Nephron Toxicity Profiling via Untargeted Metabolome Analysis Employing a High Performance Liquid Chromatography-Mass Spectrometry-based Experimental and Computational Pipeline*

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Background: Drug toxicity testing calls for in vitro assays as alternatives to animal models.

Results: OpenMS and KNIME are applicable for processing of HPLC-MS data sets to reveal metabolic changes upon chloroacetaldehyde treatment of kidney cells.

Conclusion: Most significant changes are related to oxidative stress.

Significance: Comprehensive multiomics studies support the risk assessment at an early stage of drug development.

Untargeted metabolomics has the potential to improve the predictivity of in vitro toxicity models and therefore may aid the replacement of expensive and laborious animal models. Here we describe a long term repeat dose nephrotoxicity study conducted on the human renal proximal tubular epithelial cell line, RPTEC/TERT1, treated with 10 and 35 μmol·liter⁻¹ of chloroacetaldehyde, a metabolite of the anti-cancer drug ifosfamide. Our study outlines the establishment of an automated and easy to use untargeted metabolomics workflow for HPLC-high resolution mass spectrometry data. Automated data analysis workflows based on open source software (OpenMS, KNIME) enabled a comprehensive and reproducible analysis of the complex and voluminous metabolomics data produced by the profiling approach. Time- and concentration-dependent responses were clearly evident in the metabolomic profiles. To obtain a more comprehensive picture of the mode of action, transcriptomics and proteomics data were also integrated. For toxicity profiling of chloroacetaldehyde, 428 and 317 metabolite features were detectable in positive and negative modes, respectively, after stringent removal of chemical noise and unstable signals. Changes upon treatment were explored using principal component analysis, and statistically significant differences were identified using linear models for microarray assays. The analysis revealed toxic effects only for the treatment with 35 μmol·liter⁻¹ for 3 and 14 days. The most regulated metabolites were glutathione and metabolites related to the oxidative stress response of the cells. These findings are corroborated by proteomics and transcriptomics data, which show, among other things, an activation of the Nrf2 and ATF4 pathways.

Metabolites represent the final products of all cellular and regulatory processes, providing a snap shot of the sum of all cellular process occurring at that moment (1). Consequently, to study the risks and/or consequences of exposure of humans to chemicals or drugs, untargeted metabolome analysis offers a viable approach to study the biological effects upon exposure to such compounds. However, because ethical reasons preclude the study of biological consequences of chemicals in human subjects (2), suitable models, including cultured human cells, can be employed to derive characteristic biomarkers and/or biochemical pathways that can be used for extrapolation to humans.

Untargeted metabolomics aims at analyzing the broadest possible range of metabolites present in a biological sample to obtain an unbiased view of the organization and function of biological systems and to characterize their responses to changes in their environment such as drug or chemical treatment (3–6). Cultured cells can be utilized as very discriminative sensors for external stimulation and are very well suited for studying the action of drugs and their toxicity in vitro (7, 8). In vitro cell culture systems have numerous advantages over animal models, because they are easier to control as well as to handle, and biochemical changes are easier to interpret (8, 9).

Different analytical methods such as NMR spectroscopy or MS in combination with separation by HPLC or GC are the key technologies applied to metabolomics (7, 10, 11). An NMR-based metabolic profiling method has previously been

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Metabolic Drug Toxicity Profiling by HPLC-MS

employed to screen the acute effects of six chemicals in the human renal proximal tubule epithelial cell line, RPTEC/TERT1 (12). The study showed the usefulness of metabolomics (MTX) for discrimination of toxins. A more complex experimental design was utilized for toxicity profiling of long term cyclosporine A exposures (2, 13). Cells were cultured on porous filters and treated with two concentrations of compounds for 14 days. At set intervals (1, 3, and 14 days), transcriptomics (TCX), proteomics (PTX), and MTX were performed. Cellular metabolome analysis was conducted via direct infusion electrospray ionization mass spectrometry lacking the potential of compound separation and identification via HPLC or GC, which makes qualitative and quantitative analysis quite difficult.

A comprehensive quantitative metabolic profiling therefore requires the combination of HPLC with high resolution mass spectrometry (HRMS) (14). The high information content of data generated by HRMS implies a strong need for automatable computational processing of the otherwise unmanageable amount of data (15). In addition to vendor software packages, numerous open source software tools are available for data processing like XCMS (16), MetaAlign (17), MzMine (18), PeakML/mzMatch (19), and OpenMS/TOPP (20–22). Here we present a fully automated data analysis pipeline based on OpenMS, a comprehensive open source software library for the analysis of MS data, KNIME (23), an open source workflow engine that enables visual and interactive data analysis, and R, a programming platform for statistical analysis (24). Metabolite identification is based on matching the accurate masses against the theoretical mass of databases such as Human Metabolome Database (HMDB) or METLIN, followed by confirmation of hits by fragmentation using MS/MS and mass against the theoretical mass of databases such as Human Metabolome Database (HMDB) or METLIN, followed by confirmation of hits by fragmentation using MS/MS and retention time matching using a reference standard (26).

We performed a large scale profiling study of the long term nephrotoxicity of chloroaocetaldehyde. Chloroaocetaldehyde is of particular interest as a metabolite of ifosfamide, which is a pharmaceutical used for the treatment of cancer (25). Finally, proteomics and transcriptomics data were also integrated to increase the depth of understanding of the metabolomic alterations brought about by chloroaocetaldehyde exposure.

