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Metabolomics profiling of steatosis progression in HepaRG® cells using sodium valproate

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Graphical abstract

Highlights

- Design of experiment is important in untargeted metabolomics research.
- Valproic acid invokes a typical steatosis progression in HepaRG cells.
- Adaptive responses include spermidine alteration and an upregulation of SAMe.
- New potential biomarkers include acetylcholine, creatine and spermidines.
Abstract
Non-alcoholic Fatty Liver Disease (NAFLD) is a frequently encountered Drug-Induced Liver Injury (DILI). Although this stage of the disease is reversible, it can lead to irreversible damage provoked by non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Therefore, the assessment of NAFLD is a paramount objective in toxicological screenings of new drug candidates. In this study, a metabolomic fingerprint of NAFLD induced in HepaRG® cells at four dosing schemes by a reference toxicant, sodium valproate (NaVPA), was obtained using liquid-liquid extraction followed by liquid chromatography and accurate mass-mass spectrometry (LC-AM/MS). The combination of a strict design of experiment with a robust detection method, applied on sodium valproate, validated the possibilities of untargeted metabolomics in hepatic toxicological research.

Distinctive patterns between exposed and control cells were consistently observed, multivariate analyses selected up to 200 features of interest, revealing hallmark NAFLD-biomarkers, such as diacylglycerol and triglyceride accumulation and carnitine deficiency. Initial toxic responses show increased levels of S-adenosylmethionine and mono-acetylspermidine in combination with only a moderate increase in triglycerides. New specific markers of toxicity have been observed, such as spermidines, creatine, and acetylcholine. The described design of experiment provides a valuable metabolomics platform for mechanistic research of toxicological hazards and identified new markers for steatotic progression.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
</tr>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosyl-diphosphate</td>
</tr>
<tr>
<td>AM/MS</td>
<td>Accurate Mass/Mass spectrometry</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosyl-triphosphate</td>
</tr>
<tr>
<td>BHT</td>
<td>Butyl-hydroxytoluene</td>
</tr>
<tr>
<td>CAWG</td>
<td>Chemical Analysis Working Group (metabolomics society)</td>
</tr>
<tr>
<td>Cer</td>
<td>Ceramide</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DIL</td>
<td>Concentration 1/10 of IC10</td>
</tr>
<tr>
<td>DILI</td>
<td>Drug-Induced Liver Injury</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
</tr>
<tr>
<td>HAc</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic Interaction Liquid Chromatography</td>
</tr>
<tr>
<td>HMDB</td>
<td>Human Metabolome Database</td>
</tr>
<tr>
<td>IC₁₀</td>
<td>Inhibitory concentration 10 %</td>
</tr>
</tbody>
</table>
IPA  Isopropanol
LC   Liquid Chromatography
LPC  Lysophosphatidylcholine
LPE  Lysophosphatidylethanolamine
MeOH Methanol
MFE  Molecular Feature Extractor algorithm
MFG  Molecular Formula Generator algorithm
mRSD Median Relative Standard Deviation
MS   Mass Spectrometry
MSI  Metabolomics Standards Initiative
MS/MS Tandem mass spectrometry
MTT  (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium
NAFLD Non-alcoholic fatty liver disease
NASH Non-alcoholic Steato-Hepatitis
NH₄Ac Ammonium acetate
(NH₄)₂CO₃ Ammonium carbonate
NH₄F  Ammonium formate
NR   Neutral red
NRU  Neutral red uptake
PBS  Phosphate Buffer Saline
PC   Phosphatidylcholine
PCA  Principal Component Analysis
PE   Phosphatidylethanolamine
PLS-DA Partial Least Squares Discriminant Analysis
QC   Quality Control
QTOF Quadrupole Time of Flight Mass spectrometer
RP-LC Reversed Phase Liquid Chromatography
RSD  Relative Standard Deviation
SAMe S-adenosylmethionine
SM   Sfingomyelin
TCA  Tri-Carboxylic Acid cycle
TG   Triglyceride
VIP  Variable Importance Projection
VLDL Very-light Density Lipoprotein
NaVPA Sodium valproate

**Keywords:** Valproic acid, steatosis, HepaRG, Liquid Chromatography-Mass spectrometry, metabolomics, *in vitro*
1. Introduction

Adverse drug reactions form an important risk when patients are exposed to pharmaceuticals. Liver toxicity is one of the most frequent targets of toxicity since the liver is the central organ involved in the biotransformation and excretion of xenobiotics. Its high metabolic capacity makes it a strong, yet vulnerable organ; therefore the detection of liver toxicity is a priority in pre-clinical testing \(^1,2\).

