Supplementary Materials

Title: Metabolomic fingerprinting and systemic inflammatory profiling of asthma COPD overlap (ACO)

Authors: Nilanjana Ghosh\textsuperscript{a}, Priyanka Choudhury\textsuperscript{a}, Sandeep Rai Kaushik\textsuperscript{b}, Rakesh Arya\textsuperscript{b}, Ranjan Nanda\textsuperscript{b}, Parthasarathi Bhattacharyya\textsuperscript{c}, Sushmita Roychowdhury\textsuperscript{d}, Rintu Banerjee\textsuperscript{e}, Koel Chaudhury\textsuperscript{a}\textsuperscript{*}

Affiliations:

\textsuperscript{a}School of Medical Science and Technology, Indian Institute of Technology Kharagpur (India)
\textsuperscript{b}Translational Health Group, International Centre for Genetic Engineering and Biotechnology New Delhi (India)
\textsuperscript{c}Institute of Pulmocare and Research Kolkata (India)
\textsuperscript{d}Apollo Gleneagles Hospital Kolkata (India)
\textsuperscript{e}Department of Agricultural and Food Engineering, Indian Institute of Technology Kharagpur (India)

Corresponding author: Dr. Koel Chaudhury, Ph.D

Professor, School of Medical Science & Technology (SMST)
Indian Institute of Technology Kharagpur
Kharagpur - 721302, India

Email for all correspondence: koel@smst.iitkgp.ac.in; koeliitkgp@gmail.com
Ph: +913222-283572
Materials and Methods

GC-MS Data Acquisition

Two μl of derivatized serum sample was loaded using splitless mode to RTx-5 column (5% diphenyl, 95% dimethylpolysiloxane; 30 m × 0.25 mm ×0.25 μm; Restek USA) in a GC–MS (7890A GC, 5975 MSD from Agilent Technologies, USA) for separation using an automatic liquid sampler (7683B ALS, Agilent, USA). Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The front inlet temperature was fixed at 250 °C during injection; temperature gradients of 50 to 150 °C (ramp of 10 °C/min) and 150 to 310 °C (ramp of 7 °C/min) with a hold time of 3 min between two ramps and after reaching final temperature were used. Electron ionization (EI) mode was fixed at −70 eV with a scan range of 35 to 600 m/z. Maximum scan speed was 5 spectra/sec with a 6 min solvent delay. The ion-source temperature and quadrupole temperatures were fixed at 230 and 150 °C, respectively. Sample introduction to data acquisition parameters (both GC separation and mass spectrometry) were controlled using ChemStation software (Agilent Technologies, USA), and the run time was 38.43 minutes per sample. Instrument performance over time and metabolite extraction efficiency were evaluated using peak area and retention time of internal standard in samples and quality controls (QCs). Further, as quality check, a mixture of metabolite standards at a known concentration (25 ng/10 μl) was injected after every 8 samples.

Data Pre-processing

Before analysis, the sample codes were opened by a team member not participating in sample processing and GC–MS data acquisition. GC-MS metabolite profiles were processed using Agilent Chemstation data analysis software. Chromatographic processing such as integration and convolution was performed in Agilent ChemStation software using automatic spectral deconvolution (AMDIS) algorithm. The detectable spectral features after background subtraction were annotated using NIST 14 standard mass spectral database (NIST, Gaithersburg, MD). Consistent metabolites with minimum 30% base peak intensity were considered for quantitation. Features with database matching percentage above 80% were only considered for further analysis. After chromatographic integration, peak areas of corresponding metabolites were noted. The peak areas of annotated features were extracted as a data matrix in .CSV file format.

Metabolomic data analysis using multivariate and univariate statistical analysis

Initially, the peak area metabolomic dataset generated post pre-processing was filtered. Features with >50% missing values were removed from the data. The resulting data were
further normalised to constant sum, log transformed and mean scaled using Metaboanalyst 4.0. These data pre-processing strategies such as transformation and scaling help in making features comparable. This normalised data was further subjected to univariate (UVA) and multivariate statistical analysis (MVA).

