Toxicogenomics of precision-cut liver slices for prediction of human liver toxicity

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ACUTE TOXICITY OF CCL\textsubscript{4} BUT NOT OF PARACETAMOL INDUCES A TRANSCRIPTOMIC SIGNATURE OF FIBROSIS IN PRECISION-CUT LIVER SLICES

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ABSTRACT

In rat in vivo, both paracetamol (APAP) and carbon tetrachloride (CCl₄) induce liver necrosis, but long-term treatment with CCl₄, in contrast to paracetamol, causes liver fibrosis. The aim of this study was to perform transcriptomic analysis to compare the early changes in mRNA expression profiles induced by APAP and CCl₄ in the rat precision-cut liver slice model (PCLS) and to identify early markers that could predict fibrosis-inducing potential.

Microarray data of rat PCLS exposed to APAP and CCl₄ was generated using a toxic dose based on decrease in ATP levels. Toxicity pathway analysis using a custom made fibrosis-related gene list showed fibrosis as one of the predominant toxic endpoints in CCl₄-treated, but not in APAP-treated PCLS. Moreover, genes which have a role in fibrosis such as alpha-B crystallin, jun proto-oncogene, mitogen-activated protein kinase 6, serpin peptidase inhibitor and also the transcription factor Kruppel-like-factor-6 were up-regulated by CCl₄, but not by APAP. Predicted activation or inhibition of several upstream regulators due to CCl₄ is in accordance with their role in fibrosis.

In conclusion, transcriptomic analysis of PCLS successfully identified the fibrotic potential of CCl₄ as opposed to APAP. The application of PCLS as ex vivo model to identify early biomarkers to predict the fibrogenic potential of toxic compounds should be further explored.
2.1 INTRODUCTION

Paracetamol (APAP) and carbon tetrachloride (CCl₄) are two well-known model hepatotoxins. The mechanism of liver toxicity for both compounds is a multicellular phenomenon [66, 67]. Chronic exposure to CCl₄ in vivo leads to necrosis and subsequently to fibrosis. In contrast, APAP induces necrosis but no fibrosis [68]. It remains to be established why CCl₄ induces fibrosis and APAP does not. The elucidation of this difference could lead to more insight into the mechanism of fibrosis and may also be used to find new early biomarkers for fibrosis. If such differences in the mechanism of injury between APAP and CCl₄ could be mimicked in vitro, this would enable the study of these processes in man by using human tissue. This would allow prediction of fibrogenic effects in man, thereby circumventing the issue of possible species differences, which arise when using animal models.

The study of fibrosis in vitro requires a model with an intact liver architecture that can mimic the multicellular mechanism of this process. One such model is precision-cut liver slices (PCLS), as it has all the different liver cell types in their original architecture. This PCLS model system has been validated extensively for over a decade [69, 70, 71, 72, 73]. Although the process of fibrosis in vivo is considered to be the result of chronic exposure, it has been shown that CCl₄ treatment leads to induction of biomarkers for hepatic stellate cell activation in liver slices, as early as after 16 hours of exposure, indicating that the early phase of fibrosis can be detected in this ex vivo system [72, 74]. In addition, in PCLS, the other cell types involved in fibrosis, such as hepatocytes, endothelial and Kupffer cells, remain viable and functional during culture and can be activated, as has been shown in studies using endotoxin [75, 76, 77, 78] or bile acids [79, 80]. As the validation of the rat PCLS model is greatly supported by comparison with in vivo data, which is largely confined to animal studies and is scarcely available for human liver, we studied the gene expression in rat PCLS after treatment with CCl₄ and APAP. Although we do not make the comparison with in vivo data in this paper, we know that in rats in vivo CCl₄ induces liver necrosis and fibrosis and that APAP does induce only necrosis. Microarray analysis of rat liver treated with CCl₄ and APAP in vivo and in PCLS, using a commercial gene expression in vivo database, showed that rat PCLS can predict the toxicity and at least part of the pathology observed in vivo [71]. Data of human PCLS are currently being collected and will be analyzed and published in the future.

