The Oral Bioavailability and Metabolism of Midazolam in Stable Critically Ill Children: A Pharmacokinetic Microtracing Study

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Midazolam is metabolized by the developmentally regulated intestinal and hepatic drug-metabolizing enzyme cytochrome P450 (CYP) 3A4/5. It is frequently administered orally to children, yet knowledge is lacking on the oral bioavailability in term neonates up until 1 year of age. Furthermore, the dispositions of the major metabolites 1-OH-midazolam (OHM) and 1-OH-midazolam-glucuronide (OHMG) after oral administration are largely unknown for the entire pediatric age span. We aimed to fill these knowledge gaps with a pediatric [^{14}C]midazolam microtracer population pharmacokinetic study. Forty-six stable, critically ill children (median age 9.8 (range 0.3–276.4) weeks) received a single oral [^{14}C]midazolam microtracer (58 (40–67) Bq/kg) when they received a therapeutic continuous intravenous midazolam infusion and had an arterial line in place enabling blood sampling. For midazolam, in a one-compartment model, bodyweight was a significant predictor for clearance (0.98 L/hour) and volume of distribution (8.7 L) (values for a typical individual of 5 kg). The typical oral bioavailability in the population was 66% (range 25–85%). The exposures of OHM and OHMG were highest for the youngest age groups and significantly decreased with postnatal age. The oral bioavailability of midazolam, largely reflective of intestinal and hepatic CYP3A activity, was on average lower than the reported 49–92% for preterm neonates, and higher than the reported 21% for children> 1 year of age and 30% for adults. As midazolam oral bioavailability varied widely, systemic exposure of other CYP3A–substrate drugs after oral dosing in this population may also be unpredictable, with risk of therapy failure or toxicity.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Through developmental changes in cytochrome P450 (CYP)3A activity in the intestine and liver the oral bioavailability of midazolam in children > 1 year is lower than that in preterm neonates.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ What are the oral bioavailability of midazolam and the systemic exposures to midazolam and the major metabolites 1-OH-midazolam (OHM) and 1-OH-midazolam-glucuronide (OHMG) in a population of term neonates up to 6 years of age?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Using a microtracer population pharmacokinetic approach we established that the oral bioavailability of midazolam was 66% (25–85%). While the systemic exposure to OHM and OHMG decreased with increasing age, the systemic exposure to midazolam remained constant.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Our study emphasizes that children are at an increased risk of subtherapeutic or toxic exposure of midazolam and potentially other CYP3A substrates when dosed orally. The oral [^{14}C]microtracer study design could be considered for less well-studied compounds to ultimately improve safety and efficacy of pediatric drug therapy.

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Midazolam is a short-acting benzodiazepine that is widely used in pediatric hospital practice for various indications, including the induction of anesthesia by oral administration.[1,2] When an orally administered drug is subject to intestinal and/or hepatic drug metabolism, variation in its metabolism is an important determinant of bioavailability and systemic clearance of that drug.

Oral bioavailability is defined as the fraction of the administered oral dose reaching the systemic circulation unchanged and importantly depends on the absorption and first-pass metabolism by both intestinal and hepatic drug-metabolizing enzymes. Cytochrome P450 (CYP)3A is a drug-metabolizing enzyme family, abundant in both the liver and the gut, which contributes to the first-pass metabolism of many orally administered drugs.[3] CYP3A consists of the three main isoforms CYP3A4, CYP3A5, and CYP3A7, for which the substrate specificity differs.[3,4] *In vitro* studies have shown that the CYP3A7 abundance in the liver declines rapidly after birth and that the abundance of CYP3A4 in the liver and in the gut increases with increasing age.[5-7] CYP3A5 is polymorphically expressed, with a stable expression from fetus to adult. This developmental pattern of CYP3A4 expression seen in *in vitro* studies is supported by pharmacokinetic (PK) data of CYP3A-substrate drugs. The benzodiazepine midazolam is a well-validated CYP3A probe with substrate specificity for CYP3A4/5 and almost none for CYP3A7.[8,9] In preterm infants (gestational age 26–31 weeks and postnatal age 3–13 days), oral midazolam clearance was markedly lower (0.16 L/hour/kg vs. 3.0 L/hour/kg) and oral bioavailability higher (49–92% vs. 21%) than in children beyond 1 year of age.[10-12] These findings suggest developmentally lower intestinal and/or hepatic CYP3A activity in preterm neonates. Midazolam is one of the many CYP3A4/5 substrates frequently administered to children.[3] Hence, this developmental pattern in CYP3A4/5–mediated systemic and presystemic metabolism may imply that safe and effective systemic exposure of oral doses of not only midazolam, but also other CYP3A4/5 substrates, may not be reached.

