Analysis of the mouse brain metabolome following the disruption of the gut-brain axis

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Overview

- Disruption of the gut-brain axis through bacterial infection and treatment with antibiotic was analysed using reverse phase LC-MS/MS analysis of mouse brain tissue extracts.
- A number of metabolites were found to be statistically significantly different between control and treated groups. An in-house accurate mass MS/MS library was used to help identify significant metabolites.
- To increase confidence in identification of early eluting compounds by reverse phase, an additional LCMS/MS method using HILIC separation was employed.

1. Introduction

The 'gut-brain axis' is considered to be a bi-directional communication pathway between gut microbiota and the brain by means of neural, endocrine, immune, and humoral links. In this study the gut-brain axis was disrupted by changing the gut microbiome using a bacterial pathogen (*Clostridioides difficile* infection; CDI) treated with an antibiotic (metronidazole, an antibiotic that can transfer between the blood brain barrier and in common with other antibiotics known to have a negative effect on gut microbiome). The impact of changing the gut microbiome on the brain metabolome was determined using untargeted metabolite phenotyping by high resolution LC-MS/MS.

2. Materials and Methods

Thirteen week old male C57BL/6 mice were exposed to a mixture of antibiotics (kanamycin, gentamicin, colistin, metronidazole, and vancomycin) for 4 days. One day later, they were given injections of clindamycin and then challenged 1 day later with a bacterial pathogen (*Clostridioides difficile* non-toxinogenic (TCDA-, TCDB-)). Three days after infection, the mice received the antibiotic treatment (metronizadole 500mg/ 100mL) daily in drinking water for a further 10 days. Tissues were weighed and extracted in methanol:isopropanol:water 1:1:2 v/v/v in a ratio of tissue:solvent 3:1. Following sonication, homogenisation and centrifugation, supernatants were collected, and pellets were re-extracted in methanol:MTBE 1:3 v/v. All supernatants were evaporated to dryness.

Reverse Phase LC Separation (cycle time 35 mins)

- Samples reconstituted in methanol; pooled QC analysed throughout batch
- Acquity C18 BEH (2.1x100mm 1.7µm); 50ºC, flow rate 0.4 mL/min
- Binary gradient: water + 0.1% formic acid; acetonitrile + 0.1% formic acid

HILIC LC Separation (cycle time 18 mins)

- Samples reconstituted in 90% acetonitrile analysed to support metabolite ID
- Shim-pack Velox HILIC (2.1x100mm 2.7µm); 40ºC, flow rate 0.3 mL/min
- Binary gradient; water + 10mM ammonium formate 0.1% formic acid, and acetonitrile:water+ 10mM ammonium formate 0.1% formic acid [92:8]

Mass Spectrometry Detection QTOF LCMS-9030; external mass calibration; ESI+/-

- MS mass scan m/z 65-1010; 100 msecs
- DIA-MS/MS mass scans m/z 40-1000; 33 msecs for each precursor isolation window; isolation width 35 Da; collision energy spread 5-55V; 27 mass scan events. Scan cycle time 0.99 second (28 mass scans in total).

Data processing

- Untargeted processing used MS-Dial. MetaboAnalyst was used to find statistically significant features (ANOVA p<0.05) which were annotated to metabolomics standards initiative (MSI) levels 1-2 where possible.

3. Results

3.1 Untargeted differential analysis

- MS-Dial was used to detect and align chromatographic components in all samples.
- Ion signals present in less than 80% of QCs or at RSD>20% were filtered out as well as signals present in less than 1/3 of biological samples.
- Significant features detected were determined by ANOVA (p<0.05) using MetaboAnalyst.

Early elution feature extraction
Retention times for different metabolite classes
0-3 mins; nucleotides, amino acids, purines, pyrimidines and associated derivatives.
2-6 mins; bile acids
0-8 mins; carnitines

Reverse phase method developed primarily for lipid signals
Retention times for different metabolite classes
6-10 mins; LPLs
7-16 mins; Fatty acids and related compounds 8-12 mins; MGs
22 mins; cholesterol
22-30 mins; PLs, SLs and DGs

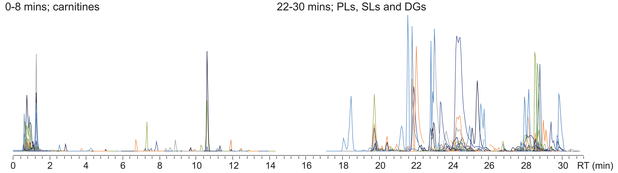

Figure 1. Mass chromatograms of ion signals found to be statistically different by ANOVA (p<0.05, FDR corrected) between groups of brain extracts (ESI+).

3.2 Metabolite identification

Significant features were analysed in LabSolutions Insight. MSI level 1 identifications were made by comparison to an in-house MS/MS library of authentic standards (Figure 2) and MSI level 2 annotations were made by comparison to online repositories such as MassBank, mzCloud and LipidMaps.