In the present study we performed further transcriptomic analysis from the data of the above-mentioned experiments with the rat PCLS model to characterise the gene expression profiles induced by APAP and CCl₄ and to elucidate whether a gene expression pattern related to early fibrosis could be detected for CCl₄ but not for APAP. We performed a comparison analysis with respect to the regulated genes, and also analysed upstream regulators, which could possibly be responsible for the observed gene expression changes. If prediction of long-term toxicity appears to be feasible at an early time point using PCLS, this model would contribute greatly to reducing and refining animal experimentation and to reducing costs of toxicity testing.
2.2 Materials and Methods

Microarray data of APAP and CCl₄ from our earlier published transcriptomic study using rat PCLS was used [71]. In these experiments rat PCLS were exposed to CCl₄ [72, 74] and APAP [71]. RNA was isolated and the RNA processing and hybridization was performed as described [72, 74, 71]. These methods are described only briefly here; see for details these two references.

Rat Liver Slice Preparation    Rat livers from male Wistar rats (Harlan, Zeist, The Netherlands) were harvested under anesthesia with isoflurane and stored at 4°C in University of Wisconsin organ preservation solution (UW, Dupont Critical Care, Waukegan, IL, USA) until slicing (max 15 min). Precision-cut liver slices (diameter 8 mm, thickness 250 µm) were prepared using a Krumdieck tissue slicer in ice-cold Krebs-Henseleit buffer, pH 7.4, supplemented with glucose to a final concentration of 25 mM, saturated with carbogen (95% O₂/5% CO₂). Slices were stored at 4°C in UW until the start of the experiment [71].

Rat Liver Slice Experiments    Slices were pre-incubated individually in 6-well culture plates, each slice in 3.2 ml Williams Medium E with glutamax-1 (Gibco, Invitrogen, Paisley, Scotland) supplemented with 25 mM D-glucose and 50 g/ml gentamycin (Gibco, Invitrogen) (WEGG medium) under carbogen atmosphere at 37°C for 1 hour, while gently shaken (90 times/min). After pre-incubation the slices were transferred to fresh WEGG medium. The experiments with APAP were performed in 6-well plates with 3.2 ml medium [71], while CCl₄ slices were incubated in 25 ml Erlenmeyer flasks containing 5 ml medium [72, 74]. The toxic dose was selected at a 60 to 90% decrease in ATP levels with respect to corresponding controls. The PCLS were exposed to 2.5 mM APAP. CCl₄ was spotted in an amount of 5 µl on a filter paper, which was attached to the stopper with a needle and was situated above the liquid phase as described earlier [72, 74]. Since the toxic concentration of CCl₄ at 16 h already showed a relatively large ATP depletion, this 16 h time point was chosen in contrast to 24 h APAP samples. Three different livers were used for each experiment; for RNA isolation 3 slices were pooled and quickly frozen in liquid nitrogen and stored at -80°C.

Isolation of RNA for Microarray Analysis    Total RNA was isolated from three combined slices from each experiment with the use of Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration and quality was determined with use of the NanoDrop (ND-1000 spectrophotometer) and the agilent technology (Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano kit). Before processing the purity of RNA was determined by measuring E₂₆₀/E₂₈₀ and the Agilent RNA Integrity number (RIN) [81]. The E₂₆₀/E₂₈₀ values from all samples were >1.8. The RIN values were >8, with one exception: one 16 h control for CCl₄ with a RIN value of 7.8. If necessary, RNA was cleaned-up by extraction with phenol/chloroform/isoamylalcohol, followed by a second extraction with chloroform/isoamylalcohol and precipitation with ethanol and lithium chloride.

RNA Processing and Hybridization    Double-stranded cDNA was synthesized from 5 µg total RNA using the Custom Superscript ds cDNA synthesis kit (Invitrogen, Carlsbad, CA) and used as a template for the preparation of biotin-labeled cRNA with use of the Bioarray HighYield RNA Translabeling kit (T7) (Enzo Life Sciences, Inc,
2.2 Materials and Methods

After fragmentation at 1µg/µl according to the manufacturer’s protocol, biotin-labeled cRNA (10 g) was hybridized at 45 C for 16-17 hours to the RGU34A array (Affymetrix, Santa Clara, CA). Following hybridization, the arrays were washed, stained with phycoerythrin-streptavidin conjugate (Molecular Probes, Eugene, OR), and the signals were amplified by staining the array with biotin-labeled anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) followed by phycoerythrin-streptavidin. The arrays were laser scanned with a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions.

**Microarray Data Pre-processing and Analysis**

Normalization of the microarray data was performed by RMA normalization using MicroarrayRUS v.1.0 software [82]. Control genes are removed from the rest of the analysis. Rank product analysis of the normalized data was performed using MicroarrayRUS v.1.0 software [83]. A list of differentially expressed genes was created using criteria of fold change of 1.5 and multiple hypothesis adjusted p-value 0.05 and used as input for pathway analysis in Ingenuity software.

