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'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans

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ABSTRACT

Acetaminophen is the primary cause of acute liver toxicity in Europe/USA, which led the FDA to reconsider recommendations concerning safe acetaminophen dosage/use. Unfortunately, the current tests for liver toxicity are no ideal predictive markers for liver injury, i.e. they only measure acetaminophen exposure after profound liver toxicity has already occurred. Furthermore, these tests do not provide mechanistic information. Here, 'omics techniques (global analysis of metabolomic/gene-expression responses) may provide additional insight.

To better understand acetaminophen-induced responses at low doses, we evaluated the effects of (sub-)therapeutic acetaminophen doses on metabolite formation and global gene-expression changes (including, for the first time, full-genome human miRNA expression changes) in blood/urine samples from healthy human volunteers.

Many known and several new acetaminophen-metabolites were detected, in particular in relation to hepatotoxicity-linked, oxidative metabolism of acetaminophen. Transcriptomic changes indicated immune-modulating effects (2 g dose) and oxidative stress responses (4 g dose). For the first time, effects of acetaminophen on full-genome human miRNA expression have been considered and confirmed the findings on mRNA level.

'Omics techniques outperformed clinical chemistry tests and revealed novel response pathways to acetaminophen in humans. Although no definitive conclusion about potential immunotoxic effects of acetaminophen can be drawn from this study, there are clear indications that the immune system is triggered even after intake of low doses of acetaminophen. Also, oxidative stress-related gene responses, similar to those seen after high dose acetaminophen exposure, suggest the occurrence of possible pre-toxic effects of therapeutic acetaminophen doses. Possibly, these effects are related to dose-dependent increases in levels of hepatotoxicity-related metabolites.

Introduction

An extensively studied hepatotoxicant in humans is acetaminophen (APAP), a readily available over-the-counter drug of which the exact pharmacological mode of action is still partially unknown. APAP is the most common cause of acute liver toxicity in Europe and the US (Kuehn, 2009; Lee, 2007). Therefore, the FDA recently reconsidered recommendations concerning the single adult APAP dose and the maximum total dose for 24 h. Also, packaging and labeling regulations were changed and elimination of prescription APAP combination products has been recommended (FDA, 2009b; FDA, 2009a; FDA, 2011).

Diagnosis of liver toxicity is based on evaluating liver function through serum parameters such as alanine aminotransferase (ALT). Unfortunately, none of these tests represent ideal biomarkers since they are positive only when pronounced liver injury has already occurred (Watkins et al., 2006; Watkins, 2009). This implies that these assays cannot predict the onset or severity of liver injury. In this respect, the more sensitive 'omics techniques (analysis of genomic and metabolomic responses to chemical challenges) may contribute (Beger et al., 2010; Harrill and Rusyn, 2008; Vinayavekhin et al., 2010; Vlaanderen et al., 2010). Significant gene expression changes in rat blood cells upon administration of APAP were detected well

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below liver damage levels as diagnosed by classical parameters (Bushel et al., 2007). The additional advantage of these technologies is their ability to study changes at a molecular level. This provides additional information on compound-specific modes-of-action which may precede classic, often toxicological endpoints (Kroeger, 2006).

Rodent-based data on *in vivo* metabolomic/transcriptomic responses in liver (Aleksunes et al., 2007; Beyer et al., 2007; Coen et al., 2004; Heinloth et al., 2007; Kikkawa et al., 2006; Morishita et al., 2006), plasma (Aleksunes et al., 2007; Coen et al., 2004), blood (Bushel et al., 2007), serum (Chen et al., 2008; Chen et al., 2009) and/or urine (Chen et al., 2008) after APAP exposure are abundantly available. Data on transcriptomic responses to low, therapeutic APAP doses in humans however are scarce. The transcriptome responses in lymphocytes from a few human APAP suicide cases, which provide insight in overtly toxic responses, are similar to those described in animal studies (Bushel et al., 2007). Fannin et al. (2010) showed that in humans a 4 g single dose of APAP caused changes in the transcriptome of peripheral blood cells, mainly involving the down-regulation of oxidative phosphorylation genes. These gene expression changes are similar to those seen after high doses of APAP. At these high doses these responses are associated with APAP-induced toxicity and liver injury. However, in the study of Fannin et al. overt hepatotoxicity did not occur. In addition, it is of interest to note that, although the liver is the primary site for APAP toxicity, the blood gene expression profile can actually be used as a surrogate to monitor liver function/toxicity upon APAP administration (Heinloth et al., 2007; Huang et al., 2010; Fannin et al., 2010; Umbright et al., 2010) and even APAP dose levels (Bushel et al., 2007).

In addition, changes in microRNA (miRNA) expression profiles in plasma and/or liver after high dose APAP exposure were recently investigated in 2 rodent studies and 1 human study (Fukushima et al., 2007; Starkey Lewis et al., 2011; Wang et al., 2009). In rodents, changes in miRNA expression profiles were observed hours before any other toxic effects on morphology, or changes in ALT or lactate levels could be detected. To our knowledge, no data on full-genome human miRNA expression responses after APAP intake are currently available.

In view of the FDA's considerations mentioned above, we aimed at exploring the effects of low APAP doses in humans. We hypothesized that blood transcriptomics (both mRNA and miRNA analysis) and blood/urine metabolomics are sufficiently sensitive and robust for this purpose. Furthermore, this is the first study which considers human full-genome miRNA expression-profile changes as possible indicators of APAP-induced molecular effects.

