# Preanalytical Pitfalls in Untargeted Plasma Metabolomics of Endocrine Hypertension

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## Background

Despite considerable morbidity and mortality, numerous cases of endocrine hypertension (EHT), which includes primary aldosteronism (PA), pheochromocytoma and paraganglioma (PPGL), and Cushing's syndrome (CS), remain undetected. ENSAT-HT is a project which aims to establish a multi-omics screening method for EHT. We used untargeted Nuclear Magnetic Resonance (NMR) and Ultra High-Performance Liquid Chromatography – Quadrupole Time of Flight Mass Spectrometry (UHPLC-QTOF-MS) metabolomics to distinguish EHT from primary HT (PHT).

## AIM

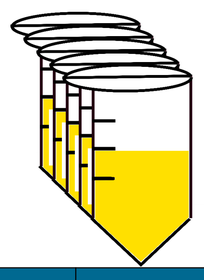
To identify biomarkers as screening tools for the different forms of EHT by analyzing ENSAT-HT plasma samples, and to investigate potentially confounding effects of various origin, using sample and patient metadata.

## Method #1: $^1$NMR

Spectroscopy: We recorded and processed spectra on our Bruker DRX AVANCE spectrometer operating at 500.13 MHz, according to our NMR method as reported and previously applied [1,2].

Data analysis: We employed

- PCA for investigating the strongest tendencies within the data
- PLSDA to separate groups defined by confounders
- Sparse PLSDA to separate disease groups



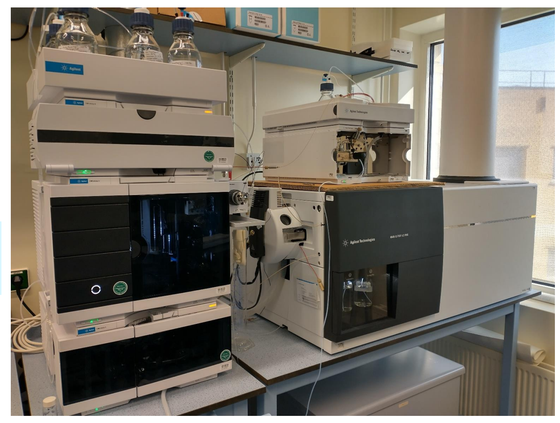
| GROUP | PLASMA NMR/QTOF |
|---|---|
| CS | 33/20 |
| PA | 104/65 |
| PHT (controls) | 106/66 |
| PPGL | 94/60 |

Samples were collected from biobanks across 13 centers, with patients sampled at different time points, resulting in significantly different sample ages amongst centers.

## Method #2: UHPLC-QTOF-MS

Samples were run on an Agilent QTOF 6545, according to a previously published method [3]. Samples were prepared according to a methanol precipitation protocol in 6 batches and were analyzed in an antiparallel fashion to account for drift.

Data Processing was largely the same as with NMR, including peak picking and batch correction.



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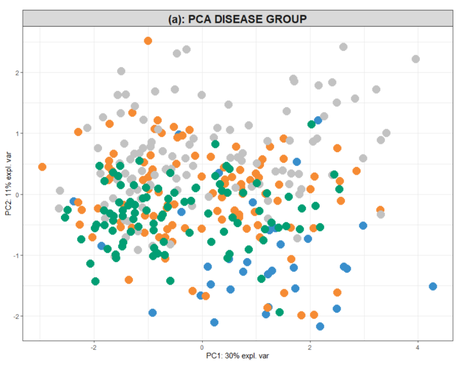


