient. First, monoclonal rabbit anti-MPO (Abcam ab10165) diluted 1:800 in PBS was plated overnight on black, clear-bottomed high-binding microtiter plates (Greiner). Wells were blocked with 75 µl assay buffer for 2 hours prior to washing and addition of MPO standard (EMD) or samples for 1 hour at 37 °C. Wells were washed and activity was measured by the addition of 50 µM Amplex Red (Invitrogen), 20 µM hydrogen peroxide and 50 mM sodium bromide in 50 mM sodium phosphate buffer, pH 7.4 with final 0.1% DMSO. Fluorescence was measured at 544<sub>Ex</sub>/590<sub>Em</sub> at 3, 6, 9 and 12 minutes on SpectraMax iD3 instrumentation (medium photomultiplier tube gain, 1.0 mm read height from top). Wells were washed, probed with 1:500 polyclonal rabbit anti-MPO (Abcam ab9535) at 37 °C for 1 hour, washed again, probed with 1:5000 biotin-conjugated goat anti-rabbit IgG (Agilent-Dako E034201-6) for 1 hour with assay buffer containing 0.05% Tween-20, washed again, incubated with 1:1000 ExtrAvidin-alkaline phosphatase (Sigma) for 1 hour, washed a final time and developed with 1-Step para-

nitrophenyl phosphate solution (ThermoFisher). Absorbance at 405 nm was measured at 10, 20, 30 and 40 minutes. Four-parameter logistic (4PL) curve fits were used for sample calibration. For samples quantifiable within multiple dilutions, the result assayed closest to its 4PL inflection point was selected.

#### *MPO Western blot*

Protein from pooled BALF, lysed human neutrophils from two healthy adults and human MPO standard (EMD) were reduced with 50 mM beta-mercaptoethanol at 80 °C for 15 minutes in 1X final concentration of Laemmli buffer (Bio-Rad). We then loaded 1.7 µg of protein or 1-100 ng of pure MPO per well on a 15-well 4-20% Mini-PROTEAN TGX gel (Bio-Rad). Proteins were transferred to a polyvinylidene difluoride membrane using a Trans-Blot turbo set to 'Mixed' size range optimization. MPO was developed using 1:1,000 polyclonal rabbit anti-MPO (Abcam ab9535) followed by 1:10,000 goat anti-rabbit-conjugated IRDye 680RD (Li-Cor 925-68071). The membrane was imaged using a 3.0 intensity level.

#### *Total protein measurement*

Neat CF BALF was assayed to measure total soluble protein in triplicate using the BCA Protein Assay Kit (Pierce). Bovine serum albumin diluted in 0.9% saline with 0.05% NaN<sub>3</sub> (Pierce) was used as the reference standard from 31.25 to 2,000 µg/ml.