**Gene Expression Dynamics Analysis**

Gene Expression Dynamics Inspector (GEDI) transforms high-dimensional gene expression data into distinct two-dimensional (2D) color patterns. The graphical output of GEDI gives the visual representation of the samples. The metagene signature of each sample is represented in a grid of 26 × 25 tiles; each of the tiles contains genes that are highly correlated with each other. The tiles are arranged such that each tile is also correlated with the adjacent tiles. Thus, it allows a global first-level analysis of the data to observe the response due to the effect of a drug. Pattern analysis of the data was performed by GEDI software (default settings) to understand the global transcriptomic changes induced by compounds [84].

**Pathway and Network Analysis**

Ingenuity Pathway Analysis (“http://www.ingenuity.com/products/ipla”) was used to determine pathways and networks that could describe the toxicity of APAP and CCl4. Rat liver tissue specific pathway analysis was performed.

**Custom-Made Toxlist Analysis**

Custom-made toxlists were also considered for analysis along with toxlists present in the Ingenuity knowledgebase. Custom fibrosis toxlists were created based on our observation that the toxlist for hepatic fibrosis in IPA does not include all the known genes related to the fibrotic process. Custom-made toxlists for fibrosis were generated by using the information from different sources such as SABioscience fibrosis PCR chip (“http://www.sabiosciences.com/rt_pcr_product/HTML/PARN-120Z.html”) and an in-house custom made chip with genes related to collagen synthesis and breakdown [85]. SABioscience provides a custom fibrosis PCR array chip containing genes related to fibrosis. Finally a combined gene list is generated from the genes of IPA fibrosis toxlist, SABioscience and in-house custom array of collagen-metabolism related genes. IPA uses Fisher exact test to calculate statistical significance.

**Upstream Regulator Analysis**

Upstream Regulator Analysis was performed to identify the upstream regulators that may be responsible for the observed gene expression
changes. IPA predicts which upstream regulators are likely to be activated or inhibited, which in turn could explain the gene expression changes observed in the dataset. IPA makes predictions on upstream regulators using a z-score algorithm [86]. The z-score value is calculated using the gene expression patterns of the genes downstream of an upstream regulator. P-value of overlap indicates the statistical significance of genes in the dataset that are downstream of the upstream regulator but unlike the z-score, it does not take into consideration up or down-regulation of genes in dataset. Upstream regulators with a z-score greater than 2 or smaller than -2 and p-value of 0.05 were considered significant and their role in fibrosis was studied.

**CAUSAL NETWORK ANALYSIS** Causal Networks are small hierarchical networks of regulators that control the expression of the regulated genes. This helps to identify novel upstream regulators, because they influence the expression either directly or indirectly via intermediate regulators [86]. Causal networks generated by IPA with an activation z-score greater than 2 or smaller than -2 and p-value of overlap 0.05 were considered significant and their role in fibrosis was studied.

2.3 RESULTS

**GENE EXPRESSION DYNAMICS ANALYSIS** The expression values of all the replicates of each group were averaged to make a representative self-organizing map of each group using GEDI (Fig. 4). The regions where gene expression patterns are different are indicated in boxes (as in A, B, C, D).

The change in color of the cells in those boxes can be interpreted as change in expression intensity of genes in those cells. The APAP control (A) group at 24h is similar to the CCl₄ control group at 16h (C), although there are slight differences, which may be ascribed to a difference in incubation time. Expression patterns of slices treated with each of the compounds were considerably different from their corresponding controls. Although visually small differences were noticed between the APAP treated (B) and CCl₄ treated (D) group, the gene expression intensity levels were different in those highlighted regions. Since each tile in the GEDI map contains a group of correlated genes, such minor differences could indicate the difference between compounds. Thus, GEDI analysis revealed evidence on the global level that APAP and CCl₄ have induced characteristic expression profiles, which can be explained possibly by the different mechanisms of toxicity of both compounds.