A key aspect in toxicology is the determination of safe concentration levels for acute and chronic exposures\(^3\). However, conventional tests do not provide additional insight in the mechanism of toxicity\(^4\)–\(^6\). Modern toxicology aims at mechanistic interpretations of adverse drug reactions using alternative tools to accurately assess the hazards associated with xenobiotics\(^4\). The fingerprinting of toxicological events allows the accurate determination of mechanisms of the toxicological outcome.

Metabolomics, the study of small organic molecules, provides the most downstream information of biochemical pathways, providing a summary of the eventual outcome after exposure to xenobiotics\(^7\)–\(^9\). Since metabolomics do not measure proteins or genes, the phenotypical toxicological response on metabolite level can be investigated as an alternative to gene or protein alterations on a (sub)histological level\(^5,6\). Furthermore, in the context of the Adverse Outcome Pathway approach, metabolomics may provide novel markers of toxicity that can be related to the molecular initiating events of the adverse effect under study\(^10\).

This study investigated the intracellular endogenous metabolic profiles of HepaRG cells, a human metabolically competent hepatic cell line\(^11,12\), exposed to sodium valproate (NaVPA) at different time points and dosage schemes to observe acute and sub-chronic effects on two different dosing levels.

NaVPA is an anti-epileptic drug which is frequently prescribed to reduce the number of epileptic seizures\(^1,13\). Due to its structural similarity with fatty acids, it competes with the endogenous lipid metabolism, provoking a hazard to induce non-alcoholic fatty liver disease (NAFLD) or steatosis\(^14\).

The metabolomic fingerprint of NaVPA as a reference toxicant for steatosis validates the set-up of a designed experimental platform including the in vitro exposure model, cell extraction and metabolomic analysis of the cell extracts. Multivariate analyses are used to select the signals related to exposure; further identification using physicochemical characteristics such as the accurate mass, MSMS spectra and retention times to generate a list of endogenous metabolites\(^15,16\). With the obtained information, a biochemical interpretation of the observed results can be inferred, allowing an accurate determination of pathways involved in the potential toxicological hazards associated with the xenobiotic of interest.
2. Materials and methods

2.1 Standards and chemicals

Cryopreserved differentiated HepaRG® cells (HPRGC10) and recommended culture media and supplements (Basal Hepatic Cell Medium (MIL600), Thawing/Plating/General Purpose Medium Supplement with antibiotics (ADD670) and Additives for Maintenance/Metabolism with antibiotics (ADD620)) were obtained from Biopredic International (Rennes, France). Collagen type 1 was obtained from Corning (Wiesbaden, Germany). Dry ice was purchased from Strombeek IJsfabriek (Strombeek, Belgium). Ultrapure (milliQ) water was obtained by the use of a PURELAB device from Elga LabWater (Tienen, Belgium). Phosphate-buffered saline (PBS) was prepared on site at the research group In Vitro Toxicology and Dermato-Cosmetology (IVTD, VUB, Belgium).

From Thermo Scientific (Rochester New York, VS) the Lab-Tek Chamber Slide w/Cover Permanox Slide Sterile 2 well were purchased. Sterile cell scrapers with sharp edge (type GBO 541070) were purchased from Greiner Bio-One (Vilvoorde, Belgium). The equipment for the use of the TC10 Automated Cell Counter (Cell Counting Slides for TC10™/TC20™ Cell Counter, Dual-Chamber en Trypan Blue Dye, 0.40 % solution) were purchased from BioRAD Laboratories (Temse, Belgium).

Ammonium acetate (for analysis, >98 %) (NH₄Ac), formic acid (for analysis, >98 %) (FA), acetic acid (glacial, anhydric for analysis, 100 %) (HAc), chloroform (for analysis) (CHCl₃), ammonium carbonate (extra pure), 2-propanol (for analysis) (isopropanol, IPA) and neutral red (NR) were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile (HPLC grade) (ACN) and methanol (LC-MS grade) (MeOH) were obtained from Fisher (Loughborough, VK). Sodium Valproate (NaVPA), ammonium formiate (97 %) (NH₄F), L-ascorbic acid (BioXtra, >99 %), butylhydroxytoluene (>99 %) (BHT) and ethylenediaminetetraacetic acid (trace metal basis, 99,995 %) (EDTA) were purchased from Sigma-Aldrich (Steinheim, Germany).