Experimental Procedures

Chemicals and Materials—Acetonitrile for LC-MS was purchased from VWR (Radnor, PA). Reagent grade formic acid, methanol, and chloroaocetaldehyde were obtained from Sigma-Aldrich. High purity water was produced using a Milli-Q Integral 3 purification system from Merck Millipore. Standard substances used for identification were obtained from Merck (amino acids) or from Sigma-Aldrich (all other compounds).

Cell Culture—The human renal proximal tubule cell line RPTEC/TERT1 was obtained from Evercyte GmbH (Vienna, Austria). The RPTEC/TERT1 cells (26) were cultured on 1-μm PET 24-mm tissue culture inserts for MTX and PTX, and on 0.2-μm aluminum oxide 25 mm inserts for TCX and differentiated in a serum-free hormonally defined medium for a minimum of 10 days before exposure commencement (2). During this time, the cells cover the surface of the filter, become contact-inhibited and quiescent, and develop a stable barrier function of approximately 150 Ohm·cm² (27). Because no further proliferation takes place after differentiation, each well has the same number of cells (28). The mature monolayers were exposed to either a low (10 μmol·liter⁻¹) or high (35 μmol·liter⁻¹) concentration of chloroaocetaldehyde or control medium, on both the apical and the basolateral sides. The cells were treated every 24 h for 14 days and lysed on days 1, 3, and 14 in ice-cold methanol for MTX/PTX or RLT buffer (RNasea mini kit; Qiagen) for TCX. Epithelial monolayer integrity was monitored every day by transepithelial electrical resistance measurement and showed no breach in monolayer integrity and thus no significant alteration in cell numbers throughout the experiments at all time points and all treatments (transepithelial electrical resistance results for these experiments have been previously published in Ref. 29). All experiments were conducted on three biological replicates.

Metabolite Extraction and HPLC-MS Sample Preparation—The cell culture medium was removed, and the cells were washed twice with ice cold PBS (Sigma-Aldrich), followed by an additional very fast washing step with ammonium bicarbonate (185 mmol·liter⁻¹, 289 mM, pH 7.8; Sigma-Aldrich). To preserve metabolites, 750 μl of ice-cold methanol containing deuter-alanine (50 μmol·liter⁻¹) as an internal standard, was added. Two wells were pooled in an Eppendorf tube, vortexed, and sonicated with an in-probe sonicator from Branson Ultrasonics Corporation (Danbury, CT) for 20 s to fully homogenize the sample. Finally, the cell lysates were centrifuged at 4 °C and 14,000 rpm for 10 min. The supernatant was used for MTX and was stored at −80 °C until analysis.

For HPLC-MS analysis, the samples were thawed and 1:5 diluted with MilliQ water. Additionally, a pool sample was prepared by blending 20 μl of each available sample followed by the same dilution step. This pool sample was used as quality control (QC) in the sample queues and was injected after every ninth run. The sample injection order was randomized.

High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)—An HPLC system (Model Accela II; Thermo Fisher Scientific, Bremen, Germany) was coupled to an Orbitrap mass spectrometer (Model Exactive; Thermo Fisher Scientific) equipped with a heated electrospray ion source operating in the positive or negative ion mode. Reversed phase HPLC separations were performed with a 100 × 2.1-mm inner diameter Hypersil Gold aQ column (Thermo Fisher Scientific) packed with 1.9-μm particles. Water (A) and acetonitrile (B) each containing 0.10% (v/v) formic acid were used as eluents. The flow rate was 0.30 ml·min⁻¹, and gradient elution was carried out as follows: 100% A for 1.5 min followed by a linear gradient to 100% B in 6.5 min and holding for 2 min. In the end, a re-equilibration step was performed for 3.0 min at 0.0% B. The total run time was 13 min. The column temperature was maintained at 30 °C, and 2.7 μl of sample was injected per run.

3The abbreviations used are: MTX, metabolomics; Nh2f2, nuclear factor E2-related factor 2; TCX, transcriptomics; PTX, proteomics; QC, quality control; PCA, principal component analysis; LIMMA, linear models for microarray assays; HMDB, Human Metabolome Database; ATF4, activation transcription factor 4; ASNS, asparagine synthetase (glutamine-hydrolyzing); HRMS, high resolution mass spectrometry.
An electrospray ionization heater temperature of 350 °C was chosen and sheath gas and auxiliary gas flow rates were set to 20 and 5.0 arbitrary units in positive mode and to 35 and 10 in the negative mode, respectively. A sprayer voltage of 3.0 kV was applied, and the resolution was set to 50,000. The mass range was split into a low mass range m/z 50–200 and a high mass range m/z 200–1000 and measured in two separate runs. The MS parameters were tuned for each method and polarity separately with selected metabolites. For the low mass range the best intensities were achieved with a capillary voltage of 25 V, tube lens voltage of 70 V, and skimmer voltage of 14 V for positive and negative mode with changed polarity. The high mass range measurements were conducted with a capillary voltage of 55 and –45 V, tube lens voltage of 115 and –105 V, and skimmer voltage of 22 and –24 V for positive and negative mode, respectively.