**GENE SELECTION BY RANK PRODUCT ANALYSIS** The rank product analysis method has been showed to perform well to identify the regulated genes, particularly for datasets that have a low number of samples or a high level of noise [83]. Regulated genes were selected using a criterion of fold change of 1.5 and adjusted p-value of < 0.05 using the MicroarrayRUS program. The Vennplex program [87] was used to observe the commonly up- or down-regulated or contra-regulated genes. Contra-regulated genes are those that are up-regulated in one compound and down-regulated in another compound. In total 174 genes were deregulated in the case of CCl₄ and 116 genes were deregulated in the case of APAP, while 63 genes were similarly regulated in the presence of both compounds, of which 19
Figure 4: Global Transcriptomic changes induced by APAP and CCl\textsubscript{4}

Comparative Gene Expression Dynamics Inspector (GEDI) analysis of averaged values of expression values from biological replicates of APAP control (A) and treated (B), CCl\textsubscript{4} control (C) and treated (D) samples. The Gene density map (E), indicates the number of genes in each cell (white cells indicate the absence of genes in those cells). The expression intensity is indicated in (F). Regions of difference are indicated with squares.

genes were up-regulated and 44 genes were down-regulated. No contra-regulated genes were found as shown in Fig. 5.

The list of genes that are specifically deregulated by either APAP or CCl\textsubscript{4}, and also the genes that are regulated in common due to APAP and CCl\textsubscript{4} treatment are shown in Supplementary data S3.

**Custom Fibrosis Toxlist Analysis**

Custom fibrosis toxlist analysis was performed using IPA with the toxlists obtained from the different sources described in the materials and methods section. Toxlists regulated with a p-value of < 0.05 were considered significant (corresponds to -log [p value] of 1.3). This analysis indicated that the set of genes in the fibrosis toxlist from SABioscience was significantly regulated by CCl\textsubscript{4} but not by APAP (Fig 6). The other custom toxlists were not found to be significantly regulated as a whole. However, differences between the effects of CCl\textsubscript{4} and APAP were found for some individual genes for the other toxlists as well as for the combined fibrotic toxlist.

Genes such as JUN, LITAF, MAPK6, PLAT and SERPINE1 from the SABioscience fibrotic toxlist were regulated by CCl\textsubscript{4}.

In Table 2 the genes from the fibrosis toxlists that are specifically deregulated in CCl\textsubscript{4} are listed. Except for PLAT, all the genes mentioned in Table 2 that are known to play a role in the fibrotic process are specifically deregulated by CCl\textsubscript{4} and not by APAP. Furthermore, the markers specific for hepatic stellate cells such as KLF6 and CRYAB are up-regulated by CCl\textsubscript{4}. 

2.3 Results

![Image of Figure 4: Global Transcriptomic changes induced by APAP and CCl\textsubscript{4}](image-url)
Acute toxicity of CCl₄ but not of paracetamol induces a transcriptomic signature of fibrosis in precision-cut liver slices.

**Figure 5:** Venn diagram comparison of regulated genes between APAP and CCl₄. Genes regulated with a fold change criterion of 1.5 and multiple hypothesis adjusted p-value of <0.05 were compared. Genes regulated due to CCl₄ and APAP are indicated in yellow and blue circles respectively and the genes regulated in common are indicated in the region of interaction between yellow and blue circles.

**Figure 6:** Custom fibrosis toxlist analysis of APAP and CCl₄ regulated genes. Horizontal line indicates the threshold corresponding to p-value of <0.05 and ND means that no regulated genes were found in common with the corresponding toxlist and hence overrepresentation was not assessed.
Table 2: Fibrosis related genes, which are specifically regulated in case of CCl₄

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Fold change</th>
<th>Role in fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAT *</td>
<td>Tissue plasminogen activator</td>
<td>2</td>
<td>PLAT increases extracellular matrix (ECM) degradation [88, 89]</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>Serpin peptidase inhibitor</td>
<td>3.7</td>
<td>Progression of fibrosis [88]</td>
</tr>
<tr>
<td>MAPK6</td>
<td>Mitogen-activated protein kinase 6</td>
<td>2.5</td>
<td>MAPK pathway is known to be involved in the activation of HSC [90]</td>
</tr>
<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
<td>2.6</td>
<td>JunD is implicated in the regulation of hepatic stellate cell (HSC) activation and liver fibrosis [91, 92]</td>
</tr>
<tr>
<td>LITAF</td>
<td>Lipopolysaccharide-induced TNF factor</td>
<td>2.5</td>
<td>Induced in hepatic stellate cells [93]</td>
</tr>
<tr>
<td>KLF6</td>
<td>Kruppel-like factor 6</td>
<td>11</td>
<td>Induced in response to early fibrosis [94]</td>
</tr>
<tr>
<td>CRYAB</td>
<td>Crystallin, alpha B</td>
<td>10.5</td>
<td>Induced in activated hepatic stellate cells [95]</td>
</tr>
</tbody>
</table>