In order to reveal the pattern and draw conclusion from the metabolomics study, the normalised data files were independently subjected to MVA using SIMCA 13.0.1 software (Umetrics, Sweden) [1]. At first, principal component analysis (PCA) (an unsupervised multivariate statistical approach) was used to generate an overview of data distribution across samples and detect possible outliers. PCA was also performed on the QC samples to ensure the data quality was not compromised. Subsequently, supervised multivariate statistical tools i.e. partial least squares discriminant analysis (PLS-DA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were applied to enhance group separation. Parameters including R2 (goodness of the fit), Q2 (predictive ability), and analysis of variance testing of cross validated predictive residuals (CV-ANOVA) score were used to detect robustness of the OPLS-DA model [2,3]. The model robustness was evaluated by performing permutation of the model and comparing it with 200 randomly permutated models. Significant metabolites for group separation in OPLS-DA model were identified using variable importance in the projection (VIP) score. Metabolites with VIP score above 1.3 were considered relevant for group discrimination.

In addition to MVA, UVA was performed to assess statistically significant differences in the expression levels of these metabolites. Statistical significance of the metabolites between the groups was obtained using one way analysis of variance (ANOVA) (Dunnett’s post hoc test) or Kruskal–Wallis test (Dunn’s post hoc test) (GraphPad Prism version 7.00 for Windows, GraphPad Software, San Diego, CA, USA). Statistical significance was considered to be p ≤0.05. The metabolites were also adjusted for multiple hypothesis testing using FDR correction using Metaboanalyst 4.0. Fold change (FC) was calculated for ACO vs asthma and ACO vs COPD. Metabolites common to both ACO vs COPD and ACO vs asthma which collectively qualify the criteria of VIP, p-value, and FDR were considered significant. These significantly altered metabolites identified in the discovery phase were further validated in a fresh cohort of subjects by performing quantitative UVA post data matrix generation.
**Supplementary Fig. 1.** Representative GC/MS chromatogram of serum derived from an ACO patient

**Supplementary Fig. 2.** Partial least squares discriminant analysis (PLS-DA) is a supervised multivariate method for assessing relationship between a descriptor matrix X and a response matrix Y. It explains differences between overall class properties. PLS-DA showing optimized discrimination between obstructive lung diseases and healthy controls (R2Y=0.954 and Q2=0.923, CV-ANOVA=0)
Supplementary Fig. 3. Response permutation test (n=200) to estimate the statistical significance of the partial least squares discriminant analysis (PLS-DA) model. The R2 and Q2 values on the extreme right-hand side of the plot are of the true model, whereas the permuted model parameters are represented on the left-hand side of the plot. The correlation coefficients between true and permuted models represent the X axis. The true class has a correlation of 1.0 with itself. The true model parameters in the validation test exhibited higher values than those of the permuted models which suggests that the generated model can satisfactorily predict ACO better than chance. (a) Healthy controls vs. diseases R2=(0.0,0.349), Q2=(0.0,-0.324) (b) ACO vs. Asthma R2=(0.0,0.515), Q2=(0.0,-0.277) (c) ACO vs. COPD R2=(0.0,0.514), Q2=(0.0,-0.273) (d) ACO, Asthma and COPD R2=(0.0,0.388), Q2=(0.0,-0.439)
Supplementary Fig. 4. Partial least squares discriminant analysis (PLS-DA) is a supervised multivariate method for assessing relationship between a descriptor matrix X and a response matrix Y. It explains differences between overall class properties. PLS-DA showing optimized discrimination between asthma, COPD and ACO (R²𝑌= 0.915 and Q²=0.862, CV-ANOVA=0).

Supplementary Fig. 5. A variable importance in projection (VIP) plot for the orthogonal projections to latent structures discriminant analysis (OPLS-DA) displays the contribution of each metabolite to the models. Features having VIP cut off values >1.30 were identified as the major contributory metabolites responsible for discrimination between (a) ACO and Asthma (b) ACO and COPD.
Supplementary Fig. 6. Metabolic pathway analysis (MetPA) of ACO patients. MetPA generated pathway impact score indicates the potential pathways altered in (A) ACO vs. asthma (B) ACO vs. COPD. Degree of importance is represented by node size and colour. Pathway impact represents the potentially altered metabolic pathways. Unaltered pathways have score 0. The color of the circle (yellow to red) is based on the p-value and the radius is defined by the pathway impact values. The highest level of changes in the disease condition is indicated by large red node.

