Physiologically based pharmacokinetic modeling to predict the impact of liver cirrhosis on glucuronidation via UGT1A4 & UGT2B7/2B4 – a case study with midazolam


Pharmaceutical Sciences, Roche Pharma Research and Early Development, Roche Innovation Center Basel, Grenzacherstrasse 124, 4070, Basel, Switzerland (AO, SF, KU, NP, JK, AP, BW), Drug Delivery and Disposition Lab, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Belgium (AO, AP, PA) and BioNotus GCV, Niel, Belgium (PA). Division of Clinical Pharmacology & Toxicology, University Hospital Basel, Basel, Switzerland (SK); Department of Clinical Research, University of Basel, Basel, Switzerland (SK); Division of Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland (SK).
Running Title Page

Running Title: “PBPK modeling of liver cirrhosis impact on UGT activity”.

Corresponding author:

Agustos Ozbey

Address: Pharmaceutical Sciences, Roche Pharma Research and Early Development, Roche Innovation Center Basel, Grenzacherstrasse 124, 4070, Basel, Switzerland

e-mail: agustos.ozbey@roche.com

Text pages: 20 - page 7 to 28 (without references, from introduction to end)

Number of Tables: 2

Number of Figures: 7 (6 + graphical abstract)

Number of References: 35

Word counts (with references):

Abstract: 250 words; Significance Statement: 68 words; Introduction: 475 words; Method: 2013 words; Results: 1012 words; Discussion: 1201 words; Conclusion: 159 words.

Abbreviations: AUC$_{\text{inf}}$, area under the plasma concentration curve between 0h to infinity; B/P, blood/plasma partitioning; CP, Child-Pugh; CL$_{\text{int}}$, intrinsic clearance; CL$_{\text{r}}$, renal clearance; C$_{\text{max}}$, maximal plasma concentration; CYPs, cytochrome P450 enzymes; F.E., fold error; f$_{\text{m}}$, fraction metabolized; f$_{\text{u,p}}$, fraction unbound in plasma; HLM, human liver microsomes; Km, Michaelis-Menten constant; MR, metabolic ratio; logP, partition coefficient between octanol and water at near infinite dilution or lipophilicity; MW, molecular weight; NA, not available; PBPK, physiologically-based pharmacokinetic; Peff, effective intestinal permeability; RAF, relative activity factor; UGT, uridine glucuronosyltransferase; V$_{\text{max}}$, maximal rate of metabolism; V$_{\text{ss}}$, volume of distribution at steady state.
Abstract

Hepatic impairment, due to liver cirrhosis, decreases the activity of cytochrome P450 enzymes (CYPs). The use of physiologically-based pharmacokinetic (PBPK) modeling to predict this effect for CYP substrates has been well-established, but the effect of cirrhosis on uridine-glucuronosyltransferase (UGT) activities is less studied and few PBPK models have been reported. UGT enzymes are involved in primary N-glucuronidation of midazolam and glucuronidation of 1'-OH-midazolam following CYP3A hydroxylation. In this study Simcyp® was used to establish PBPK models for midazolam, its primary metabolites midazolam-N-glucuronide (UGT1A4) and 1'-OH midazolam (CYP3A4/3A5) and the secondary metabolite 1'-OH-midazolam-O-glucuronide (UGT2B7/2B4), allowing to simulate the impact of liver cirrhosis on the primary and secondary glucuronidation of midazolam. The model was verified in non-cirrhotic subjects before extrapolation to cirrhotic patients of Child-Pugh (CP) classes A, B, and C. Our model successfully predicted the exposures of midazolam and its metabolites in non-cirrhotic and cirrhotic patients, with 86% of observed plasma concentrations within 5th-95th percentiles of predictions and observed geometrical mean of AUCinf and Cmax within 0.7-1.43-fold of predictions. The simulated metabolic ratio (AUCglucuronide/AUCparent, MR), was calculated for midazolam-N-glucuronide to midazolam (indicative of UGT1A4 activity) and decreased by 40% (CP A), 48% (CP B), and 75% (CP C). For 1'-OH-midazolam-O-glucuronide to 1'-OH-midazolam the MR (indicative of UGT2B7/2B4 activity) dropped by 35% (CP A), 51% (CP B), and 64% (CP C). These predicted MRs were corroborated by the observed data. This work thus
increases confidence in Simcyp® predictions of the effect of liver cirrhosis on the pharmacokinetics of UGT1A4 and UGT2B7/UGT2B4 substrates.
Significance statement

This paper presents a PBPK model for midazolam and its metabolites and verifies the accurate simulation of pharmacokinetic profiles when using the Simcyp® hepatic impairment population models. Exposure changes of midazolam-N-glucuronide and 1’-OH-midazolam-O-glucuronide reflect the impact of decreases in UGT1A4 and UGT2B7/2B4 glucuronidation activity in cirrhotic patients. The approach used in this study may be extended to verify the modeling of other UGT enzymes affected by liver cirrhosis.
Graphical abstract

**Graphical abstract** Flowchart presenting the development of a PBPK model for midazolam and its metabolites and model application to evaluate UGT1A4 and UGT2B7/UGT2B4 activity changes in cirrhotic patients. The model is first developed in control subjects, with parameter-optimization to fit observed data. Then, after additional verification, the optimized model is extended to patients with liver cirrhosis by incorporating specific population modifications for Child-Pugh (CP) stages A, B and C.
1. Introduction

Hepatic impairment due to liver cirrhosis is associated with multiple pathophysiological changes, which can affect the pharmacokinetics (PK) of administered drugs. Hepatic extraction may be influenced by the liver shunt effect (Buob et al., 2011; Nardelli et al., 2020; Small et al., 2023), potentially leading to increased bioavailability of orally administrated compounds. The synthesis of plasma proteins, such as albumin and α1-acid glycoprotein, can be reduced by up to 50% (Barry et al., 1990; Johnson et al., 2010; Viani et al., 1992), which would elevate the free fraction in plasma and potentially increase the clearance and the volume of distribution. Furthermore, liver enzyme activity can be impacted. The activity of hepatic cytochrome P450 enzymes (CYPs) is known to be reduced in patients with liver cirrhosis (Delco et al., 2005; Johnson et al., 2010). However, the activity of UDP-glucuronosyltransferases (UGTs), responsible for drug clearance via glucuronidation, is not characterized well in patients with liver cirrhosis (Bigo et al., 2013; Rowland et al., 2013). An example of the influence of liver cirrhosis on UGT1A4 activity can be observed in the literature with the case study of lamotrigine, an antiepileptic drug metabolized primarily by UGT1A4, which exhibited altered pharmacokinetics in patients with liver cirrhosis (Marcellin et al., 2001). The diminished UGT1A4 activity in these individuals resulted in prolonged half-life and reduced clearance, necessitating dose adjustments for optimal therapeutic outcomes. These observations underscores the critical role of UGT1A4 in drug metabolism and highlight the need for personalized dosing strategies in the context of liver cirrhosis to ensure both efficacy and safety.
Our prior research evaluated the impact of liver cirrhosis on probe CYP substrates included in the Basel Cocktail, a formulation comprising six specific compounds: caffeine (CYP1A2), efavirenz (CYP2B6), flurbiprofen (CYP2C9), omeprazole (CYP2C19), metoprolol tartrate (CYP2D6), and midazolam (CYP3A). We employed physiologically-based pharmacokinetic (PBPK) modeling to predict PK of the probe substrates, after oral administration of the Basel Cocktail, in non-cirrhotic control subjects and cirrhotic patients, further categorized into Child-Pugh (CP) classes A, B, and C. We were able to predict the impact of liver cirrhosis on CYP-mediated metabolism, followed by proposing correction factors for dose adjustments tailored to cirrhotic patients ("Dose Adjustment in Patients with Liver Cirrhosis - Comparison of Two Different Modeling Approaches," 2023; Duthaler et al., 2022). In addition to the drug molecules themselves, the Basel Cocktail study also quantified plasma concentrations of the major circulating metabolites. Concentrations of glucuronide metabolites were estimated by calculating the difference between measurements obtained with and without beta-glucuronidase treatment of the samples (Duthaler et al., 2022). The metabolic ratio \( \frac{\text{AUC}_{\text{glucuronide}}}{\text{AUC}_{\text{parent}}} \) (MR) was considered to reflect the activity of the enzyme forming the metabolite and was used to evaluate the effect of liver cirrhosis on glucuronidation (Donzelli et al., 2014; Fuhr et al., 2007). Among the compounds under investigation, glucuronide formation could be measured for caffeine (paraxanthine), metoprolol (metoprolol-glucuronide) and midazolam (midazolam-\text{-}N\text{-}glucuronide, 1'-OH midazolam-\text{-}O\text{-}glucuronide) and showed decreased glucuronidation in CP patients. Midazolam offered the most promising opportunity for additional research due to the availability of clear PK data.
on its well-defined glucuronides, whose associated UGT enzymes were already identified. Moreover, the glucuronidation reactions appeared to be relatively enzyme-specific. Midazolam and its metabolite 1'-hydroxymidazolam are subject to glucuronidation via UGT1A4 and UGT2B7/UGT2B4, respectively (Figure 2) (Seo et al., 2010; Wessels et al., 2021), and both showed approximately 80% reduction in the MRs for glucuronide formation in Child C patients (Duthaler et al., 2022). The case example described in this paper extends the midazolam PBPK model to midazolam-\(N\)-glucuronide, generated directly through glucuronidation by UGT1A4 and to 1'-OH midazolam-\(O\)-glucuronide, generated through the glucuronidation of metabolite 1'-OH-midazolam by UGT2B7 and UGT2B4 (Johnson et al., 2023; Seo et al., 2010). The activity changes of UGT1A4 and UGT2B7/UGT2B4, were determined in the hepatic impaired populations relative to the control subjects to act as a basis for future PBPK simulations of drugs cleared by these enzymes under hepatic impairment conditions.
2. Materials and Methods