The isotope labeled standards D-tryptophane-2',4',5',6',7'-d5 (98 %), laurylic acid-12,12,12-d3 (99 %) and cholesterol-25,26,26,26-d4 (99 %) were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). The standards 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC-17:0), 1,2-diheptadecanoyl-sn-glycero-3-fosfaat (PA-17:0), N-heptadecanoyl-sphing-4-enzyme (Cer-17:0) and 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC-17:0) were purchased from Avanti Lipids (Alabaster, Alabama, VS). ATP-13C₁₀, TG-(12C₉-13C:0), lysine-13C₆-15N₂, glucose-13C₆, ADP, ATP, stearic acid, folic acid, mono-, di- and trioleylglycerol, misoprostol, cholic acid-d₄, phosphoenolpyruvate, ornithine, glutamate-d₄, leucine-d₃, adenine, glucosephosphate, citric acid, caffeine, N-acetylg glucosamine, pyridoxal-d₃, dopamine-d₄, palmitoylcarnitine, cholesteryl palmitate, succinic acid-d₄ and a standardised amino acid mix (AAS18) were purchased from Sigma-Aldrich (St. Louis, Missouri, VS).
2.2 Determination of testing concentrations

Cryopreserved differentiated HepaRG® cells were thawed and seeded in chamber slides at a density of 0.089 x 10^6 cells/well using Basal Hepatic Medium® enriched with HepaRG Thaw and Seed Supplement®. Plates were incubated for 7 days at 37 °C and 5 % CO₂. The IC_{10} dosage for a 24 h and 72 h exposure were assessed by the Neutral Red Uptake assay (NRU)\textsuperscript{17,18}.

After seven days of cultivation, the wells were divided in 2 negative control groups and 8 groups which are exposed to NaVPA at different concentrations ranging from 250 µg/mL to 10 000 µg/mL for a period of 24 h and 47 µg/mL to 10 000 µg/mL for a period of 72 h, respectively (Table SI-1). After exposure, the cells were washed and the Neutral red was dissolved in water/ethanol/HAc (49/50/1) (v/v) and absorbance was measured at 540 nm. The IC_{10} was calculated based on 4-parametric analysis of the response curves of three plates using Masterplex® QT curve-fitting software, IC_{10} exposure concentrations were set at 2 300 µg/mL and 665 µg/mL for the 24 h and 72 h exposure, respectively. Full details are available in the Supplementary Information SI-1.

2.3 Metabolomics experiments

Full details of all protocols are available in Supplementary Information SI-2.

2.3.1 Design of the experiment

False positive results are an important issue in untargeted metabolomics experiments\textsuperscript{19,20}. It is a common problem since hundreds to thousands of signals are being analysed and correlated to the biological question. To reduce the number of false positive features, the experiment has been performed in duplicate at two concentrations and two time periods, resulting in 8 datasets. Features which are significantly altered in both experiments are considered robust and represent metabolites involved in the progression of the toxicological injury. The term marker of toxicity is defined in this article as a feature which is selected concordantly with the same correlation and impact in the two replicates of at least one of the four exposure conditions of \textit{in vitro} HepaRG® cell cultures to NaVPA.

2.3.2 Seeding of the HepaRG® cultures and exposure to NaVPA

Cryopreserved differentiated HepaRG® cells were thawed and seeded in collagen-coated 2-well Lab-Tek chamber slides (Permanox, Sigma Aldrich) at a density of 1.03 x 10^6 cells/well. After seven days of cultivation, the cell cultures were visually checked for hepatocyte/biliary cell ratio and assigned to a negative control group or to any of the two exposure groups: (1) the IC_{10} (2 300 µg/mL and 665 µg/mL for 24 h and 72 h exposure, respectively) NaVPA and (2) a 1/10 dilution of the IC_{10} (DIL, 230 µg/mL and 66.5 µg/mL for 24 h and 72 exposure, respectively). In each experiment, two non-cultured chamber slides were used as blank samples.
2.3.3 Sample preparation

The cell cultures were harvested and processed by the protocol of Wu et al. and adapted by Cuykx and Mortelé et al.\textsuperscript{21,22} The cells were washed twice with PBS (37 °C) and flash frozen on liquid nitrogen. The cells were scraped from the surface with three times 200 µL of a cooled (-80 °C) 80 % (v/v) MeOH/milliQ water solution, quenching metabolism and precipitating proteins. Afterwards, the cells were collected and LLE was performed with ultrapure water, methanol, and chloroform (final solvent ratio 2/3/2 water/MeOH/CHCl\textsubscript{3}). The mixture was three times vortexed for 30 s and centrifuged at 2200 g for 7 min by an Eppendorf Centrifuge 5804 (Eppendorf, Hamburg, Germany).