The MS/MS analysis was performed with a quadrupole Orbitrap mass spectrometer (Model Q Exactive; Thermo Fisher Scientific). For fragmentation experiments an S-lens RF level of 50, a sprayer voltage of 3.5 kV, and capillary and heater temperature of 350 °C were chosen. A sheath gas flow of 50 and auxiliary gas of 15 for positive electrospray ionization mode and 40 and 10 for negative mode, respectively, were employed. In Selected ion monitoring mode (m/z 100–1000), the resolution was set to 70,000, and for the data-dependent fragmentation, a resolution of 17,500 was chosen. In both cases, the automatic gain control target was 10^6, and the maximum injection time was set to 25 ms. The quadrupole isolation window was 1.0 m/z, and a normalized collision energy of 25 arbitrary units was used. The data-dependent fragmentation settings were an isolation width 0.1% Da, resulting in an intensity threshold for fragmentation of 4 × 10^6, the apex trigger was set to 2–5 s, and molecules charged higher than 3 were excluded.

Data Evaluation—Both chromatograms and mass spectra were recorded with Xcalibur 2.2 (Thermo Fisher Scientific). The generated HPLC-MS data were further processed with ProteoWizard 3.0.4243 (30), OpenMS 1.11 (22), KNIME 2.9.2 (23), R 2.15.1 (24), and SIMCA 13.0.3 (Umetrics, Umeå, Sweden).

Transcriptome Analysis—Transcriptomic analysis was performed on Illumina® HT 12 v4 BeadChip arrays (~47,000 transcripts). Deregulated probes were identified using a moderated one-way analysis of variance with a Benjamini-Hochberg corrected p value with a cutoff value of 0.05. A moderated two-sided t test with a Benjamini-Hochberg correction for multiple testing was calculated for the remaining probes. Both steps were carried out in R with the LIMMA package. The number of differentially expressed probes was calculated for each treatment condition based on a fold cutoff of 1.5 (0.58 log2 fold) and a p value cutoff of 0.001.

Proteome Analysis—A detailed description of transcriptomics and proteomics analysis can be found in Refs. 2 and 13. Briefly, peptides for PTX were labeled after protein extraction and digestion with isobaric tags for relative and absolute quantitation (iTRAQ) and measured by HPLC-MS. The HPLC system was directly coupled to a linear ion-trap Orbitrap mass spectrometer (Model LTQ Orbitrap XL; ThermoFisher Scientific) with a nano-electrospray ionization source operated in positive ionization mode. A total of nine 4plex iTRAQ sets were analyzed, each comparing a low and a high chloroacetaldehyde dose to a control sample at a given time point (1, 3, or 14 days) with three biological replicates. Protein identification and quantification workflows were implemented in OpenMS (version 1.9) (21) and TOPPAS (31). Statistical analysis was performed using the isobar package (32) and custom R code. Significantly increased or decreased proteins were reported if two p values were below 0.05: the first was based on a Cauchy distribution fitted to the global protein ratio distribution, and the second was based on the spread of peptide ratios contributing to the protein ratio.

Results

Experimental Design—To map both concentration, as well as time-dependent effects of drug exposure, our experimental design involved treatment of the RPTEC/TERT1 cells with three different conditions, namely null control (0.0 μmol·liter⁻¹ chloroacetaldehyde, labeled C), low concentration (10 μmol·liter⁻¹ chloroacetaldehyde, labeled L), and high concentration (35 μmol·liter⁻¹ chloroacetaldehyde, labeled H). The cells were treated for 14 days with medium exchange every 24 h and were harvested at three different time points, specifically after 1, 3, and 14 days of exposure. Each experiment was performed in triplicate, which led to 3 × 3 × 3 = 27 samples to characterize the biological effects of drug treatment. Analysis of the samples was preceded with one blank injection (20% methanol in water) followed by two analyses of the QC sample (pool sample diluted 1:5 with water). The injection order of the 27 metabolite extracts was randomized; blank samples were analyzed after three metabolite extracts, whereas QC samples were injected after six metabolite extracts. This resulted in a total set 44 raw data files (27 samples, 11 blanks, and 6 QC) to be sequentially analyzed.

Data Handling, Quality Control, and Statistical Analysis—Computational workflows established in OpenMS and KNIME were utilized for the automated processing of HPLC-MS data. These open source tools allowed the detection and linking of features by means of tailor-made downstream analysis including restrictive filtering criteria to leave only those features that were detected with high confidence for further statistical evaluation.

All steps of data handling and analysis are graphically illustrated in Fig. 1. Data evaluation was performed essentially in two stages. First, all features related to potential metabolites were detected, aligned, and statistically analyzed in the full scan high resolution Orbitrap MS data to reveal features differentially regulated upon chloroacetaldehyde treatment (steps 1–14 in Fig. 1). Second, differentially regulated features were identified based on the criteria outlined in Ref. 33, also incorporating additional targeted metabolite identification experiments using MS/MS (steps 15 and 16 in Fig. 1).

The 44 raw files were converted into mzML format using the ProteoWizard tool msconvert (step 1 in Fig. 1). The individual data files were then centroided by using OpenMS PeakPicker-HiRes (step 2 in Fig. 1), followed by feature detection by means of FeatureFinderMetabo (step 3 in Fig. 1), which detects and quantifies metabolic features within an HPLC-MS map (20).
We used a noise threshold of 250 for the negative mode and 500 for the positive mode and a signal to noise ratio of 3 for feature detection. Detected features may represent metabolites in different charge states and/or as various adduct species. Feature detection was performed for each HPLC-HRMS map individually. After identifying features in all 44 HPLC-HRMS runs, corresponding features were matched using FeatureLinker-UnlabeledQT (steps 4 and 5 in Fig. 1). Features were merged into a cluster across runs if the differences in retention time and m/z were smaller than chosen thresholds (≤10 s and ≤5 ppm here).