Genes known to play a role in fibrosis and are specifically regulated in CCl₄ with corresponding fold change and their role in fibrosis. * PLAT is also regulated due to APAP.
Table 3: Upstream regulators predicted to be activated or inhibited due to CCl₄ treatment

<table>
<thead>
<tr>
<th>Upstream Regulator</th>
<th>Molecule Type</th>
<th>Predicted State</th>
<th>Activation z-score</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDN1</td>
<td>cytokine</td>
<td>Activated</td>
<td>2.2</td>
<td>4.38E-03</td>
</tr>
<tr>
<td>EGF</td>
<td>growth factor</td>
<td>Activated</td>
<td>2.7</td>
<td>1.05E-04</td>
</tr>
<tr>
<td>ERN1</td>
<td>kinase</td>
<td>Activated</td>
<td>2.2</td>
<td>1.23E-04</td>
</tr>
<tr>
<td>IL1A</td>
<td>cytokine</td>
<td>Activated</td>
<td>2.4</td>
<td>1.67E-02</td>
</tr>
<tr>
<td>IL1B</td>
<td>cytokine</td>
<td>Activated</td>
<td>2.2</td>
<td>1.71E-06</td>
</tr>
<tr>
<td>NUPR1</td>
<td>transcription regulator</td>
<td>Activated</td>
<td>2.8</td>
<td>2.11E-02</td>
</tr>
<tr>
<td>STAT1</td>
<td>transcription regulator</td>
<td>Activated</td>
<td>2</td>
<td>5.33E-02</td>
</tr>
<tr>
<td>TGFA</td>
<td>growth factor</td>
<td>Activated</td>
<td>2.4</td>
<td>2.53E-04</td>
</tr>
<tr>
<td>TGFB1</td>
<td>growth factor</td>
<td>Activated</td>
<td>3.8</td>
<td>6.40E-04</td>
</tr>
<tr>
<td>TNF</td>
<td>cytokine</td>
<td>Activated</td>
<td>3.6</td>
<td>1.00E-09</td>
</tr>
<tr>
<td>ACOX1</td>
<td>enzyme</td>
<td>Inhibited</td>
<td>-2.6</td>
<td>1.38E-11</td>
</tr>
<tr>
<td>HNF1A</td>
<td>transcription regulator</td>
<td>Inhibited</td>
<td>-2.6</td>
<td>4.55E-10</td>
</tr>
<tr>
<td>HNF4A</td>
<td>transcription regulator</td>
<td>Inhibited</td>
<td>-3.0</td>
<td>7.28E-09</td>
</tr>
<tr>
<td>PPARA</td>
<td>ligand-dependent nuclear receptor</td>
<td>Inhibited</td>
<td>-2.6</td>
<td>7.66E-22</td>
</tr>
<tr>
<td>PPARD</td>
<td>ligand-dependent nuclear receptor</td>
<td>Inhibited</td>
<td>-2.6</td>
<td>1.28E-10</td>
</tr>
<tr>
<td>PPARG</td>
<td>ligand-dependent nuclear receptor</td>
<td>Inhibited</td>
<td>-2.4</td>
<td>8.27E-07</td>
</tr>
<tr>
<td>RXRA</td>
<td>ligand-dependent nuclear receptor</td>
<td>Inhibited</td>
<td>-2.6</td>
<td>6.74E-09</td>
</tr>
<tr>
<td>TFAM</td>
<td>transcription regulator</td>
<td>Inhibited</td>
<td>-2</td>
<td>1.18E-04</td>
</tr>
</tbody>
</table>

Upstream regulators with their corresponding molecular type, predicted activation or inhibition state, activation z-score and p-value of overlap.

**Upstream Regulator Analysis**

Upstream Regulator Analysis revealed that several regulators are involved in controlling the expression of the genes regulated by CCl₄ and APAP treatment. These regulators and their target molecules (regulated genes) are shown in Table 3 and Table 4 respectively. ACOX1, HNF4A and HNF1A are predicted to be inhibited and TGFB1 is predicted to be activated due to treatment with both compounds. However, PKD1, AKT1, BRCA1, FGF2 and TP53 are unique to APAP treatment and PPARA, NUPR1, IL18, STAT1, TGFA, TNF, PPARD, PPARG, RXRA, TFAM, EDN1, EGF, IL1A and ERN1 are unique to CCl₄ treatment.