Two times 400 µL of the polar and two times 100 µL of the non-polar fraction were recovered. A QC was created by collecting 40 µL and 20 µL aliquots of all samples for the polar and non-polar phases respectively.\textsuperscript{7} The polar fraction was dried under vacuum using a Savant Speedvac concentrator SVC 100 H (Savant, Thermo Scientific, USA), the apolar fraction was evaporated using a nitrogen stream. Full details about the original protocol are mentioned in SI-2.

2.3.4 Quality Control (QC)

According to Dunn et al., QC samples were used to observe the analytical variation throughout the experiment and to correct for shifts in signal intensities\textsuperscript{7}. Aliquots of all samples were taken and homogenized in a QC-pool. This pool was used to equilibrate the LC-MS system, one QC-sample for the analysis was injected every four samples to monitor the system performance. The variation within this QC-group was then used to observe the quality of the system.

2.3.5 LC-MS analysis

LC-MS analysis was performed according to previous established protocols, details of the four analytical runs are available in SI-3 \textsuperscript{23}. The non-polar fraction was analysed in two runs (one positive, one negative mode) on a Kinetex XB-C18 (150 x 2.1 mm; 1.7 µm particle size, Phenomenex, Utrecht, the Netherlands). Mobile phase composition consisted of mixtures methanol, isopropanol and water with ammonium acetate (pH 6.7) and mixtures of acetonitrile, isopropanol and water with an acetate buffer (pH 4.2) for negative and positive mode, respectively. The polar fraction was analysed in positive mode with a HILIC system using an iHILIC column (100 x 2.1 mm; 1.8 µm particle size, HILICON, Umeå, Sweden) using ACN, MeOH and water with a NH\textsubscript{4}F/FA buffer (pH 3.15). In negative ionisation mode, a polymeric iHILIC Fusion Column (100 x 2.1 mm, 5 µm particle size, HILICON) was used in combination with ACN, MeOH and water with a (NH\textsubscript{4})\textsubscript{2}CO\textsubscript{3} buffer (pH 9). The columns were attached on an Agilent Infinity 1290 UPLC (Agilent Technologies, Santa Clara, USA), the detection was done with an Agilent 6530 QTOF with Agilent Jet Stream nebuliser (Agilent Technologies).

2.3.6 Data analysis
2.3.6.1 Data-quality control
Evaluating the internal m/z calibration, the continuous detection of the high and low reference masses was investigated for every analysis run. Next, the consistent detection of the internal standards in all samples was controlled to evaluate the precision and repeatability of the analysis method regarding retention time, the m/z-accuracy and the abundance ratios. Only samples where internal standards were detected and where the number of molecular features was comparable to the other samples, were considered. The absence of internal standards and molecular features in an acquired LC-MS run indicate analytical issues during the run, and therefore, the removal of the failed runs was considered to improve the quality of the final dataset. The raw data were searched for the internal standards using the Find by Formula algorithm (Agilent Technologies) with the following parameters: formula matching ± 10 ppm, expected variation 2 mDa ± 8 ppm. The results of internal standard quality control were used to set the width of parameters for further data processing.

2.3.6.2 Data-pretreatment
Acquired data were imported on the MassHunter Qualitative software (Agilent Technologies, v 2.06.00). Molecular features representing the extracted metabolites were searched using the Molecular Feature Extractor (Agilent Technologies). The algorithm searches for m/z values with a chromatographic profile and groups them together with possible isotopes and adducts. The group of these ions represent possible signals generated by a single molecule. The MFE used signals that counted at least 100 counts to extract features which matched the isotopes based on the common organic molecules isotope-model. The isotopes were grouped based on a peak spacing tolerance of 0.0025 m/z ± 15 ppm. Other parameters were retained on default settings. A filter was applied to retain the molecular features with an abundance >3 000 and a score >80.
All extracted data-samples were exported and merged into a dataset with Mass Profiler Professional (Agilent Technologies, v 12.5). Features were aligned with a retention window of 0.2 min in RP-LC and 0.3 min in HILIC and a m/z window of 15 ppm. The data were Log10 transformed. Every signal was baselined to the median of all samples.
The dataset was cleaned up by blank subtraction (signals with median abundance > 10 x highest blank), a frequency filter (present in at least 80 % of samples within a group) and a variability filter (RSD <30 % within at least one exposure group) reduced noisy features.

2.3.6.3 Statistical analysis
The resulting dataset contains all qualitative features on which principal component analysis (PCA) is performed. PCA is an unsupervised multivariate statistics method that visualises the maximum variance of the samples based on their metabolome profiles, independent of the group they belong
to. The PCA scatter plots were used for quality control: low-quality samples outside the 95%-confidence interval were removed and the variance within and between the exposure groups was checked.

