Metabolic Alterations of HepaRG Cells in Response to Ethanol and TNF-α Co-exposure

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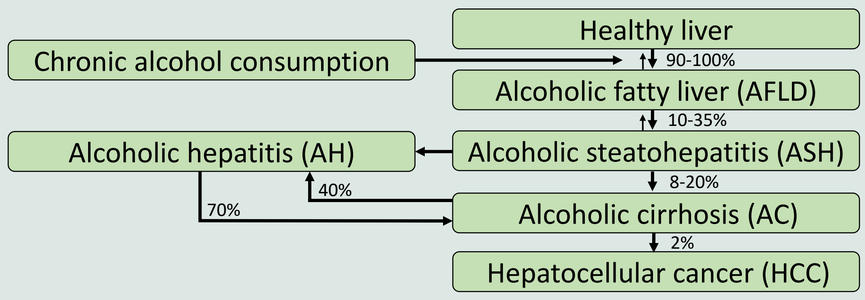
INTRODUCTION

- **Alcoholic liver disease** (ALD) is **highly prevalent but poorly identified** and characterized with lack of sensitive and specific early biomarkers
- Early-stage ALD is characterized by progressive intracellular lipid accumulation resulting in **alcoholic fatty liver disease**
- **Inflammation** of the liver is triggered by upregulation of **TNF-α** produced by Kupffer cells in response to ethanol and lipopolysaccharide exposure
- The **HepaRG** hepatic cell line provides an interesting alternative to primary hepatocytes due to low variability and long-term stability while maintaining most liver-specific functions
- As metabolic alterations are reflected in the phenotype and vice versa, metabolomics can help to identify **early-stage indicators** of alcoholic fatty liver disease

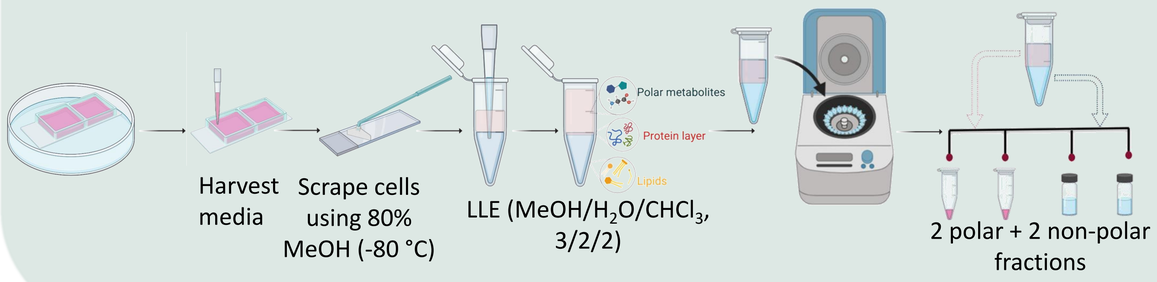


METABOLOMICS WORKFLOW

Exposure and sample preparation

- HepaRG: co-culture of hepatocyte- and biliary-like cells after 7 days of incubation
- 1 x 10^6 HepaRG cells per sample

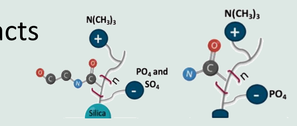
Exposure group 1 (n=7): 24h exposure to ethanol at IC_{10} (368 mM)
Exposure group 2 (n=7): 24h exposure to ethanol at IC_{10} (368 mM) + TNF-α (50 ng/mL)
Negative control (n=7): 24h incubation without ethanol and TNF-α
Extraction blank (n=3): 24h incubation without cells, ethanol and TNF-α
→ Experiment was repeated with 2nd batch HepaRG cells for **validation**



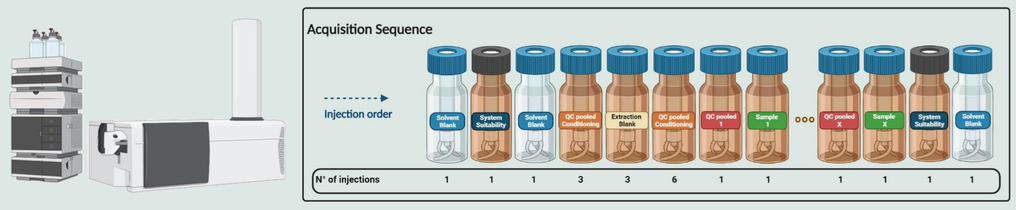
LC-MS based analyses

- **RPLC-ESI-DTIM-QToF-HRMS** (Agilent 6560) for **non-polar** extracts
  - UPLC BEH **C18** in ESI+ and ESI-
  - Drift tube ion mobility to increase annotation confidence
    - Comparison of experimental $^{DT}CCS_{N2}$
  - Iterative exclusion DDA to improve MS2 coverage