This resulted in a so-called consensus map representing all detected metabolite features across runs. The consensus map was exported as a consensus XML (step 8 in Fig. 1). This file was preprocessed with the tool FileFilter (step 6 in Fig. 1), which was used to remove features with a retention time smaller than the column flow-through time. The TextExporter (step 7 in Fig. 1) converted the file format into comma separated values for further data processing.

Differential features in the consensus XML file were then identified in a computational workflow implemented in KNIME. The consensus file contained several thousand features, most of which could be assigned to background signals and chemical noise. Therefore, all features for which the median blank intensity was more than 20% of the sample intensity (average intensity of the corresponding feature in the 11 blank analyses) were removed (step 9 in Fig. 1). To normalize the data, we performed a global normalization based on the notion that for a comparison between any two maps only a small number of features will be differentially regulated, whereas most of the features will have ratios of approximately 1:1. Utilizing this, we performed pairwise comparisons between each map and the map with the highest number of features. For each pairwise comparison, we computed all ratios, excluding the ones below 0.67 and above 1.5 (step 10 in Fig. 1). Subsequently, we computed the average intensity ratio and multiplied all feature intensities in the respective map by the inverse (robust regression normalization).

In addition, all features were removed that were not present in at least six study samples with a secondary criterion that they had to be present in at least two of the three biological replicates (step 11 in Fig. 1). The QC samples were used in the automated data filtering workflow to account for signal (in)stability. The respective feature had to be present in five of six pool samples, and the relative standard deviation of the signal had to be less than 25% to be considered as a significant feature. Steps 9–11 were carried out by R scripts organized as separate nodes in KNIME, so that the filtering and normalization could be performed automatically. Missing features in the filtered data set (~4% for positive and negative mode) were assigned an intensity value of 1 (to avoid division by 0 in the ratios), if they are absent in all three biological replicates.

Aiming for differential features, the filtered data sets (step 12 in Fig. 1) were evaluated using multivariate and univariate statistical approaches (step 13 in Fig. 1). Principal component analysis (PCA) was performed to identify grouping patterns and to see outliers using the statistics software package SIMCA (step 14 in Fig. 1). All data sets were scaled to unit variance with an inverse square root block weight prior to principal component analysis. Differentially regulated features were determined using linear models for microarray data (LIMMA) (34) using the R integration in KNIME. The p values were corrected for
Additionally, in nearly all of these cases, the log2 fold changes of features were differentially regulated both on days 3 and 14. In the negative mode, 45 features that were differentially expressed on day 3 were also differentially regulated features at days 3 and 14 for both positive and negative mode, respectively, after strict filtering (level 4). The final output of the computational workflow was a filtered feature list displaying the differentially expressed metabolites (if positively identified) or features (for unknowns) for the respective time point and treatment (step 17 in Fig. 1).

Results of Differential Metabolome Analysis—HPLC-MS data obtained from the chloroacetaldehyde nephrotoxicity study were evaluated according to the data processing workflow presented in Fig. 1, which resulted in 428 and 317 features for positive and negative mode, respectively, after strict filtering meeting the quality criteria described above. The filtered features lists were used for PCA to visually explore the data sets. No outliers were detected in the distance to model plot (DModX plot); therefore the model was built on all 27 samples. As depicted in Fig. 2, the PCA scores plot revealed a clear difference between the cell extracts treated with the high concentration of chloroacetaldehyde for 3 and 14 days (H03 and H14) compared with the other samples of the study (C01, C03, C14, L01, L03, L14, and H01). Moreover, positive and negative mode showed comparable trends as are shown in Fig. 2 (a and b).

Significantly regulated metabolites were evaluated by pairwise comparison of control to low or high concentration for each time point using LIMMA, which has a higher discriminatory power than t tests without increasing the false positive rate, especially when dealing with small sample sizes (39). The returned p values were corrected for multiple testing according to Hochberg and Benjamini (35). LIMMA clearly confirmed the qualitative findings of the initial PCA analysis. It also revealed that the majority of significant changes occurred for the high concentration treatment for 3 and 14 days (Fig. 3).

A considerable overlap could be observed between the differentially regulated features at days 3 and 14 for both positive and negative ionization mode. In positive mode, 42 of the 72 features that were differentially expressed on day 3 were also differentially expressed on day 14. In the negative mode, 45 features were differentially regulated both on days 3 and 14. Additionally, in nearly all of these cases, the log2 fold changes showed the same trend for days 3 and 14, which means that the respective feature was consistently up- or down-regulated on both time points. In most of these cases, day 14 showed more pronounced effects than day 3 (37 of 52 features for positive mode and 34 of 45 features for negative mode).

Feature Identification—All features of the filtered data set were first annotated by accurate mass search against the HMDB within a 5-ppm mass window using the OpenMS tool AccurateMassSearch. Approximately 20–30% of all features were annotated searching for [M+H]+/[M-H]-, exclusively. Allowing common electrospray ionization adducts (40), the number of annotations rose to ~50–60% of the filtered data set. Unambiguous identification was sought only for significantly regulated features, and therefore, targeted fragmentation experiments were carried out to confirm metabolite structures based on tandem mass spectra. In most cases, substance confirmation was established by comparison of retention time, mass spectrum, and tandem mass spectrum of the sample and an authentic reference standard, which led to the identification of 13 significantly regulated metabolites (level 1). The corresponding chromatographic and mass spectrometric data are collected in Tables 1 and 2. Additionally, two features could be identified based on the comparison of retention time and accurate mass.
with that of a reference substance (level 2). Finally, the tandem mass spectra of three features, for which no standard substance was available, were matched to the reference spectra contained in METLIN (37, 41) (level 3).