The oral bioavailability of midazolam has been previously studied across the pediatric age span.[10-14] However, there is a distinct knowledge gap for the age group from birth (term born) throughout infancy, i.e., < 1 year old. The classical study design to obtain data on oral bioavailability entails a crossover study in which an oral and intravenous (IV) dose of a drug are administered alternately, with a washout period in between. This design is ethically and practically challenging as children are exposed twice to therapeutic drug doses with extensive blood sampling.

An interesting approach to study oral bioavailability is by a [^{14}C] labeled microtracer, which has been shown practically and ethically feasible to study developmental changes in PK in children.[15-17] A microtracer is defined as "< 1/100th of the dose needed to reach the no observed adverse effect level or < 100 μg," concurrently administered with a therapeutic dose.[18,19] The [^{14}C]label allows quantification of extremely low plasma concentrations by accelerator mass spectrometry (AMS) in only 10–15 μL plasma.[20,21] A microtracer of an oral [^{14}C]labeled drug is administered simultaneously with therapeutic IV doses of the same unlabeled drug, allowing measuring both the oral and IV disposition in one subject at the same time and, with that, accurately quantifying the absolute oral bioavailability,[15,16] overcoming the limitations of a traditional crossover design.

Besides the oral bioavailability of midazolam, the systemic exposure to the major metabolites 1-OH-midazolam (OHM) and 1-OH-midazolam-glucuronide (OHMG) after oral dosing is also of interest, since both metabolites are pharmacologically active, although to a lesser extent than midazolam.[22] Also, a better understanding of age-related variation in metabolite disposition provides further insight in developmental pharmacology. OHM is the primary metabolite formed by CYP3A, which is further glucuronidated to OHMG by uridine diphosphate (UDP)-glucuronosyltransferase (UGT)2B4, UGT2B7, and, to a lesser extent, UGT1A4.[23,24] A high systemic exposure to OHMG may result in therapeutic effects of this metabolite despite its lower potency.[25] A report of five critically ill adults with severe renal failure showed accumulation of OHMG after continuous IV infusion of midazolam.[25] This accumulation led to prolonged sedation (assessed by Ramsey score and electroencephalographic (EEG) evaluation) that could be reversed by the use of flumazenil, which is a competitive benzodiazepine antagonist. This finding highlights the importance of knowledge on disposition of the metabolites of midazolam. The metabolism and disposition of midazolam and the primary metabolite OHM after oral dosing have been described in preterm neonates and older children,[10,13,14,26,27] but gaps remain for term neonates to children < 1 year old. Most importantly, to the best of our knowledge, data on systemic exposure of OHMG in adults and children after oral dosing are not available.

Given these considerations, we have designed and conducted an oral [^{14}C]midazolam microtracer population PK study in stable, critically ill children from 0–6 years old with the aim to answer two questions: (i) what is the oral bioavailability of midazolam; and (ii) what is the systemic exposure to midazolam and its major metabolites OHM and OHMG after oral dosing in this population.

MATERIAL AND METHODS

Setting

This multicenter study was carried out in the level III pediatric intensive care unit of the Erasmus MC–Sophia Children's Hospital, Rotterdam, the Netherlands (October 2015–March 2018) and the Radboudumc-Amalia Children's Hospital, Nijmegen, the Netherlands (May 2017–March 2018). The study was approved by the Dutch Central Committee on Research Involving Human Subjects (EudraCT 2014-003269-46). Parental written informed consent was obtained. The radiation exposure of a single microtracer was explained to the parents and legal guardians by a comparison with the yearly mean background exposure of 2.6 mSv in the Netherlands in 2013.[28] The Dutch Nuclear Research and Service Group estimated the radiation exposure for a single microtracer < 1 μSv was well below the minimal risk category 1 of the International Commission of Radiological Protection, where a maximum exposure of 100 μSv is allowed. Category 1 risk studies are considered minimal risk and ethically justified in humans when they provide new scientific knowledge.[29]

Population

Children were eligible to participate in the study when aged from birth (postmenstrual age > 36 weeks) up to 6 years of age, had medical need for sedation with continuous IV midazolam, and had an indwelling arterial or central venous line in place enabling blood sampling. To minimize interindividual variability due to critical illness or organ failure, exclusion criteria were death anticipated in 48 hours, extra corporeal

membrane oxygenation treatment, circulatory failure (defined by the administration of more than one vasopressor drug, or increase of the dose of a vasopressor drug in the last 6 hours), kidney failure (according to the pediatric Risk, Injury, Failure, Loss, End stage renal disease (pRIFLE) criteria "failure," i.e., estimated creatinine clearance decreased by 75% or a urine output of < 0.3 mL/kg/hour for 24 hours or anuria for 12 hours), liver failure (defined by aspartate aminotransferase (AST) or alanine aminotransferase (ALT) > 2 times the upper limit for age), gastrointestinal disorders, or concomitant administration of comedication known to interact with midazolam (according to the Flockhart Table[30]).