2.1 Basel cocktail clinical study in control and cirrhotic patients

The clinical study outline is summarized in supplementary materials, and full clinical study details have been published previously (Duthaler et al., 2022).

In short, 12 control, 16 CP A, 15 CP B and 5 CP C patients received the Basel cocktail (10 mg caffeine, 50 mg efavirenz, 12.5 mg flurbiprofen, 10 mg omeprazole, 12.5 mg metoprolol tartrate, and 2 mg midazolam) and the pharmacokinetics was assessed for the drug substances and key metabolites over a period of 24 hours. Baseline characteristic of patients are presented in Supp. Table 1, and a summary of measured PK parameter of midazolam and its metabolites are presented in Supp. Table 2.

2.2 In-house experimental measurements of 1'-OH-midazolam-glucuronide

The physicochemical parameters for 1'-OH-midazolam-O-glucuronide and midazolam-N-glucuronide could not be found in existing literature. Therefore, 1'-OH-midazolam-O-glucuronide was purchased from Toronto Research Chemicals (Canada) and measurements of blood/plasma (B/P) partitioning, logP, pKa, and free fraction in plasma (f_{u,p}) were made in house. Unfortunately, midazolam-N-glucuronide could not be obtained and so in silico estimates had to be used.

Protein binding was measured in cassette-mode with 1'-OH-midazolam-glucuronide, including diazepam as a control. Diazepam data were in normal ranges. Pooled, mixed gender, healthy plasma was spiked to a final concentration of 1 µM and dialyzed against Sørensen buffer in a high throughput equilibrium dialysis setup with a semipermeable
membrane (MW cutoff 12-14 kDa; Thermo Fisher Scientific) according to the manufacturer’s instructions. Experiments were performed for 5 h at 37°C and 5% CO₂. The fraction unbound in plasma (\(f_{u,p}\)) was determined according to:

\[ f_{u,p} = \frac{\text{Concentration}_{\text{buffer,final}}}{\text{Concentration}_{\text{plasma,final}}} \]

For blood/plasma partitioning, test compound was spiked in fresh human blood from a healthy male donor. Immediately after spiking, the sample was mixed and a whole blood sample was drawn. The blood aliquot was put onto a rotating shaker (Heidolph titramax) and incubated for 1 h at 37°C and 5% CO₂. Thereafter, the hematocrit was determined and a whole blood sample was drawn from the aliquot. The remaining blood was further centrifuged (3000 g, 8 minutes) to collect plasma. Blood/plasma partitioning was calculated using the following equation:

\[ \text{Blood/plasma partitioning} = \frac{\text{Concentration}_{\text{blood,final}}}{\text{Concentration}_{\text{plasma,final}}} \]

The pKa was measured using a SIRIUS T3 instrument from pION Inc. HCl (0.5 M) or KOH (0.5 M) were added to a stirred solution of 1'-OH-midazolam-O-glucuronide, during which pH was continuously measured with a precision combination glass electrode. 1'-OH-midazolam-O-glucuronide (0.5 mM) was dissolved in 1.8 mL of water containing 0.15 M KCl as background electrolyte. The pH of the sample solution was checked and then 0.5 M HCl was added to bring the pH down to the initial pH value of 2 followed by a titration with standardized base solution until pH 12 was reached. A single potentiometric titration curve was obtained and pKa results were calculated using SIRIUS T3 Refine Version 1.1.
The logP value was determined by SiriusT3 pH-metric medium logP method, which is based on a standard potentiometric titration method in the presence of octanol reported previously (Keemink et al., 2023). A weighed sample of 1 mg was dissolved in a two-phase water-octanol system and titrated from pH 2 to pH 12. The pH of each point in the titration curve was calculated using equations that contain pKa and P, and the calculated points were fitted to the measured curve by manipulating the P value. The P that provides the best fit was taken to be the measured P value.

2.3 PBPK modeling method

PBPK models were developed in Simcyp® version 21 (Certara USA, Princeton, NJ, https://www.certara.com) (Jamei et al., 2009). The modeling workflow is presented in Figure 1. Simulations were first performed for the control group and a full PBPK modeling approach was used for midazolam, its primary metabolites 1'-OH-midazolam and midazolam-N-glucuronide, while for its secondary metabolite, 1'-OH-midazolam-O-glucuronide, a minimal PBPK mode was applied. Details of midazolam metabolic pathway with the involved enzymes are described in Figure 2. Once the model had been verified in control subjects, it was extrapolated to liver patients with cirrhosis by switching to the appropriate Simcyp® population for CP A, CP B or CP C. Simcyp® provides populations for each Child-Pugh stage by appropriate modification of model parameters. Simcyp® Child-Pugh population files integrate the changes in enzyme abundance for UGT1A4, UGT2B7/2B4, and CYP3A4/5 based on data obtained from different sources. For UGT1A4 (El-Khateeb et al., 2021; Prasad et al., 2018) and
UGT2B4 (El-Khateeb et al., 2021), abundance values are obtained from \textit{in vitro} studies. For UGT2B7, abundance is back-calculated from intravenous morphine data (Hasselström et al., 1990), and for CYP3A4 (Guengerich & Turvy, 1991; Prasad et al., 2018) abundance is obtained from \textit{in vitro} studies and has been shown to be in concordance with observed \textit{in vivo} data. Additionally, physiological parameters such as liver size and perfusion, portosystemic shunt, liver expression of drug-metabolizing enzymes and drug transporters, serum albumin concentration, and renal function are adjusted for each Child-Pugh categories (Johnson et al., 2010).

\textbf{2.3.1 Midazolam model}

To support the modeling of the effect of hepatic impairment, the default “Sim-Midazolam” file from the Simcyp® library was enhanced by incorporating an advanced dissolution, absorption, and metabolism (ADAM) model and a full PBPK model. Given the clinical data available for midazolam and its metabolites in control and cirrhotic populations from the Basel cocktail study, we identified an opportunity to enhance the mechanistic aspect of the default “Sim-Midazolam” file from the Simcyp® library. Incorporating an advanced dissolution, absorption, and metabolism (ADAM) model improved the prediction of midazolam absorption since the first-order model had resulted in a significant underprediction of the observed $T_{\text{max}}$ (0.24 h vs 1.2 h) and an overestimation of the $C_{\text{max}}$ (7.5 ng/mL Vs 4.4 ng/mL) in control subjects. The ADAM model allowed us to capture the observed data better (Table 2).