Features identified as described above are referred to as metabolites. Altogether, 18 differentially regulated metabolites were found, and each of them showed a significant \( p \) value at least for H03 or H14 after performing LIMMA followed by Benjamini-Hochberg correction (Table 3).

The differentially regulated metabolites belong to different metabolite classes, such as amino acids, and glutathione pathway-related metabolites showing up-regulation, whereas purine nucleotides showed strong down-regulation. The trends of signal intensities for all 18 differentially regulated metabolites are graphically illustrated in Fig. 4. The \( p \) values and \( \log_2 \) ratios are collected in Table 3 along with the significantly regulated transcripts and proteins. The observed changes from all three omics disciplines were integrated for biological interpretation.

### Discussion

**Biological Effects of Chloroacetaldehyde Treatment Revealed by Combined TCX, PTX, and MTX—**Oxidative stress is a major component of proximal tubule injury induced by chemical oxidants or mitochondrial disrupters. Here we show that the metabolomic profiles upon chloroacetaldehyde treatment of RPTEC/TERT1 cells indicated major changes in the metabolites related to the defense against oxidative stress, in particular intermediates of the glutathione pathway, and in abundance of amino acids.

At the transcriptome level, there was a major impact on the Nrf2 and ATF4 pathways, which are involved in the oxidative stress response and the unfolded protein response, respectively. A major feature of Nrf2 stress response is to promote glutathione synthesis and recycling (2). The sharp increase in glutathione species (both reduced GSH and oxidized GSSG) was in accordance with the increase in both the catalytic and modifier subunits of the rate-limiting enzyme in the synthesis of GSH: \( \gamma \)-glutamyl cysteine synthetase (42). After 14 days of exposure

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**TABLE 1**

Identification data for compounds detected in the positive ionization mode

| Compound            | Molecular formula | \( m/z \) [M+H]* | \( t_R \) | Fragments | Error ppm |
|---------------------|-------------------|------------------|----------|-----------|-----------|
| Pyroglutamic acid   | C₅H₇NO₃          | 130.0499         | 85.47    | 84.0450   | 0.00      |
| Isoleucine          | C₆H₁₃NO₂          | 132.1019         | 106.64   | 86.0969   | 0.00      |
| Leucine             | C₆H₁₃NO₃          | 132.1019         | 114.85   | 86.0969   | 0.00      |
| Aspartic acid       | C₅H₁₀NO₄          | 134.0447         | 47.43    | 116.0346  | -0.75    |
| l-Carnitine         | C₇H₁₅NO₃          | 162.1125         | 50.76    | 74.0244   | 0.00      |
| Tyrosine            | C₉H₁₁NO₃          | 182.0812         | 127.82   | 60.0816   | 0.19      |
| Pantothenic acid    | C₉H₁₇NO₅          | 220.1178         | 263.21   | 147.0440  | -0.45    |
| Glycerophosphocholine | C₈H₁₆NO₃P       | 258.1099         | 48.85    | 104.1073  | -0.94    |
| Glutathione         | C₆H₁₁N₂O₅S       | 308.0908         | 75.35    | 179.0483  | -0.93    |
| Oxidized glutathione | C₂₀H₁₅N₂O₁₂S₂   | 613.1589         | 128.18   | 355.0732  | -0.51    |

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**FIGURE 3.** Number of significantly regulated features according to LIMMA for positive and negative mode.
to chloroacetaldehyde, both transcript and protein levels of the glutamate-cysteine ligase catalytic and modifier subunits were increased (Table 3). In addition, these two proteins were among the most impacted in the whole proteomic data set upon chloroacetaldehyde exposure.

\(\gamma\)-Glutamyltransferases (GGTs) contribute to the degradation of GSH, either by cleaving glutamate off the cysteinyl-glycine moiety or by transferring the resulting glutamyl group to a substrate protein. The transcripts of \(\gamma\)-glutamyltransferase 1, an extracellular GGT of particular importance in the proximal tubule, and GGTL2 (\(\gamma\)-glutamyltransferase light chain 2), a smaller gene encoding only a light GGT chain, were down-regulated at all time points in 35 \(\mu\)mol liter\(^{-1}\) chloroacetaldehyde treatment. A decrease in GGTL2 protein was also detected on day 14. Although it is unlikely that GGTL2 possesses GGT catalytic activity because this is carried out by the heavy GGT chain, it has been shown to bind glutamate, which could constitute a level of interference with other processes, including de novo GSH synthesis.

