Metabolic Signature of Ethanol-Induced Hepatotoxicity in HepaRG Cells by LC-MS-based Untargeted Metabolomics

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Introduction

- **Alcoholic liver disease** (ALD) is **highly prevalent but poorly identified** and characterized with lack of sensitive and specific early biomarkers for ALD
- Excessive alcohol consumption leads to progressive intracellular lipid accumulation resulting in **alcoholic fatty liver disease**
- As metabolic alterations are reflected in the phenotype and vice versa, metabolomics can help to identify **early-stage indicators** of alcoholic fatty liver disease

Objectives

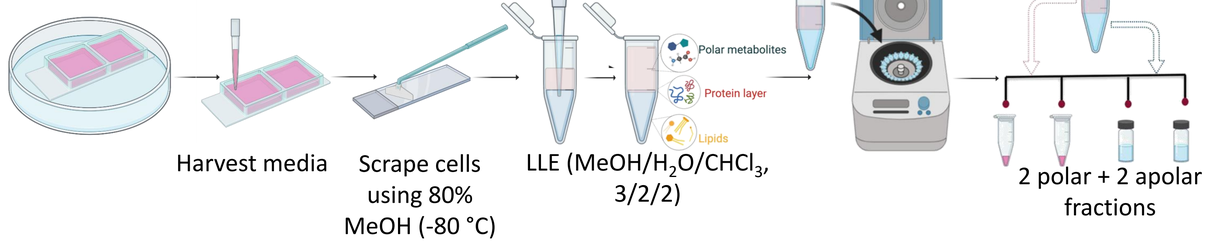
- **Mechanistic elucidation** of ethanol-induced hepatotoxicity at the cellular level using the human HepaRG liver cell line
- Generation of hypotheses on the **mechanism of action** of ALD
- Showcase potential **diagnostic biomarkers** for early ALD

Approach

Exposure conditions

- 1 x 10^6 HepaRG cells per sample
- 7 days of incubation to develop co-culture of hepatocyte-like cells and biliary-like cells
- 4 sample groups
  - Exposure to **ethanol at IC_{10}** (n = 6)
  - Exposure to **ethanol at 1/10 of IC_{10}** (n = 6)
  - Unexposed **controls** (n = 6)
  - Extraction blanks (n = 2)
- 2 exposure times
  - **24 h and 48 h**
- 2 sample types
  - **Intracellular and extracellular** extracts were harvested
- 2 batches
  - Entire experimental set-up was repeated for **validation** of metabolomics results

Sample preparation



LC-MS based metabolomics

Apolar extracts

- **RPLC-ESI-DTIM-QToF-HRMS** (Agilent 6560)
- UPLC BEH **C18** column in ESI+ and ESI-
- Drift tube ion mobility to increase annotation confidence

Polar extracts

- **HILIC-ESI-QToF-HRMS** (Agilent 6530)
- iHILIC-Fusion column in ESI+
- iHILIC-Fusion(P) column in ESI-

Worklist

- System suitability
- Conditioning
- Random injection order
- QC_{pooled} at regular intervals
- Samples in MS1
- QC in MS1/MS2/DTIM
- Iterative exclusion DDA

Data processing

Preprocessing and pretreatment

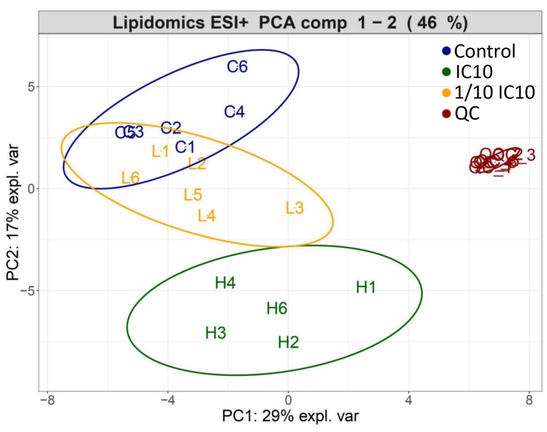
- Peak **picking** & **alignment**
- **Deisotoping**, duplicate removal & drift correction
- **Filtering** (e.g. mRSD < 30%)
- Random forest **imputation**
- Log **transformation**
- PQN **normalization**
- Pareto **scaling**