As a supervised multivariate statistical method, partial least squares-discriminant analysis (PLS-DA) was performed. The data were exported as an Excel-file and analysed by using PLS-DA in EZinfo (Umetrics). In the PLS-DA S-plots, the molecular features of interest are selected, based on the correlation of the signal variance between exposure group and the negative control group, the impact of the feature on the model based on abundance differences and a visual graphic control of the abundance differences in the plot between both groups.

All experimental settings were performed in duplicate. Both resulting lists with selected features from the same analysis method were compared. The data were matched, whereby the consistent features (same retention time and molecular mass) which were selected twice with the same effect in the same group comparison, were selected as markers of toxicity for this experimental setting.

2.3.6.4 Identification

In Mass Hunter, the signals corresponding to potential markers of toxicity were selected, the complete result set was extracted and the Molecular Formula Generator (MFG) generated a list of possible chemical formulas. The identification was based on the m/z-value, the isotope pattern, the measured retention time and the fragmentation spectra acquired during the equilibration runs. The most plausible formulas were selected, based on the biochemical plausibility, the total matching score, the ppm-deviations (max. 10 ppm) and the fitting of the isotope pattern (max. 5 % deviation for the m+1 isotope and 1 % for the m+2 isotope)\textsuperscript{15}. The measured m/z-values, combined with the MS/MS fragmentation spectra from the equilibration runs were chemically interpreted and were compared to experimentally confirmed or \textit{in silico} predicted reference spectra from the metabolite databases METLIN, LIPID MAPS, the Human Metabolome Database (HMDB) and/or ChemSpider\textsuperscript{24–29}.

The resulting levels of identification are based on the standardised reporting rules, published by the Chemical Analysis Working Group (CAWG) and the Metabolomics Standards Initiative (MSI)\textsuperscript{30,31}. Briefly, identification can be related to five levels of confidence: level 5 being the lowest which only provides an m/z value. Level 4 is a low-confident annotation and provides a chemical formula. Level 3 has been divided in two categories: annotation based on mass spectrometric characteristics of the feature and retention times without (3B) and with fragmentation spectra (3A) and is related to at least a class-wise annotation. Level 2 identification is a hit confirmed with a reliable retention time and a confirmation of the fragmentation spectra with one of the database libraries. Level 1 identification is a complete similarity with an injected reference standard. Confident identification is crucial to derive biological information of the statistical results, full confidence to level 1 is hardly possible since the
availability of standards for all biologically-relevant chemicals is logistically impossible and expensive. However, level 2 and level 3 identifications provide a reliability which allows the researchers to infer a biological interpretation to the outcome of the experiments. Metabolites with lower confidence levels in identification (level 4-5) are reported in the Supplementary Information SI-11 of the article since they have a discriminatory impact on the statistical models, but they are not included in the biochemical discussion.

3 Results
3.1 Experimental observations
After the exposure period, the cultures were evaluated. Photos were taken, representative images are depicted in Figure 1. Healthy control cultures (Fig 1, A and D) show clear clustering of hepatocytes and are clearly distinctive from the biliary cells. The DIL group after 24 h (Fig 1, B) has a slightly faded lining between the clusters and the biliary cells, but the cytological morphological differences are not always clear. After 72 h (Fig 1, E) exposure to NaVPA, differences are clear. Stress is noticeable as the hepatic clusters are less organised, but no clear cell death is observed. At the IC_{10} concentrations (Fig 1, C and F), cytological morphology was severely distorted at higher concentrations and longer exposure times, which is concordant with the dose-response paradigm. Effects were noticeable in both hepatocytes and biliary cells, showing swollen and badly defined cell borders. Cluster organisation is not visible and debris are observed. All samples were analysed within 7 days, after QC, the samples in Table SI-4 have been excluded for further analysis.

3.2 Data Quality
The calculated mRSDs (Table SI-5) were below 10 % for all QC samples in the non-polar fraction, giving a very reliable method for the detection and data-pretreatment parameters. Although the mRSDs for the polar methods were higher, they never exceeded 20 %. The mRSDs of the sub-populations increased from 10 % to 20 % for the non-polar fractions and from 15 % to 30 % in comparison to the QC, indicating biological and analytical (sample preparation) variance. The variance in the sub-cytotoxic concentration was systematically higher than the control and cytotoxic population, probably due to biological variance. The variance in the total dataset is higher, exceeding over 35 %, indicating a significant in-between variance for the majority of the extracted molecular features.

In all PCAs, the inter group variability between the three exposure groups lies in the PC1 and/or PC2 (Figures SI-6A-D; Red: Negative Control, Blue: DIL, Brown: IC_{10}). As an example, the PCA-plots of experiment 72 h-2 is shown in Figure 2. This separation among the exposure groups implicates a clear metabolic impact on the cell cultures by exposure to these subtoxic and toxic concentrations of NaVPA.
The subcytotoxic concentration is in all models more similar to the negative control group in comparison to the group exposed to the IC$_{10}$.

