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High-resolution mass spectrometry identifies delayed biomarkers for improved precision in acetaminophen/paracetamol human biomonitoring

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ABSTRACT

Paracetamol/acetaminophen (N-acetyl-p-aminophenol, APAP) is a top selling analgesic used in more than 600 prescription and non-prescription pharmaceuticals. To study efficiently some of the potential undesirable effects associated with increasing APAP consumption (*e.g.*, developmental disorders, drug-induced liver injury), there is a need to improve current APAP biomonitoring methods that are limited by APAP short half-life. Here, we demonstrate using high-resolution mass spectrometry (HRMS) in several human studies that APAP thiomethyl metabolite conjugates (S-methyl-3-thioacetaminophen sulfate and S-methyl-3-thioacetaminophen sulphoxide sulfate) are stable biomarkers with delayed excretion rates compared to conventional APAP metabolites, that could provide a more reliable history of APAP ingestion in epidemiological studies. We also show that these biomarkers could serve as relevant clinical markers to diagnose APAP acute intoxication in overdosed patients, when free APAP have nearly disappeared from blood. Using *in vitro* liver models (HepaRG cells and primary human hepatocytes), we then confirm that these thiomethyl metabolites are directly linked to the toxic N-acetyl-p-benzoquinone imine (NAPQI) elimination, and produced via an overlooked pathway called the thiomethyl shunt pathway. Further studies will be needed to determine whether the production of the reactive hepatotoxic NAPQI metabolites is currently underestimated in human. Nevertheless, these biomarkers could already serve to improve APAP human biomonitoring, and investigate, for instance, inter-individual variability in NAPQI production to study underlying causes involved in APAP-induced hepatotoxicity. Overall, our findings demonstrate the potential of exposomics-based HRMS approach to advance towards a better precision for human biomonitoring.

1. Introduction

APAP is an active ingredient in more than 600 prescription and non-prescription pharmaceuticals, and is one of the most commonly used pharmaceuticals worldwide (Kristensen et al., 2016). The medical benefits of APAP are widely recognized; nevertheless, recent experimental as well as epidemiological studies suggest APAP as a possible risk factor for certain developmental disorders in humans (*i.e.*, neurodevelopmental, reproductive and urogenital), on its own, or in combination with other environmental toxicants (Bauer et al., 2021; Kortenkamp and Koch, 2020; Kristensen et al., 2016).

In addition, APAP overdose is one of the most important causes of

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drug-induced liver injury, that can eventually lead to acute liver failure (ALF) (Gulmez et al., 2015; Lee, 2004). Data from the U.S. Acute Liver Failure Study Group registry of more than 700 patients with ALF across the US, implicates APAP poisoning in nearly 50% of all ALF. In Europe, APAP overdose was found to represent one-sixth of all-cause ALF (Lee, 2004). Overall, APAP overdose, even without suicidal intent, represents a large proportion of ALF leading to registration for transplantation (Gulmez et al., 2015).

Efficient monitoring of APAP in epidemiological or clinical studies to study these potential undesirable effects (*i.e.*, developmental disorders or hepatotoxicity at recommended doses) is often hampered by recall bias or the fact that concentrations of circulating APAP or those of its main excreted phase II metabolites (*i.e.*, the glucuronide and sulfate conjugates) are usually undetectable rapidly after APAP intake due to its short half-life (Bauer et al., 2021; Watkins et al., 2006). Hence, identifying new APAP biomarkers that could provide a more reliable history of APAP ingestion in both blood and urine would provide more precision to study its association with developmental disorders or to study hepatotoxicity in populations with predisposing factors (Michaut et al., 2014).

Recently, we demonstrated that the APAP metabolism in human, in particular the oxidative pathways, is probably more complex than previously thought (David et al. 2021a). Using exposomics-based high-resolution mass spectrometry (HRMS), we showed that N-acetyl-p-benzoquinone imine (NAPQI)-derived metabolites produce significant signals of conjugated thiomethyl metabolites such as S-methyl-3-thio-acetaminophen sulfate (S-CH_3-APAP-S) via an overlooked pathway called the thiomethyl shunt (Cooper et al., 2011; David et al., 2021a). NAPQI is a highly reactive intermediate formed predominantly via the cytochrome P450 2E1 that has been linked to hepatocellular injury due to its electrophilic nature and its covalent binding to cysteine molecules on liver proteins (Athersuch et al., 2018; McGill and Jaeschke 2013). Moreover, we showed that the detected thiomethyl metabolites exhibited delayed peak concentrations probably due to a late reabsorption after the enterohepatic circulation (David et al., 2021a). To date, the production of these conjugated thiomethyl metabolites from the glutathione-derived metabolites (*i.e.*, mercapturate and cysteine conjugates) has been largely ignored in human, and considered to be negligible (*i.e.*, <1% of the initial dose (Klutch et al., 1978)).

Here, we postulate that the conjugated thiomethyl metabolites S-methyl-3-thioacetaminophen sulfate and S-methyl-3-thio-acetaminophen sulphoxide (SO-CH_3-APAP-S) could provide a more reliable history of APAP ingestion due to their delayed excretion compared to conventional APAP metabolites. We first studied the excretion rates of these NAPQI-derived thiomethyl metabolites in two observational studies with pregnant women who consumed APAP at recommended doses. We then studied the importance of these biomarkers to monitor NAPQI detoxification and elimination in patients after acute intoxication. Finally, we used *in vitro* liver models (HepaRG cells and primary human hepatocytes) as well as enteric bacteria to provide a better understanding of the formation of these thiomethyl metabolites (in particular to identify their precursors), and a better understanding of the site where the biotransformation occurs. Overall, using a combination of observational human studies and *in vitro* studies, our findings support the relevance of these thiomethyl metabolites to improve the precision of APAP biomonitoring in epidemiological studies or to study the elimination of the toxic fraction after acute APAP intoxication and assess inter-individual variability in the production of NAPQI in clinical studies.

2. Material and methods

2.1. Study design

The excretion rates of APAP metabolites were studied in three different observational studies and an interventional study. Details on protocols and timing of biological sample collections after APAP intake for each study are provided in Table S1. Briefly, the first observational study (section 3.1) was a longitudinal one involving the collection of urine samples over two days from five pregnant women that consumed APAP. No intervention was performed for ethical reasons, and differences in dosage (500 mg vs 1 g), brands (Doliprane, Dafalgan, Paracetamol Mylan), and co-administration with other pharmaceuticals were observed between intakes and pregnant women (Table S2). Urine samples of these women were collected at the hospital at different time points per day, after one (pregnant woman 1), two (pregnant women 2, 3 and 4), or four (pregnant woman 5) APAP intakes. Overall, 57 urine samples were collected, corresponding to 10, 9, 13, 13, and 12 urine collections per day for pregnant women from 1 to 5, respectively. This study was approved by the appropriate ethical committees [CPP (Comité de Protection des Personnes Sud-Est) and CNIL (Commission Nationale de l'Informatique et des Libertés).

The cross-sectional observational study (section 3.2) included 20 pregnant women from the PELAGIE birth cohort (Chevrier et al., 2011). In this study, 10 pregnant women who declared no APAP intake versus 10 pregnant women who declared pharmaceutical consumption (including APAP) over the last 3 months were selected. These women were asked to collect, at home, the first-morning void urine sample into 10-ml vials (containing nitric acid to prevent bacterial multiplication) and to complete a questionnaire including information on drug intake. They returned the questionnaire and the urine sample to the research laboratory by local mail in a self-addressed stamped package. Informed consent was obtained from all participants and this study was approved by the appropriate ethical committees indicated above CPP and CNIL (Biological Research & Collection BRC Identification number: 2020-A00478-31).

The third observational study involved 13 individuals (11 women and 2 men) who were admitted to hospital for suspicion of acute APAP intoxication. The protocol of antidote treatment consisted of N-acetylcysteine (NAC) administration at 150, 50 and 100 mg/kg body weight at +1 h, +2–5 h and + 6–22 h after hospitalization, respectively. Information of age of patients, Alanine transaminase (ALT) and Aspartate aminotransferase (AST) measurements, percentages of total protein and factor V is provided in Table S3. Informed consent was obtained from all participants in the study and the study was approved by the Scientific Ethical Committee of Rennes Hospital in France (Avis n° 22.185).

Data from a previous interventional study (David et al., 2021a) were also used to compare the excretion rate of APAP metabolites with those obtained during the first longitudinal observational study. Briefly, the study recruited 4 healthy men aged 30–60 years at the Department of Pharmacy and Department of Biology, University of Copenhagen. Exposure to APAP was performed over four days with an administration of 1 g of APAP on the third day. Urine samples were collected +1 h, +2h (urine only), +4h, +6h after intake, and in the morning of the subsequent two days (+24 and 48 h, urine and blood plasma). All individuals provided informed consent to participate in the study. The study protocol was in compliance with the Helsinki Declaration and was approved by the Regional Scientific Ethical Committees of Copenhagen in Denmark (Protocol nr.: 17003845; and as part of trial H-17002476).

2.2. Targeted analyses of APAP in urine samples

Targeted analyses were used to analyse urine samples from the cross-sectional observational study (section 3.2) to identify pregnant women with APAP concentrations within therapeutic doses (and compare these data with questionnaires and non-targeted results).

APAP concentrations analyzed as free APAP after hydrolysis of β-glucuronide conjugates (*i.e.*, corresponding to free APAP + APAP glucuronide) using a LC-MS/MS method were measured for each pregnant woman and are represented in ng/ml. Twenty μL of urine were transferred to a glass insert (Teknolab Sorbent, Kungsbacka, Sweden) of a 96-well Rittner plate (Teknolab Sorbent). Ten μL of ammonium acetate (pH 6.5) and 0.01 mL glucuronidase (*Escherichia coli,* Roche Diagnostics

(CAS No 9001–45-0), ≥80 U/mg protein) were added, and the solution was incubated at 37 °C for 90 min. After addition of 0.025 mL of MilliQ water and 0.3 mL of internal standard, the plates were centrifuged for 10 min at 3000 rpm, prior to injection. Extracts were analysed for free APAP after glucuronide deconjugation using LC-MS/MS. A 4 μm C18 column (2.1 mm i.d. ×50 mm, Genesis Lightning) was used before the injector to reduce the interference of contaminants during the mobile phase. A 1.7 μm C18 column (2.1 mm i.d. ×100 mm; Fortis Technologies) was used for separation, and the mobile phases were water and acetonitrile with 0.1% v/v formic acid. The samples were analysed on a Shimadzu UFLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a QTRAP5500 (triple quadrupole linear ion trap mass spectrometer) equipped with a TurboIon Spray source (AB Sciex, Framingham, MA, USA), in duplicates. All runs included at least 10 blank samples, used for a chemical noise subtraction step for all biological samples. Excellent linearity was seen for the calibration standards ranging from 10 to 1000 ng/mL in acetonitrile/water (50:50). The correlation coefficient (r^2) observed was above 0.994. LOD and LOQ were determined by analysis of 10 different blank toluene samples spiked with internal standards. The limit of detection (LOD) was defined as three times the standard deviation of the concentration corresponding to the peak at the same retention time as the analyte in the chemical blanks (n = 10) as described in Krais et al. (2023). Equally, the limit of quantification (LOQ) was defined as ten times the standard deviation. The determined LOD was 2 ng/ml.