TGFB1, a growth factor known to play an important role in hepatic fibrosis during hepatic stellate cell activation, is predicted to be activated due to both APAP and CCl₄. However, 28 genes regulated by CCl₄ treatment are causally linked to activation of TGFB1, whereas only 19 genes are causally linked due to APAP treatment, resulting in a much lower p value for CCl₄ than for APAP. Mechanistic networks for visualizing the causal link between TGFB1 activation and their corresponding target molecules are shown in Fig. 7 and 8.
2.3 RESULTS

Figure 7: Upstream regulator TGFB1 and corresponding regulated target genes due to CCl4 treatment. TGFB1 is predicted as activated and early fibrotic genes such as JUN, MAPK6, PLAT SERPINE1, are causally linked to TGFB1. Their up-regulation due to CCl4 is consistent with TGFB1 activation.

Regulated genes are highlighted in red or green color, based on up or down-regulation respectively; the intensity of the color increases with degree of fold change. The upstream regulator (TGFB1) is indicated as activated (orange) or inhibited (blue) with the color intensity increasing with the confidence level. Orange lines with arrowheads at the end of interactions indicate activation, while blue lines with bars indicate inhibition. Yellow lines indicate inconsistent findings and grey line indicate effect not predicted.
Figure 8: Upstream regulator TGFβ1 and corresponding regulated target genes due to APAP treatment

TGFβ1 is predicted as activated but none of the fibrosis related genes are causally linked to TGFβ1 except for PLAT. Regulated genes are highlighted in red or green color, based on up or down-regulation respectively; the intensity of the color increases with degree of fold change. The upstream regulator (TGFβ1) is indicated as activated (orange) or inhibited (blue) with the color intensity increasing with the confidence level. Orange lines with arrowheads at the end of interactions indicate activation, while blue lines with bars indicate inhibition. Yellow lines indicate inconsistent findings and grey line indicate effect not predicted.
Table 4: Upstream regulators predicted to be activated or inhibited due to APAP treatment

<table>
<thead>
<tr>
<th>Upstream Regulator</th>
<th>Molecule Type</th>
<th>Predicted State</th>
<th>Activation z-score</th>
<th>p-value of overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1</td>
<td>kinase</td>
<td>Activated</td>
<td>2.2</td>
<td>4.77E-03</td>
</tr>
<tr>
<td>BRCA1</td>
<td>transcription regulator</td>
<td>Activated</td>
<td>2.4</td>
<td>2.67E-04</td>
</tr>
<tr>
<td>FGF2</td>
<td>growth factor</td>
<td>Activated</td>
<td>2.2</td>
<td>4.45E-02</td>
</tr>
<tr>
<td>TGFB1</td>
<td>growth factor</td>
<td>Activated</td>
<td>2.2</td>
<td>1.27E-03</td>
</tr>
<tr>
<td>TP53</td>
<td>transcription regulator</td>
<td>Activated</td>
<td>2.0</td>
<td>1.32E-02</td>
</tr>
<tr>
<td>ACOX1</td>
<td>enzyme</td>
<td>Inhibited</td>
<td>-2.6</td>
<td>1.80E-04</td>
</tr>
<tr>
<td>HNF1A</td>
<td>transcription regulator</td>
<td>Inhibited</td>
<td>-2.0</td>
<td>2.16E-09</td>
</tr>
<tr>
<td>HNF4A</td>
<td>transcription regulator</td>
<td>Inhibited</td>
<td>-2.9</td>
<td>1.33E-04</td>
</tr>
<tr>
<td>PKD1</td>
<td>ion channel</td>
<td>Inhibited</td>
<td>-2</td>
<td>2.34E-02</td>
</tr>
</tbody>
</table>

Upstream regulators with their corresponding molecular type, predicted activation or inhibition state, activation z-score and p-value of overlap.