The Basel Cocktail study used a novel formulation, the so-called CombiCap, which consists of the commercially available solid formulations of each compound, which was
transformed into mini-tablets and encapsulated (Camblin et al., 2016). *In-vivo* trials indicate that the CombiCap formulation has equivalent characteristics to market-available formulations (Camblin et al., 2016). The CombiCap formulation was modeled in Simcyp® as an immediate-release solid formulation. However, initial simulations in control patients displayed an absorption phase which was faster than observations and so a lag time of 0.2 h was incorporated and the mean residence time in the stomach was increased from 0.27 h to 0.5 h. Midazolam solubility in water was obtained from PubChem (Hines et al., 2002) and its intestinal permeability was estimated based on permeability measured in Caco-2 cells (P_{app}). Calibration of Caco-2 P_{app} to human jejunum permeability (P_{eff} of 4.24×10^{-4} cm/s) was performed using a reference P_{app} value of midazolam, available as reference drug in Simcyp®, with known P_{eff} (Tolle-Sander et al., 2003). The clinically observed volume of distribution (V_{ss}) of 0.88 L/kg from the default model was retained in the full PBPK model by adjusting the tissue-to-plasma partitioning coefficients (Kp) predicted with the Rodgers and Rowland method using a scalar of 0.325 (Kupferschmidt et al., 1995). An identical Kp scalar was used for simulating PK profiles in different CP populations. The f_{u,p} in the default Simcyp® model is 0.032, but the measured f_{u,p} for control subjects in the Basel cocktail study was 0.0179 and so this value was used in our model (Duthaler et al., 2022).

Midazolam is metabolized by CYP3A4 and CYP3A5 into 1’-OH-midazolam and 4-OH-midazolam (Wessels et al., 2021) and is also directly glucuronidated to midazolam-N-glucuronide via UGT1A4 (Figure 2) (Wessels et al., 2021). The Simcyp® model parameters for these enzymatic pathways and renal excretion were retained, but in order to compensate for the decrease of f_{u,p} by a factor of ~2, CL_{int} and the renal
clearance (\(CL_r\)) were increased by 2-fold. The fraction metabolized (\(f_m\)) and fraction excreted in urine, were conserved. The details of the input parameters are provided in Table 1.

Additional validation of the midazolam model was performed with clinical data obtained after both single-dose intravenous and oral administrations. This validation covered four distinct single-dosing regimens for each administration route (Supp. Figure 1 and Supp. Figure 2). Furthermore, drug-drug interaction (DDI) validation was performed using clinical data from studies with IV or oral administration of midazolam as the victim compound and erythromycin, ketoconazole and ritonavir as perpetrators (Supp. Table 3 and Supp. Table 4).

2.3.2 1'-OH-midazolam model

Midazolam is oxidized to 1'-OH-midazolam via CYP3A4 and CYP3A5 (Figure 2) (Seo et al., 2010; Zhu et al., 2008). Molecular weight, \(\log P\), \(pK_a\), and blood/plasma partitioning for 1'-OH-midazolam were obtained from the literature (Nguyen et al., 2016) whereas the \(f_{u,p}\) of 0.0492 was the value measured in the control subjects of the Basel Cocktail study (Duthaler et al., 2022). Distribution was modeled using the Rodgers and Rowland method with a \(K_p\) scalar of 2.88 applied to recover the published \(V_{ss}\) of 1.71 L/kg (Johnson et al., 2023). 1'-OH-midazolam is glucuronidated to 1'-OH-midazolam-N-glucuronide and 1'-OH-midazolam-O-glucuronide via UGT1A4 and UGT2B7/UGT2B4, respectively, and a minor oxidation via CYP3A4 to 1',4-di-OH-midazolam also contributes to its elimination (Figure 2) (Seo et al., 2010; Zhu et al., 2008).
In characterizing the elimination parameters, the $\text{CL}_{\text{int}}$ values due to CYP3A4 and UGT1A4/2B7/2B4 were recalculated using the methodology proposed by Johnson et al. (Johnson et al., 2023) whereby the fraction metabolized ($f_m$) by UGT enzymes is based on the relative activity factor (RAF) method. The $\text{CL}_{\text{int}}$ for the oxidation into 1’-4-di-OH-midazolam, associated with CYP3A4, was taken from the human liver microsome (HLM) experiments conducted by Nguyen et al. (Nguyen et al., 2016). Similarly, the $\text{CL}_{\text{int}}$ for total glucuronidation was sourced from Nguyen et al. (Nguyen et al., 2016). To determine the UGT1A4 and UGT2B7 RAFs, we performed a literature search to identify pertinent studies and selected the most recent ones for use in this work. Thus, one study from Busse et al. (2020) (Busse et al., 2020) was used for UGT1A4 and UGT2B7 and a study from Lapham et al. (Lapham et al., 2020), was used for UGT2B4. These studies described UGT enzyme activities in pooled mixed-gender HLM and recombinant systems, using midazolam, zidovudine, and ertugliflozin as probe substrates for UGT1A4, UGT2B7 and UGT2B4, respectively. The RAF values, representing the ratio of $\text{CL}_{\text{int}}$ in HLM to that in recombinant systems, were as follows: 0.48 (UGT1A4), 1.12 (UGT2B7), and 10 (UGT2B4). Subsequently, these RAFs were applied to the recombinant $\text{CL}_{\text{int}}$ values for 1’-OH-midazolam, measured by Zhu et al. (Zhu et al., 2008) and Seo et al. (Seo et al., 2010) to calculate the unbound intrinsic clearance ($\text{CL}_{\text{int},u}$) in HLM for UGT1A4, UGT2B4, and UGT2B7. The $f_m$ values of 0.12 for UGT1A4, 0.82 for UGT2B4, and 0.06 for UGT2B7 were derived and finally, a uniform retrograde optimization of $\text{CL}_{\text{int}}$ values, preserving the $f_m$ values, was made based on the observed data in control patients. The final model parameters are provided in Table 1. We verified the performance of our 1’-OH-midazolam model using the pediatric dataset employed.
by Johnson et al. (Johnson et al., 2023). This dataset comprises three distinct studies presenting pediatric clinical data of 1'-OH-midazolam PK following the ontravenous administration of midazolam. Our model showed good simulation of pediatric clinical data with predicted/observed ratios of mean $C_{\text{max}}$ and AUC$_{\text{inf}}$ within the 1.2-0.67 fold range (Supp. Table 5, Supp. Table 6).

### 2.3.3 1'-OH-midazolam-O-glucuronide modeling parameters

1'-OH-midazolam-O-glucuronide is the main secondary metabolite of midazolam biotransformation and is formed via UGT2B7/UGT2B4 (Figure 2) (Seo et al., 2010; Zhu et al., 2008). The elimination of 1'-OH-midazolam-O-glucuronide from the blood was assumed to be only renal (Heizmann & Ziegler, 1981; Zhu et al., 2008) and CL$_r$ was estimated to fit the clinical plasma concentrations of control patients. The V$_{ss}$ was predicted with the Rodgers and Rowland method (Rodgers et al., 2005; Rodgers & Rowland, 2006). Blood/plasma partitioning, logP, pKa, and f$_{u,p}$ of 1'-OH-midazolam-O-glucuronide were measured in-house, as described in Methods. The input parameters are provided in Table 1.