- **HILIC-ESI-QToF-HRMS** (Agilent 6530) for **polar** extracts
  - iHILIC-Fusion (silica, zwitterionic) in ESI+
  - iHILIC-Fusion(P) (polymer, zwitterionic) in ESI-



- **Quality Assurance (QA)/Quality Control (QC) procedures**
  - Pooled QC samples, system suitability mixture, usage of internal standards, SOPs,…



Data preprocessing and pretreatment

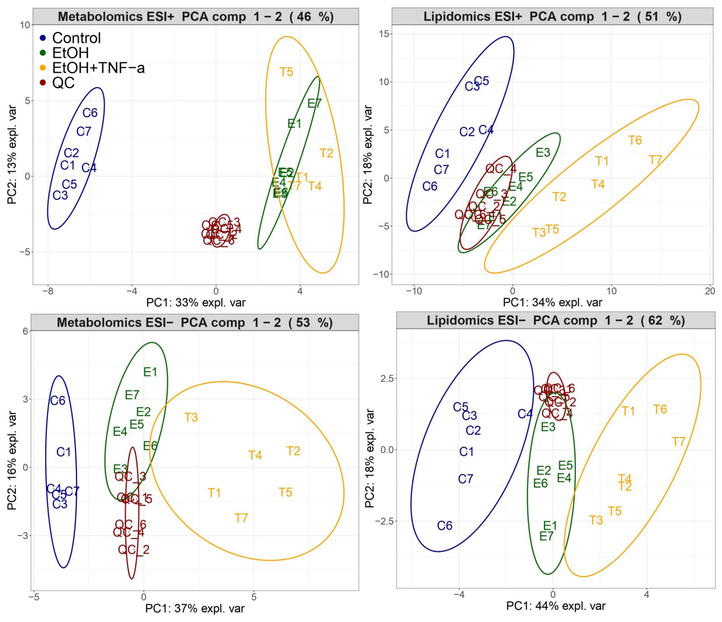
- Peak **picking, alignment**, **deisotoping**, duplicate removal & drift correction
- **Filtering** (e.g. mRSD intensity < 30%)
- Random forest **imputation** for missing values
- Log **transformation**, probalistic quotient **normalization** & Pareto **scaling**

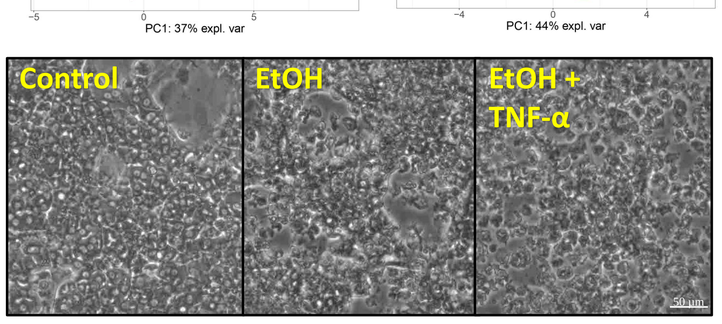
Statistics and annotation

- **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, LipidMatch, LipidHunter, LipoStar for annotations & **manual confirmation**