2.3. Non-targeted analyses

2.3.1. Chemicals

The list of standards used for the annotation of APAP metabolites and the 15 labelled internal standards (IS) spiked in samples for the untargeted analyses and their respective suppliers are provided in Supplementary Table S4. All solvents were high-performance liquid chromatography grade, purchased from Biosolve Chime (Dieuze, France). Strata-X Polymeric Reversed Phase cartridges (200 mg, 3 mL) were supplied by Phenomenex (Le Pecq, France).

2.3.2. UHPLC-ESI-QTOFMS analyses

Urine samples and medium samples from the *in vitro* liver models and the enteric bacteria were analyzed using HRMS-based non-targeted methods as described in David et al. (2021a). Briefly, all samples were spiked with the mix of 15 internal standard, then urine samples (500 μl), and media from HepaRG cells, hepatocyte samples (300 μl), and microbiota samples (150 μl) were diluted to 1 mL of HPLC grade water, acidified with 1% formic acid and extracted using Strata-X SPE. Blood plasma samples spiked with the mix of internal standard were extracted using the protein precipitation (PPT) method described in Chaker et al. (2022). Briefly, PPT Protein precipitation was carried out using a 3:1 (v/v) cold methanol to matrix ratio. Samples were left at −20 °C for 1 h to improve protein removal. Centrifugation was performed at 4 °C and 13,300 rpm for 20 min, after which supernatants were collected and evaporated to dryness under vacuum. Samples were recovered in 80:20 (v/v) ultrapure water to acetonitrile mixture.

All sample extracts were profiled using an Exon UHPLC system (AB SCIEX, USA) coupled to an AB SCIEX X500R Q-TOF-MS system (SCIEX, Canada), equipped with a DuoSpray ion source as described in David et al. (2021a). As a first step, all the samples were analyzed in a full-scan experiment (50–1100 Da) in both −and +ESI modes. MS/MS mass fragmentation information for chemical elucidation was obtained by further analysis of selected samples in sequential window acquisition of theoretical mass spectrum (SWATH) data-independent acquisitions. The Q1 isolation window strategy was generated using the SCIEX Variable Window Table from previously acquired MS data on the specific sample of interest in order to optimize window widths across the entire *m/z* range. SWATH experiments were performed in both – and + mode with parameters: MS1 accumulation time, 80 ms; MS2 accumulation time, 30 ms; collision energy, 35 eV; collision energy spread, 15 eV; cycle time, 469 ms; mass range, *m/z* 50–900. Ten SWATH windows were selected in the method (with window widths ranging from 25 to 280 Dalton). Quality controls for HRMS-based analyses are described in the Supplementary information.

2.3.3. Quality control

In order to evaluate and control for potential background contaminants, one workup sample (*i.e.*, extraction with HPLC grade water instead of sample) per analytical batch was prepared and injected. Quality control (QC) samples, consisting of pools of all biological samples, were injected all along the analytical batch in order to monitor for UHPLC-ESI-TOF-MS repeatability and sensitivity. To ensure there was no presence of remaining traces in the UHPLC system from previous batches, solvent blank samples that could affect subsequent analytical runs, solvent blank samples (acetonitrile/H_2O (20:80)) were also injected within the analytical batch. Sample batch order of injection was as follows: first injections corresponded to blank samples (workup and solvent), followed by the injection of a QC sample six times consecutively, and then all samples from the batch, randomly distributed in sets of 5, interspersed each time with a periodic QC to monitor the analytical sensitivity and repeatability.

2.3.4. Data processing and annotation

The obtained LC-MS raw files (wiff2 format) were converted into mzXML format using MSConvert GUI (Palo Alto, CA, USA) using the Proteowizard open-source software (Chambers et al., 2012). Converted data were then imported to MATLAB computer and visualization environment (Release 2021b, The Mathworks, Inc., Natick, MA, USA) and analysed with MSroi (Pérez-Cova et al., 2021) chemometrics strategy using the MATLAB MSroi app (Pérez-Cova et al., 2021). This approach was employed for data compression, feature detection and filtering. In short, MSroi enables significant data compression in the spectral dimension without losing their instrumental spectral accuracy. The main parameters used for the regions of interest (ROI) procedure in this work were a mass error tolerance of 10 ppm, a minimum threshold value of 1000, and a minimum peak width of 10 s, and ROIs *m/z* values were calculated with the median of the *m/z* values determined for each chromatographic peak. The outcome of the ROI approach consists of a vector including the list of relevant *m/z* ROI values (according to the previously mentioned parameters), and a data matrix with the MS intensities at the selected ROIs (for all considered retention times and samples). The graphical interfase allows the visualization of the ROI features. The ROI vectors and MSroi matrix containing information of the features were used for multivariate and univariate analysis in MATLAB environment in order to identify APAP metabolites after APAP, acetaminophen cysteine (APAP-Cys), acetaminophen mercapturate (APAP-NAC), and acetaminophen glutathione (APAP-SG) exposures. Principal component analysis (PCA) was performed using the PLS Toolbox (Eigenvector Research Inc., Wenatchee, WA, USA) as a first step to examine QC clustering, sample separation and to identify any analytical or biological outliers. Partial least squares-discriminant analysis (PLS-DA) was then performed and by the use of VIP scores, the compounds showing higher VIP score were selected as potential biomarkers of exposure.

The discriminating markers between exposed and control groups identified through multivariate analyses were included in a suspect list. The suspect list included all the APAP metabolites found in a previous study of the same authors (David et al., 2021a) and also included other metabolites available in the literature (Chen et al., 2008). As explained in a previous study, the identities of the expected APAP metabolites were determined from accurate mass, isotopic fit, fragmentation data obtained from SWATH acquisition and from comparison with standard compounds when available or spectra available in online libraries or the literature (David et al., 2021a). In all cases, metabolite annotation was based on recommendations by Schymanski et al. (2014) (Supplementary

Table S6).

2.3.5. Accurate integration of identified and annotated markers

Peak integration of all APAP metabolites was performed manually using the SCIEX OS Analytics tool to ensure accuracy in the study of the excretion rates of APAP metabolites for each individual. The integrated peak area of individual markers was normalized to a corrective factor based on the total peak area measured with MarkerView (this corrective factor was calculated by dividing the total peak area of each sample to that of a reference sample) (David et al, 2021a). MATLAB (Release 2021b, The Mathworks, Inc., Natick, MA, USA) was used to generate plots.

2.4. Cell culture and treatment of HepaRG cells and human hepatocytes

Highly differentiated human HepaRG cells from passages 13 to 16 were cultured as described (Le Vee et al., 2013). Briefly, cells plated at a density of 2×10^5 cells/cm^2 in 12-well tissue culture-treated polystyrene plates, were first grown for 2 weeks in Williams' E medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% (vol/vol) fetal calf serum (FCS) (Cytiva, Velizy-Villacoublay, France), 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml bovine insulin (Sigma–Aldrich, Saint-Quentin-Fallavier, France), 2 mM glutamine (Invitrogen) and 50 μM hydrocortisone hemisuccinate (Upjohn, Paris La Défense, France). Cells were next cultured for an additional 2-weeks period in the same medium supplemented with 2% (vol/vol) dimethyl sulphoxide (DMSO), in order to get a full differentiation status of the cells (Bchini et al., 1990) before their use for metabolism activity assay. This differentiated status of DMSO-treated cell cultures was routinely checked by their phase-contrast microscopy analysis, demonstrating the presence of hepatocyte-like islands and the formation of bile canaliculi, as previously reported (Le Vee et al., 2013).

For establishing human hepatocyte cultures, fresh or cryopreserved human hepatocytes, prepared from human liver fragments, were obtained from Biopredic International (Saint-Grégoire, France). No differences were observed between fresh and frozen hepatocytes regarding their potential to metabolize APAP and the other tested metabolites. Cells were directly (for fresh hepatocytes) or after thawing (for cryopreserved hepatocytes) suspended at a concentration of 1×10^6 cells/ml in a seeding medium composed of Williams' E medium, supplemented with 10% (vol/vol) FCS, 5 μg/ml bovine insulin, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells were then seeded on 24-well tissue culture-treated polystyrene plates at a density of 2×10^5 cells/cm^2. After 24 h, the medium was discarded, and hepatocytes were cultured in a maintenance medium composed of seeding medium, supplemented by 50 μM hydrocortisone hemisuccinate and 2% (vol/vol) DMSO, as previously described (Le Vee et al., 2015). The culture medium was routinely renewed every 2–3 days and the cells were used for metabolism activity assays after one week of culture.

For cell treatment by APAP precursors, cells were first incubated with 50 μg/ml of APAP or APAP-Cys in the maintenance medium described above, during 0.5 h, 2 h, 6 h, 24 h, or 48 h, to optimize the incubation time required to detect APAP metabolites. Cells were next exposed for 24 h to 50 μg/ml of APAP-Cys, APAP-SG, or APAP-NAC.