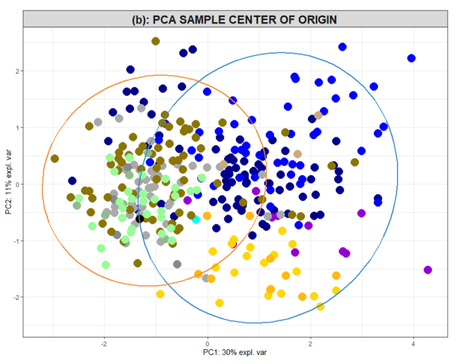
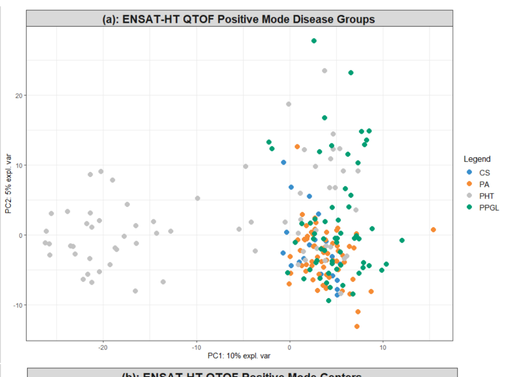




**Figure 1:** PCA score plots derived from the NMR dataset. In plot (a), samples were colored according to disease group (CS, PA, PHT or PPGL), whereas in plot (b), samples were colored according to the centers in which they were collected. Though a distinction is clear in plot (a) between EHT and PHT, samples were strikingly different from center to center, forming two main clusters, C1 (left) and C2 (right).

| Metabolite | NMR Peaks (ppm) | Dataset | Reason* | Center 1 PHT/ CLUSTER 2/ HIGH SAMPLE AGE |
|---|---|---|---|---|
| Acetylcarnitine | 3.177 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↓ |
| Creatine | 3.021, 3.917 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↑ |
| Dimethyl sulfone | 3.137 | PA-PHT, PPGL-PHT | PLSDA SAMPLE AGE | ↑ |
| Glucose | 5.220, 5.227 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↓ |
| Glutamate | 2.047, 2.060, 2.075, 2.095, 2.103, 2.108, 2.113, 2.122, 2.132, 2.140, 2.145, 2.325, 2.332, 2.341, 2.356 | PA-PHT, PPGL-PHT | Center 1 PHT, PLSDA CLUSTER, SAMPLE AGE | ↑ |
| Glutamine | 2.095, 2.103, 2.108, 2.113, 2.122, 2.132, 2.140, 2.145, 2.418, 2.428, 2.433, 2.444, 2.449, 2.460 | PA-PHT, PPGL-PHT | Center 1 PHT, PLSDA CLUSTER, SAMPLE AGE | ↓ |
| Glycerol | 3.555, 3.567 | PA-PHT | PLSDA CLUSTER, SAMPLE AGE | ↑ |
| Glycine | 3.548 | PA-PHT, PPGL-PHT | PLSDA SAMPLE AGE | ↓ |
| Lactate | 1.321, 1.307, 4.080, 4.094, 4.108, 4.121 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↑ |
| Methanol | 3.346 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↓ |
| Methionine | 2.122 | PA-PHT, PPGL-PHT | Center 1 PHT, PLSDA SAMPLE AGE | ↓ |
| Ornithine | 3.041, 3.057 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↑ |
| Pyruvate | 2.356 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↓ |
| Unknown metabolite | 3.284 | PA-PHT, PPGL-PHT | PLSDA CLUSTER, SAMPLE AGE | ↑ |

**Tables:** Features found to be related to confounders. All listed NMR metabolites (left) were also related to disease group discrimination, as were QTOF features (right) highlighted in **bold**.