Several metabolic routes lead to the production of glutamate, the first building block in GSH synthesis. One source is the conversion of pyroglutamic acid (aka 5-oxoproline) to glutamate by OPLAH (5-oxoprolinase (ATP-hydrolyzing)). Although glutamate levels remained very stable over time except for a mild decrease on day 14 and pyroglutamatic acid levels were strongly increased on days 3 and 14, the mRNA levels of OPLAH were drastically decreased on days 3 (2.5 \(\log_2\) fold) and 14 (\(\log_2\) fold), suggesting a role of OPLAH in pyroglutamic acid accumulation. However, it is difficult to conclude on the impact of these transcriptional changes on OPLAH protein levels, because OPLAH was not detected in the proteomic analysis. Pyroglutamic acid itself can be formed nonenzymatically from glutamine, glutamate, and \(\gamma\)-glutamyl proteins (GGT products) but can also be the result of the degradation of \(\gamma\)-glutamyl cysteine (GSH synthesis intermediary) into cysteine and pyroglutamic acid by the enzyme \(\gamma\)-glutamylcystotransferase (43), which was up-regulated at the transcript level on day 1 only (0.63 \(\log_2\) fold over control). An increased production of this metabolite would withdraw \(\gamma\)-glutamyl-cysteine from glutathione synthesis but provide free cysteine, possibly for de novo protein synthesis. Fig. 5 shows the glutathione pathway containing the information of all three omics levels.

In addition to the glutathione pathway, Nrf2 also up-regulates a set of target enzymes involved in the enzymatic elimination of reactive oxygen species. In the transcriptomic data set, the genes for heme oxygenase 1 and NAD(P)H dehydrogenase quinone 1, encoding reactive oxygen species detoxifying enzymes, glutathione reductase, and members of the thioredoxin system that reduce oxidized thiols on proteins (thioredoxin and thioredoxin reductase 1) were all up-regulated. At the protein level, heme oxygenase 1 was strongly increased at all time points, whereas NAD(P)H dehydrogenase quinone 1 was increased on days 3 and 14.

Another pathway affected by chloroacetaldehyde is the unfolded protein response, in particular the branch governed by the transcription factor ATF4. We have previously shown that under certain cellular stresses, Nrf2 and ATF4 cooperate in GSH recycling. ATF4 drives the transcription of genes involved in the entry and biosynthesis of amino acids, the production of tRNA synthetases that present the amino acids to the ribosome during translation. ATF4 primes the cell to reactivate translation via up-regulation of eukaryotic initiation factor genes. Genes related to the response to endoplasmic reticulum stress (or unfolded protein response), notably driven by ATF4, were also strongly affected by chloroacetaldehyde, particularly on day 14. Among the up-regulated ATF4 targets were several amino acid transporters (SLC7A11 (solute carrier family 7; anionic amino acid transporter light chain, xc system; member 11); SLC7A5 (solute carrier family 7; amino acid transporter light chain, L system; member 5); SLC3A2 (solute carrier family 3; amino acid transporter light chain, xc system; member 5); and SLC1A5 (solute carrier family 1; amino acid transporter light chain, L system; member 5)), asparagine synthetase, and the glycyl-tRNA synthetase (GARS). On day 14, the tRNA synthetases for asparagine, leucine, and threonine were down-regulated (asparaginyl-tRNA synthetase, leucyl-tRNA synthetase, and threonyl-tRNA synthetase, respectively). Substrates of the up-regulated transporters include isoleucine, leucine, tyrosine, and glutamine, all of which were increased in the metabolomics measurements, and alanine, which was decreased. Interestingly, although the levels of the substrates of asparagine synthe-

### TABLE 2

| Compound                  | Molecular formula | \(m/z\) [M-H]\(^{-}\) | \(t_R\) | Fragments   | Error \(ppm\) |
|---------------------------|-------------------|------------------------|--------|-------------|--------------|
| Alanine                   | \(C_5H_{10}N_2O_2\) | 88.0404                | 47.4   |             | 0.00         |
| O-Phosphoethanolamine     | \(C_5H_{10}N_2O_2\) | 140.0119               | 46.6   | 122.907     | 0.71         |
| Glutamate                 | \(C_5H_{10}N_2O_2\) | 145.0620               | 47.63  |             | 0.47         |
| Glutamate                 | \(C_5H_{10}N_2O_2\) | 146.046                | 48.67  | 102.057     | 0.68         |
| AMP                       | \(C_9H_{15}N_3O_5P_2\) | 346.0563               | 74.80  | 128.035     | 1.37         |
| ADP                       | \(C_9H_{15}N_3O_5P_2\) | 426.0228               | 87.10  | 158.9254    | 1.73         |
| CDP-Ethanolamine          | \(C_{11}H_{20}N_4O_2P_2\) | 445.0538              | 50.20  | 201.9671    | 1.53         |

- \(m/z\): Mass-to-charge ratio
- \(t_R\): Retention time
- Fragments and Error: Data for compounds detected in the negative ionization mode.
tase were increased (glutamine) and decreased (aspartic acid) at both time points, the levels of the two products of the reaction (glutamic acid and asparagine) were comparable with time-matched controls, suggesting a tight regulation of the levels of these two amino acids and a probable consumption of glutamine and aspartic acid by other processes.

In addition to these impacts on glutathione and amino acids, the metabolomics data set revealed changes in more isolated compounds (see Table 3).