2.5. Enteric bacteria

Five gut bacterial strains were selected including *Escherichia coli* (Ecoli), *Enterococcus faecalis* (efae), *Agathobacter rectalis* (arec), *Phocaeicola vulgatus* (pvul) and *Bifidobacterium longum* (blon). The bacterial strains used in this study were selected to be representative of each phylum present in the gut microbiome (Klünemann et al., 2021; Shalon et al., 2023). *Agathobacter rectalis* DSM 17629, *Phocaeicola vulgatus* DSM 1447, *Bifidobacterium longum subsp*. Longum DSM 20219 were provided by DSMZ Gmbh, Braunschweig, Germany. *Escherichia coli* DSM 1576 and *Enterococcus faecalis* ATCC19433 were provided by CIP (Institut Pasteur Collection, Paris, France). Cultures were performed in GAM medium (Gifu anaerobic broth, Fisher scientific, Illkirch, France), at 37 °C with agitation under anaerobic conditions generated by Genbag anaer (Biomerieux, Marcy l'Etoile, France). Growth kinetics were performed for each strain to identify the time required to reach the exponential phase. For this purpose, a multiskan FC spectrophotometer (Thermo Scientific, Illkirch, France) was used to measure the optical density at 620 nm.

For exposure experiments, culture growth was stopped during the exponential phase in order to prepare lysates or strains. For lysates preparation *(31)*, cultures were centrifuged (10000 g, 15 min), washed twice with potassium phosphate buffer (100 mM, pH 7.0), resuspended in borate buffer (50 mM, pH = 8.4), and broken by mechanical lysis with glass beads in a tissue lyser (Qiagen, Courtaboeuf, France) 10 min, 50 Hz. The extracts were obtained after removing the beads by centrifugation. The lysates were exposed to 50 μg/ml of APAP and APAP-Cys for 20 min. For strain preparation, cultures were centrifuged at 8000g for 3 min, washed in PBS (pH = 7), and resuspended in the same buffer. The E coli strain was exposed to 50 μg/ml of APAP and APAP-Cys for 30 min, 2 h, 6 h, and 24 h. Then all strains were exposed to 50 μg/ml APAP and APAP-Cys for 24 h.

2.6. Statistical analysis

Univariate analyses were performed to compare fold changes, calculate the areas under the curve (AUC) and determine statistical significance. AUCs were calculated using trapezoidal numerical integration (trapz function) from MATLAB. Statistics Toolbox™ from MATLAB (Eigenvector Research Inc., Wenatchee, WA, USA) was used to assess statistical significance using a one-way ANOVA *t*-test, considering $P < 0.001$ statistically significant, followed by a multiple comparison test (multcompare) to perform pairwise comparisons, based on the Tukey-Kramer critical value.

3. Results

3.1. Thiomethyl metabolites are consistently detected in pregnant women exposed to APAP

Thiomethyl metabolites such as S-CH_3-APAP are produced from NAPQI-glutathione conjugates via the thiomethyl shunt pathway, which involves the activity of a cysteine-conjugate beta lyase (CCBL) to cleave the C-S bond of cysteine conjugate, such as APAP-Cys (Cooper and Pinto, 2006) (Fig. 1). This reaction yields ammonia, pyruvate and a reactive thiol (SH-APAP) which can be subsequently methylated with an active form of methionine (Cooper et al., 2011). To date, these thiomethyl metabolites of APAP have been neglected in human as they were estimated to account for less than 1% of the initial dose in humans (Klutch et al., 1978; Slattery et al., 1989).

We recently demonstrated using HRMS-based analytical methods that significant signals can be observed for the conjugated forms of S-CH_3-APAP (*i.e.*, S-CH_3-APAP-S) and its sulphoxide (*i.e.*, SO-CH_3-APAP-S) in urine from male volunteers who ingested 1 g of APAP (David et al., 2021a). Delayed excretion rates were observed for these thiomethyl metabolites (peak time at 24 h after APAP intake) compared to conventional APAP metabolites (peak time 2–4 h after intake) (Fig. 2A).

In order to replicate our previous results on other human populations, we first monitored and compared the excretion of S-CH_3-APAP-S and SO-CH_3-APAP-S in urine samples collected from five pregnant women (PW) in their 2nd trimester of pregnancy, up to 2 days after a single or repeated APAP intake (n = 57). APAP is the most commonly used pharmaceutical during pregnancy (worldwide, more than 50% of pregnant women are estimated to use APAP (Bauer et al., 2021; Kristensen et al., 2016)), and the main cause of drug overdose during this period (Wilkes et al., 2005). Areas under the curve (AUCs) based on normalised areas obtained from LC-HRMS were calculated for all APAP metabolites (table S6) previously annotated using MS/MS structural

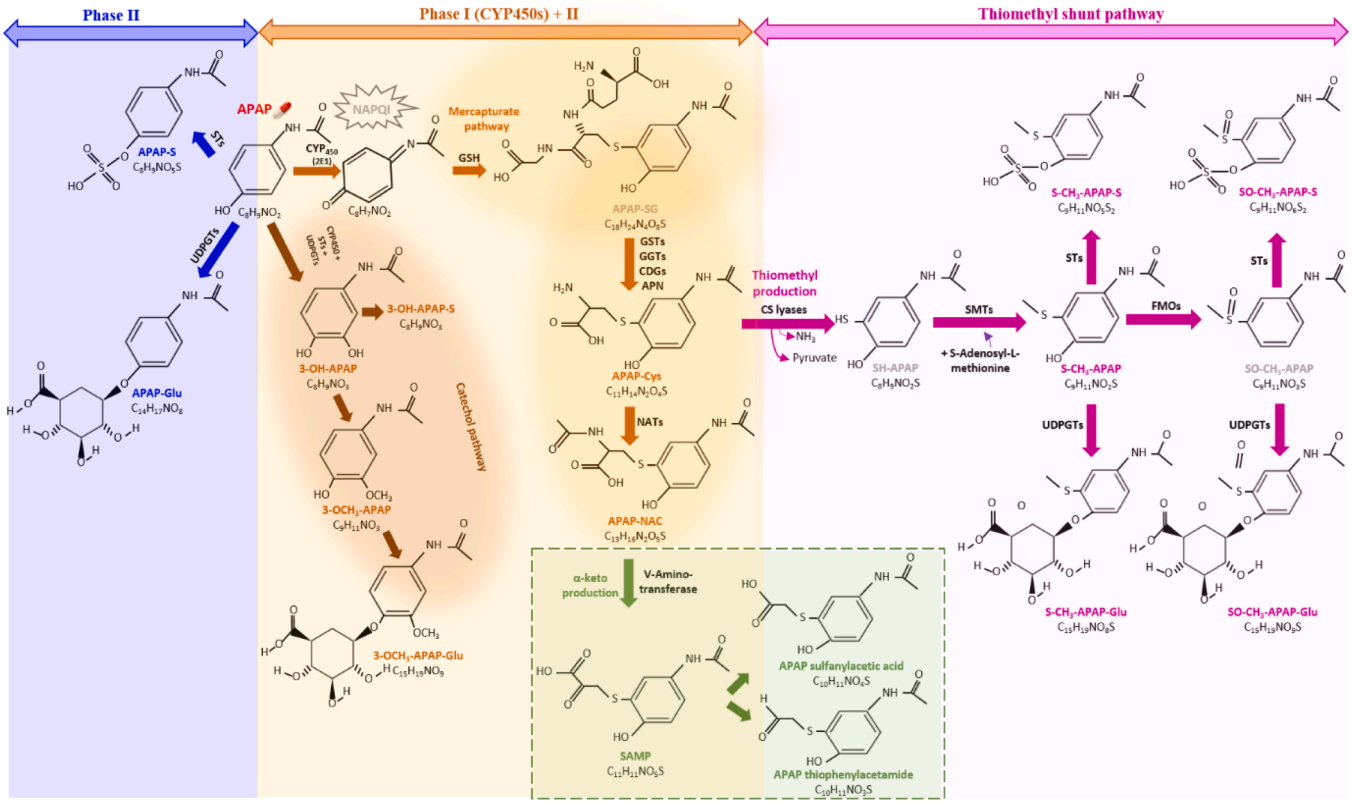

Fig. 1. Main pathways involved in human APAP metabolism including the thiomethyl shunt recently highlighted using HRMS based non-targeted analyses (David et al., 2021a)) and structures of the main APAP metabolites detected in negative mode using a UHPLC-ESI-QTOF. All metabolites were annotated based on MS2 confidence levels using a reference standard when available (see Table S5). APAP metabolites in grey are known precursors but not detected during this experiment because of their lability or low levels. APAP: acetaminophen; APAP-S: APAP sulfate; APAP-Glu: APAP glucuronide; NAPQI: N-acetyl-p-benzoquinone imine; 3-OH-APAP: 3-hydroxyacetaminophen; 3-OH-APAP-S: 3-hydroxyacetaminophen sulfate; 3-OCH_3-APAP: 3-methoxyacetaminophen; 3-OCH_3-APAP-Glu: 3-methoxyacetaminophen glucuronide; APAP-SG: acetaminophen glutathione; APAP-Cys: 3-(Cystein-S-yl)acetaminophen; APAP-NAC: Acetaminophen mercapturate; NAC-O-APAP: Acetaminophen mercapturate sulfoxide; SH-APAP: 3-mercaptoacetaminophen; SH-APAP-Glu: 3-mercaptoacetaminophen glucuronide; SH-APAP-S: 3-mercaptoacetaminophen sulfate; S-CH_3-APAP: S-methyl-3-thioacetaminophen; S-CH_3-APAP-S: S-methyl-3-thioacetaminophen sulfate; S-CH_3-APAP-Glu: S-methyl-3-thioacetaminophen glucuronide; SO-CH_3-APAP: S-methyl-3-thioacetaminophen sulphoxide; SO-CH_3-APAP-S: S-methyl-3-thioacetaminophen sulphoxide sulphate; SO-CH_3-APAP-Glu:: S-methyl-3-thioacetaminophen sulphoxide glucuronide; STs: sulfotransferases; UDPGTs: uridine diphosphoglucuronyltransferases; GSTs: glutathione S-transferases; GGTs: gamma glutamyltransferases, CGDs: cysteinylglycine dipeptidases; NATs: N-acetyltransferases; CS lyases: Cysteine S-conjugate β-lyases; SMTs: S-methyl-transferases; FMOs: flavin-dependent monooxygenases; APN: aminopeptidase N.

elucidation (David et al., 2021a) to assess differences in the diagnostic signals produced by this analytical technique.