### 2.3.4 Midazolam-N-glucuronide modeling parameters

Midazolam-N-glucuronide formation enzyme kinetics via the primary glucuronidation of midazolam by UGT1A4 (Seo et al., 2010; Wessels et al., 2021) were taken from the Simcyp® Sim-midazolam file (Hyland et al., 2009). Since no measured data were reported and midazolam-N-glucuronide was not available, the f$_{u,p}$, pKa, logP, and blood-plasma partitioning values were estimated from the chemical structure with the software
ADMET Predictor version 10.3 (Simulations Plus Inc., USA, Lancaster, CA, http://www.simulations-plus.com). A sensitivity analysis of these parameters was then performed to assess the impact of uncertainties on the predictions. The elimination of midazolam-N-glucuronide was assumed to be only renal (Tian et al., 2019). A full PBPK approach was applied to predict the $V_{ss}$ with the Rodgers and Rowland method (Rodgers et al., 2005; Rodgers & Rowland, 2006). A middle-out approach was applied to fit with the clinical PK profiles in the control subjects by estimation of a Kp scalar and renal clearance (CL$_r$) using the sensitivity analysis module. The input parameters are provided in Table 1.

2.4 Simulation settings

Simulations were conducted using the same number of individuals per group in the clinical study, with 10 to 20 trials (total number of individuals to result in not less than 100 virtual subjects). The distribution of demographic characteristics, such as age, gender distribution, and body weight, were set-up to match as closely as possible with those observed in the actual clinical subjects (Supp. Table 1). Plasma PK profiles were simulated and the geometric mean of the area under the curve from zero to infinity ($AUC_{inf}$) and the maximal plasma concentration ($C_{max}$) were calculated for the 100 virtual individuals, for midazolam and its metabolites and compared with geometric mean of the observed clinical values (Duthaler et al., 2022). The simulations were assessed by comparing the observed plasma concentrations to the simulated median and 5$^{th}$-95$^{th}$ quartiles of the predicted concentration profiles.
2.5 PK parameter calculation and metabolic ratio (MR) estimation as a surrogate for UGT1A4 and UGT2B7/UGT2B4 activity

AUC<sub>inf</sub> values for midazolam and its metabolites were calculated via non-compartmental analysis using the linear trapezoidal method, while maximum plasma concentration (C<sub>max</sub>), and corresponding time (T<sub>max</sub>) were obtained directly from the observed and predicted plasma concentration–time profiles. The software PKanalix (version 2019R1; Lixoft SAS, Abtony, France) was used to calculate the PK parameters of observed data, while simulated AUC<sub>inf</sub> and C<sub>max</sub> were obtained directly from Simcyp®.

The metabolic ratio AUC<sub>inf</sub>, glucuronide/AUC<sub>inf</sub>, non-glucuronidated precursor was used to reflect the activity of the UGT enzymes (Donzelli et al., 2014; Fuhr et al., 2007).
3. Results

3.1 Revised midazolam model validation with additional clinical data and DDI studies

The revised midazolam model provided good predictions of the additional clinical observation after IV and oral administration, with observed data falling within the 5th-95th quartiles of the predicted concentration profiles (Supp. Figure 1 and Supp. Figure 2). Additionally, the revised midazolam model demonstrated good performance in predicting DDI effects (Supp. Table 3 and Supp. Table 4). Following co-administration with erythromycin, three out of four $C_{\text{max}}$ ratios and three out of four $AUC_{\text{inf}}$ ratios for erythromycin fell within the 0.70 to 1.43-fold range. With ketoconazole co-administration, all $C_{\text{max}}$ and $AUC_{\text{inf}}$ ratios were within this range. However, for ritonavir, $C_{\text{max}}$ data was unavailable, while $AUC_{\text{inf}}$ ratios were 0.58 and 1.53.

3.2 Comparison of model simulations to observed concentrations in all patient groups

PBPK model simulations demonstrated good accuracy for both midazolam and its metabolites in control and cirrhotic patients. In control non-cirrhotic subjects, 90% of the observed plasma concentrations for midazolam and its metabolites fell within the simulated 5th-95th percentiles in 120 virtual control non-cirrhotic subjects (Figure 3). Furthermore, the fold-error values (observed/predicted) of $AUC_{\text{inf}}$ and $C_{\text{max}}$ were within the range of 0.7-1.43, as detailed in Table 2.

In the cirrhotic populations, good model performance is maintained with 82% of observed plasma concentrations for the CP A, CP B, and CP C classes falling within the simulated 5th-95th percentiles for both midazolam and its metabolites, see Figures 4, 5,
and 6. Nevertheless, an overestimation of the $AUC_{inf}$ of midazolam and midazolam-$N$-glucuronide is observed in the CP B stage patients (Table 2) and an overestimation of midazolam $C_{max}$ is seen in CP B patients. Underestimation of the $C_{max}$ values for 1'-OH-midazolam is apparent in CP C patients and in CP A and CP C for midazolam-$N$-glucuronide (Table 2).

### 3.3 Sensitivity analysis for 1'-OH-midazolam-O-glucuronide and midazolam-$N$-glucuronide

Details of sensitivity analysis results are presented in Supp. Table 7. Measured logP, pKa, $f_{u,p}$, and B/P values for 1'-OH-midazolam-O-glucuronide are reported in Table 1. To assess the influence on the PK of 1'-OH-midazolam-O-glucuronide of these parameters, we performed a parameter sensitivity analysis, which revealed that these parameters had only a minimal effect on the $AUC_{inf}$ and $C_{max}$ predictions. Specifically, varying the B/P ratio from 0.6 to 1.4, for a hematocrit of 43% for males and 38% for female, resulted in a 5% impact on $C_{max}$ and no impact on $AUC_{inf}$. As for $f_{u,p}$, a $\pm 1.5$-fold variation of the parameter led to a 8% impact on $C_{max}$ but had no discernible impact on $AUC_{inf}$. When we subjected the logP parameter to a 10-fold variation, there were no noticeable effects on either $C_{max}$ or $AUC_{inf}$. Similarly, for both acidic and basic pKa values, a 1 pKa unit variation did not impact $C_{max}$ and $AUC_{inf}$.

Similarly, a sensitivity analysis was also performed for midazolam-$N$-glucuronide, for which logP, pKa, B/P, and $f_{u,p}$ were estimated via ADMET predictor® based on the chemical structure of the compound, with the recorded values detailed in Table 1. Varying the B/P ratio from 0.6 to 1.4, resulted in a 13% impact on $C_{max}$ and no impact...
on AUC_{inf}. As for f_{u,p}, a ±1.5-fold variation led to a 22% impact on C_{max} and a 3% impact on AUC_{inf}. When we subjected the logP parameter to a 10-fold variation, there were no effects on either C_{max} or AUC_{inf}. Similarly, for both acidic and basic pKa values, a 1 pKa unit variation did not impact C_{max} and AUC_{inf}.

3.4 Comparison of the simulated and observed impact of liver cirrhosis on exposure of midazolam and metabolites

The geometric mean of observed and predicted AUC_{inf} and C_{max} of midazolam and its metabolites in control and cirrhotic patients are shown in Table 2.

The geometric mean of observed AUC_{inf} for midazolam showed a 1.77-fold, 2.61-fold, and 6.35-fold increase in CP A, CP B, and CP C subjects compared to the control, while simulations predicted increases of 1.89-fold, 4.44-fold, and 7.41-fold, respectively. For 1'-OH-midazolam, the geometric mean of observed AUC_{inf} increased by 2.03-fold, 4.79-fold, and 9.15-fold in CP A, CP B, and CP C subjects in comparison to the control, while simulations predicted increases of 1.94-fold, 3.02-fold, and 5.58-fold, respectively. For glucuronides, 1'-OH-midazolam-glucuronide, showed an increase of 1.38-fold, 1.29-fold, and 1.94-fold in geometric mean of observed AUC_{inf}, while predicted decreases in CP A, CP B, and CP C subjects were 1.24-fold, 1.52-fold, and 2.16-fold, respectively. AUC_{inf} of midazolam-N-glucuronide, displayed a 1.32-fold, 1.38-fold, and 1.41-fold increase observed in CP A, CP B, and CP C subjects compared to the control, while predictions showed changes of 1.27-fold, 2.61-fold, and 2.16-fold, respectively (Table 2).
The geometric-mean of observed $C_{\text{max}}$ for midazolam showed a 1.43-fold, 1.31-fold, and 2.52-fold increase in CP A, CP B, and CP C subjects compared to the control, while simulations predicted increases of 1.60-fold, 2.74-fold, and 3.30-fold, respectively. For 1'-OH-midazolam, the geometric-mean of observed $C_{\text{max}}$ increased by 1.93-fold, 2.78-fold, and 4.01-fold in CP A, CP B, and CP C subjects in comparison to the control, while simulations predicted increases of 1.63-fold, 1.79-fold, and 1.91-fold, respectively. For glucuronides, 1'-OH-midazolam-glucuronide, showed a decrease of 0.98-fold, 0.71-fold, and 0.63-fold in geometric mean of observed $C_{\text{max}}$, while predicted decreases in CP A, CP B, and CP C subjects were 0.86-fold, 0.68-fold, and 0.51-fold, respectively. $C_{\text{max}}$ of midazolam-N-glucuronide, displayed a 1.25-fold, 1.32-fold, and 1.69-fold increase observed in CP A, CP B, and CP C subjects compared to the control, while predictions showed changes of 0.81-fold, 1.18-fold, and 0.69-fold, respectively (Table 2).