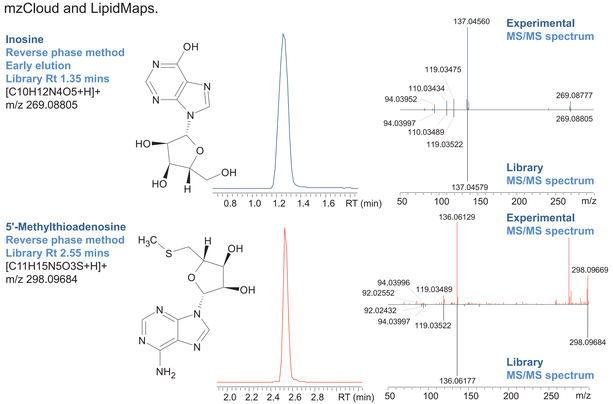

Figure 2. To increase reporting confidence particularly for early eluting features, MS/MS product ion spectra were matched against an in-house MS/MS data repository acquired with authentic standards.

3.3 Biological effects of CDI and metronidazole

Figure 3 highlights 20 of the most significant metabolites from the ANOVA analysis that could be identified to MSI level 1 or 2 in ESI+ and ESI- datasets. Log10(p) values ranged from 2-7 and all were significant at p<0.05 after FDR correction. Data curated using LabSolutions Insight.

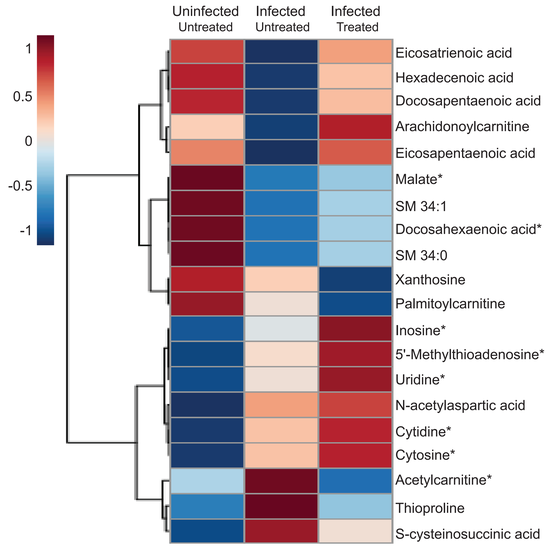

Figure 3. Heatmap created using MetaboAnalyst showing some of the most significant phenotypic differences in brain extracts from uninfected mice and mice infected with *Clostridium difficile* that were treated or not with metronidazole. Significance determined by ANOVA p<0.05 (FDR corrected). Metabolites marked with * are those identified to MSI level 1 by comparison to authentic standards.

Additive effects of CDI and metronidazole on disruption of the gut-brain axis

- Infection and treatment had an additive effect on increasing the relative concentrations of nucleosides including cytidine, cytosine, inosine, uridine and 5'-methylthioadenosine.
- Xanthosine and palmitoylcarnitine showed the reverse trend, which were diminished by infection and further diminished by metronidazole treatment.

Effects of CDI on gut-brain axis restored (fully or in part) by metronidazole treatment

- Free fatty acids, arachidonoylcarnitine, malate, SM 34:0 and SM 34:1 were diminished by infection and restored to some extent with metronidazole treatment.
- Acetylcarnitine , thioproline, N-acetylaspartic acid and S-cysteinosuccinic acid showed the opposite trend – infection caused these metabolites to increase while metronidazole lowered concentrations.

3.4 Increasing confidence in metabolite identification

- Reporting confidence can be markedly increased by comparing with product ion MS/MS spectra from an authentic reference material. For highly polar, low molecular weight metabolites which elute between 0-1 mins, reporting confidence was further enhanced by HILIC LC-MS/MS analysis (Figure 4).
- The HILIC LC-MS/MS method was optimized for highly polar metabolites and identification was confirmed by authentic reference material (product ion MS/MS spectra and retention time data acquired under HILIC conditions).

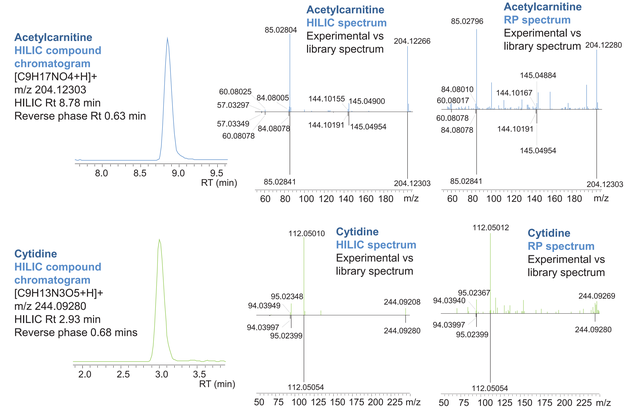

Figure 4. HILIC mass chromatograms for acetylcarnitine and cytidine as examples of highly polar metabolites eluting with retention times of 0.63 and 0.68 mins by reverse phase LC-MS/MS. Confidence in metabolite identification was further enhanced by a library search with authentic reference material acquired using the HILIC LC-MS/MS method.

4. Conclusions

- In this study, the gut microbiome was disrupted by infecting mice with the bacterial pathogen *Clostridioides difficile* treated with the antibiotic metronidazole. The effect of changing the gut microbiome on the brain metabolome was analysed by high resolution LC-MS/MS using an untargeted workflow.
- Infection and treatment had a significant effect on the relative concentrations of many metabolites including nucleosides, free fatty acids, carnitines and carboxylic acids
- Metabolites were identified to MSI level 1 by comparison to an in-house MS/MS library of authentic standards or to MSI level 2 by comparison to online repositories (MassBank, mzCloud, LipidMaps).
- To increase reporting confidence in highly polar compounds which elute very early in reverse phase analysis, a HILIC method was used to confirm identification and significance.