Supplementary Table 1. Pathway analysis using MetPA shows potential pathways involved in ACO vs. Asthma. The table shows the total number of metabolites involved in a pathway, hits, p-values, Holm-adjusted p-values, FDR and impact of the respective pathways.

| Pathway                                         | Total Cmpd | Hits | Raw p   | -log(p)   | Holm adjust | FDR      | Impact  |
|-------------------------------------------------|------------|------|---------|-----------|-------------|----------|---------|
| Fructose and mannose metabolism                 | 20         | 1    | 1.88E-11| 24.699    | 7.88E-10    | 3.94E-10 | 0       |
| Amino sugar and nucleotide sugar metabolism     | 37         | 1    | 1.88E-11| 24.699    | 7.88E-10    | 3.94E-10 | 0       |
| alpha-Linolenic acid metabolism                 | 13         | 1    | 7.00E-06| 11.87     | 0.00028     | 9.80E-05 | 0.3333  |
| Arachidonic acid metabolism                     | 36         | 1    | 2.16E-05| 10.745    | 0.000841    | 0.000226 | 0.3135  |
| Neomycin, kanamycin and gentamicin biosynthesis | 2          | 1    | 2.74E-05| 10.506    | 0.00104     | 0.00023  | 0       |
| Alanine, aspartate and glutamate metabolism     | 28         | 2    | 3.95E-05| 10.14     | 0.001461    | 0.000276 | 0       |
| Citrate cycle (TCA cycle)                       | 20         | 1    | 6.64E-05| 9.6202    | 0.002389    | 0.000307 | 0.03273 |
| Arginine biosynthesis                           | 14         | 1    | 7.32E-05| 9.5227    | 0.002561    | 0.000307 | 0       |
| Purine metabolism                               | 65         | 1    | 7.32E-05| 9.5227    | 0.002561    | 0.000307 | 0       |
| Cysteine and methionine metabolism              | 33         | 1    | 8.05E-05| 9.4273    | 0.002656    | 0.000307 | 0.02184 |
| Sphingolipid metabolism                         | 21         | 1    | 8.05E-05| 9.4273    | 0.002656    | 0.000307 | 0       |
| Glycerophospholipid metabolism                  | 36         | 1    | 9.98E-05| 9.212     | 0.003095    | 0.000349 | 0.01324 |
| Aminoacyl-tRNA biosynthesis                     | 48         | 8    | 0.000446| 7.7152    | 0.013381    | 0.001296 | 0.16667 |
| Glycine, serine and threonine metabolism        | 33         | 4    | 0.000461| 7.6824    | 0.013381    | 0.001296 | 0.48704 |
Supplementary Table 2. Pathway analysis using MetPA shows potential pathways involved in ACO vs. COPD. The table shows the total number of metabolites involved in a pathway, hits, p-values, Holm-adjusted p-values, FDR and impact of the respective pathways.