Methods

Human subjects

The experimental protocol was approved by the Medical Ethics Committee of the University Hospital Maastricht and all participants signed an informed consent. Subjects were healthy, aged 48.4 ± 4.5, 5 males and 2 females, 1 being a smoker. Two weeks prior and during the intervention subjects did not take any additional medication.

Study design and sample collection

Subjects took 0.5, 2 or 4 g of APAP over 24 h in the form of crushed 500 mg APAP tablets (Healthypharm) through oral self administration; daily doses were spread over equal amounts and taken at 6 h intervals. Interference due to repeated APAP exposure was prevented by a wash-out period of at least 2 weeks between experimental rounds. All subjects successfully completed the study, with the exception of one subject (a 49 year old non-smoking female), who participated in the 0.5 g and 2 g dose-round and was replaced by another subject (41 year old non-smoking male) in the 4 g dose-round. Sampling was performed as indicated in Fig. 1. The time at which experimental rounds were started was kept constant to minimize the effects of diurnal variation between rounds.

Clinical chemistry

Plasma from each donor collected before (T0) and after (T25) APAP intake was analyzed for; ALT, total bilirubin, alkaline phosphatase, gamma glutamyl transpeptidase, lactate dehydrogenase and albumin using standard clinical liver chemistry tests.

Metabolomic analyses

For analysis of serum and urine samples, a combination of NMR, LC–MS and/or GC–MS has been used (Lommen et al., 2011a).

Urine. A mixture of urine:acetone (1:4) was prepared (3-fold). Samples were centrifuged; supernatant was collected, dried under nitrogen and reconstituted (30 mg/ml) in 99.95% D_2O. Urinary excretion of APAP and its metabolites was normalized against measured urinary concentrations of androstanetriol/androstan-diol-one-glucoronide, etiocholanone-glucoronide and androstenetriol-glucoronide which are expected to be constant. Part of the sample was stored for LC–MS analysis. To the remainder 1 M phosphate buffer (90:10; v:v; pH = 7) in 99.95% D_2O was added for NMR analysis.

Serum. From serum of coagulated blood (30 min., room temperature) a polar and an apolar metabolite fractions were extracted as follows:

Polar fraction. Preparation is identical to the procedure as described for urine samples (see above), with the exception that the reconstituted sample was extracted with deuterated chloroform to obtain a lipid free sample. From the resulting D_2O fraction a part was kept for LC–MS analysis. The NMR sample was prepared as with urine.

Apolar fraction. To 1 ml of serum, 10 ml of CD_3OD:$CDCl_3$ in a proportion 2:1 (v:v) was added and centrifuged. The chloroform-phase was collected, dried under nitrogen, reconstituted in 1 ml of deuterated chloroform and re-extracted with 99.95% D2O. A fraction of this sample was used for NMR analysis and the remainder was stored for GC–MS analysis after derivatization.

Data analyses/metabolite identification. NMR data were pre-processed and aligned using an in-house developed program (Lommen et al., 1998). GC–MS and LC–MS data were pre-processed and aligned by means of metAlign (Lommen, 2009).

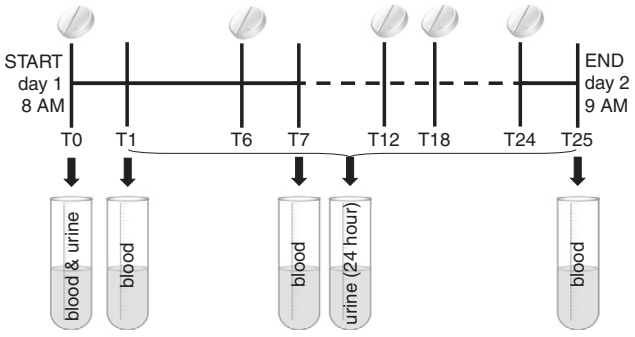

Fig. 1. Sampling and dosing schedule. Pills indicate APAP dosing time points, tubes indicate sampling of blood and/or urine at the corresponding time point (T in hours) for each dose round. In the 4 g APAP dose round, blood samples for transcriptomic analyses were not collected on T1 and T7.

Untargeted univariate analyses. A combination of high fold change (FC) and relatively strict p-values was used (FC≥2–10, $p<0.01$) to exclude selection of false positives from the differential data sets due to the high inter-individual variation and a limited number of samples. Signals complying with these conditions were further identified based on literature (Bales et al., 1984; Chen et al., 2008). Statistically significant masses selected from LCMS analysis were identified by means of LC-nanomate-Orbitrap for elemental composition analysis of MS^n fragments (Rijk et al., 2009; Ruiz Aracama et al., submitted for publication).

Targeted analyses. A list of masses of interest was loaded into Search LCMS (Lommen et al., 2011b) to create a file containing the amplitudes of these masses.

Metabolite visualization. Pathvisio, a pathway visualization tool, was used to create a metabolic map (van Iersel et al., 2008). This metabolic map was based on available literature (Chen et al., 2008; Daykin et al., 2002). LC–MS data were averaged per group level ($n=6$) and visualized per test fluid (polar serum fraction and urine), time point and dose. For visualization purposes only, all 0 values were manually set to 8 (which is almost equal to the lowest measured value). Then al values were log-transformed resulting in a data range with a minimum of 3 and a maximum of 17.