**Causal Network Analysis**

Causal Network Analysis reveals small hierarchical networks of interacting regulators, which explains the gene expression changes observed in the dataset. In the case of treatment with CCl₄, many of these networks involve multiple regulators combined with a master regulator. For example HNF₄A interacts with HNF₁A and CTNNB₁, which in turn have causal relations with 23 genes in the network. All the significant networks which are predicted to be activated or inhibited due to both APAP and CCl₄ are reported in supplementary data S2. There were 24 causal networks predicted as activated or inhibited in case of CCl₄ but only five such significant networks are reported in the case of APAP. Although the overall number of regulated genes affected by exposure to the toxic concentration of APAP or CCl₄ was more or less the same, there were large differences with respect to the resulting causal networks.
2.4 DISCUSSION

In this study, we performed the comparative analysis of the gene expression profiles of rat PCLS induced by APAP and CCl₄, which are known to induce toxicity by different mechanisms. Comparison was performed using gene expression patterns, regulated genes, and pathway and upstream regulator analysis of regulated genes.

Pattern analysis using GEDI revealed characteristic expression patterns due to a toxic concentration of each of the compounds with respect to the corresponding control (Figure 4). The relatively small differences between the APAP and CCl₄ induced expression patterns could be due to the different mechanisms of toxicity, including the onset of fibrosis due to CCl₄. Similarities in the changes in expression patterns may be explained by the fact that both compounds induce necrosis after short-term treatment, which was concluded previously both for CCl₄ after 16 h and for APAP after 24 h by ToxShield prediction [71].

Comparison of the regulated genes showed that there is good overlap among the regulated genes and there is also a significant number of genes uniquely regulated due to either APAP or CCl₄ (Figure 5). Some of those genes uniquely regulated due to CCl₄ treatment include fibrosis related genes (Table 2). With the exception of PLAT, these fibrosis related genes were not found to be regulated by treatment of rat PCLS with the necrosis-inducing compounds iproniazid and bromobenzene either (data not shown).

Bovenkamp et al. showed that in non-treated rat liver slices, the expression of hepatic stellate cell specific markers such as αB-crystallin, KLF6 and heat shock protein 47 remained constant during incubation for 24 h, indicating quiescence of HSC. In contrast, incubation with CCl₄ led to a time- and dose-dependent increase in mRNA expression of these markers. In accordance with these findings, in our microarray analysis, αB-crystallin, KLF6 and HSP47 were also up-regulated [72, 74]. Regulation of these hepatic stellate cell markers indicates initiation of the fibrotic processes in CCl₄ treated slices.

The growth factor TGFβ₁ plays a key role in fibrosis via hepatic stellate cell activation. When we focused on genes involved in the TGFβ₁ signaling pathway and their change in expression due to treatment with APAP or CCl₄, we found that more genes involved in the TGFβ₁ pathway are regulated by CCl₄ than by APAP: 19 target genes in the dataset have an expression direction consistent with activation of TGFβ₁ due to CCl₄ treatment (Figure 7), in contrast only 13 target genes have an expression direction consistent with activation of TGFβ₁ due to APAP treatment (Figure 8). This observation gives an indication of early fibrotic processes activated within 16 h due to a toxic concentration of CCl₄. From the TGFβ₁ network resulting from regulated genes due to APAP or CCl₄ treatment, it can be seen that, in the case of exposure to CCl₄, genes that have a clear role in fibrosis such as JUN, LITAF, MAPK6, PLAT and SERPINE1 are causally linked to TGFβ₁ and are up-regulated, but in case of exposure to APAP, only PLAT is up-regulated and causal relation to TGFβ₁ is seen. It has been reported that TGF- induces SERPINE₁ expression via pSmad2L/C signaling and promotes extracellular matrix deposition in myofibroblasts, thereby accelerating liver fibrosis [96]. This observation indicates a substantial involvement of TGFβ₁ in the toxicity process initiated by CCl₄ but not by APAP.

Upstream regulator analysis revealed several regulators that control the expression of regulated genes. Predicted activation or inhibition of those regulators and their relation with hepatic fibrosis is outlined here. While a few upstream regulators were activated or inhibited similarly due to APAP and CCl₄ treatment, some regulators were particularly activated...
or inhibited by either APAP or CCl$_4$. Toxic concentrations of APAP and CCl$_4$ are known to induce necrosis in short-term treatment; however, transcriptomic analysis in this study revealed significant differences with respect to genes involved in the toxicity process.