3.5 Metabolic ratio and prediction of enzyme activity changes in cirrhotic patients

The MRs, calculated as the total AUC$_{\text{inf}}$ ratios of midazolam-N-glucuronide/midazolam and 1'-OH-midazolam-O-glucuronide/1'-OH-midazolam, serve as indicators of the metabolic activities of UGT1A4 and UGT2B7/2B4, respectively. For UGT1A4, the observed metabolic rates showed a 32%, 73%, and 79% reduction in CP A, CP B, and CP C subjects, respectively, compared to the control. Simulations predicted reductions of 26%, 47%, and 78% for the same groups (Figure 7). Regarding UGT2B7/2B4, the observed reductions in metabolic rates were 35%, 51%, and 64% in CP A, CP B, and CP C subjects compared to the control. Simulations predicted reductions of 40%, 48%, and 75%, respectively (Figure 7).
4. Discussion

In this study, we have described a PBPK model for midazolam, its two primary metabolites, midazolam-N-glucuronide, and 1'-OH-midazolam, and the secondary metabolite, 1'-OH-midazolam-O-glucuronide. We included a mechanistic ADAM absorption model for midazolam and full-PBPK models for midazolam, midazolam-N-glucuronide, and 1'-OH-midazolam. We optimized the model using clinical data from non-cirrhotic control patients, verified the model with additional literature data for midazolam, and then extended the model to patients with cirrhosis enabling us to predict the PK of midazolam and its metabolites in these specific patient populations.

Sensitivity analyses were conducted to assess the influence of variations in B/P, f_{u,p}, logP, and pKa on the PK (C_{max} and AUC_{inf}) of 1'-OH-midazolam-O-glucuronide and midazolam-N-glucuronide. These parameters were measured in-house for 1'-OH-midazolam-O-glucuronide, while for midazolam-N-glucuronide, they were predicted based on the chemical structure. Hence, we consider it necessary to perform sensitivity analyses to explore the potential impact of uncertainties in these measured and predicted parameters. Insensitivity of 1'-OH-midazolam-O-glucuronide and midazolam-N-glucuronide AUC_{inf} to B/P and f_{u,p} variation was as expected since for both compounds, renal excretion was considered as the only elimination pathway and the CL_r was directly estimated from the observed PK profiles in control subjects. In addition, f_{u,p} and B/P increase led to reduced C_{max} and increased V_{ss} for both compounds, ultimately having no impact on AUC_{inf}. Furthermore, we noted minimal effects of logP on V_{ss} prediction within the studied ranges for values below logP = 3. For 1'-OH-
midazolam-O-glucuronide and midazolam-N-glucuronide, with logP values of 0.2 and -0.736, respectively, we considered a logP exceeding 3 as an excessive upper bound for sensitivity evaluations. However, we can highlight that when logP exceeded 3, logD switched to a positive value for both glucuronides, and a pronounced impact on $V_{ss}$ prediction was observed, subsequently influencing $C_{\text{max}}$ and $AUC_{\text{inf}}$ predictions. Additional sensitivity analysis were conducted to evaluate the impact of enzyme abundances on 1'-OH-midazolam exposure predicted via PBPK modeling. According to the $f_m$ calculation with the RAF method, UGT2B4 can be considered as the main enzyme metabolizing 1'-OH-midazolam ($f_m=82\%$). Based on this, a sensitivity analysis on the impact of CYP3A4 and UGT2B4 abundances on the AUC of 1'-OH-midazolam (for a fixed value of abundance of other enzymes) was performed (Supp. Table 8). The range of the sensitivity analysis was selected based on the abundance of CYP3A4 and UGT2B4 in Simcyp® healthy volunteers and CP C populations. Results indicated a stronger impact of UGT2B4 abundance change on predicted 1'-OH-midazolam $AUC_{\text{inf}}$, than CYP3A4 abundance changes. Therefore, concerning the PBPK modeling predictions, we can consider UGT2B4 abundance as a more impactful parameter and the main enzyme influencing the predicted 1'-OH-midazolam exposure.