Transcriptomic analyses

mRNA/miRNA isolation. Total RNA was isolated from whole blood (PAXgene Blood RNA kit, PreAnalytiX/Qiagen), treated with DNase and globin reduced (GLOBINclear kit, Ambion) according to the manufacturer's protocols. Total RNA for miRNA analyses was isolated using the RiboPure-Blood kit (Ambion) according to the manufacturer's protocol which included a DNase digestion. Integrity of all RNA was checked on a Bioanalyzer (Agilent).

mRNA/miRNA microarray analyses. All data are MIAME compliant and the raw micro-array data have been deposited in the GEO database (GSE30418 — Low dose human APAP exposure).

mRNA. cRNA targets were prepared according to the Affymetrix protocol for an input of 250 ng RNA. cRNA was hybridized (Affymetrix Human Whole Genome U133A plus 2 GeneChip) according to the manufacturer's recommended procedures. After hybridization GeneChips were washed/stained using a fluidics station (Affymetrix) and scanned (GeneArray scanner, Affymetrix).

miRNA. 1 μg of total blood cell RNA was labeled (miRCURY_LNA microRNA-array kit_V11.0, Exiqon) according to the manufacturer's protocol (single color labeling) and scanned (Genepix_4000B scanner with software version 3.0 (Axon)). Expression of miR-19a, miR-19b and miR-374a was confirmed with RT-PCR (TaqMan MicroRNA Assays, Applied Biosystems) according to the manufacturer's protocol. FC's were calculated according to the $^{\Delta\Delta}$Ct-method, using U6 snRNA for normalization (Livak and Schmittgen, 2001). The miRNA array of one donor (subject E) was aberrant in intensities from all others and was therefore excluded from further analyses.

mRNA-array statistical analyses. After quality control, data were re-annotated (EntrezGene customCDF_V13) (Lin et al., 2011; Dai et al., 2005). Next, the data were RMA normalized and filtered for expressed genes using present/absent-calls obtained from the MAS5 algorithm; only data with less than 2 absent calls/reporter over the 6 arrays/time point were included. In the 4 g APAP dose round, blood sampling was not preformed on T1 and T7 for mRNA analyses, instead additional samples on T0 and T25 were collected to enable miRNA analyses as will be described below. Significant Analyses of Micro-arrays (SAM) was performed to find differentially expressed genes (DEGs) (Shi et al., 2006; Tusher et al., 2001). SAM analyses were performed per time point (two class paired analyses with FC≥1.5 and a false discovery rate (FDR)≤0.1, for 0.5 g and 2 g at T1, T7 and T25 for 4 g at T25 only). In addition, an analysis over time (Time Course, TC) was performed (one class time course, FDR≤0.1, for 0.5 g and 2 g only). Finally, significantly altered biological pathways were identified using MetaCore (GeneGO, $p<0.05$, only pathways with at least 5 gene products were taken into consideration). Pathway analyses appoint individual genes to pathways consisting of biologically related genes representing a certain biological function/effect, which not only enhances statistical power, but also enables easier interpretation of gene-expression data at a higher hierarchical level of cellular function.

miRNA-array statistical analyses. Genepix data files were imported into the statistical program R (Dessau and Pipper, 2008). After a thorough quality control all datapoints were background-corrected, log-transformed, filtered for human miRNAs and quantile normalized (after 4 g T25 only). MiRNAs have 4 technical replicates/chip, when at least 3 out of 4 possible signals were present, mean expression values were calculated. Only miRNAs with $p<0.05$ (*t*-test) and FC≥1.5 were considered to be differentially expressed. For these miRNAs, a target scan was performed (Microcosm) to identify target genes. Expression of mRNA products of these target genes (target mRNA) was checked by linking to the corresponding mRNA arrays (4 g at T25). Only target genes of which the mRNA product was significantly differentially expressed in the opposite direction of the miRNA were taken into consideration. These target genes were arranged into categories according to their involvement in cell processes (taken from GeneCards-V3, similar to mRNA analyses).

Results

Clinical chemistry

Averaged data from all individuals ($n=6$) showed no response toward liver toxicity in any of the clinical liver tests (student's *t*-test T0 vs. T25, all $P>0.05$, raw data available in Supplementary Table A).

Metabolomics

Urine

A total of 23 masses could be identified; all were derived from APAP (Supplementary Table B). Several metabolites could be detected at all doses; APAP as mother compound, APAP-glucuronide, APAP-sulfate, cysteine-APAP and N-acetylcysteine-APAP (Supplementary Table B). The latter 2 compounds are derivatives of NAPQI, which is thought to cause the toxic effects of high APAP doses through protein-adduct formation leading to oxidative stress and finally liver damage (Dahlin et al., 1984). There was no statistical evidence for secondary (non-APAP) changes in urine.

Serum

Polar metabolite fraction. The serum dataset showed less significant differences than urine samples (Supplementary Table B). As in urine,

Fig. 2. Schematic visualization of APAP metabolic pathway. The group averages ($n=6$) for each metabolite per dose, time point and sample material are visualized as in the example. Gray boxes; not measured/detected. Increase in a metabolite is pictured from green (no increase, equals a numerical value of 3 on a log-scale) to yellow, orange and red (high increase, maximum value equals 17 on a log-scale). Raw data are available in supplementary table B.