We confirmed that significant signals for S-CH_3-APAP-S and SO-CH_3-APAP-S could be detected in all PWs (AUC for combined metabolites represented an average 13 ± 9% of all AUCs, table S6, Fig. 2B), and that inter-individual variabilities were evident (combined AUCs for S-CH_3-APAP-S and SO-CH_3-APAP-S ranged from 5 to 6% (PW 5 and 3) to 21–24% (PW 4 and 1) of all AUCs (table S6)). Overall, the phase II glucuronide conjugation was the dominant pathway in all PWs in terms of signals observed with our HRMS-based method (average AUCs of 48 ± 5%), followed by those related to the catechol metabolites (average AUCs of 20 ± 6%), the mercapturic pathway-related metabolites (average AUCs of 15 ± 5%), and then the thiomethyl conjugates. Altogether, the AUCs based on signals observed for NAPQI-derived metabolites (*i.e.*, from the mercapturic pathway and the thiomethyl shunt combined) represent up to 28% of all AUCs. The signals from thiomethyl metabolites can even be dominant in some cases compared to those observed from the mercapturic pathways (*e.g.*, for PW1 and PW4), showing the importance of these thiomethyl metabolites to monitor more accurately the total excretion of the toxic NAPQI using current LC-ESI-MS-based methods.

We also confirm the potential of S-CH_3-APAP-S and SO-CH_3-APAP-S to identify inter-individual variability in hepatotoxic NAPQI excretion, in particular when they are combined with mercapturic metabolites (their combined AUCs varied by twice as much, from 19 to 41% of all AUCs for the 5 PWs). The inter-individual variabilities observed for the excretion of S-CH_3-APAP-S and SO-CH_3-APAP-S in PWs are similar to the ones observed in the interventional study in men (Fig. 2A, table S7). The inter-individual variabilities observed for the 5 PW could be related to the individual intrinsic ability to generate NAPQI-derived metabolites (genetic factors), and/or could be influenced by drug co-administration (table S2) or other environmental factors (including other xenobiotics (Pristner and Warth 2020)) involved in CYP2E1 induction (*e.g.*, nutrition and fasting) (Michaut et al., 2014).

A delayed concentration peak was observed for thiomethyl metabolites compared to others in the PWs (from 2 to 20 h, Fig. 2B), even though the delay was more variable and sometimes lower than those observed in the interventional study (20–22 h, Fig. 2A). The lack of control over the initial intake and other factors like co-administration or repeated intakes may have influenced the kinetics observed in this observational study with PWs.

Overall, our results confirm that these thiomethyl metabolites: i) are stable biomarkers that can be consistently detected with comparable diagnostic sensitivity as phase II metabolites; ii) present delayed peak excretions compared to conventional phase II metabolites after APAP intake, and iii) show inter-individual variability (even at therapeutic doses) that could be related to the individual intrinsic ability to generate NAPQI-derived metabolites (genetic factors), and/or could be

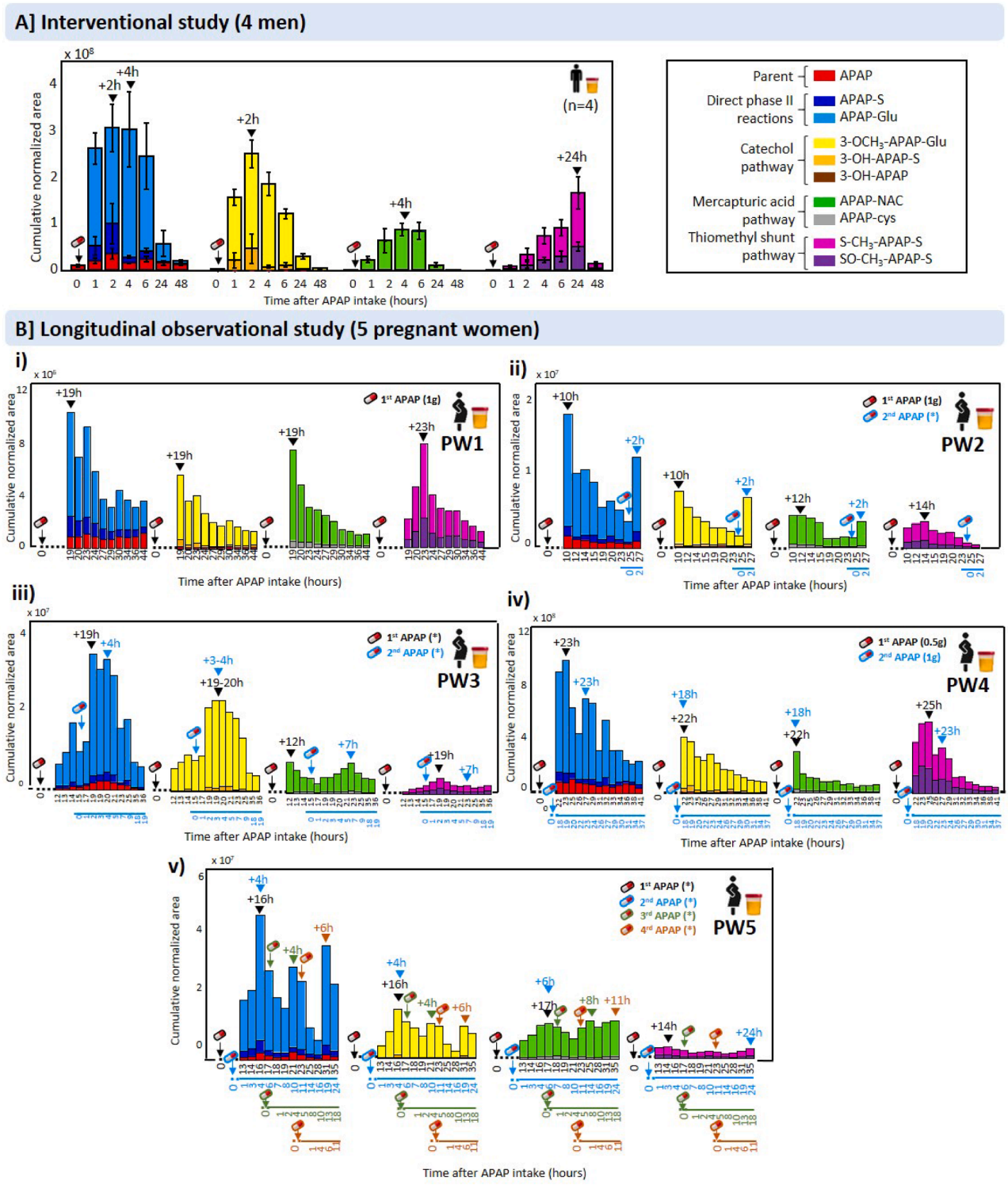

Fig. 2. Kinetics of excretion of free APAP, phase II metabolites (APAP-Glu and APAP-S), catechol metabolites (3-OCH_3-APAP-Glu, OH-APAP-S, and OH-APAP), glutathione derived metabolites (APAP-NAC and APAP-Cys) and thiomethyl metabolites (S-CH_3-APAP-S and SO-CH_3-APAP-S) detected using UHPLC-ESI-QTOF-MS analyses in A) urine of 4-men (mean ± SEM) before intake (baseline, n = 12) and at different time points after intake of 1 g APAP (interventional study (David et al., 2021a)) and B) urine of 5 pregnant women (PW) from the longitudinal observational study (plots i to v) at different time points after intake (0 h) of ≥ 1 APAP doses (represented with different colors in the figure). In all the plots, the integrated peak areas of individual markers were normalised to the total peak area as explained in the experimental section. Medications containing APAP and other co-administration were taken by the 5 pregnant women: PW1: containing APAP (1x doliprane 1 g), co-administration (1x betamethasone, 2x Vicks inhaler); PW2: containing APAP (2x paracetamol), co-administration (3x esomeprazole, 2x plaquenil 400 mg and 2x leucocetirizine 5 mg); PW3: containing APAP (2x doliprane), co-administration (2x omeoprazol, 3x spasfon, 1.5x dolormyl); PW4: containing APAP (1x doliprane 0.5 mg, 1x doliprane 1 g), co-administration (3x mupirocine 2% pomade, 1x mupirocine); PW5: containing APAP (6x dafalgan), co-administration (2x levothyrox, 4x timoferol, 2x magnesium, 2x gestarelle, 2x esomeprazole) (for more information see Table S2). Annotation: To facilitate reading, for PW5, despite the intake of 6 APAP doses, only the time periods of the first 4 doses of APAP have been represented in the figure.*= unknown amount of APAP.

influenced by other environmental factors.

3.2. Thiomethyl metabolites inform on the timing of APAP intakes

We then monitored the presence of these thiomethyl metabolites in a cross-sectional study using urine collected from 10 PWs who reported APAP use and 10 PW who did not (based on questionnaires). In addition to questionnaires and urinary LC-HRMS profiling, we also used conventional targeted LC-MS/MS to identify accurately PWs with urinary APAP concentrations within therapeutic doses.

The 10 PWs that did not report APAP consumption had basal APAP urinary concentrations (median of 125 ng/ml, table S8) (Fig. 3A) similar to urinary environmental concentrations already observed in the general population (medians of 61.7 ng/ml and 100 ng/ml in the German and Danish populations, respectively) (David et al., 2021a; Modick et al., 2014; Nielsen et al., 2015). The sources of environmental exposure to APAP have been linked to APAP precursors such as aniline and 4-aminophenol (two large-volume intermediates in industrial processes), and/or indirect APAP exposure through environmental sources (Kristensen et al., 2016; Modick et al., 2014; Modick et al., 2016; Vorkamp et al., 2021). In these PWs, the thiomethyl metabolites could only be detected in one PW (*i.e.*, PW3). The presence of low detectable levels of S-CH_3-APAP-S and SO-CH_3-APAP-S in PW3 could be related to a higher APAP environmental exposure (*i.e.,* $1.4{\cdot}10^3$ ng/ml as opposed to the median of 125 ng/ml), or more simply to a recall bias.

For PWs who reported APAP consumption, large differences in the distribution of APAP metabolites and their intensities measured by the HRMS-based method could be observed among the 10 PWs. These differences in distribution and intensities can be explained by different times of urine collection after the initial intake as the targeted analyses revealed that only two PWs (11 and 19) had APAP concentrations within therapeutic ranges. Interestingly, the comparison of results from targeted and non-targeted LC-HRMS-based analyses demonstrate the ability of thiomethyl metabolites to inform on the timing of APAP ingestion (*i.e.*, based on the fact that thiomethyl metabolites have delayed kinetics compared to other conventionally investigated APAP metabolites).