The final PBPK model replicated PK profiles in both control and cirrhotic patients. The $AUC_{\text{inf}}$ and $C_{\text{max}}$ values for midazolam and its metabolites in control and cirrhotic (CP) patients were predicted with good accuracy, mostly falling within a range of 0.7-1.43-fold of the predicted values. Exceptions were midazolam and midazolam-$N$-glucuronide $AUC_{\text{inf}}$ in CP B, midazolam $C_{\text{max}}$ in CP B, 1'-OH-midazolam $C_{\text{max}}$ in CP C, and midazolam-$N$-glucuronide $C_{\text{max}}$ in CP A, which were predicted within a 0.5-2-fold
interval. Also 1'-OH-midazolam-\textit{O}-glucuronide \( C_{\text{max}} \) in CP C, which was underpredicted by 2.39-fold (Table 2). A tendency of the model to under-predict later plasma concentrations could be seen for midazolam-\(N\)-glucuronide starting from hour 12 and 1'-OH-Midazolam-\textit{O}-glucuronide starting after hour 4 in controls (Figure 3). Similarly, an under-prediction of concentration at hour 24 was observed for 1'-OH-midazolam in CP A and CP B populations (Figure 4 and Figure 5). Additionally, even if a consistent decrease in UGT1A4 clearance was predicted (Supp. Figure 3), our prediction indicated an inconsistent increase in midazolam-\(N\)-glucuronide AUC\textsubscript{inf} of 1.27, 2.61, and 2.16-fold (Table 2). The AUC\textsubscript{inf} of midazolam-\(N\)-glucuronide is impacted by multiple parameters, such as, the formation rate via UGT1A4, which is impacted by the UGT1A4 abundance in liver or the renal excretion, which is impacted by the \( f_{u,p} \) and the glomerular filtration rate (GFR). The liver cirrhosis impacts all these parameters at different level and understanding the nonlinear increase in midazolam-\(N\)-glucuronide AUC\textsubscript{inf} would be possible by enhancing the model's predictive capacity through the incorporation of more data. For instance, obtaining precise data on the renal excretion rate of the glucuronides could not only enhance predictions regarding their pharmacokinetics but also enable a top-down approach to optimize the abundance of UGT1A4 and UGT2B7/2B4 in cirrhotic patients. This affirms the utility of the Simcyp\textsuperscript{®} hepatic impairment populations for simulating CYP3A oxidation and UGT1A4 and UGT2B7/2B4 glucuronidation. Exceptions were midazolam and midazolam-\(N\)-glucuronide AUC\textsubscript{inf} values for CP B stage patients with fold error (F.E.) of 0.53 and 0.57, respectively, and the \( C_{\text{max}} \) of midazolam in CP B patients with a F.E. of 0.50. Also, the \( C_{\text{max}} \) of 1'-OH-midazolam and midazolam-\(N\)-glucuronide in CP C patients were underestimated, with F.E. values of
1.71 and 2.39, respectively and $C_{\text{max}}$ of midazolam-$N$-glucuronide in CP A patients was underestimated, with F.E. values of 1.50. The predicted significant reduction in metabolic ratios for midazolam-$N$-glucuronide and 1'-$\text{OH}$-midazolam-$O$-glucuronide of 40% and 35%, in CP A, 48% and 51% in CP B and 75% and 65%, in CP C patients was in line with clinical observations and can be attributed to decreased glucuronidation activity of UGT1A4 and UGT2B7/2B4 with hepatic impairment. In addition, the calculated $f_m$ values (from available in-vitro literature data) for 1'-$\text{OH}$-midazolam metabolism (0.12 for UGT1A4, 0.82 for UGT2B4, and 0.06 for UGT2B7), suggest that UGT2B4 primarily drives the formation of 1'-$\text{OH}$-midazolam-glucuronide. Hence, the significant decrease in the MR ratio observed for UGT2B7/2B4 can be attributed mainly to the reduced activity of UGT2B4. Moreover, the AUC$_{\text{inf}}$ of 1'-$\text{OH}$-midazolam is higher in CP-A, CP-B, and CP-C compared to the control. This suggests that the reduction in activity of UGT1A4/2B4/2B7, responsible for eliminating 1'-$\text{OH}$-midazolam, might be more important than that of CYP3A, which is responsible for 1'-$\text{OH}$-midazolam formation (Table 2 and Supp. Figure 3). This supports the relevance of the decline in the hepatic abundance of these metabolizing enzymes according to the CP status as captured within Simcyp®. For example, drops of 69%, 68%, 80%, and 82% are seen for CYP3A4/5, UGT2B7, UGT2B4, and UGT1A4 between control and CP C patients in enzymes hepatic abundance in Simcyp® (Supp. Figure 3). Additionally, as measured data on UGT2B7 abundance in the kidneys of cirrhotic patients is not available in literature, it was held constant at 48 pmol/mg-protein across all populations in Simcyp® (Supp. Figure 3). However, despite the increase in the predicted relative contribution of renal CL$_{\text{int}}$ through UGT2B7—rising from 0.1% in control subjects to 1.5% in CP C
patients—this effect is marginal and does not impact the overall accuracy of predictions in this study. We also assessed the ratio of \( \text{AUC}_{1'-\text{OH-midazolam}}/\text{AUC}_{\text{midazolam}} \) (Supp. Figure 4). This ratio exhibited an increasing trend in clinical data up to the CP B state, followed by a decline in CP C patients. In contrast, our PBPK modeling predicted a slight decrease in the ratio until the CP B state, followed by a slight increase in CP C patients. Since the concentrations of 1'-OH-midazolam are influenced not only by changes in CYP3A4/5 activity but also by concurrent alterations in UGT2B7/2B4 activity, the ratio (1'-OH-midazolam/midazolam) is not a straightforward indicator of enzyme activity changes in cirrhotic patients. In the context of liver cirrhosis evaluation, the MR serves as a significant parameter, providing valuable insights into changes in enzyme activity. However, additionally to the MR, the concentration profiles have to be considered to gain a broader understanding of how liver cirrhosis affects compound exposure. Regarding 1'-OH-midazolam and midazolam, the observed concentrations increase with liver cirrhosis, a trend well captured by our PBPK model (Table 2, Figure 3-6). Additionally, we evaluated the change in the predicted fraction of midazolam metabolized (\( f_m \), \%) by CYP3A4, CYP3A5, UGT1A4 in the liver and UGT1A4 in the kidney and 1'-OH-midazolam metabolized by UGT2B4/2B7/1A4 in the liver. The predicted \( f_m \) varied slightly between the control and CP populations, but even if the hepatic CL (L/h) decreased significantly with the liver cirrhosis state, the relative \( f_m \) of each enzyme stayed consistent across each population for both compounds (Supp. Table 9).

We consider that the modeling workflow used in this study can be extended to investigate the behavior of other UGT enzymes, enabling a better validation of PBPK
models for alterations in cirrhotic patients and facilitating more precise dosage adjustments for UGT substrates. However, gaps in understanding remain. One such gap is how hepatic impairment impacts transporter activities, the impact of liver cirrhosis on the hepatic transporter has been studied (Armani et al., 2023), but more research is needed for renal transporter. In the case of midazolam, the glucuronide metabolites are excreted in the urine, and in the model presented in this study, the renal CL was obtained via top-down fitting to clinical data. However, a future opportunity for improvement would be to apply a bottom-up approach that includes transporter PK data for the glucuronides allowing evaluation of the impact of liver cirrhosis on transporter activity. Another gap is the limitations in the available data to support UGT enzymes abundance changes in CP populations. Additional proteomics measurements or top-down optimizations should be considered to increase the precision of UGT enzymes abundance change in cirrhotic patients and the PBPK modeling of its substrates. The impact of hepatic impairment on the expression of UGT enzymes beyond the liver should also be acknowledged. For example, the kidney can be more important for isoforms with high kidney expression such as UGT1A9 and UGT2B7 (Bigo et al., 2013). In vitro studies using human kidney microsomes (HKM) and recombinant UGT systems revealed that glucuronidation of various compounds within the human kidney was driven mainly by UGT1A9 and UGT2B7 (Knights et al., 2013). For instance, UGT2B7 plays a significant role in the N-glucuronidation of carbamazepine. Edaravone exhibited a 5.4-fold higher CL\textsubscript{int} through glucuronidation in HKM compared to HLM, primarily driven by UGT1A9 and secondarily by UGT2B7. Morphine formed glucuronide metabolites (M3G and M6G) primarily via UGT2B7 in HKM, while UGT1A9 predominantly handled
furosemide’s kidney glucuronidation with a minor UGT2B7 contribution. Moreover, mycophenolic acid, propofol, gemfibrozil, and valproic acid, and various nonsteroidal anti-inflammatory drugs, such as flufenamic, mefenamic, or niflumic acid, showed significant glucuronidation within the human kidney, largely mediated by UGT1A9 and UGT2B7 (Knights et al., 2013). It is also important to acknowledge the potential impact of intestinal metabolism for orally administered drugs. For midazolam, the simulated fraction that escapes gut metabolism ($F_g$) increases from 52% in control subjects to 81% in CP C patients because the cirrhotic status decreases gut metabolism with a reduction of approximately 50% in the abundance of CYP3A4 and CYP3A5 in the intestine between control and CP C patients. Since multiple UGT enzymes are expressed in the intestine (e.g. UGT1A1/7/8/9, UGT2B7/17) this could also play a role for UGT substrates where intestinal metabolism is important. For midazolam, no significant intestinal glucuronidation was predicted. However, literature data indicates that for raloxifene, for example, intestinal UGT enzymes significantly influenced oral bioavailability. Raloxifene undergoes glucuronidation via UGT1A1, UGT1A8, UGT1A9, and UGT1A10, and presents a mere 2% oral bioavailability. This low bioavailability is the result of intestinal metabolism primarily driven by UGT1A9 and UGT1A10, leading to a pronounced intestinal first-pass effect (Mizuma, 2009; Ritter, 2007). Another example is ticagrelor, for which in vitro studies in human intestinal microsomes indicated a glucuronidation activity and additional evaluation in recombinant system confirmed the implication of UGT1A9 (main) and UGT1A7, UGT1A3, UGT1A4, UGT1A1, UGT2B7 and UGT1A8 as enzymes involved in its metabolism (Liu et al., 2021).
To further validate PBPK modeling for UGT substrates in cirrhotic patients, there is a need for more data on the alterations in hepatic and extra-hepatic UGT enzyme levels in cirrhotic patients. Additional information about the activity of UGT1A9 and UGT2B7 in the kidneys and intestines, as well as UGT1A10 in the intestines in cirrhotic patients would improve the precision of PBPK modeling for compounds which undergo notable extra-hepatic metabolism. In addition, human PK data for such UGT substrates and their glucuronide metabolites in cirrhotic patients are needed to allow the validation of the PBPK models.
5. Conclusion

Similarly to CYP enzymes, UGT enzymes may be impacted by liver cirrhosis. In this project, we developed and verified a comprehensive PBPK model for midazolam and its metabolites, in both non-cirrhotic control and cirrhotic patient populations. The model exhibited a good degree of accuracy, capturing pharmacokinetic profiles and verifying the suitability of Simcyp® hepatic impairment populations for simulating CYP3A-mediated oxidation and UGT1A4 and UGT2B7/UGT2B4 mediated glucuronidation. Our modeling approach can be extended to explore the behavior of other UGT enzymes affected by liver cirrhosis. We anticipate that the reporting of additional hepatic impairment study data for drugs cleared via glucuronidation by specific UGT enzymes will enable validation of PBPK modeling for additional UGT enzymes and lead to improved dose adjustment predictions for a greater range of drugs. In addition, it is important to highlight the significant extra-hepatic activity of UGT enzymes and the need for further research to characterize changes in extra-hepatic enzyme abundance and activity in cirrhotic patients.
Declarations section

Acknowledgements:
Special thanks to Aynur Ekiciler, who ordered, stocked and prepared the 1'-OH-midazolam-glucuronide for in house measurements.