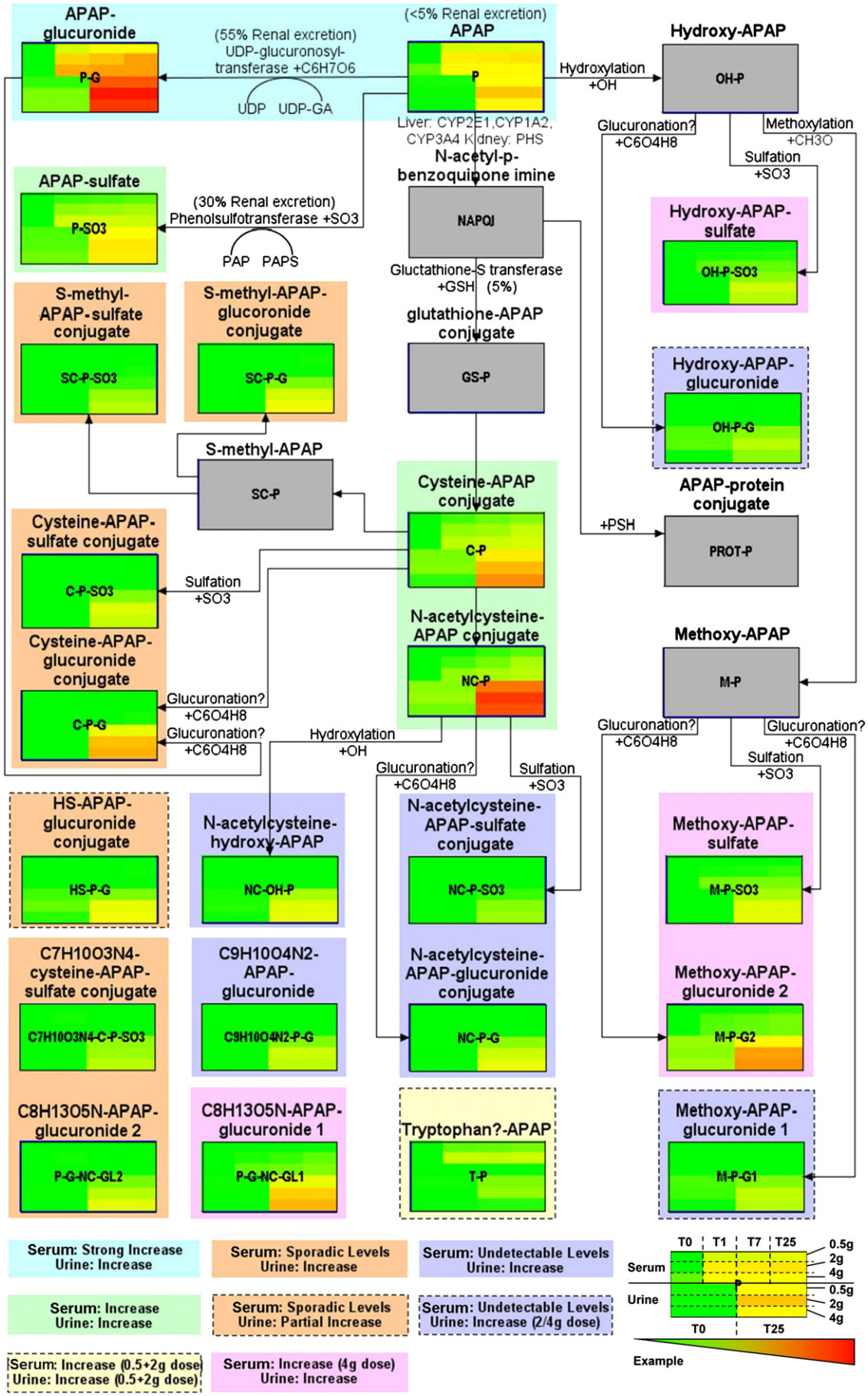
APAP-glucuronide
P-G
(55% Renal excretion)
UDP-glucuronosyl-transferase +C6H7O6
UDP UDP-GA
(<5% Renal excretion)
APAP
P
Hydroxylation +OH
Hydroxy-APAP
OH-P
Liver: CYP2E1,CYP1A2, CYP3A4 Kidney: PHS
Glucuronation? +C6O4H8
Methoxylation +CH3O
Sulfation +SO3
APAP-sulfate
P-SO3
(30% Renal excretion)
Phenolsulfotransferase +SO3
PAP PAPS
N-acetyl-p-benzoquinone imine
NAPQI
Hydroxy-APAP-sulfate
OH-P-SO3
Gluctathione-S transferase +GSH (5%)
S-methyl-APAP-sulfate conjugate
SC-P-SO3
S-methyl-APAP-glucoronide conjugate
SC-P-G
glutathione-APAP conjugate
GS-P
Hydroxy-APAP-glucuronide
OH-P-G
S-methyl-APAP
SC-P
Cysteine-APAP conjugate
C-P
APAP-protein conjugate
+PSH
PROT-P
Cysteine-APAP-sulfate conjugate
C-P-SO3
Sulfation +SO3
N-acetylcysteine-APAP conjugate
NC-P
Methoxy-APAP
M-P
Cysteine-APAP-glucuronide conjugate
C-P-G
Glucuronation? +C6O4H8
Hydroxylation +OH
Sulfation +SO3
HS-APAP-glucuronide conjugate
HS-P-G
N-acetylcysteine-hydroxy-APAP
NC-OH-P
N-acetylcysteine-APAP-sulfate conjugate
NC-P-SO3
Methoxy-APAP-sulfate
M-P-SO3
C7H10O3N4-cysteine-APAP-sulfate conjugate
C7H10O3N4-C-P-SO3
C9H10O4N2-APAP-glucuronide
C9H10O4N2-P-G
N-acetylcysteine-APAP-glucuronide conjugate
NC-P-G
Methoxy-APAP-glucuronide 2
M-P-G2
C8H13O5N-APAP-glucuronide 2
P-G-NC-GL2
C8H13O5N-APAP-glucuronide 1
P-G-NC-GL1
Tryptophan?-APAP
T-P
Methoxy-APAP-glucuronide 1
M-P-G1
Serum: Strong Increase
Urine: Increase
Serum: Sporadic Levels
Urine: Increase
Serum: Undetectable Levels
Urine: Increase
Serum: Increase
Urine: Increase
Serum: Sporadic Levels
Urine: Partial Increase
Serum: Undetectable Levels
Urine: Increase (2/4g dose)
Serum: Increase (0.5+2g dose)
Urine: Increase (0.5+2g dose)
Serum: Increase (4g dose)
Urine: Increase
T0 T1 T7 T25
Serum
Urine
0.5g
2g
4g
T0 T25
Example