ACOX1, a ROS producing enzyme, is down-regulated due to CCl$_4$ treatment in our study. A mouse study established the role of ACOX1 in fibrosis showing that ACOX1 knockout mice develop fibrosis $[97, 98]$. ACOX1 is down-regulated due to CCl$_4$ treatment and also 17 genes which have causal relation to it are regulated. In contrast, in the case of exposure to APAP, ACOX1 expression is not altered. HNF$_4$A and HNF$_1$A transcription factors are predicted to be inhibited due to both APAP and CCl$_4$ treatment. HNF$_4$A is involved in differentiation of hepatocytes and is known to be down-regulated in fibrosis $[99]$. Furthermore, HNF$_1$A also plays a role in the differentiation of hepatocytes. Therefore, the predicted inhibition of HNF$_4$A and HNF$_1$A due to both APAP- and CCl$_4$-induced necrosis is possibly related to their general role in the process of differentiation of hepatocytes after induction of necrosis. Inhibition of PPARG and RXRA activity, which is predicted due to CCl$_4$ treatment but not to APAP, has been shown to lead to hepatic stellate cell proliferation $[100]$. PPARD agonistic activity leads to anti-fibrotic effects in a fibrosis mouse model induced with CCl$_4$ $[101]$. These results are in accordance with ours, supporting the fibrogenic potential of CCl$_4$, which is not seen in APAP. PPARA, known to attenuate oxidative stress, one of the most important processes responsible for fibrosis, and to have anti-fibrotic effects in a rat study $in vivo$ $[102]$, is predicted to be inhibited by treatment with CCl$_4$. Thus, the predicted inhibition of PPARA due to CCl$_4$ also accords with its fibrotic effect. TFAM, a mitochondrial transcription factor, is predicted to be inhibited in the case of CCl$_4$ treatment in PCLS, whereas it was observed to be activated in maintenance of quiescent hepatic stellate cells $[103]$. No such predicted inhibition of TFAM was seen due to APAP treatment. PKD1, which is predicted to be inhibited in the case of treatment with APAP but not CCl$_4$, has not been described as playing a potential role in fibrosis up to now. NUPR1, a transcription factor regulating apoptosis is up-regulated due to CCl$_4$ and was found to be involved in fibrotic changes due to CCl$_4$ treatment in mice $[104]$. ERN1 which is known to play a role in the adaptive response to endoplasmic reticulum (ER) stress is also predicted to be activated due to CCl$_4$ $[105]$. In addition, inflammatory cytokines such IL1A, IL1B and TNF are predicted to be activated due to CCl$_4$. TNF is also known to play a role in HSC proliferation. Overall, transcription factors such as PPARD, PPARA, TFAM, NUPR1, ERN1 and TNF, which are all known to play a role in fibrosis, are predicted to be activated or inhibited only due to CCl$_4$.

Causal network analysis revealed many causal networks which with either one regulator or with groups of interconnected regulators, may have accounted for the observed gene expression changes. Comparison of the causal networks regulated by APAP and CCl$_4$ revealed interesting differences since many of the networks were specifically found to be activated or inhibited by CCl$_4$, whereas in the case of APAP few such networks were observed. For instance, causal network containing NOCR2 transcription factor as master-regulator in connection with other intermediate regulators such as AR, ESR1, HNF$_4$A, NCOR$_2$, NR4A1, PGR, PPARG, RXRA, THR2 and VDR could account for the observed expression changes of 35 genes regulated due to treatment with CCl$_4$. TGFB1 was shown to up-regulate NCOR2 expression $[106]$. Causal networks helps to identify novel upstream regulators such as NCOR2 with possible role in hepatic fibrosis, which in-turn helps to derive mechanistic hypothesis. Since the aim of this paper was to explore the differences between APAP and CCl$_4$ in fibrosis, the causal networks were not explored further in detail.
A recent paper reported the characterization of the proteins involved in hepatic stellate cell activation by CCl₄ in vitro [107]. When we compared the genes regulated in our transcriptomic analysis with the proteins regulated due to CCl₄ treatment in hepatic stellate cells, 17 genes were found in common with the proteins including CRYAB and SERPINE1. Furthermore, a comparison with genes regulated by APAP treatment and proteins involved in HSC activation revealed that 11 are similarly regulated, but none of them overlap with the fibrosis related genes (Supplementary data S3).

In conclusion, in this study we focused on the changes in gene expression profiles due to treatment of PCLS with APAP or CCl₄ and found that those changes reflect the characteristic difference between these compounds in their ability to induce liver fibrosis after chronic dosing in vivo. This study indicates that transcriptomic analysis of PCLS can be used to identify the fibrotic potential of toxic compounds after short-term exposure. Further studies with more fibrotic and non-fibrotic compounds are needed to verify this finding and to identify a set of biomarkers that can be used in drug-induced toxicity screening.

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