Hence, urinary targeted analyses and HRMS-based profiles allowed to distinguish PWs who had urine presumably collected < 6 h after APAP ingestion (*e.g.*, PW11) (Fig. 3A). This PW had APAP urinary concentrations within those generated by therapeutic ranges ($1.3{\cdot}10^6$ ng/ml) and an HRMS-based profile matching what was observed during the first 6 h of the 4-men interventional study (*i.e.*, direct phase II conjugates and catechols' signals higher than those of thiomethyl metabolites) (David et al., 2021a). Likewise, urinary targeted analyses and HRMS-based profiles allowed to distinguish PWs for whom urine collection was performed between 6 and 24 h after APAP ingestion (*e.g.*, PWs 13 and 15). These PWs had urinary APAP concentrations below therapeutic ranges but higher than basal concentrations ($2.1{\cdot}10^4$ – $8.4{\cdot}10^4$ ng/ml, respectively) while HRMS-based metabolite profiles (*i.e.*, thiomethyl metabolites signals higher than direct phase II conjugates and catechols) are consistent with the profiles observed 24 h after APAP intake in the interventional study.

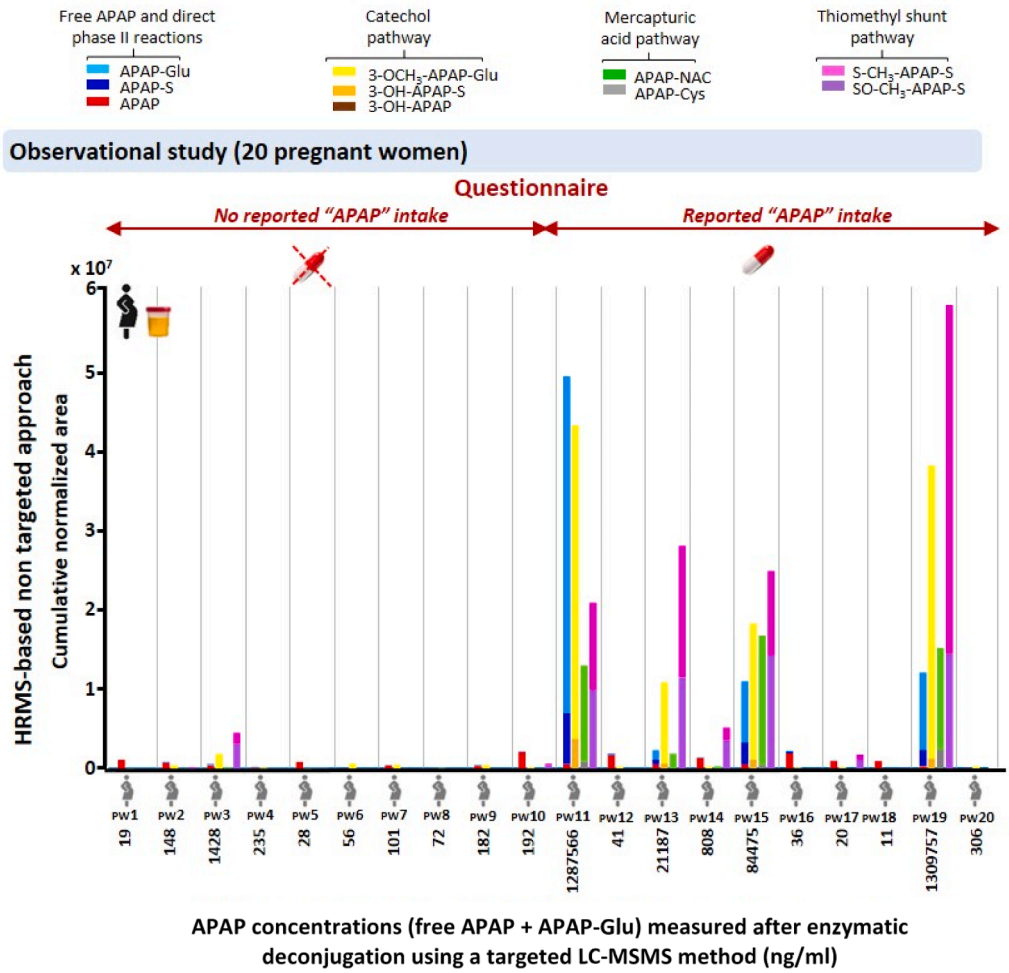

Fig. 3. Non-targeted HRMS-based profiles of APAP metabolites versus targeted APAP concentrations (free APAP + APAP-Glu, ng/ml) measured after enzymatic deconjugation using a targeted LC-MS/MS method in urine samples from 20 pregnant women from the PELAGIE cohort (including 10 women who declared "no APAP intake" versus 10 women who declared "APAP intake").

Furthermore, LC-HRMS-based analyses also provided additional observations compared to targeted analyses and/or the questionnaires. In particular, the presence of thiomethyl metabolites helped detect intentional APAP intake that occurred ~ 24–48 h before sampling. At this time point, environmental basal APAP urinary concentrations (David et al., 2021a; Modick et al., 2014) are interfering with the assessment of APAP therapeutic use since APAP and its glucuronide conjugate have nearly disappeared from urine. However, the presence of S-CH_3-APAP-S and SO-CH_3-APAP-S, still detectable using LC-HRMS at sufficient levels, allows to confirm a previous intake greater than 24 h before urine collection (*e.g.*, PWs 14 and 17).

We also observed one peculiar LC-HRMS-based metabolite profile for PW19 who reported APAP use, which could serve to identify individuals that had repeated APAP intakes. PW 19 had APAP urinary concentrations within the range of those related to therapeutic use ($1.3{\cdot}10^6$ ng/ml) while both levels of short-term metabolites (*e.g.*, 3-OCH_3-APAP-Glu) and long-term metabolites (*i.e.*, S-CH_3-APAP-S and SO-CH_3-APAP-S) detected with LC-HRMS were high (Fig. S1). This combination would suggest that urine was collected after a course of both recent (<6 h) and late intakes (>24 h). Furthermore, unusually high levels of S-CH_3-APAP-S and SO-CH_3-APAP-S but also other conjugates and intermediates forms of thiomethyl metabolites were found. This is the case of conjugated glucuronide forms (*i.e.*, S-methyl-3-thioacetaminophen glucuronide [S-CH3-APAP-Glu], S-methyl-3-thioacetaminophen sulphoxide glucuronide [SO-CH3-APAP-Glu], the intermediate conjugated 3-thioacetaminophen glucuronide [SH-APAP-Glu], and non-conjugated S-CH_3-APAP, Fig. 3B). The high levels of metabolites related to the thiomethyl shunt suggest an excessive production of NAQPI-derived glutathione conjugates (unseen so far in the other individuals). However, it is unclear if this abundance of thiomethyl derivatives is related to a specific susceptibility of this individual to generate NAPQI metabolites, to the pregnancy itself (as sex steroids could be involved in the regulation of CYP2E1(Konstandi et al., 2013)) or to an accumulation of these metabolites in urine over time due to repeated intakes.

Currently, standard monitoring methods analyze APAP and the phase II conjugates (*e.g.*, APAP-Glu) as free APAP after enzymatic deconjugation, but they are limited by their respective short half-lives. Our results suggest that the integration of the thiomethyl biomarkers in these monitoring methods would allow to study the ratio of the short-term metabolites (*i.e.*, phase II glucuronide and conjugated methoxy APAP) over the long-term ones (*i.e.*, thiomethyl metabolites), and could extend the window of exposure assessment (*i.e.*, when APAP and its glucuronide conjugate have nearly disappeared from urine), and consequently provide a better idea of the timing of APAP intake in epidemiological studies. Furthermore, we also show that studying the ratio of short-term metabolites over long-term ones could also serve to identify individuals that have repeated APAP intakes (*i.e.,* individuals with high levels of both short-term and long-term metabolites).

3.3. Thiomethyl metabolites allow monitoring the elimination of the toxic fraction after acute intoxication

We then monitored the presence of S-CH_3-APAP-S and SO-CH_3-APAP-S in blood plasma samples collected from individuals who were admitted to hospital for suspicion of acute APAP intoxication (n = 13, 11 women and 2 men) to study if these biomarkers could help to diagnose acute intoxication and monitor the elimination of the toxic NAPQI over time in intoxicated patients.

Based on hospital protocol, the antidote (NAC, weight-based dosage) was administered in 8 out of 13 patients, sometimes at several time points. The decision to administer the antidote was based on measurements of blood serum APAP concentrations performed using absorption spectrophotometric measurement COBAS® assays (Roche Diagnostic®) and clinical expertise of emergency doctors. All individuals we selected had repeated collections of blood samples (n = 12 with 2 repeated blood samples and n = 1 with 3 repeats) in order to study the excretion of S-CH_3-APAP-S and SO-CH_3-APAP-S signals over time as an indicator of NAPQI elimination.

Blood serum APAP concentrations measured in these patients ranged from 4 to 284 mg/L (median 52 mg/L), and correlated positively with APAP signals measured with the HRMS-based method ($r^2 = 0.89$). The repeated blood samples collected allowed observing significant reductions in APAP concentrations over time (*i.e.*, from 24% of initial concentration up to 97% for the repeated sample) for 11 out of 13 patients.

Our results confirmed that the reduction of APAP concentration in blood observed over time in intoxicated patients was always associated with an increase in thiomethyl metabolites signals (mainly as S-CH_3-APAP-S) (Fig. 4). In 4 out of 11 patients (*i.e.*, I, J, K and M), the signals observed for thiomethyl metabolites were equivalent or higher than the ones observed for free APAP, indicating that a high proportion of reactive NAPQI was generated from the APAP overdose in these patients, and then reabsorbed into the systemic circulation before being eliminated. For these 4 patients with equivalent or higher thiomethyl metabolite signals than the ones observed for APAP, the timing of the second blood collection was always >11 h (between 11 h and 42 h), and the percentage of APAP reduction >90%. Signals of thiomethyl metabolites were always low for blood samples collected less than 6 h apart, showing that elimination of NAPQI (either after conjugation to glutathione or the antidote) is also subject to a delay in case of acute intoxication.

Hence, our results confirm that both thiomethyl metabolites provide significant signals in blood with a delayed kinetic in addition to urine at both therapeutic doses (see (David et al., 2021a)) and after acute intoxication as shown here. While measuring free APAP concentrations in blood remains the ideal way to diagnose acute intoxication just after the overdose, these results also show the potential of these thiomethyl conjugate markers to serve as relevant clinical markers when the patients are admitted more than one day after the overdose, and that free APAP has nearly disappeared from blood. Furthermore, we also show that these thiomethyl conjugates can also serve to monitor the elimination over time of the toxic NAPQI into a non-toxic forms.