Table 1
Cell processes affected after intake of 2 g and 4 g of APAP. MetaCore pathways that were significantly affected were counted and divided over general cell processes. For each cell process, the number of corresponding effected pathways was scored as % of total pathways.

	APAP dose/time point				
Cell process	2 g/ T1	2 g/ T7	2 g/ T25	2 g/Time Course	4 g/ T25
Immune response	29	0	56	33	13
Lipid metabolism	7	0	6	0	13
Cholesterol transport	0	0	11	0	25
Apoptosis and survival/ oxidative stress/DNA damage	0	0	11	20	25
Drug metabolism/pain	36	0	0	7	0
Other processes	29	100	17	40	25

all masses were derived from APAP, but their levels were much lower. Again, there was no evidence for secondary changes.

Apolar metabolite fraction

Statistical analysis did not show significant differences in chemical profiles upon APAP treatment.

Pathway visualization

Data from urine and polar serum metabolites were visualized in a metabolic pathway (Fig. 2, based on raw data available in Supplementary Table B). Five of these APAP metabolites, 3 glucuronide products, an APAP-sulfate conjugate and what seems to be an APAP-tryptophan product, have never been described before (Supplementary Table B, highlighted in gray). APAP itself and APAP-glucuronide are most strongly elevated. In general, a dose-dependent and time-dependent effect is apparent, especially in serum. With an increase in APAP-dose, relatively more cysteine-, hydroxy- and methoxy-metabolites were formed.

Transcriptomics

mRNA expression

DEGs. After intake of 4 g of APAP (T25 time-point was analyzed only) transcriptomics analyses resulted in 285 DEGs (FDR = 0.004). At lower APAP doses. Intermediate time points were also considered. Intake of 2 g of APAP resulted in DEGs at T1 (16, FDR = 0.095), T7 (2, FDR = 0.000), T25 (254, FDR = 0.016) and over time (3484, FDR = 0.094). After intake of 0.5 g of APAP, DEGs were detected at T25 (7, FDR = 0.056) and over time (2, FDR = 0.000) only. In general, almost all genes regulated at a specific time point were also affected over time.

Pathway analyses

8 pathways were regulated at T25 after 4 g of APAP (see Supplementary Table C). Intake of 2 g of APAP resulted in 14, 3, 18 and 15 pathways to be significantly affected at T1, T7, T25 and over time (TC) respectively. No biological pathways were significantly regulated at any specific time point or over time after 0.5 g of APAP.

Cell processes

Each significant pathway was categorized according to the cell process to which it relates (Supplementary Table C, third column). Table 1 shows the percentage of affected pathways over total pathways at that particular dose/time point. After intake of 2 g of APAP, mainly immune response pathways were regulated, including "macrophage inhibitory factor mediated glucocorticoid regulation", "signaling of several interleukins, CD40" and "T-helper17 cell related processes". This response changed after intake of 4 g of APAP toward pathways involved in "apoptosis and survival/oxidative stress/DNA damage" (mainly oxidative phosphorylation) and "cholesterol transport" (mainly cholesterol and Sphingolipids transport). The category "other processes" in Table 1 contains several pathways which could not be scored within any of the other categories. These pathways mainly represent very general (blood) cell processes.

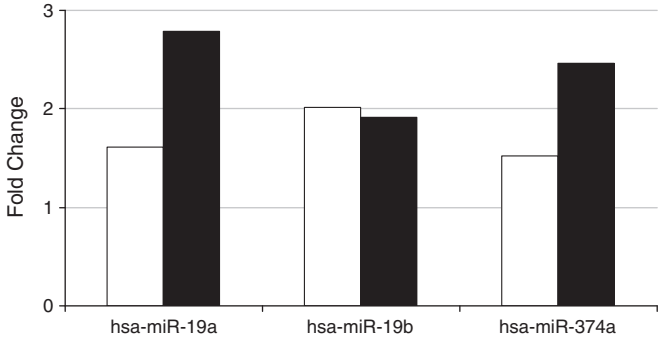

Fig. 3. Confirmation of miRNA expression. Fold changes of miRNA expression (T0 vs. T24) according to microarray analyses (white bars) were confirmed by RT-PCR (black bars).

miRNA expression

Differentially expressed miRNAs. Blood miRNA levels were determined after intake of 4 g of APAP (T25); 11 miRNAs were significantly regulated. Differential expression of 3 miRNAs was confirmed by means of RT-PCR (Fig. 3). 89 mRNA products of predicted target genes appeared significantly expressed and negatively correlated to its targeting miRNA (expression profiles are presented in Fig. 4).

miRNA target function/cell processes. Target gene functions were grouped according to the cell process to which it relates (Supplementary Table D) and scored as percentage over total cell processes (Table 2). Categories for cell processes were kept similar to the categories used in mRNA analysis as much as possible. As with mRNA analysis, "immune response" and "apoptosis and survival/oxidative stress/DNA damage" were the most dominantly present process categories.