| Pathway                                                                 | Total Cmpd | Hits | Raw p       | Holm adjusted p | FDR      | Impact     |
|------------------------------------------------------------------------|------------|------|-------------|-----------------|----------|------------|
| Valine, leucine and isoleucine degradation                             | 40         | 4    | 3.08E-16    | 1.30E-14        | 0.02264  |
| Fructose and mannose metabolism                                        | 20         | 1    | 1.30E-06    | 5.33E-05        | 1.82E-05 | 0          |
| Amino sugar and nucleotide sugar metabolism                            | 37         | 1    | 1.30E-06    | 5.33E-05        | 1.82E-05 | 0          |
| Linoleic acid metabolism                                               | 5          | 1    | 2.33E-05    | 0.000907        | 0.000231 | 1          |
| Glyoxylate and dicarboxylate metabolism                                | 32         | 4    | 2.74E-05    | 0.001043        | 0.000231 | 0.30689   |
| Propanoate metabolism                                                  | 23         | 3    | 3.99E-05    | 0.001475        | 0.000279 | 0          |
| Pathway                                      | Rank | Value | Start  | End    | Start | End  | Start | End  | Start | End  |
|----------------------------------------------|------|-------|--------|--------|-------|------|-------|------|-------|------|
| Arginine biosynthesis                        | 14   | 1     | 8.96E-05 | 9.3199 | 0.003226 | 0.00471 | 0     |
| Purine metabolism                            | 65   | 1     | 8.96E-05 | 9.3199 | 0.003226 | 0.00471 | 0     |
| Glyceraldehyde / Gluconeogenesis              | 26   | 1     | 0.000304 | 8.0987 | 0.010333 | 0.001277 | 0    |
| Pyruvate metabolism                          | 22   | 1     | 0.000304 | 8.0987 | 0.010333 | 0.001277 | 0    |
| Neomycin, kanamycin and gentamicin biosynthesis | 2    | 1     | 0.000346 | 7.9693 | 0.01107  | 0.001321 | 0    |
| Porphyrin and chlorophyll metabolism         | 30   | 1     | 0.000787 | 7.1477 | 0.024388 | 0.002743 | 0    |
| Primary bile acid biosynthesis               | 46   | 2     | 0.000849 | 7.0714 | 0.025472 | 0.002743 | 0.05823 |
| Cysteine and methionine metabolism           | 33   | 1     | 0.001228 | 6.7023 | 0.035615 | 0.003439 | 0.02184 |
| Sphingolipid metabolism                      | 21   | 1     | 0.001228 | 6.7023 | 0.035615 | 0.003439 | 0    |
| Citrate cycle (TCA cycle)                    | 20   | 1     | 0.005571 | 5.1902 | 0.15041  | 0.014623 | 0.03273 |
| Steroid biosynthesis                         | 42   | 3     | 0.008848 | 4.7275 | 0.23005  | 0.02186  | 0.087 |
| Alanine, aspartate and glutamate metabolism  | 28   | 2     | 0.009964 | 4.6088 | 0.2491   | 0.023132 | 0    |
| Glycine, serine and threonine metabolism     | 33   | 4     | 0.011458 | 4.4691 | 0.27499  | 0.023132 | 0.48704 |
| Valine, leucine and isoleucine biosynthesis  | 8    | 4     | 0.011553 | 4.4608 | 0.27499  | 0.023132 | 0    |
| Aminocetyl-tRNA biosynthesis                 | 48   | 8     | 0.011566 | 4.4597 | 0.27499  | 0.023132 | 0.16667 |
| Glycerophospholipid metabolism               | 36   | 1     | 0.017918 | 4.022  | 0.37628  | 0.034207 | 0.01324 |
| Arginine and proline metabolism              | 38   | 1     | 0.083341 | 2.4848 | 1        | 0.15219  | 0.0778 |
| Inositol phosphate metabolism                | 30   | 1     | 0.1265   | 2.0675 | 1        | 0.20435  | 0.12939 |
| Phosphatidylinositol signaling system        | 28   | 1     | 0.1265   | 2.0675 | 1        | 0.20435  | 0.03736 |
| Ascorbate and aldurate metabolism            | 8    | 1     | 0.1265   | 2.0675 | 1        | 0.20435  | 0    |
| alpha-Linolenic acid metabolism              | 13   | 1     | 0.21509  | 1.5367 | 1        | 0.32718  | 0.33333 |
| Fatty acid biosynthesis                      | 47   | 2     | 0.23369  | 1.4537 | 1        | 0.32718  | 0.01473 |
| Fatty acid elongation                        | 39   | 1     | 0.2337   | 1.4537 | 1        | 0.32718  | 0    |
| Fatty acid degradation                       | 39   | 1     | 0.2337   | 1.4537 | 1        | 0.32718  | 0    |
| Biosynthesis of unsaturated fatty acids      | 36   | 6     | 0.24336  | 1.4132 | 1        | 0.32972  | 0    |
| Pentose phosphate pathway                    | 22   | 2     | 0.27644  | 1.2858 | 1        | 0.36282  | 0.04712 |
| Galactose metabolism                         | 27   | 4     | 0.29421  | 1.2234 | 1        | 0.36475  | 0.07387 |
| Starch and sucrose metabolism                | 18   | 2     | 0.29528  | 1.2198 | 1        | 0.36475  | 0.47093 |
| Butanone metabolism                          | 15   | 2     | 0.4916   | 0.7101 | 1        | 0.58992  | 0    |
| Glutathione metabolism                       | 28   | 2     | 0.55905  | 0.58152| 1        | 0.64768  | 0.09582 |
| Arachidonic acid metabolism                  | 36   | 1     | 0.57057  | 0.56111| 1        | 0.64768  | 0.3135 |
| Synthesis and degradation of ketone bodies   | 5    | 1     | 0.6028   | 0.50618| 1        | 0.66625  | 0    |
| Glycerolipid metabolism                      | 16   | 1     | 0.76231  | 0.2714 | 1        | 0.82095  | 0.09346 |
| Steroid hormone biosynthesis                 | 85   | 1     | 0.78623  | 0.24051| 1        | 0.82554  | 0.00528 |
| Pantothenate and CoA biosynthesis            | 19   | 1     | 0.83508  | 0.18022| 1        | 0.85545  | 0    |
| Selenocompound biosynthesis                  | 20   | 1     | 0.96683  | 0.033738| 1        | 0.96683  | 0    |
**Supplementary Fig. 7.** Metabolite set enrichment analysis (MSEA) tool of Metaboanalyst shows the most altered metabolic pathways in the serum of ACO patients as compared to (a) asthma and (b) COPD. Pathways are shown in order of decreasing statistical significance from top to bottom (red to yellow) with length of the bars indicating their estimated fold enrichment.