3.4. The liver produces thiomethyl metabolites from glutathione conjugates

We then used *in vitro* liver models (HepaRG -HRG- and primary human hepatocytes -PHH) to provide a better understanding of the formation of these thiomethyl metabolites (in particular to identify their precursors), and a better understanding of the site where the biotransformation occurs. The biotransformation that leads to the delayed excretion of thiomethyl metabolites via the thiomethyl shunt pathway has been shown to occur in rodents via the enterohepatic circulation and biliary excretion of the glutathione-derived conjugates into the intestine (Gemborys and Mudge, 1981). However, it remains unclear whether the thiomethyl shunt involved in the formation of S-CH_3-APAP-S and SO-CH_3-APAP-S is preferentially occurring in the liver (before excretion of thiomethyl metabolites in the bile), or via the microbiota (Cooper and Hanigan, 2010; Larsen, 1985).

We exposed HRG cells (4 independent experiments) and PHH (3 independent experiments, from 3 independent donors) to APAP and APAP-Cys, separately, at 50 μg/ml. APAP was used as a positive control to study the ability of these tests to study APAP metabolism, but also to determine whether the cells could produce thiomethyl metabolites *in vitro* from APAP, which implies multiple successive steps, and the involvement of several enzymes (*i.e.*, CYP2E1 > glutathione S-transferases > 3 γ-glutamyltransferase > dipeptidases > CCBL > thiomethyltransferase). The second experiment involved exposure to APAP-Cys as it is known as the main precursor of CCBL in the thiomethyl shunt pathway (Cooper et al., 2011). A 24 h exposure time was selected for both HRG cells and PHH as the optimal time of exposure, as indicated by kinetic studies using several time points (Fig. S2).

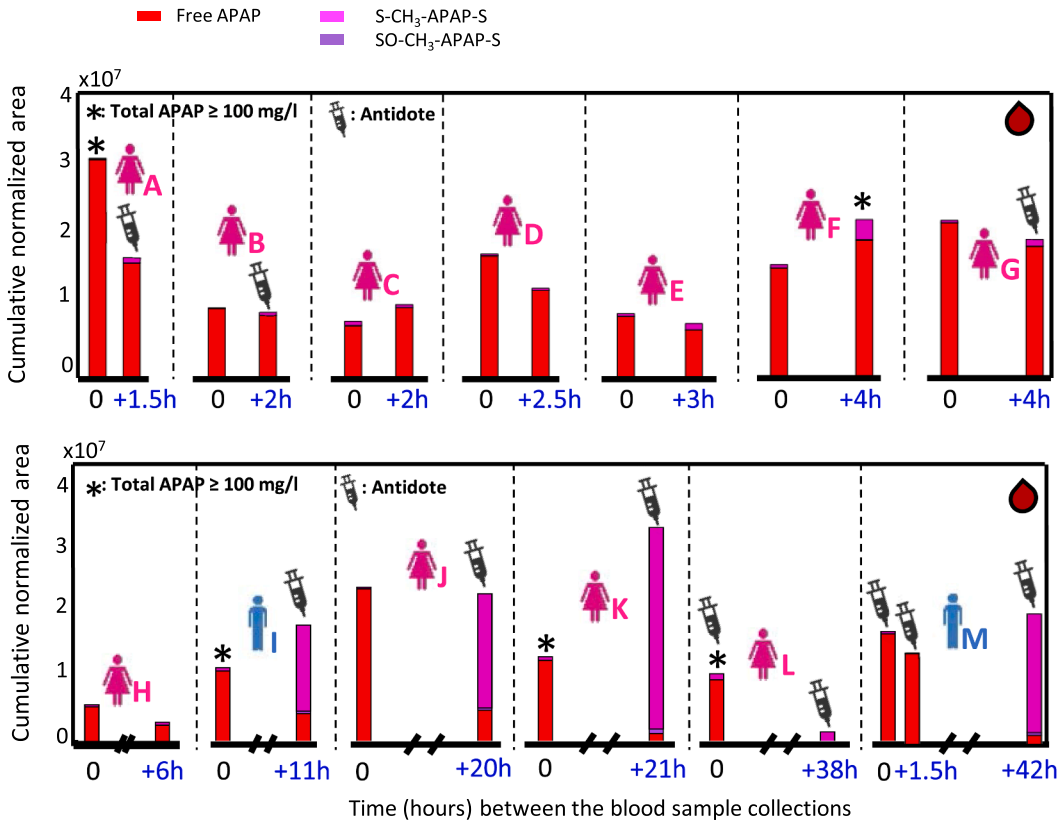

Fig. 4. Distribution profiles of free APAP and thiomethyl shunt metabolites measured using UHPLC-ESI-HRMS in the repeated blood plasma samples collected from patients admitted to hospital for suspicion of acute APAP intoxication (n = 13, 11 women and 2 men). Patients treated with N-acetylcysteine (NAC) antidote are indicated in the figure.

All metabolites detected *in vivo* could be detected after exposure to APAP for 24 h in HRG cells and PHH. In both models, levels of thiomethyl metabolites were low (normalised area of S-CH3-APAP-S and SO-CH3-APAP-S represent about 2 and 1% in PHH and HRG of all detected metabolite's areas, respectively) compared to direct phase II conjugates (Table 1). They, therefore, differed from the higher thiomethyl signal observed *in vivo* (see Figs. 2 and 3). As mentioned previously, the formation of thiomethyl metabolites is dependent on NAPQI production by CYP2E1. Hence, the low levels of thiomethyl metabolites observed after APAP exposure *in vitro* are probably associated to the limited cytochrome P450 capabilities of these models to generate NAPQI metabolites via CYP2E compared to *in vivo* hepatocytes. This seems to be consistent with the fact that higher levels of thiomethyl metabolites were observed in PHH than in HRG cells since CYP2E1 activity in PHH is known to be higher than in HRG cells (Kanebratt and Andersson, 2008). In agreement with this, higher levels of the catechol oxidative metabolite 3-OCH_3-APAP-Glu were also observed in PHH compared to HRG cells.

We then exposed HRG cells and PHH to APAP-cysteine for 24 h to study their ability to generate thiomethyl metabolites. We confirmed here that APAP-cysteine is an important and direct precursor of thiomethyl metabolites since the conjugated forms S-CH_3-APAP and SO-CH_3-APAP were the dominant metabolites detected in both models. High levels of both sulfate conjugates (as observed *in vivo*) but also glucuronide conjugates (*i.e.*, S-methyl-3-thioacetaminophen glucuronide [S-CH_3-APAP-Glu]) could be detected (normalised total area of S-CH_3-APAP-S and SO-CH_3-APAP-S represent 69 and 70 % of all metabolites in PHH and HRG, respectively, Table 1). Hence, we showed that both liver models produce CCBL enzymes that have the ability to cleave the C-S bond of cysteine conjugates, which tends to demonstrate the involvement of the liver in the formation of these thiomethyl metabolites.

Furthermore, HRMS-based non-targeted analyses using MS/MS structural elucidation helped identify other relatively minor metabolites related to APAP-Cys such as S-(5-acetylamino-2-hydroxyphenyl)

Table 1
Production of the main APAP metabolites detected after exposure (24 h) to APAP and APAP-Cys in primary human hepatocytes (PHH) and HepaRG cells (HRG) using LC-ESI-HRMS (expressed in percentages calculated using the HRMS normalized area of detected metabolites, mean ± SEM).

Main detected	APAP exposure	
APAP metabolites	PHH (N = 3, n = 4)	HRG (N = 4, n = 3)
APAP-Glu	43 ± 10 %	64 ± 25 %
APAP-S	37 ± 7 %	32 ± 11 %
3-OCH_3-APAP-Glu	14 ± 8 %	2 ± 1 %
3-OH-APAP	3 ± 0.5 %	1 ± 0.2 %
APAP-NAC	n.d.	0.5 ± 0.1 %
3-OH-APAP-S	1 ± 0.5 %	0.3 ± 0.1 %
S-CH_3-APAP-S	1 ± 0.2 %	0.2 ± 0.1 %
SO-CH_3-APAP-S	1 ± 0.1 %	0.1 ± 0.1 %
	APAP-Cys exposure	
	PHH (N = 3, n = 4)	**HRG (N = 4, n = 3)**
S-CH_3-APAP-S	47 ± 5 %	54 ± 12 %
SO-CH_3-APAP-S	22 ± 4 %	16 ± 3 %
APAP-NAC	16 ± 1 %	5 ± 0.3 %
S-CH_3-APAP-Glu	10 ± 4 %	22 ± 3 %
SAMP	4 ± 0.5 %	2 ± 0.5 %
Acetamide	1 ± 0.5 %	1 ± 0.2 %

N = number of independent experiments, n = number of replicates within each independent experiment. Phase II conjugates and catechol metabolites were not detected after exposure to APAP-Cys and were not included in the calculation of percentages.

mercaptopyruvic acid (SAMP), an APAP metabolite potentially formed by the transamination reaction of APAP-cysteine (see Fig. 1), previously observed in mouse (Chen et al., 2008). The conversion of a cysteine S-conjugate to the corresponding alpha-keto acid occurring by transamination with a suitable alpha-keto acid acceptor has already been observed for other compounds conjugated to NAC (Cooper and Hanigan 2010). One other minor metabolite was annotated as N-(4-hydroxy-3-((2-oxoethyl)thio)phenyl)acetamide. This minor metabolite could also be detected *in vivo* in the men and PWs, but at much lower levels than the thiomethyl metabolites (AUCs for SAMP in the men and PW correspond to<4% of all combined AUCs *in vivo,* see tables S6 and S7). Furthermore, the peak excretion of these minor metabolites were observed earlier than those of thiomethyl metabolites in the controlled study *in vivo* with the 4 men (*i.e.*, between 2 and 6 h as opposed to 24 h, see Fig. S3), suggesting that they are not involved in the thiomethyl shunt.