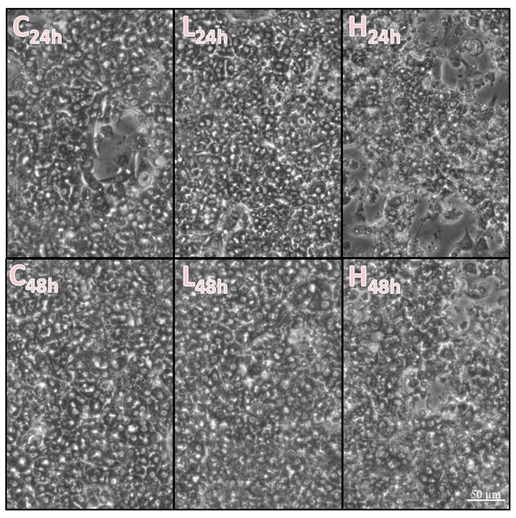
Statistics and annotation

- **PLS-DA & random forest** binary classifier
  - -VIP > 1 & MDA > 0.1
- Mann-Whitney U – Student t
  - -**p < 0.05 & FC > 5|< 0.2**
- Boxplots to confirm feature importance
- MS-DIAL, MS-Finder, MassBank, NIST, METLIN, GNPS, LipidMatch, LipidHunter for annotations & **manual confirmation**