### TABLE 3
Differentially regulated metabolites, proteins, and mRNA transcripts upon chloroacetaldehyde treatment

| HMDB entry | Metabolites | FC_H03 | p_H03 | FC_H14 | p_H14 |
|------------|-------------|--------|-------|--------|-------|
| HMDB00267  | Pyroglutamic acid; [M+H]+ | 3.38 | 0.006 | 1.83 | 0.003 |
| HMDB00687  | L-Leucine; [M+H]+ | 0.69 | 0.047 | 0.92 | 0.011 |
| HMDB00172  | L-Isoleucine; [M+H]+ | 0.64 | 0.001 | 0.96 | 0.010 |
| HMDB00151  | L-Asparatic acid; [M+H]+ | -1.98 | 0.002 | -3.44 | 0.0001 |
| MDB00062  | L-Galaine; [M+H]+ | 0.27 | 0.367 | -2.35 | 0.002 |
| HMDB00158  | L-Tyrosine; [M+H]+ | 0.40 | 0.191 | 0.72 | 0.035 |
| HMDB00210  | Pantothenic acid; [M+H]+ | 0.54 | 0.094 | 0.98 | 0.048 |
| HMDB00086  | Glycerophosphocholines; [M+H]+ | 0.69 | 0.0070 | 0.75 | 0.029 |
| HMDB00125  | Glutathione; [M+H]+ | 2.46 | 0.0020 | 3.39 | 0.0002 |
| HMDB00337  | Oxidized glutathione; [M+H]+ | 2.80 | 0.1230 | 2.86 | 0.0005 |
| HMDB00161  | L-Alanine; [M+H]+ | -1.54 | 0.003 | -3.13 | 0.003 |
| HMDB00224  | O-Phosphoethanolamine; [M+H]+ | 0.03 | 0.892 | -1.33 | 0.006 |
| HMDB00641  | L-Glutamine; [M+H]+ | 0.97 | 0.008 | 0.61 | 0.027 |
| HMDB00089  | Cytidine; [M+H]+ | 2.03 | 0.013 | 2.61 | 0.001 |
| HMDB00045  | Adenosine monophosphate; [M+H]+ | -2.06 | 0.044 | -1.29 | 0.008 |
| HMDB01397  | Guanosine monophosphate; [M+H]+ | -1.74 | 0.035 | -0.75 | 0.042 |
| HMDB01341  | ADP; [M+H]+ | -0.93 | 0.192 | -1.02 | 0.026 |
| HMDB01564  | GDP-Enzymehemoglobin; [M+H]+ | 0.09 | 0.680 | -1.04 | 0.004 |

**Acession Proteins**

| P07099 | epoxide hydrolase 1, microsomal | 0.85 | 0.207 | 1.68 | 0.042 |
| P48506 | glutamate-cysteine ligase, catalytic subunit | - | - | 2.63 | 0.024 |
| P48507 | glutamate-cysteine ligase, modifier subunit | - | - | 2.46 | 0.035 |
| Q14300 | gamma-glutamyltransferase light chain 2 | - | - | 0.053 | 0.290 |
| OT75046 | butyrobetaine (gamma), 2-octanoylglycerol | 0.96 | 0.098 | 0.84 | 0.034 |
| P69091 | hemoglobin (deoxyhemoglobin) | 2.42 | 0.022 | 2.33 | 0.026 |
| P15559 | NAD(P)H dehydrogenase, quinone 1 | 1.54 | 0.035 | 2.33 | 0.031 |

**Gene ID Genes**

| OPLAH | 5-oxoprolinase (ATP-hydrolyzing) | -2.00 | 1.7E-16 | -1.06 | 4.7E-10 |
| GSS | glutathione synthetase | 0.11 | - | -0.01 |
| GPX1 | glutathione peroxidase 1 | 0.89 | 1.5E-09 | 1.92 | 2.4E-12 |
| GPX2 | glutathione peroxidase 2 (gastrointestinal) | 1.98 | 4.5E-14 | 2.61 | 8.3E-17 |
| TNDC12 | thioredoxin domain containing 12 (endoplasmic reticulum) | 0.90 | 3.5E-03 | 1.05 | 3.0E-08 |
| GT1 | gamma-glutamyltransferase 1 | 0.98 | 2.1E-06 | 0.00 | 3.3E-05 |
| GTLTC2 | gamma-glutamyltransferase light chain 2 | -1.08 | 8.6E-05 | -0.61 | 7.1E-05 |
| PANK1 | pantothenate kinase 1 | -0.46 | 4.8E-04 | -0.75 | 6.9E-08 |
| BBOX1 | butyrobetaine (gamma), 2-octanoylglycerol | 0.23 | 3.5E-08 | -2.00 | 3.4E-15 |

**Gene ID Genes (Nrff targets)**

| TNFR1 | thioredoxin 1 | 1.88 | 1.3E-11 | 2.64 | 4.9E-21 |
| HMNOX1 | hemoglobin (deoxyhemoglobin) | 1.80 | 2.5E-13 | 2.96 | 8.8E-19 |
| NQO1 | NAD(P)H dehydrogenase, quinone 1 | 3.11 | 8.4E-17 | 2.29 | 1.8E-16 |
| GCLM | glutamate-cysteine ligase, modifier subunit | 1.35 | 3.4E-14 | 1.55 | 2.4E-18 |
| SOD1 | SOD1 | 1.18 | 3.8E-11 | 1.29 | 6.2E-16 |
| TXN | thioredoxin | 0.73 | 9.6E-08 | 1.19 | 2.6E-13 |
| GCLC | glutamate-cysteine ligase, catalytic subunit | 0.87 | 3.9E-06 | 0.45 | 3.6E-07 |
| GSR | glutathione reductase | 0.48 | 1.7E-08 | 0.35 | 2.3E-09 |