Because group discrimination is already possible with unsupervised methods, the use of a PLS-DA model was considered a reliable method. Model quality is defined by $R^2$ and $Q^2$ values present in Tables SI-7 and SI-8. $R^2$ values were high for every model, $Q^2$ values achieved good levels (0.7 and higher).

### 3.3 Selection of potential markers of toxicity

The selection based on the reported criteria revealed many features of interest within each single experiment, as reported in Figures SI-9 and SI-10 (up to 50% of the features in the non-polar fraction), but only a limited fraction of them was concordant between both experiments. Of the selected signals, further identification refined the number of metabolites: due to different adduct formation, few significant features could be grouped together into one metabolic feature. In the end, 92 and 113 metabolites have been selected as reliable signals for NaVPA-induced NAFLD in a 24 h and 72 h time frame, respectively. A full list of all markers for each condition (24 h vs 72 h) is available in SI-11, a summary of all markers with a reliable level of annotation confidence (3 or higher) has been provided in Table 1.

During a 24 h exposure, upregulation of lipid species was especially observed for the IC$_{10}$ concentration, except for phosphatidylycholines (PC) and phosphaticylethanolamines (PE). Polar metabolites, such as carnitine, acetylcholine, taurine and S-adenosylmethionine (SAMe) are downregulated, acetylspermidines were more abundantly present. The DIL group showed similar, yet less distinctive patterns. When HepaRG cells were exposed for longer periods (72 h), a massive upregulation of triglycerides (TG) and especially heavy-chain TGs noticed during the exposure at both concentrations. Also poly-unsaturated acyl-chains were more frequently incorporated in the TGs. TGs with short and saturated chains were present in lower abundances. PEs were systematically less abundant, also one lyso PE (18:1) and one PC (16:0/18:1) were downregulated. Diacylglycerols (DG) were more abundantly present, but not in the same order of magnitude as the TGs. Of the polar compounds, the similar patterns were visible as in the short-term exposure: a downregulation of acetylcholine, carnitine and creatine with an upregulation of diacetyl spermidine. Mono-acetylspermidine was however not upregulated and SAMe showed an upregulation at lower concentrations. Further potential markers included ornithine and a citric acid -N-sugar.

### 4 Discussion

#### 4.1 Design of Experiments

Since hepatotoxicity is one of the main reasons for drugs to be retracted from the market, a reliable platform for pre-clinical liver toxicity screening is an ideal tool to reduce costs. The human HepaRG*
liver cell line is a recent product with growing interest for a wide range of applications. The non-existent biological variance of cell lines (one single donor) is an advantage in comparison to samples from in vivo studies, since it decreases the number of replicates needed to observe important changes in the cellular profile. The low cost, easy cultivation and low ethical restriction makes competent cell lines an attractive tool for a first quick screening of new chemical entities\textsuperscript{32,33}. The HepaRG\textsuperscript{®} cell line is derived from a human hepatocarcinoma. The cells can differentiate into hepatocytes and biliary-like cells. In in vitro environments, the hepatocytes cluster together in lobule-like structures, mimicking the in vivo situation\textsuperscript{12,34,35}. Furthermore, the cell line is genetically and metabolically stable and shows biotransformation competence, making it an easy and reliable alternative for primary human hepatocytes\textsuperscript{35-37}.

The testing concentrations for the metabolomics experiments were first determined with the Neutral Red Uptake assay. The viability assay determines cell viability by measuring the uptake of Neutral Red in the lysosomes, a cell organelle associated with catalysis and breakdown of unwanted proteins and structures. The lysosome is not associated with mitochondrial activity, making it more suitable for the dose estimation of sodium valproate, which is a known mitotoxicant than energy dependent viability assays, such as ATP-ase and MTT assays\textsuperscript{18,38}.

The simulation of an acute (24h) and sub-acute (72h) dosage allows the investigation of the impact of short and long-time processes. Since many compounds are dosed in a chronic scheme, the repeated dose 72 h gives a more accurate toxicological profile than a single-dose study, as proven by previous transcriptomics experiments by Rodrigues et al\textsuperscript{39}.