Data availability:
The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Funding:
The study was conducted without external funding.

Conflict of interest disclosure:
None of the authors reports any interest of conflict regarding this study.

Authors’ contribution:
Performed data analysis: Agustos C. Ozbey, Janneke Keemink, Bjoern Wagner
Wrote or contributed to the writing of the manuscript: Agustos C. Ozbey, Alessandra Pugliano, Janneke Keemink, Bjoern Wagner, Stephan Krähenbühl, Pieter Annaert, Stephen Fowler, Neil Parrott, Kenichi Umehara.

Key points:
Evaluation of glucuronidation in cirrhotic patients, PBPK prediction of midazolam and its metabolites in control and cirrhotic patients

Keywords:
PBPK, midazolam, glucuronidation, pharmacokinetics, metabolic ratio (MR), liver cirrhosis
References


Mizuma, T. (2009). Intestinal glucuronidation metabolism may have a greater impact on oral bioavailability than hepatic glucuronidation metabolism in humans: a study with raloxifene, substrate for UGT1A1, 1A8, 1A9, and 1A10. *International journal of pharmaceutics*, 378(1-2), 140-141.


Figure 1 Flowchart presenting the development and application of PBPK modeling and the use of generated data, using Simcyp® version 21, in order to evaluate the activity changes for UGT1A4 and UGT2B7/UGT2B4. The modeling strategy starts with control group and extends the model to liver cirrhosis patients (CP) by incorporating specific population modifications for different Child-Pugh stages.

Figure 2 Midazolam primary and secondary metabolic pathways mediated by CYP and UGT enzymes (Seo et al., 2010; Wessels et al., 2021).

Figure 3 Observed (data points) individual and simulated (black line) median plasma concentration-time profiles of midazolam and its metabolites: 1'-OH-midazolam, midazolam-N-glucuronide, 1'-OH-midazolam-O-glucuronide after an oral dose of 2 mg in control patients. Data are presented in linear and semi-log scale; the grey lines represent the 5th and 95th percentiles of the simulated population.

Note: midazolam and 1'-OH-midazolam plasma concentrations were directly measured while midazolam-N-glucuronide and 1'-OH-midazolam-O-glucuronide plasma concentration were derived via treatment with β-glucuronidase (more information available in Supp. materials)

Figure 4 Observed (data points) individual and simulated (black line) median plasma concentration-time profiles of midazolam and its metabolites: 1'-OH-midazolam, midazolam-N-glucuronide, 1'-OH-midazolam-O-glucuronide after an oral dose of 2 mg in Child-Pugh A cirrhotic patients. Data are presented in linear and semi-log scale; the grey lines represent the 5th and 95th percentiles of the simulated population.

Note: midazolam and 1'-OH-midazolam plasma concentration were directly measured while midazolam-N-glucuronide and 1'-OH-midazolam-O-glucuronide plasma concentration were derived via treatment with β-glucuronidase (more information available in Supp. materials)

Figure 5 Observed (data points) individual and simulated (black line) median plasma concentration-time profiles of midazolam and its metabolites: 1'-OH-midazolam,
midazolam-N-glucuronide, 1'-OH-midazolam-O-glucuronide after an oral dose of 2 mg in Child-Pugh B cirrhotic patients. Data are presented in linear and semi-log scale; the grey lines represent the 5th and 95th percentiles of the simulated population.

Note: midazolam and 1'-OH-midazolam plasma concentration were directly measured while midazolam-N-glucuronide and 1'-OH-midazolam-O-glucuronide plasma concentration were derived via treatment with β-glucuronidase (more information available in Supp. materials)

**Figure 6** Observed (data points) individual and simulated (black line) median plasma concentration-time profiles of midazolam and its metabolites: 1'-OH-midazolam, midazolam-N-glucuronide, 1'-OH-midazolam-O-glucuronide after an oral dose of 2 mg in Child-Pugh C cirrhotic patients. Data are presented in linear and semi-log scale; the grey lines represent the 5th and 95th percentiles of the simulated population.

Note: midazolam and 1'-OH-midazolam plasma concentration were directly measured while midazolam-N-glucuronide and 1'-OH-midazolam-O-glucuronide plasma concentrations were derived via treatment with β-glucuronidase (more information available in Supp. materials)

**Figure 7** Box plots of the metabolic ratios (MRs) calculated for observed patients (normal: N=12, CP A: N=16, CP B: N=15, CP C: N=5) in the Basel Cocktail study and simulated individuals (normal N=12x10, CP A: N = 16x10, CP B: 15x10, CP C: 5x20) in Simcyp®. The MR is calculated as the ratio of total AUC of midazolam-N-glucuronide to midazolam, representative of UGT1A4 activity in control and cirrhotic population (A) and total AUC ratio 1'-OH-midazolam-O-glucuronide to 1'-OH-midazolam, representative of UGT2B7/UGT2B4 activity in control and cirrhotic populations (B).

The geometrical mean of the simulated MR for midazolam-N-glucuronide to midazolam (indicative of UGT1A4 activity) decreased by 40% (CP A), 48% (CP B), and 75% (CP C). For 1'-OH-midazolam-O-glucuronide to 1'-OH-midazolam the geometrical mean of the MR (indicative of UGT2B7/2B4 activity) dropped by 35% (CP A), 51% (CP B), and 64% (CP C). These predicted MRs were corroborated by the observed data.
<table>
<thead>
<tr>
<th></th>
<th>midazolam</th>
<th>midazolam-N-glucuronide</th>
<th>1'-OH-midazolam</th>
<th>1'-OH-midazolam-O-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MW (g/mol)</strong></td>
<td>325.8</td>
<td>501.9</td>
<td>342</td>
<td>517.9</td>
</tr>
<tr>
<td><strong>logP</strong></td>
<td>3.53&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-0.736&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.59 (Johnson et al., 2023)</td>
<td>0.20&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>pKa</strong></td>
<td>6 (base)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>3.55&lt;sup&gt;2&lt;/sup&gt; (acid) ; 2.27&lt;sup&gt;2&lt;/sup&gt; (base)</td>
<td>13.6 (acid) ; 3.63 (base) (Nguyen et al., 2016)</td>
<td>2.81&lt;sup&gt;3&lt;/sup&gt; (acid) ; 4.23&lt;sup&gt;3&lt;/sup&gt; (base)</td>
</tr>
<tr>
<td><strong>B/P</strong></td>
<td>0.603&lt;sup&gt;3&lt;/sup&gt; (Allonen et al., 1981; Heizmann et al., 1983)</td>
<td>0.944&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1 (assumed) (Johnson et al., 2023; Nguyen et al., 2016)</td>
<td>0.613&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>fu&lt;sub&gt;p&lt;/sub&gt; (%)</strong></td>
<td>1.79&lt;sup&gt;2&lt;/sup&gt; (Duthaler et al., 2022) (binding to albumin)</td>
<td>36&lt;sup&gt;2&lt;/sup&gt; (Duthaler et al., 2022) (binding to albumin)</td>
<td>4.92 (Duthaler et al., 2022) (binding to albumin)</td>
<td>35.4&lt;sup&gt;2&lt;/sup&gt; (assumed to bind to albumin)</td>
</tr>
<tr>
<td><strong>Caco-2 cell permeability (10&lt;sup&gt;6&lt;/sup&gt; cm/s)</strong></td>
<td>0.17&lt;sup&gt;2&lt;/sup&gt; (Parhizkar et al., 2017)</td>
<td>0.88 (Kupferschmidt et al., 1995)</td>
<td>1.27&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.71 (Johnson et al., 2023)</td>
</tr>
<tr>
<td><strong>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</strong></td>
<td>37&lt;sup&gt;2&lt;/sup&gt; (Tolle-Sander et al., 2003)</td>
<td>0.88</td>
<td>1.27&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.71 (Johnson et al., 2023)</td>
</tr>
</tbody>
</table>