Discussion

The currents test for liver toxicity cannot measure APAP exposure without the occurrence of liver toxicity. 'Omics techniques seem promising with respect to their sensitivity and specificity in measuring molecular effects. We therefore hypothesize that 'omics techniques are sensitive and specific enough to measure the effects of low, (sub-)therapeutic APAP doses in humans. To test this hypothesis, transcriptomic (mRNA and miRNA) and metabolomic analyses were performed on blood samples (transcriptomics) and serum/urine samples (metabolomics) from healthy volunteers taking a low doses of APAP over 24 h. In addition, classic clinical liver toxicity tests were performed. While no effects of APAP intake could be detected by any of the classic liver tests, 'omics techniques were able to identify responses to low APAP doses which were similar to those seen after high APAP doses known to cause adverse health effects.

Metabolomic analysis demonstrated that urine samples contained more APAP-derived metabolites in comparison to serum samples. This can be explained by the fact that metabolites accumulate in urine. NAPQI, hydroxy-APAP and methoxy-APAP are oxidative metabolites that all have been associated with hepatotoxic effects of APAP (Dahlin et al., 1984; Chen et al., 2008; Wilson et al., 1982). Especially NAPQI-protein adducts are believed to be key metabolites in causing inflammation-mediated liver toxicity through oxidative stress after exposure to high doses of APAP (Dahlin et al., 1984). NAPQI-protein adducts can be detected in blood/urine when they are released from hepatic cells after lyses due to severe toxicity. Although no NAPQI, methoxy-APAP or hydroxy-APAP metabolites itself

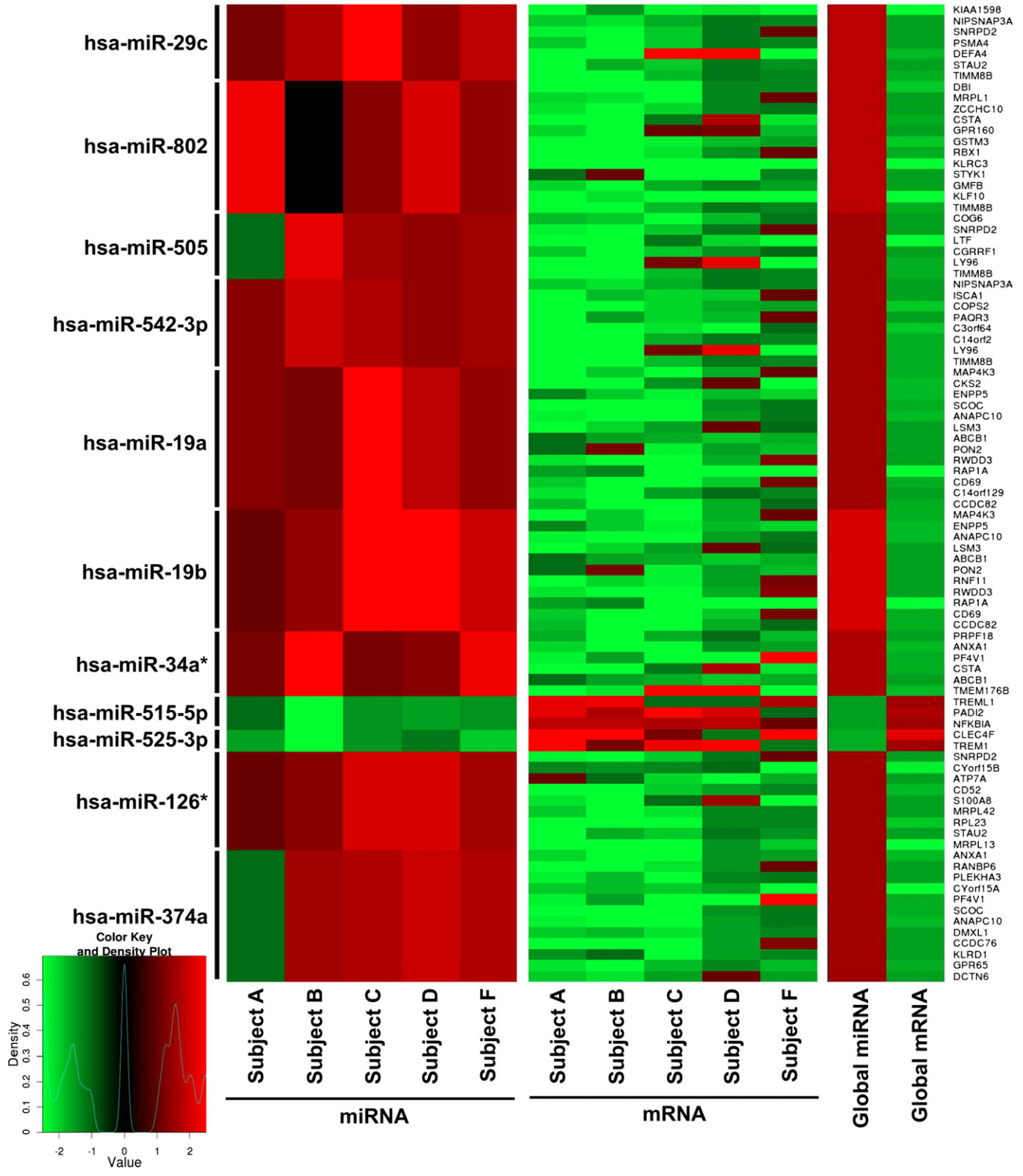

Fig. 4. Fold changes of miRNAs and their negatively correlated mRNA targets. From left to right: miRNAs, individual miRNA expression (FC) per subject, individual target mRNA expression (FC) per subject, group average (global) miRNA/target mRNA expression and target mRNA gene name. Red and green mean up and down regulation respectively after intake of 4 g APAP over 24 h.