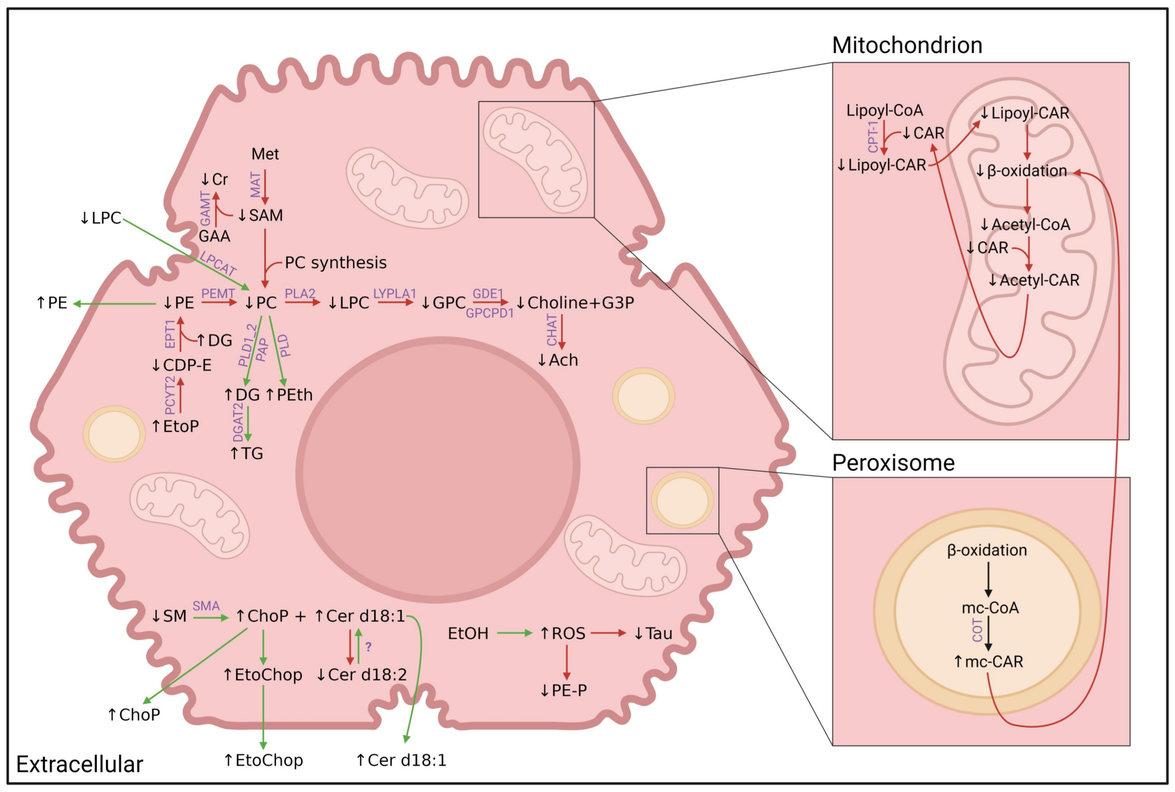
Results and Discussion

PCA and microscopic evaluation

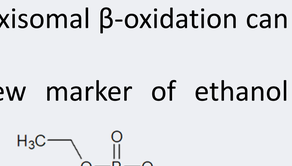




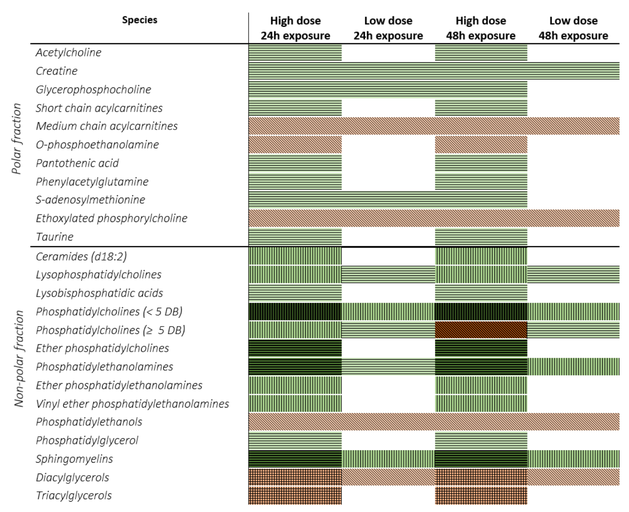
- PCA plots indicate **strong metabolic impact of ethanol exposure**
  - Clear separation IC_{10} (H) – CTL (C)
  - More overlap 1/10 IC_{10} (L) - C
- Morphological differences C - H
  - **Faded lining** polarized hepatocyte colonies
  - **Impaired organization** of hepatic clusters & accumulated debris
- No clear morphological differences C - L



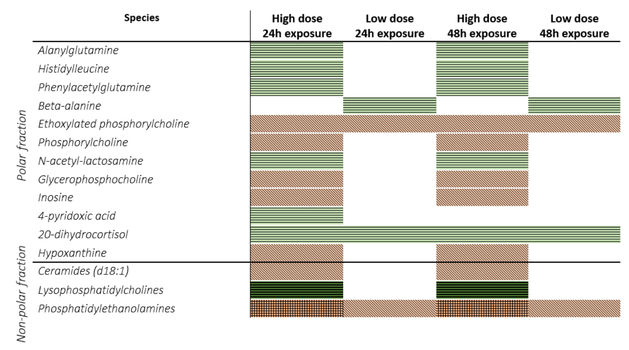
- 94 altered intracellular metabolites & 23 altered extracellular metabolites
- **↓PC**: ↓formation due to ↓conversion from PE and ↓methyl transfer due to ↓SAM, ↑consumption for production of PEth, DG and subsequent TG
- **↓LPC & GPC**: ↓PC catabolism and ↑intrahepatic LPC uptake for compensation
- **↓PE**: corresponds to ↑precursors EtOP and DG, ↑extracellular PE due to ↑secretion
- **↓SM**: ↑hydrolysis to ChOP and Cer (d18:1)
- **↓Cer (d18:2)**: unknown mechanism
- **↓CAR**: ↓biosynthesis impairs mitochondrial β-oxidation
- **↑medium chain CAR**: incomplete oxidation products of peroxisomal β-oxidation can not be processed by impaired mitochondrial β-oxidation
- **Ethoxylated phosphorylcholine (EtoChoP)** might be a new marker of ethanol exposure
  - Absence in control samples
  - Similar fold change as PEth 16:0_18:1
  - Unlike PEth 16:0_18:1, EtoChoP was found intra- and extracellularly



Intracellular sample fraction



Extracellular sample fraction



Color									
Number of species	>10	6-10	3-5	1-2	0	1-2	3-5	6-10	>10
Abundance	Lower	Lower	Lower	Lower	NA	Higher	Higher	Higher	Higher

Conclusions

- Minor effect of exposure time
- Major effect of exposure concentration
- Multiple altered metabolites consistent with steatotic image
- Cer (d18:2), Cr, EtOP, Ach and medium chain CAR as additional markers of toxicity
- EtoChOP is a potential new marker of ethanol exposure

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