A very important addition in the current study is the inclusion of a batch-replicate. Since many studies only perform their experiment once, such results provide often a vast range of potential markers of toxicity. False positive results are often found during univariate and multivariate testing procedures, which can be countered by false discovery rate (FDR)-correction and cross-validation, respectively. However, FDR-correction can contribute to the attrition of less obvious markers which only show small differences, which can have a major impact on the final outcome of the cell (e.g. hormones). The design of experiments in this article does include small significant differences, as long as they are reliable (being present in both replicates). The removal of non-concordant features includes the risk of false-negatives as well, especially for the non-polar fraction. The incorporation of extra carbons or desaturations (e.g. C16 vs C18 or C22:4 vs C22:5 acyl chains) results in different m/z values, resulting in non-concordant features. This risk can be anticipated by using alternative pattern mining techniques, observing metabolic groups rather than species independently. For our current article, consistent responses were preferred since they provide more confidence in the biological interpretation. Therefore, the approach employed in the current study is more reliable than the
inclusion of FDR and cross-validation of a single dataset on its own. The thresholds for marker selection was not based on the Variable Importance of Projection (VIP) only, but rather on which metabolites showed similar correlations and trends across both replicates. Although this initially increases the risk for false positive results when only one experiment is used, the number of false negative metabolites gets lowered, while the validation with the second experiment rejects the false positive markers initially retained by the low selection thresholds. As shown in Figure SI-9 and SI-10, initial markers get further narrowed down to a relative small subset of 200 markers of toxicity.

The low percentage of concordant metabolites (especially for the 72 h) can be related to the increased variability in combination with the variance filter of 30% within a sample group during extended exposure-times. For the polar fraction, the responses and trends have been re-investigated, showing false negative results for diacetylspermidine and SAMe. These markers of toxicity have been re-integrated in the final table, because of their key positions in cell biochemistry.

Current shortcoming are mentioned for the polar fraction in negative ionisation mode: the method seems not to monitor the metabolites efficiently. Only a limited number of metabolites was found concordantly of interest. Furthermore, no effects have been observed for typical differences such as disruptions in the TCA cycle. Possible explanations are poor peak shapes and retention time shifts observed during the data processing.

4.2 Markers of Toxicity

After exposure of HepaRG® cells to sodium valproate, redundancies were observed between all models, schematically shown in Figure 3. Neutral lipids such as triglycerides and ceramides were upregulated, in combination with a downregulation of carnitine. The inhibition of beta-oxidation by NaVPA reduces free carnitine, increasing the fatty acid pool. The free fatty acids are incorporated in lipid species (TG and ceramides). In a short time-window (24 h), the lipid accumulation results in a moderate alteration of the profile. Longer exposures (24 h → 72 h) to higher concentrations (1/10 of IC_{10} → IC_{10}) result in extreme inhibition of the oxidation, providing more and longer acyl chains to be incorporated in the cell. A remarkable alteration is the decreased incorporation of saturated fatty acids, while heavy, unsaturated acyl-chains were more often included in the triglyceride species. The presence of long-chain poly-unsaturated acyl chains are suspected to be related to the activation of arachidonic acid metabolism pathways. The observed downregulation of PEs could be related to excretion of the lipid species in VLDL-type chylomicrones^{40,41}. However, this has not been proven since the extracellular medium has not been analysed.

S-Adenosylmethionine (SAMe) is a key metabolite in one-carbon metabolism. The upregulation of SAMe in the DIL group at 72 h of exposure indicates an adaptive response, which eventually results in a decrease after exposure at long and high concentrations. SAMe is thus an important metabolite to
follow-up the progression in the development of NAFLD. The reduced presence of this SAMe might be linked to the presence of odd-chain acyls present in multiple lipid species and the increase in spermidines. Probably, the mono-acetyl spermidine is further acetylated to diacetyl spermidine in the chronic exposure, resulting in a paradoxically lower concentration at sub-chronic exposures in comparison to acute exposures.

Small polar molecules such as acetylcholine and creatine were systematically observed as being downregulated, but they could not be fitted in a known pathway of toxicity. These metabolites might represent potentially new specific markers of toxicity for NAFLD, but confirmatory studies with other reference steatogenic toxicants, such as tetracycline and tamoxifen, are necessary to validate these metabolic changes and confirm the hypothesis of new markers of NAFLD.

Sodium valproate is known to induce steatosis through inhibition of the mitochondrial activity: the structural analogy with fatty acids prevents beta-oxidation through inhibition of the carnitine-shuttle and Coenzyme A-bonding, reducing fatty acid transport in the mitochondrion, respectively. The downregulation of carnitine is a hallmark of NaVPA intoxications. The similar backbone to fatty acids results in a competition for binding spots to carnitine to be transferred into the mitochondria through the carnitine shuttle. In the mitochondria, NaVPA inhibits beta-oxidation of endogenous acyls through the same mechanism, resulting in reduced oxidative phosphorylation. Furthermore, sodium valproate has been related to the induction of mitochondrial permeability transition pore opening and to the inhibition of triglyceride excretion.