were identified within this study, most likely because of fast conversion due to their reactive nature, their derived structures were readily detectable. With an increase in APAP-dose, relatively more of the cysteine-, hydroxy- and methoxy-derived metabolites were detected (especially in urine). In addition, 5 so far unknown APAP metabolites were detected; 3 glucuronide products, an APAP-sulfate conjugate and what seems to be an APAP-tryptophan product. The concentration of these metabolites in general seems to increase with APAP dose and time (especially in urine). Further elucidation of the exact structure of these metabolites could possibly add to our understanding of the still

Table 2
Cell processes of target genes of oppositely regulated miRNAs after intake of 4 g of APAP. Cell processes of significantly regulated and oppositely correlated miRNA target genes as described by GeneCards V3, scored as % of total target genes.

	APAP dose/time point
Cell process	4 g/T25
Immune response	24
Lipid metabolism	3
Cholesterol transport	0
Apoptosis and survival/oxidative stress/DNA damage	12
Drug metabolism/pain	2
mRNA/tRNA related	10
Other processes	35
Unknown	15

partially unknown mechanism-of-action of acetaminophen. So, far we do not know of any other study describing as many metabolites at such low APAP dose levels.

Whole-blood mRNA expression demonstrates dose-related molecular effects after intake of APAP doses ≥2 g/24 h. The early response (T1/T7) to an APAP dose of 2 g/24 h mainly involves the regulation of processes related to "drug metabolism/pain" and "immune response" as well as some "lipid metabolism" processes (see Table 1). Closer evaluation of the actual pathways involved in these cell processes revealed that many significantly altered pathways can be directly linked to the known efficacy of APAP. Among others, these pathways involve prostaglandin-E2 and prostaglandin-1/-2 biosynthesis. APAP is believed to exert its effect through cyclooxygenase-1/-2 and possibly cyclooxygenase-3 (Vane, 1971; Davies et al., 2004). Arachidonic acid is released in the early stages after trauma of cellular membranes. Cyclooxygenase enzymes transform arachidonic acid to prostaglandins, which in turn cause pain and fever.

At T25 after intake of 2 g of APAP, the affected cell processes shifted toward processes related to an immune response. Among the immune-related processes are macrophage inhibitory factor (MIF)-mediated glucocorticoid regulation, signaling of interleukin (IL)-1, IL-17, IL-23 and CD40, cytokine production by T-helper (Th) 17 cells and Th17 differentiation. MIF has been suggested to act as a pro-toxicant signal in drug-induced liver injury (Bourdi et al., 2002). Th17 cells have been described only recently and were shown to be potent inducers of tissue inflammation. Also Th17 cells have been linked to several liver diseases including alcohol-induced liver disease, non-alcoholic steatohepatitis and hepatitis (Hammerich et al., 2011). Th17 cells are known to secrete IL-17, a very potent pro-inflammatory cytokine (Korn et al., 2009). However, also other immune cells like macrophages and neutrophils are known to produce IL-17 (Benghiat et al., 2009; Song et al., 2008). One study has mentioned an increased serum concentration of IL-17 in patients with APAP-induced acute liver failure, but IL-17 concentrations showed high inter-individual variability and were not uniformly present (Li et al., 2010). Besides IL-17, also IL-1, IL-23 and CD40 are among the regulated processes after 2 g APAP at T25, which can all be linked to Th17 cells since their expression seems to be required for the differentiation of Th17 cells (Iezzi et al., 2009; Korn et al., 2009). In addition, these interleukins also have other important roles in the regulation of the immune system and are therefore not exclusively linked to Th17 cells. For instance, IL-1 specifically has been proposed to be a biomarker for liver toxicity (Akbay et al., 1999; Lacour et al., 2005). Although several immune-modulating effects of APAP in humans have been suggested before (Baken et al., 2008; Doran et al., 1989; Graham et al., 1990; Harrill et al., 2009; Prymula et al., 2009), no definitive conclusion about possible immunotoxic effects of APAP can be drawn from the present study. Nevertheless, there are clear indications that responses from the immune system, similar to those that would be expected after high, toxic doses of APAP, are triggered after intake of a low, therapeutic dose of APAP.