We then compared the production of thiomethyl metabolites after exposure to APAP (positive control), and 3 of its potential direct precursors: the glutathione conjugate (APAP-SG), APAP-Cys, and APAP-NAC for 24 h in HRG cells and PHH (Fig. 5). All glutathione-derived metabolites produced significantly higher levels of S-CH_3-APAP-S and SO-CH_3-APAP-S than APAP ($p < 0.001$ in HRG cells and PHH, respectively), which is in agreement with our previous observations. As for the glutathione-derived metabolites, APAP-Cys and APAP-SG produced higher and similar levels of S-CH_3-APAP-S and SO-CH_3-APAP-S compared to APAP-NAC. The thiomethyl shunt involves the activity of CCBL to cleave cysteine S-conjugates, and subsequent methylation with an active form of methionine to produce thiomethyl metabolites (Cooper et al., 2011; Gemborys and Mudge, 1981), meaning that APAP-SG and APAP-NAC were probably biotransformed to APAP-Cys beforehand. Interestingly, APAP-NAC produced high levels of the SAMP and N-(4-hydroxy-3-((2-oxoethyl)thio)phenyl)acetamide compared to APAP-SG and APAP-Cys. Such difference was more evident in HRG cells, where the production of the latter metabolites by APAP-SG and APAP-Cys was almost non-existent, but less significant in PHH, where there was a significant production of SAMP by APAP-SG (ANOVA's $p < 0.001$).

These results confirm the ability of *in vitro* liver models to generate the thiomethyl metabolites from all glutathione-derived metabolites (APAP-SG, APAP-Cys and APAP-NAC). As we did not observe differences in the kinetics of formation between conventional APAP metabolites and the thiomethyl metabolites *in vitro*, it is likely that the delayed excretion of the thiomethyl metabolites observed *in vivo* is due to a late reabsorption into the systemic circulation after the enterohepatic circulation. These results demonstrate the important role of the thiomethyl shunt pathway, together with the mercapturic pathway, in the detoxification and the elimination of the reactive NAPQI metabolite, and suggest that the production of this reactive metabolite may be underestimated in humans.

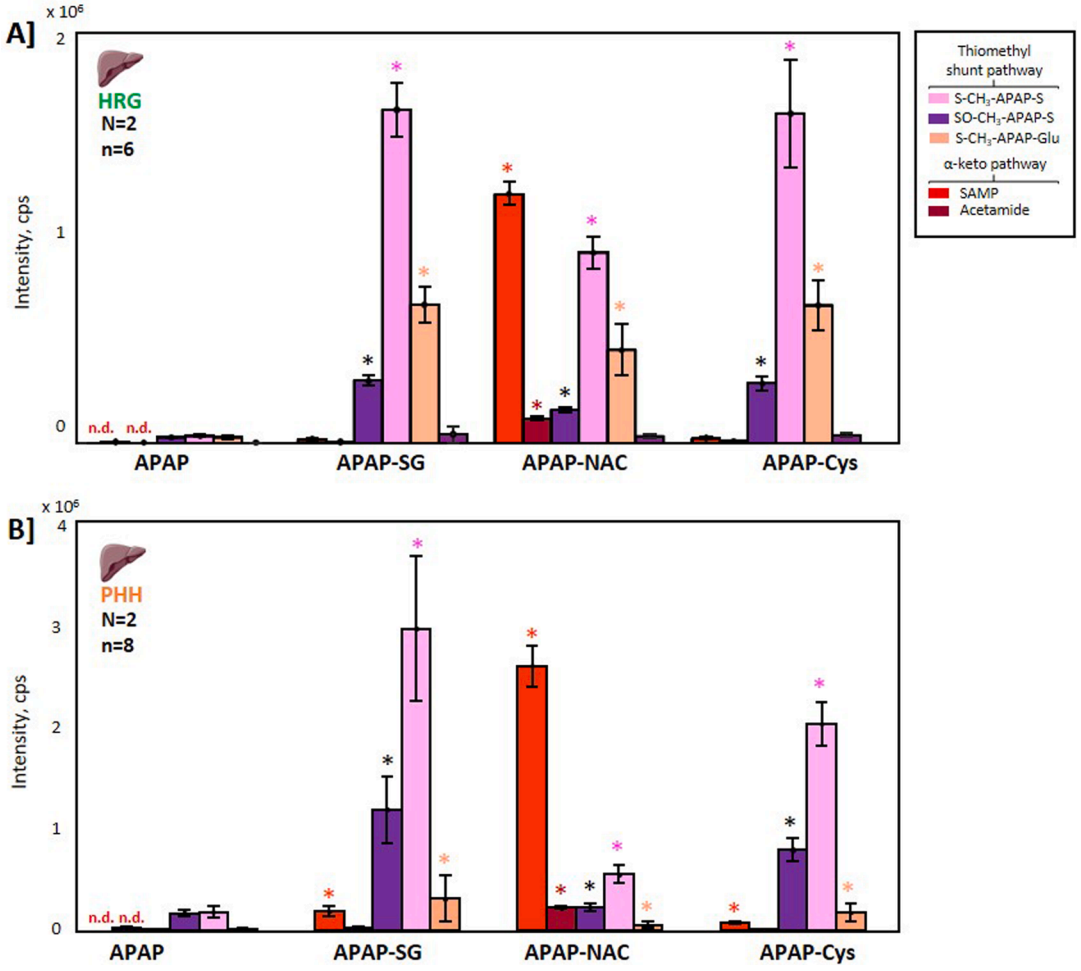

Fig. 5. Production of thiomethyl metabolites (mean ± SEM) in A] HepaRG cells (HRG) and B] primary human hepatocytes (PHH) after exposure to APAP (positive control) and their main potential precursors: APAP-SG, APAP-NAC and APAP-Cys at 50 μg/ml. One-way ANOVA followed by multiple comparisons test is used to indicate statistical differences between APAP-SG, APAP-NAC, and APAP-Cys treatments versus APAP (*$p < 0.001$). N = number of independent experiments, n = number of replicates within each independant experiments.

3.5. Selected enteric bacteria formed very low levels of thiomethyl metabolites

The contribution of the gastrointestinal microbiota to produce thiomethyl metabolites, after biliary excretion has been previously studied in germ-free and neomycin-treated mice. The results suggested that the gut microbiota could play an important role in the C-S cleavage of APAP-Cys to form S-CH_3-APAP-S and SO-CH_3-APAP-S (Mikov, 1994). In agreement with that, several studies have shown that many enteric bacteria contain CCBL (Cooper and Hanigan 2010; Larsen, 1985).

Here, we selected five bacterial strains representative of different phyla colonizing the human gut (*i.e., Agathobacter rectalis, Phocaeicola vulgatus, Bifidobacterium longum, Escherichia coli,* and *Enterococcus faecalis*), and that previously displayed CCBL activity for some of them (Cooper and Hanigan 2010; Larsen, 1985). The bacterial strains were exposed to APAP (positive control) and APAP-Cys, separately, at 50 μg/ml for 24 h in anaerobic conditions.

In these conditions, the exposure of the bacterial strains to APAP generated direct phase II conjugates (APAP-Glu and APAP-S) and the conjugated catechol oxidative metabolite 3-OCH_3-APAP-Glu, demonstrating the ability of bacterial strains to metabolize APAP in these experimental conditions. However, much lower levels of these metabolites were generated here compared to the levels observed in liver models.

As opposed to the liver models, exposing the bacterial strains to APAP-Cys, the main precursor of CCBL, did not generate the formation of thiomethyl metabolites S-CH_3-APAP-S and SO-CH_3-APAP-S. However, the SAMP, N-(4-hydroxy-3-((2-oxoethyl)thio)phenyl)acetamide acid metabolite, observed in the liver models and *in vivo,* could be generated in presence of all bacterial strains, and in some cases at higher levels compared to the liver models (*i.e.,* higher production of SAMP by *E.coli* and of N-(4-hydroxy-3-((2-oxoethyl)thio)phenyl)acetamide by *E. coli* and *P. vulgatus*), suggesting a potential involvement of the microbiota in the production of this alpha-keto acid from APAP-Cys (Fig. 6). Moreover, a metabolite corresponding to the alpha-keto pathway, named the 2-(5-acetamido-2-hydroxyphenyl)sulfanylacetic acid was also detected in most of strains after APAP-Cys exposure.

Since the results we observed could be related to the inability of these tests to reproduce the same conditions as the ones observed in the gastrointestinal tract for the exposure experiments (*i.e.*, in terms of pH or even a community-based response), we then exposed APAP and APAP-Cys to lysates from all these bacterial strains obtained after cell