**Supplementary Table 3.** Data related to the quantitative enrichment analysis (QEA) using metabolite set enrichment analysis (MSEA) tool. Potential pathways involved in ACO vs. asthma shows total compounds, hits, p values and false discovery rates (FDR).

| Pathway                              | Total Cmpd | Hits | Statistic Q | Expected Q | Raw p     | Holm p    | FDR       |
|--------------------------------------|------------|------|-------------|------------|-----------|-----------|-----------|
| Amino sugar and nucleotide sugar metabolism | 37         | 1    | 49.228      | 1.4706     | 1.88E-11  | 5.44E-10  | 2.75E-10  |
| Fructose and mannose metabolism      | 20         | 2    | 48.349      | 1.4706     | 1.90E-11  | 5.44E-10  | 2.75E-10  |
| Neomycin, kanamycin and gentamicin biosynthesis | 2          | 1    | 23.24       | 1.4706     | 2.74E-05  | 0.000739  | 0.000265  |
| Steroid biosynthesis                 | 42         | 2    | 21.664      | 1.4706     | 5.50E-05  | 0.001431  | 0.000292  |
| Arginine biosynthesis                | 14         | 1    | 21.058      | 1.4706     | 7.32E-05  | 0.001829  | 0.000292  |
| Purine metabolism                    | 65         | 1    | 21.058      | 1.4706     | 7.32E-05  | 0.001829  | 0.000292  |
| Cysteine and methionine metabolism   | 33         | 1    | 20.843      | 1.4706     | 8.05E-05  | 0.001851  | 0.000292  |
| Sphingolipid metabolism              | 21         | 1    | 20.843      | 1.4706     | 8.05E-05  | 0.001851  | 0.000292  |
| Glycerophospholipid metabolism       | 36         | 1    | 20.358      | 1.4706     | 9.98E-05  | 0.002097  | 0.000322  |
| Aminoacyl-tRNA biosynthesis          | 48         | 8    | 16.457      | 1.4706     | 0.000446  | 0.00892   | 0.001118  |
| Glycine, serine and threonine metabolism | 33      | 3    | 16.829      | 1.4706     | 0.000461  | 0.00892   | 0.001118  |
| Valine, leucine and isoleucine       | 8          | 4    | 16.754      | 1.4706     | 0.000463  | 0.00892   | 0.001118  |
Table 4. Data related to the quantitative enrichment analysis (QEA) using metabolite set enrichment analysis (MSEA) tool. Potential pathways involved in ACO vs. COPD shows total compounds, hits, p values and false discovery rates (FDR).