At T25 after 4 g of APAP, a shift appears toward a different response. This response includes processes involved in "apoptosis and survival/oxidative stress/DNA damage". In fact, the most significantly regulated process at the latter dose is "oxidative phosphorylation", which is down-regulated. In general, liver toxicity due to high, toxic APAP doses is attributed to oxidative stress and mitochondrial dysfunction caused by reactive metabolites formed during drug metabolism. This process is associated with the down-regulation of genes involved in oxidative phosphorylation (Fannin et al., 2010; Han et al., 2010; Katyare and Satav, 1989), as is also seen within this study. Therefore, our study indicates that therapeutic APAP doses cause gene-expression changes in oxidative stress-related processes which may indicate a toxicological risk for liver injury. A recent study focusing on the effects of a bolus dose of 4 g of APAP on gene expression changes in human blood cells, confirms our findings in that APAP modifies the transcriptome signature by down-regulation of oxidative phosphorylation genes (Fannin et al., 2010). The authors from this study concluded that these changes resembled those observed in patients who overdosed on APAP and also those changes seen in rats receiving toxic APAP doses. These findings support the idea of a possible pre-toxic response to low, therapeutic doses of APAP proceeding, but eventually not resulting in actual profound liver injury. The liver is a robust, regenerative organ with a large over-capacity in a normal, healthy human. Therefore low levels of liver toxicity, if occurring, would probably be dealt with by this organ without any notable complications for the individual.

MiRNAs target mRNAs, which usually induces translational repression and mRNA instability. Based on target gene mRNA product analyses, processes affected by miRNAs at T25 after 4 g of APAP showed a very similar pattern as those found after mRNA pathway analyses. Regulated miRNAs appeared to be associated with "apoptosis and survival/oxidative stress/DNA damage" and "immune response". Thereby, the miRNA data confirmed the findings on mRNA level.

So far we know of only 2 rodent studies and 1 human study exploring the effects of toxic APAP doses on miRNA expression levels (Fukushima et al., 2007; Starkey Lewis et al., 2011; Wang et al., 2009). MiRNAs are suggested to have an important role in toxicological mechanisms and consequently are proposed to represent key molecules for toxicity expression (Fukushima et al., 2007). Also, circulating miRNAs are suggested to be sensitive and informative biomarkers for drug-induced liver injury (Starkey Lewis et al., 2011; Wang et al., 2009). In both before mentioned rodent studies, changes on miRNA level were visible before any other toxicological changes in morphology, ALT or lactate could be detected. After a high APAP dose, miR-29c and miR-19b were both down-regulated in mouse liver, while in the plasma both these miRNAs were up-regulated (Wang et al., 2009). Our study demonstrated both miR-19b and miR-29c to be up-regulated in blood cells after a relatively low APAP dose. Furthermore, miR-542-3p was shown to be up-regulated in rat livers (Fukushima et al., 2007), which is comparable to our results obtained in blood after 4 g of APAP. In humans, hsa-miR-122 and hsa-miR-192 circulating in blood plasma have been suggested as possible biomarkers for acute liver injury induced by high APAP dose ingestion (Starkey Lewis et al., 2011). Both miRNAs were significantly up-regulated in plasma of patients suffering from APAP induced acute liver injury, i.e. in patients with elevated ALT levels. Most likely, these miRNAs are released from the liver due to toxic insults. Especially hsa-miR-122, which expression is liver specific, would otherwise not occur in plasma (Liang et al., 2007). In the current study, ALT levels were not elevated and neither were hsa-miR-122 nor hsa-miR-192 levels. Although hsa-miR-192 did almost pass the selection criteria for significance with a fold change of 1.76 and a p-value of 0.097. In the current study the expression pattern of miRNAs in blood cells was included, which is not the case in the study of Starkey Lewis et al. Therefore the outcome of both studies cannot be directly compared. Nevertheless, the increase of hsa-miR-192 in plasma could

(partly) be caused by increased expression of this miRNA in white blood cells and leakage from these cells due to toxicity. This phenomenon seems to precede leakage of hsa-miR-122 from liver cells. Taken together, not only on the metabolomic and mRNA level, but also on the miRNA level, responses to low, therapeutic APAP doses resembled those seen after high, toxic APAP doses.

In summary, we have shown that 'omics techniques are able to measure a response to short term, low dose APAP intake by human volunteers on mRNA and miRNA in blood cells and on serum and urine metabolite level. 'Omics thereby outperforms the classical clinical chemistry tests which were not able to detect any response to low dose APAP exposure. Furthermore, several new APAP metabolites which have not yet been described in literature were identified. Intake of 2 g of APAP resulted in a diverse metabolite pattern, including cysteine-, hydroxy- and methoxy-metabolites that have been associated with APAP-related hepatotoxicity after high APAP dose exposure. Transcriptomic changes indicated dose-specific immune-modulating effects, which cannot be fully explained yet, but at least show that low dose APAP exposure does trigger an immune response. This response could possibly be related to a mild version of liver damage, but eventually does not lead to severe liver injury. A possibly cause for liver damage after low APAP doses could be related to the oxidative APAP metabolites associated to hepatotoxicity that were measured at these doses. 24 h after ingestion of 4 g of APAP, a metabolomic response very similar as seen after 2 g was detected, but with higher levels of hepatotoxicity-related metabolites. An increase in oxidative metabolite levels is associated with the gene-response specific for this dose demonstrating changes in oxidative stress-related pathways. In addition, effects of APAP administration on miRNA expression confirmed the findings on mRNA level concerning both the effects of APAP on immune responses and the effects of APAP on apoptosis/oxidative stress/DNA damage.

The individual factors which influence APAP susceptibility remain still partially unknown. Our study – having in itself insufficient numbers of volunteers to evaluate this – may provide a model for exploring such inter-individual variability in drug responses, since the data revealed a wealth of biological information indicating effects both related to efficacy and possible toxicity of APAP. Therefore, studies like this one could help in finding answers to the questions that are currently posted about the social and medical issues around APAP use and its still partially unknown mechanism-of-action. However, larger sample numbers will then have to become available.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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