**Elimination:**

|                          | CYP3A4: 1'-OH: V<sub>max</sub> = 10.5* ; Km = 2.16 4-OH: V<sub>max</sub> = 10.4* Km =31.8 | CYP3A5: 1'-OH: V<sub>max</sub> = 39.4* Km = 4.16 4-OH: V<sub>max</sub> = 8.06* Km =38.4 | UGT1A4: V<sub>max</sub> = 890* (pmol/min/mg); Km = 40.3 | CL<sub>r</sub> = 23.3<sup>4</sup> (Tian et al., 2019) |

|                          | CL<sub>int,CYP3A4</sub> = 83.3* CL<sub>int,UGT1A4</sub> = 1488* (Busse et al., 2020; Lapham et al., 2020; Seo et al., 2010; Zhu et al., 2008) | CL<sub>int,UGT2B7</sub> = 109* CL<sub>int,UGT2B4</sub> = 1488* (Busse et al., 2020; Lapham et al., 2020; Seo et al., 2010; Zhu et al., 2008) | CL<sub>r</sub> = 0.166 (Rodgers & Rowland prediction (Rodgers et al., 2005; Rodgers & Rowland, 2006)) | CL<sub>r</sub> = 43.0<sup>4</sup> (Heizmann & Ziegler, 1981; Zhu et al., 2008) |

<sup>1</sup>Obtained from the midazolam PBPK model present in the Simcyp® library.
<sup>2</sup>Structural based prediction via ADMET predictor
<sup>3</sup>Measured in-house
<sup>4</sup>Fitted to clinical observations in control patients

* midazolam and 1'-OH-midazolam CL<sub>int</sub> calculation are detailed in Method
** The typical CL<sub>r</sub> in 20-30 years old healthy male is scaled to the population of interest with the demographic parameters of the population of interest (age, gender, body weight, serum creatinine, glomerular filtration rate)

Abbreviations: B/P, blood/plasma partitioning, CL<sub>int</sub>, intrinsic clearance CL<sub>r</sub>, renal clearance; fu<sub>p</sub>, fraction unbound in plasma; Km, Michaelis-Menten constant; logP, partition coefficient between octanol and water at near infinite dilution or lipophilicity; MW, molecular weight; Peff, intestinal permeability; V<sub>max</sub>, maximal rate of metabolism V<sub>ss</sub>, volume of distribution at steady state
Table 2 Geometric mean values of observed (Obs.) and predicted (Pred.) AUC$_{\text{inf}}$ (ng/mL.h) and C$_{\text{max}}$ (ng/mL) for midazolam and its metabolites: 1'-OH-midazolam, midazolam-N-glucuronide, 1'-OH-midazolam-O-glucuronide, in control and cirrhotic populations. The corresponding fold-error (F.E.) (obs/pred) and the fold changes as ratios (hepatic impaired / control) are also shown. Standard deviations of predictions are presented between parentheses.

<table>
<thead>
<tr>
<th>AUC$_{\text{inf}}$ (ng/mL.h)</th>
<th>midazolam</th>
<th>1'-OH-midazolam</th>
<th>1'-OH-midazolam-glucuronide</th>
<th>midazolam-N-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.3</td>
<td>14.7 (14.9)</td>
<td>0.90</td>
<td>4.13</td>
</tr>
<tr>
<td>CP A</td>
<td>23.5</td>
<td>27.8 (25.5)</td>
<td>0.85</td>
<td>8.37</td>
</tr>
<tr>
<td>CP B</td>
<td>34.7</td>
<td>65.2 (49.0)</td>
<td>0.53</td>
<td>19.8</td>
</tr>
<tr>
<td>CP C</td>
<td>84.4</td>
<td>109 (77.2)</td>
<td>0.77</td>
<td>37.8</td>
</tr>
</tbody>
</table>

| CP A /Control | 1.77 | 1.89 | 2.03 | 1.91 | 1.38 | 1.24 | 1.32 | 1.15 |
| CP B /Control | 2.61 | 4.44 | 4.79 | 3.02 | 1.29 | 1.52 | 1.38 | 2.25 |
| CP C /Control | 6.35 | 7.41 | 9.15 | 5.58 | 1.94 | 2.16 | 1.41 | 1.85 |

| C$_{\text{max}}$ (ng/mL) | | | | | | |
|--------------------------|-----------|------------------|-----------------------------|-------------------------|
| Control | 4.44 | 4.30 (3.25) | 1.03 | 1.49 | 1.83 (0.79) | 0.81 | 20.7 | 30.5 (5.55) | 0.70 | 0.277 | 0.285 (0.231) | 0.97 |
| CP A | 6.36 | 6.90 (3.98) | 0.92 | 2.87 | 2.99 (1.17) | 0.96 | 20.3 | 26.1 (6.07) | 0.78 | 0.347 | 0.231 (0.138) | 1.50 |
| CP B | 5.83 | 11.8 (4.60) | 0.50 | 4.14 | 3.28 (1.00) | 1.26 | 14.6 | 20.8 (5.33) | 0.70 | 0.365 | 0.336 (0.187) | 1.09 |
| CP C | 11.2 | 14.2 (4.37) | 0.79 | 5.97 | 3.49 (1.16) | 1.71 | 13.0 | 15.7 (5.10) | 0.83 | 0.469 | 0.196 (0.115) | 2.39 |

| CP A /Control | 1.43 | 1.60 | 1.93 | 2.67 | 0.98 | 0.86 | 1.25 | 0.81 |
| CP B /Control | 1.31 | 2.74 | 2.78 | 2.97 | 0.71 | 0.68 | 1.32 | 1.18 |
| CP C /Control | 2.52 | 3.30 | 4.01 | 3.30 | 0.63 | 0.51 | 1.69 | 0.69 |

Abbreviations: AUC$_{\text{inf}}$, area under the plasma concentration curve between 0h to infinity; CP, Child-Pugh; F.E., fold-error, Obs., observed values; Pred., predicted values
PBPK modeling workflow

**Figure 1**

- **Model building in control subjects**
  - PBPK model building for midazolam, 1-OH-midazolam, 1-OH-midazolam-O-glucuronide and midazolam-N-glucuronide in control

- **Final optimized model in control subjects**
  - For midazolam, 1-OH-midazolam, 1-OH-midazolam-O-glucuronide and midazolam-N-glucuronide

- **Parameter optimization**

- **Verification of predictions with control data**

- **Verification of the optimized midazolam model with additional IV and oral clinical concentration-time profiles and victim DDI studies from literature**

- **Extrapolation to cirrhotic patients**
  - Switch to the appropriate population for CP A, CP B, and CP C.
  - Verification of predictions with observation in CP patients

- **Evaluation of liver cirrhosis impact on glucuronides' systemic exposure & Metabolic ratio for UGT1A4 and UGT2B7/UGT2B4**
  - Calculation of the metabolic ratio \( \frac{\text{AUC}_{\text{glucuronide}}}{\text{AUC}_{\text{parent}}} \) in all populations: indicative of enzyme activity changes
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7