**Gene ID Genes (ATF4 targets)**

| SLC37A11 | solute carrier family 7, member 11 | 2.23 | 6.4E-15 | 2.97 | 3.0E-19 |
| SLC7A5 | solute carrier family 7, member 5 | 0.48 | 4.9E-03 | 2.95 | 2.2E-16 |
| SLC7A2 | solute carrier family 7, member 2 | 1.62 | 1.1E-12 | 1.88 | 3.0E-14 |
| ANS2 | asparagus synthetase (glutamine-hydrolyzing) | 0.76 | 4.0E-03 | 1.32 | 1.5E-06 |
| GARS | glycyl-RNA synthetase | 0.39 | 2.0E-02 | 0.61 | 3.5E-10 |
| TARS | threonyl-RNA synthetase | 0.86 | 7.1E-01 | 0.31 | 2.4E-02 |
| LARS | leucyl-RNA synthetase | -0.21 | 1.5E-02 | -0.64 | 1.0E-09 |
| NARS | asparaginyl-RNA synthetase | -0.15 | 1.7E-01 | -0.79 | 2.5E-09 |

*Compound ID was established by accurate mass, fragment spectra and retention time of a reference substance (level 1).

*Compound ID was established by accurate mass and retention time of a reference substance (level 2).

*Compound ID was established by accurate mass and fragment spectra (level 3) (according to standards proposed in Ref. (33)).

*Reported values are log2 ratios relative to controls.

*p value calculated using R package LIMMA followed by Benjamini-Hochberg correction.

*Average sample p value calculated using R package isobar.

*p value calculated using moderated t test followed by Benjamini-Hochberg correction.
metabolites of particular interest to proximal tubule cells, namely carnitine and pantothenic acid. Upon chloroacetaldehyde exposure, we observed a strong down-regulation of L-carnitine at day 14, as well as of γ-butyrobetaine hydroxylase, the enzyme catalyzing the formation of L-carnitine from γ-butyrobetaine, at genome and proteome levels. γ-Butyrobetaine hydroxylase is highly expressed in the proximal tubule in vivo and is also increased in RPTEC/TERT1 upon contact inhibition and dedifferentiation (28). Additionally we have shown that this gene is one of the most strongly and frequently deregulated compounds in a study with 9 chronic nephrotoxins (27). A decrease in γ-butyrobetaine hydroxylase expression suggests an impairment of fatty acid shuttling into the mitochondria, which would result in a reduction in fatty acid beta oxidation rates. For fatty acids to be transported by the carnitine shuttle, they must first be conjugated to CoA, which is primarily produced via the degradation of pantothenic acid, initiated by the pantothenate kinase 1 enzymes. Pantothenic acid was increased upon chloroacetaldehyde treatment and because it is not thought to be produced by mammalian cells. Increased intracellular level of pantothenic acid is thus likely due to the impaired fatty acid transport as less CoA, and therefore less pantothenic acid is consumed. This alteration suggests a decrease in the capacity of the cells to perform beta oxidation, which could be caused by reactive oxygen species induced mitochondrial injury or other types of mitochondrial injury.

Interestingly we have previously reported a dose- and time-dependent increase in supernatant lactate in RPTEC/TERT1 cells exposed to chloroacetaldehyde, which may point to a shift to glycolysis as an energy source (44). Supplementation in both carnitine and pantothenic acid has been shown to protect cells against oxidative stress (45–47). Wojtczak and Slyshenkov (47) also described for Ehrlich ascites tumor cells that pantothenic acid was only protective when de novo glutathione synthesis was enabled, thus suggesting a direct role for pantothenic acid in glutathione metabolism regulation.

Taken together, the results demonstrate that deep mechanistic insights of chemically induced cellular injury can be
achieved using differentiated cells together with standardized experimental and computational workflows. We describe the first application of the new computational tool FeatureFinder-Metabo in combination with automated filtering routines implemented in KNIME for large scale metabolomics profiling studies employing HPLC-HRMS. The developed workflows return a list of trustworthy and statistically relevant metabolite features that could be mapped onto several pathways relevant for the biological response to drug treatment. Integrating metabolomics with proteomics and transcriptomics data helped corroborate these findings and allowed a better understanding of the influenced pathways.

The overall picture reveals that chloroacetaldehyde applied at a high concentration of 35 μmol·liter⁻¹ for an extended exposure period (more than 3 days) has considerable effects in the form of oxidative stress, endoplasmic reticulum stress (unfolded protein response) and a possible impairment of fatty acid shuttling into the mitochondria of RPTEC/TERT1 cells.

**Author Contributions**—C. G. H., P. J., W. D., and O. K. conceived and coordinated the study and wrote the paper. C. R., R. R., and M. R. designed, performed, and analyzed the metabolomics experiments and wrote the paper. S. R. and P. H. designed, performed, and analyzed the proteomics and transcriptomics experiments, respectively. A. L., A. W., and P. J. performed the cell culture experiments and worked on the biological interpretation of the data. C. R., R. R., O. K., and C. G. H. designed and implemented the computational workflows in this study. All authors reviewed the results and approved the final version of the manuscript.

**FIGURE 5.** Influence of chloroacetaldehyde treatment on glutathione metabolism. The bar plots show the mean metabolite signal intensities for control, as well as high concentration treatment at days 3 and 14. The error bars represent 95% confidence intervals deduced from biological triplicates. The regulation of involved enzymes is indicated by ○, which indicates unchanged (arbitrary cutoff 0.5- and 1.5-fold change), or ↑, which indicates increased/up-regulated, or ↓, which indicates decreased/down-regulated) on the right side for TCX and on the left side for PTX (for all three time points comparing control to high concentration). If a corresponding gene or protein was not detected, it is marked with ×. Fold change values for metabolites, proteins, and genes are given in Table 3.
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