As a result, lipid breakdown and elimination are prevented, accumulating fatty acids. The cell responds by incorporating the overload of acyls into non-polar acylated structures, such as triglycerides and ceramides resulting in NAFLD ballooning, a state prior to NASH.

Arachidonic acid is known to be a precursor of inflammatory species, such as the prostaglandins. The upregulated production of arachidonic acid provides an increased presence of arachidonic acids and other downstream metabolites, such as docosa-hexenoic, which are incorporated into the observed heavy-chain triglycerides. Similar effects have been observed by Wells and Ma.

Van Ginneken et al. mentioned also the incorporation of odd-chain acyls in the lipid species due to an upregulation of the one-carbon metabolism. SAMe is known to be involved in methyl transferring reactions and is a crucial metabolite in many regulatory biochemical methylation reactions. The initial upregulation might be explained by the abundance of acyls to be metabolised, the downregulation can be related to the incorporation of these methylene groups in odd-carbon acyl chains which are further stored in the triglycerides.

The observed alterations in lipid metabolism matched perfectly with the typical toxicological impact described in literature, suggesting a very good sensitivity of metabolomics in the research of mechanistic pathways of hepatotoxicity. Furthermore, additional metabolites were altered which could
not immediately be related to the lipid beta-oxidation. The alterations of these metabolites correlate with the hepatotoxic effects, but further research should investigate causality to validate the potential of the metabolites to be used as markers of toxicity in risk assessment studies.

5. Conclusions

Metabolomics is a powerful research tool to investigate fingerprints related to toxicological profiles. However, a proper design of experiment is essential to reduce false positive and false negative results. The investigation of sodium valproate as a steatogenic reference toxicant on HepaRG® cells using four different dosage schemes and experimental replication efficiently reduced false positive results. The toxicological fingerprint obtained from the different dosing schemes followed a toxicological paradigm, resulting in more severe responses at higher concentrations and longer exposure times. The selected markers of toxicity matched with literature: decreased carnitine, SAMe and PEs in combination with an upregulation of neutral heavy-chain lipids represent a typical steatotic profile. Extra potentially specific markers of toxicity have been observed, such as spermidines, creatine, and acetylcholine. Initial toxic responses can be distinguished from severe responses by increased levels of SAMe, the increase of mono-acetyl spermidine and a moderate increase in triglycerides. Severe intoxications bear the hallmark of increase in unsaturated triglycerides, decreased PEs, SAMe and carnitine levels and an upregulation of di-acetyl spermidine. The successful in vitro untargeted metabolomics approach validates the possibilities of untargeted metabolomics fingerprinting to assess the mode of action of a toxicant.

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Conflicts of interest

The authors declare no conflicts of interest
References

3. ECHA.
41. Donato MT, and Gómez-Lechón MJ. Curr Drug Metab [Internet]. 2012;1160–73.
52. Schofield Z, Reed MA, Newsome PN, Adams DH, Günther UL, and Lalor PF. World J Gastroenterol [Internet]. 2017;2685.
Table 1: Summary of up- and down-regulated annotated markers of toxicity after a 24h and 72h exposure. A black line separates the unique molecules from the non-unique (family based) alterations.

<table>
<thead>
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<th></th>
<th>24 h</th>
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<td>IC_{10}</td>
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<tr>
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Cer: ceramide, DG: diacylglycerol with more than (>) or less than (<) 36 carbons divided over both acyl chains, LPE: lysophosphatidylethanolamine. PC: phosphatidylcholine, PE: Phosphatidylethanolamine, PUFA: containing mostly unsaturated fatty acids, SAMe: S-adenosyl methionine, SFA: containing mostly saturated fatty acids, SM: Sfingomyeline, TG: triglyceride with more than (>) or less than (<) 50 carbons divided over all acyl chains. Colours represent the degree of up- (green) or down-regulation (red).
**Figure 1:** Photos of the *in vitro* cell cultures at a 10x20 amplification as an example of cell morphology at different stages. A-C: control, DIL (1/10 dilution of $IC_{10}$) and $IC_{10}$ after 24 h of exposure, respectively and D-F: control, DIL and $IC_{10}$ after 72h of exposure, respectively.
Figure 2: PCA-plots of each platform (non-polar fraction in negative and positive ionisation mode, polar fraction in negative and positive ionisation mode) for experiment 72 h-2 after 72 h of exposure as an example of all PCA-plots mentioned in SI-6. Red: Negative Control, Blue: DIL, Brown: IC$_{10}$. 