| mzmed | rtmed | High in | Possibilities | CID | Level of Identification Rigor |
|---|---|---|---|---|---|
| **130.0863** | 3.271 | Center 3 | Furanone derivative. | - | 3 |
| **175.1155** | 5.874 | Center 10 | - | - | 4 |
| **195.0263** | 1.768 | Center 3 | - | - | 4 |
| **203.0289** | 3.165 | Center 4 | - | - | 4 |
| **205.0834** | 8.811 | Center 1 | Carboxylic acid with tropyllium ion. | - | 3 |
| **233.0784** | 4.321 | Center 4 | PEG | - | 2 |
| **246.1882** | 6.488 | Center 1 | Glu-Val/ gamma Glu-Val | - | 3 |
| **247.1286** | 3.512 | Center 1 | Leu-Leu | 76807 | 2 |
| **248.1317** | 3.513 | Center 1 | >> | >> | 2 |
| **259.1148** | 6.469 | Center 3 | - | - | 4 |
| **270.1993** | 11.584 | Center 4 | - | - | 4 |
| **288.2068** | 8.291 | Center 3 | - | - | 4 |
| **293.1437** | 8.344 | Center 4 | PEG | - | 2 |
| **293.2473** | 15.488 | Center 1 | - | - | 4 |
| **296.1523** | 8.345 | Center 4 | PEG | - | 2 |
| **302.0804** | 11.485 | Center 3 | - | - | 4 |
| **303.2315** | 15.194 | Center 1 | - | - | 4 |
| **305.1980** | 8.616 | Center 4 | PEG | - | 2 |
| **307.1741** | 8.617 | Center 4 | PEG | - | 2 |
| **310.2011** | 8.967 | Center 3 | - | - | 4 |
| **311.2041** | 9.384 | Center 3 | - | - | 4 |
| **317.2472** | 15.613 | Center 1 | - | - | 4 |
| **329.1873** | 8.864 | Center 4 | PEG | - | 2 |
| **333.1881** | 7.687 | Center 3 | - | - | 4 |
| **337.1704** | 8.862 | Center 4 | PEG | - | 2 |
| **357.2397** | 15.618 | Center 1 | - | - | 4 |
| **373.2325** | 14.554 | Center 1 | - | - | 4 |
| **373.2347** | 14.358 | Center 1 | - | - | 4 |
| **375.2139** | 13.363 | Center 1 | - | - | 4 |
| **383.2552** | 15.784 | Center 1 | - | - | 4 |
| **384.2049** | 9.286 | Center 4 | PEG | - | 2 |
| **389.1737** | 13.908 | Center 1 | - | - | 4 |
| **414.7625** | 9.662 | Center 4 | PEG | - | 2 |
| **448.2675** | 9.827 | Center 4 | PEG | - | 2 |
| **470.2803** | 9.979 | Center 4 | PEG | - | 2 |
| **536.3261** | 13.713 | Center 1 | - | - | 4 |
| **559.1506** | 3.074 | Center 3 | Inosine | 135398641 | 1 |



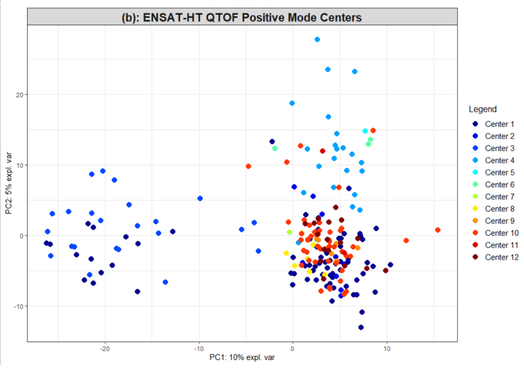




**Figure 2**: The PCA scores plots of the dataset collected from UHPLC-QTOF-MS in positive mode, colored by (a) disease group and (b) sample center of origin. Center 1 and Center 3 PHT samples form a separate cluster from all other study samples.

## Discussion

NMR: Cluster 2 plasma samples harvested from whole blood possibly after a precentrifugation delay in cold temperature [4], possible delay between plasma harvesting and storage at room temperature [4] for Center 1 PHT samples, methanol likely an impurity in Center 8 & 9 samples. Similar patterns to our sample age signature in literature after prolonged plasma storage at -80°C [5].

QTOF features identified: Leu-Leu - internal study links to freeze-thaw cycles, Inosine - precentrifugation delay [6], PEG - Polyethylene glycol ions reported in [7] found in Center 4 samples.

## Conclusion

Our study did not result in robust EHT biomarkers, due to the lack of adequate solutions and international consensus for containing the bias caused by preanalytical factors. This need should be covered by decisions on study design requirements for future multicenter metabolomics studies, with respect to future as well as published research findings on the effects of preanalytical conditions.

### References

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