RESULTS & DISCUSSION

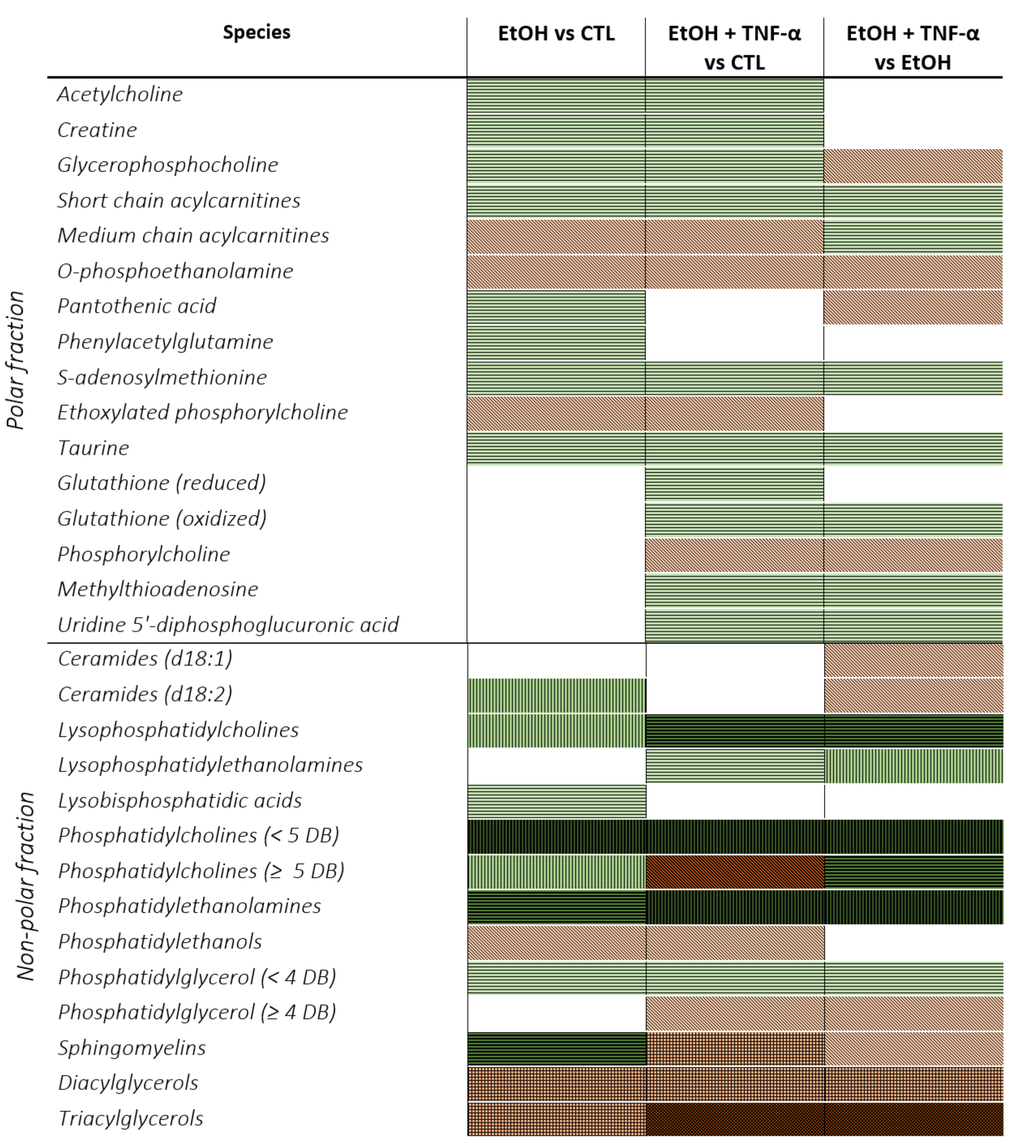
PCA and microscopic evaluation





- PCA plots indicate **strong metabolic impact of ethanol and ethanol + TNF-α exposure**
  - Clear separation all groups (except overlap EtOH – EtOH+TNF-α in polar ESI+)
- Morphological differences control vs. exposed cells
  - **Faded lining** polarized hepatocyte colonies
  - **Impaired organization** of hepatic clusters

Metabolic alterations

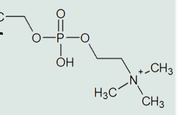


Color									
Number of species	>10	6-10	3-5	1-2	0	1-2	3-5	6-10	>10
Abundance	Lower	Lower	Lower	Lower	NA	Higher	Higher	Higher	Higher

- **↓Short chain carnitines (C_2-C_5)**: ↓biosynthesis impairs mitochondrial β-oxidation
- **↑Medium chain carnitines (C_6-C_{13})**: 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
- **↓Phosphatidylcholines**: ↓formation due to ↓conversion from phosphatidylethanol-amines and ↓methyl transfer due to ↓S-adenosylmethionine, ↑consumption for production of phosphatidylethanol, diglycerides and subsequent triglycerides
- **↓Phosphatidylethanolamines**: corresponds to ↑precursors O-phosphoethanolamine and diglycerides
- **Number of double bonds** determines direction of alteration (phosphatidylcholines & phosphatidylglycerols)
- **↑Triglycerides** strongly influenced by inflammation

CONCLUSIONS

- Metabolomic alterations (>100 annotations) can **distinguish** hepatic **exposure** to ethanol and co-exposure to ethanol and TNF-α
- **Steatotic image** is **enlarged** by co-exposure to inflammatory cytokine **TNF-α**
- **EtoChOP** is a potential new marker of ethanol exposure



- Future perspectives
  - Elucidate metabolic footprint
  - Deepen biological interpretations
  - In vivo exploration

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HepaRG & EtOH | Polar methods | Non-polar methods