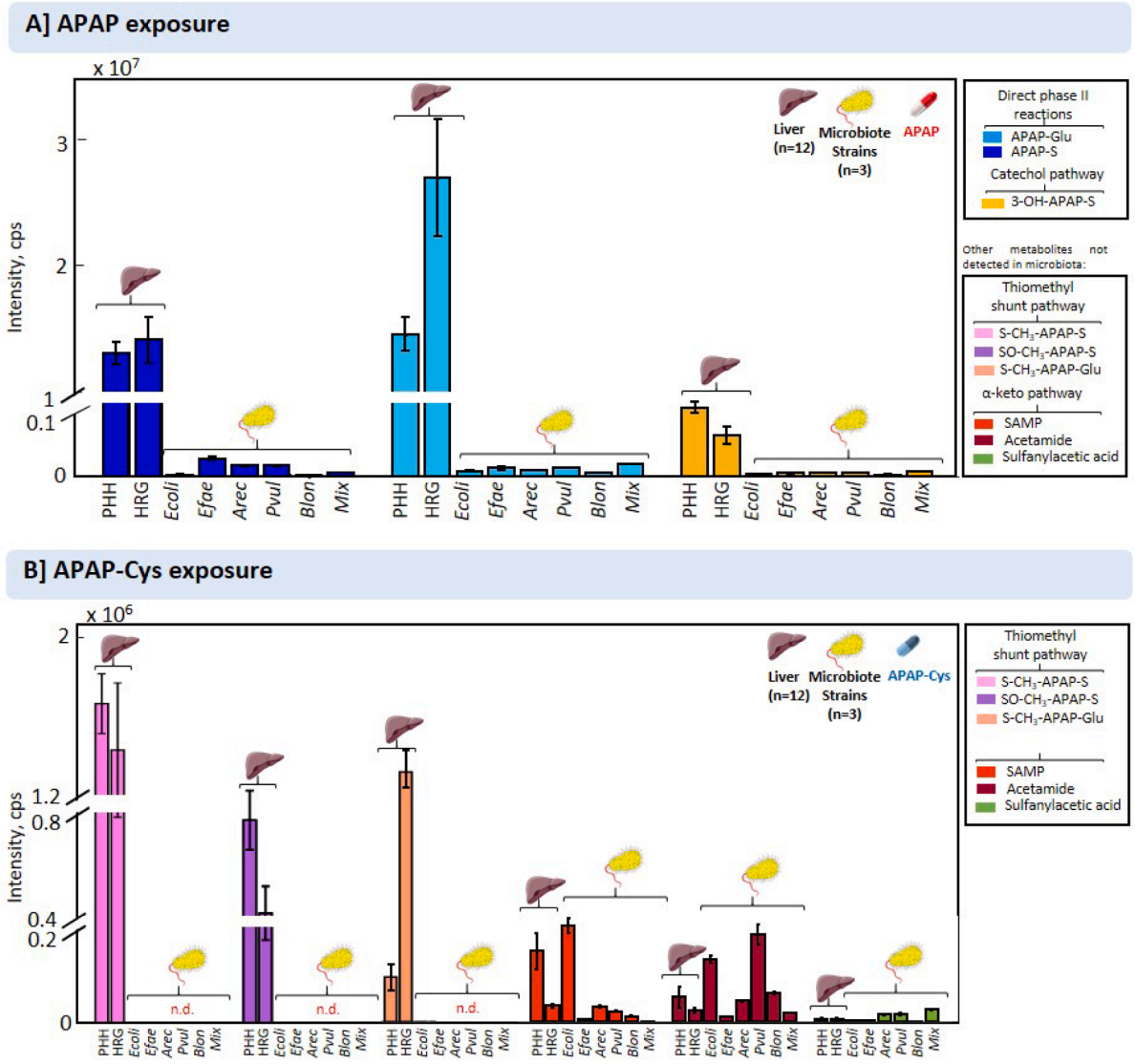

Fig. 6. Production of APAP metabolites by HepaRG cells (HRG), primary human hepatocytes (PHH), and 5 types of intestinal microbiome strains after 24 h exposure to A] free acetaminophen (APAP) and to B] APAP-Cys both at 50 μg/ml. *Escherichia coli* (Ecoli), *Enterococcus faecalis* (Efae), *Agathobacter rectalis* (Arec), *Phocaeicola vulgatus* (Pvul), *Bifidobacterium longum* (Blon) and a mixture of the five strains (Mix). n = total number of replicates.

homogenizations using sonication (Schwiertz et al., 2008). Interestingly, low levels of S-CH_3-APAP-S (but no SO-CH_3-APAP-S) were observed after exposure to both APAP and APAP-Cys, suggesting that an active form of CCBL is present in these bacterial strains. No significant differences in the production of S-CH_3-APAP-S were observed between the bacterial strains (Fig S4).

Even though these results suggest that the tested bacterial strains do not display a predominant role in the thiomethyl shunt as opposed to the liver, further experiments with the gut microbiota (*i.e.,* with other bacterial strains but also with more similar conditions than the ones observed in the gastrointestinal tract including studying community-based response), will be needed to confirm these results.

4. Discussion

APAP is an active ingredient in more than 600 prescription and non-prescription pharmaceuticals and is one of the most commonly used pharmaceuticals. The medical benefits of APAP are widely recognized. Nevertheless, it has been suggested that chronic use of APAP during the pregnancy could be associated with developmental disorders in human (Bauer et al., 2021). APAP overdose is also known to be an important cause of ALF worldwide (Lee, 2004). In this study, we provide evidence of the utility of new biomarkers that could serve to improve APAP biomonitoring methods in epidemiological and clinical studies. More specifically, we replicate previous results observed in men and show that the thiomethyl metabolites S-CH_3-APAP-S and SO-CH_3-APAP-S are stable biomarkers consistently detected in urine from pregnant women after APAP intake, with a delayed excretion compared to conventional metabolites. We also demonstrate that high signals for S-CH_3-APAP-S and SO-CH_3-APAP-S can be increasingly detected over time in blood collected after APAP acute intoxication, suggesting that these markers could serve as relevant clinical markers when the patients are admitted more than one day after the overdose and that free APAP concentrations have nearly disappeared from blood. Using *in vitro* liver models, we show that these thiomethyl metabolites are directly linked to the elimination of the hepatotoxic NAPQI metabolites since S-CH_3-APAP-S and SO-CH_3-APAP-S are the main metabolites formed after exposure to NAQPI-derived metabolites (glutathione, cysteine, and mercapturate conjugates) in both PHH and HRG cells. Exposure on liver models and enteric bacteria suggests that the liver could be one of the main sites of biotransformation of these metabolites, even though further experiments on the microbiota will be needed to confirm these results. The kinetics of formation of the thiomethyl metabolites observed *in vitro* suggest that the delayed excretion observed *in vivo* is likely related to a late reabsorption into the systemic circulation after the enterohepatic circulation.

Based on our observations, we believe that the measurement of these thiomethyl metabolites in epidemiological and clinical studies could serve to provide a more reliable history of APAP ingestion by studying the ratio of the short-term metabolites (*i.e.*, phase II glucuronide conjugates and conjugated methoxy APAP) over the long-term ones (*i.e.*, thiomethyl metabolites). In particular, these markers could improve human APAP biomonitoring in epidemiological studies as they allow extending the assessment of APAP therapeutic use even 24–48 h after intake when environmental APAP urinary concentrations are interfering with basal APAP urinary concentrations observed at this time after the intake, or when APAP glucuronide conjugates have nearly disappeared from urine. Importantly, these biomarkers could also help identify individuals that had repeated APAP intakes (*i.e.,* individuals with high levels of short-term metabolites and long-term metabolites) as we observed for a specific PW, which would allow to identify more easily chronic users potentially more at risk to develop undesirable effects.

Potential clinical applications for these markers include i) a better typing of patients after APAP acute intoxication (*i.e.*, to monitor more precisely the kinetic detoxification process through the appearance of thiomethyl metabolites); ii) a better typing of chronic users to study risks of developing hepatotoxicity at the highest recommended doses (even though this is still controversial there is accumulating evidence that the maximum recommended dose can induce mild-to-moderate hepatic cytolysis); and iii) identification of inter-individual-variability in NAPQI production and consequently identify individuals or populations with underlying predispositions (*e.g.,* pre-existent induction of CYP2E1) to develop APAP-induced liver injuries (Athersuch et al., 2018; Michaut et al., 2014).

The current study presents limitations inherent to the production of HRMS-based semi-quantitative data. Hence, these data cannot be used to extrapolate the accurate percentage of excretion of the thiomethyl metabolites in APAP metabolism. Nevertheless, our quality control data provide confidence for the sample-to-sample comparison made to study the kinetics of the excretion of individual metabolites at different time points. The comparison of the relative contributions of APAP metabolites based on their analytical responses is therefore relevant for application in human biomonitoring given that similar platforms (LC-MS) and ion sources (*i.e.*, electrospray ion sources) are usually used for APAP biomonitoring.

Further toxicological investigations would also help to understand if the thiomethyl shunt acts only as a pathway for NAPQI detoxification and excretion, or if it can contribute to liver or renal toxicity in case of APAP overdose through the generation of intermediates with highly reactive sulfur-containing fragment as observed with environmental contaminants (*e.g.*, trichloroethylene) (Cooper et al., 2011). In one particular PW (*i.e.*, PW 19), unusually high levels of thiomethyl metabolites, and their intermediates were detected. Non-conjugated S-CH_3-APAP and potential reactive intermediates (SH-APAP detected as glucuronide conjugate) were detected in the urine of this PW. The toxicity related to the generation of these reactive thio- intermediates remains unclear, in particular in the case of acute intoxication, and has never been studied so far in the case of APAP. Moreover, S-CH_3-APAP was previously described as being equipotent with aspirin and more potent than APAP as an analgesic in the mouse writhing test, even though its duration of action was much shorter than the parent compound even at twice the dose (Hertz and Deghenghi, 1983). Hence, it is not clear how this S-CH_3-APAP metabolite could contribute to the analgesic effects when produced in large quantities in humans.

In conclusion, this study highlights the role of the thiomethyl shunt pathway in APAP metabolism to produce stable thiomethyl metabolites from the NAPQI-derived metabolites that are excreted in both urine and blood. These biomarkers could be used in future epidemiological studies to improve the biomonitoring of APAP use, as well as in clinical studies to improve the diagnosis of acute intoxication and follow the elimination of NAPQI. Importantly, these results demonstrate the important role of the thiomethyl shunt pathway, together with the mercapturic pathway, in the detoxification and the elimination of the reactive NAPQI metabolite, and suggest that the production of this reactive metabolite may be underestimated in humans. Future steps include the synthesis of pure standards for S-CH_3-APAP-S and SO-CH_3-APAP-S that are not currently commercially available and the development of high-throughput targeted methods. Further validations in terms of specificity, sensitivity, and reproducibility in larger populations (including children as well) will be required for these new markers before applications in routine practice. Finally, with this study, we also demonstrated the utility of HRMS exposomics-based approach to uncover new biomarkers that provide a better understanding of the kinetic of elimination of a drug/xenobiotic, and advance a step forward towards improved precision for human biomonitoring of the chemical exposome (David et al., 2021b).

CRediT authorship contribution statement

Eva Gorrochategui: Writing – review & editing, Writing – original draft, Visualization, Investigation, Methodology, Conceptualization. **Marc Le Vee:** Writing – review & editing, Investigation, Methodology.

Habiba Selmi: Writing – review & editing, Visualization, Investigation, Methodology. **Anne Gérard:** Writing – review & editing, Visualization, Investigation, Methodology. **Jade Chaker:** Writing – review & editing, Methodology. **Annette M. Krais:** Writing – review & editing, Methodology. **Christian Lindh:** Writing – review & editing, Methodology. **Olivier Fardel:** Writing – review & editing, Investigation, Methodology. **Cécile Chevrier:** Writing – review & editing, Methodology. **Pierre Le Cann:** Writing – review & editing, Methodology. **Gary W. Miller:** Writing – review & editing, Funding acquisition. **Robert Barouki:** Writing – review & editing, Funding acquisition. **Bernard Jégou:** Methodology, Investigation, Funding acquisition, Visualization. **Thomas Gicquel:** Writing – review & editing, Investigation, Methodology. **David Kristensen:** Writing – review & editing, Investigation, Methodology. **Arthur David:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Visualization, Investigation, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.108299.

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