| Metabolite Pathway                                         | Total Cmpd | Hits | Statistic Q | Expected Q | Raw p       | Holm p       | FDR         |
|-----------------------------------------------------------|------------|------|-------------|------------|-------------|-------------|-------------|
| Fructose and mannose metabolism                           | 20         | 2    | 32.195      | 1.5625     | 3.19E-07    | 9.25E-06    | 9.25E-06    |
| Amino sugar and nucleotide sugar metabolism               | 37         | 1    | 31.249      | 1.5625     | 1.30E-06    | 3.64E-05    | 1.88E-05    |
| Glyoxylate and dicarboxylate metabolism                   | 32         | 3    | 24.52       | 1.5625     | 2.74E-05    | 0.000741    | 0.000265    |
| Arginine biosynthesis                                     | 14         | 1    | 21.765      | 1.5625     | 8.96E-05    | 0.00233     | 0.00052     |
| Purine metabolism                                         | 65         | 1    | 21.765      | 1.5625     | 8.96E-05    | 0.00233     | 0.00052     |
| Glycolysis / Gluconeogenesis                               | 26         | 1    | 18.836      | 1.5625     | 0.000304    | 0.007294    | 0.001254    |
| Pyruvate metabolism                                       | 22         | 1    | 18.836      | 1.5625     | 0.000304    | 0.007294    | 0.001254    |
| Neomycin, kanamycin and gentamicin biosynthesis            | 2          | 1    | 18.521      | 1.5625     | 0.000346    | 0.007611    | 0.001254    |
| Glutathione metabolism                                    | 28         | 1    | 16.501      | 1.5625     | 0.000787    | 0.016521    | 0.002239    |
| Porphyrin and chlorophyll metabolism                      | 30         | 1    | 16.501      | 1.5625     | 0.000787    | 0.016521    | 0.002239    |
| Primary bile acid biosynthesis                            | 46         | 2    | 15.015      | 1.5625     | 0.000849    | 0.016521    | 0.002239    |
| Cysteine and methionine metabolism                        | 33         | 1    | 15.392      | 1.5625     | 0.001228    | 0.022106    | 0.00274     |
| Sphingolipid metabolism                                   | 21         | 1    | 15.392      | 1.5625     | 0.001228    | 0.022106    | 0.00274     |
| Glycine, serine and threonine metabolism                  | 33         | 3    | 9.7149      | 1.5625     | 0.011457    | 0.18331     | 0.020963    |
Valine, leucine and isoleucine biosynthesis

Aminoacyl-tRNA biosynthesis

Steroid biosynthesis

Glycerophospholipid metabolism

Arginine and proline metabolism

Ascorbate and aldarate metabolism

Inositol phosphate metabolism

Phosphatidylinositol signaling system

Galactose metabolism

Starch and sucrose metabolism

Steroid hormone biosynthesis

Pantothenate and CoA biosynthesis

Alanine, aspartate and glutamate metabolism

Selenocompound metabolism

Valine, leucine and isoleucine degradation

Supplementary Table 5. Comparisons of serum immunological mediator levels among asthma, COPD ACO and healthy controls. The levels were measured using Magnetic Luminex Assay-Human Premixed Multi-Analyte Kit based on the Luminex xMAP technology. All values are expressed as mean ± standard deviation (SD). One-way ANOVA (Dunnett’s post hoc test) or Kruskal–Wallis test (Dunn’s post hoc test) was conducted for pairwise comparison.
|        | IL-2       | 199.8±58.17 | 326.3 ±49.24 | 188.3 ±67.08 | 111 ±51.01 | *** | ns | * |
|--------|------------|-------------|--------------|--------------|------------|-----|----|---|
| IL-4   | 51.35±2.049| 53.71 ±5.999| 46.85 ±8.022 | 52.2 ±1.875 | ns         | *   | ns |   |
| IL-13  | 3327±465.8 | 3593 ±595.8 | 2255 ±808.7  | 2107 ±501.7 | ns         | **  | ***|   |
| IL-1α  | 102.4±26.12| 118.4 ±9.093| 158 ±11.67   | 79.11 ±5.749| ns         | ****| ** |   |
| IL-21  | 206.6±11.85| 179 ±18.38  | 191.6 ±26.38 | 176.2 ±23.66| *          | ns  | *  |   |
| IL-23  | 1595±428.2 | 895.5 ±327.3| 1307 ±441.8  | 661.6 ±165.5| **         | ns  | ****|   |
| Periostin | 599.3±133.2| 611.8 ±136.6| 381.6 ±114.8 | 606.8 ±104.9| ns         | **  | ns |   |
| TSLP   | 23.89±1.726| 34.38 ±7.033| 20.48 ±3.037 | 18.83 ±2.787| ****       | ns  | *  |   |
| IL-8   | 270.5±81.69| 172.3 ±51.45| 256.7 ±58.65 | 106.7 ±17.57| **         | ns  | ****|   |
| Eotaxin| 1392±383.4 | 1440 ±241.5 | 993.2 ±209.2 | 918.2 ±381.3| ns         | *   | *  |   |

ns-not significant; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.0001

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