Study design

A single [^{14}C]midazolam (20.3 (14.1–23.6) ng/kg; 58 (40–67) Bq/kg; 0.25 mL/kg) dose was administered as an oral microtracer via the enteral feeding tube to ensure delivery in the gastrointestinal tract, followed by either 1 or 2 mL of saline or food to ensure rinsing of the tube. The IV therapeutic midazolam dose was prescribed by the treating physician for clinical purposes and was adjusted on the guidance of validated sedation scores and according to a standardized sedation titration protocol. According to this protocol, midazolam bolus doses varied between 0.05–0.2 mg/kg and the continuous infusion rate between 0.05–0.3 mg/kg/hour. Blood samples were taken pre-microtracer administration and around 0.5 hours, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, and 24 hours after administration of the [^{14}C]midazolam microtracer to ensure that the PK of the oral absorption phase was captured. The maximum number of blood samples for the study was limited to eight per subject, and the maximum amount of blood could not exceed the guidelines by the European Medicines Agency (EMA) (up to 1% of calculated circulating blood volume).[31] The blood samples were centrifuged and plasma was stored at −80°C until analysis.

Midazolam

Midazolam for therapeutic infusion was manufactured and compounded by the Pharmacy A15 (Gorinchem, The Netherlands) under good manufacturing practice conditions. [^{14}C]midazolam was synthesized by Selcia Ltd (Shelley, United Kingdom) at a specific activity of 1033 MBq/mmol (equal to 2.85 MBq/mg). The chemical name is 8-chloro-6-(2-fluorophenyl)-1-methyl-^{4}H-[1-^{14}C]imidazo[l,5-a][l,4] benzodiazepine hydrochloride, and it was brought in ethanol solution (96%). In the department of Radiology and Nuclear Medicine at the VU University Medical Center (Amsterdam, The Netherlands), the solution was further diluted to the required concentration with sodium chloride 0.9% solution (Fresenius Kabi, Zeist, The Netherlands) under good manufacturing practice conditions. The final [^{14}C]midazolam concentration was 210–270 Bq/mL with 1 Bq = 0.31 ng [^{14}C]midazolam.

Measurements

[^{14}C]midazolam, [^{14}C]OHM and [^{14}C]OHMG plasma concentration quantification. *Plasma sample extraction and Ultra Performance Liquid Chromatography Separation.* The ultra performance liquid chromatography and AMS (see paragraph '*AMS analysis*') qualifications were performed in accordance with the recommendation of the European Bioanalytical Forum.[32] Methanol (200 µL, containing unlabeled midazolam, OHM, and OHMG) was added to 15 µL plasma samples in order to precipitate proteins and to extract the test substance using protein precipitation plates (Phenomenex, Utrecht, the Netherlands). Each run consisted of samples and eight calibrator levels (180, 60, 20, 10, 5, 2.5, 1.25, and 0.625 Bq/L) in duplicate, plus three different quality control (QC) levels (135, 7.5, and 0.625 Bq/L) in duplicate to quantify midazolam, OHM, and OHMG. Subsequently, 30 µL extract was evaporated to dryness, redissolved in 30 µL 1 mM ammonium formate in water + 5% acetonitrile and 25 µL was injected on the ultra performance liquid chromatography. The fractions where midazolam, OHM, and OHMG eluted from the column were collected for each sample, transferred to a tin foil cup, evaporated to dryness, and analyzed using combustion-carbon dioxide-AMS. Each series was accompanied by two calibration lines at eight levels, and QCs in triplicate at three levels. Accuracy and precision complied with the European Bioanalysis Forum criteria of 20% of 2/3 of the QCs.

AMS analysis. [^{14}C]levels were quantified as described before.[16,33] The tin foil cups (see paragraph '*Plasma sample extraction and Ultra Performance Liquid Chromatography Separation*') were combusted on an elemental analyzer (Vario Micro; Elementar, Langenselbold, Germany). Generated CO_2 was transferred to an in-house–developed gas interface, composed of a zeolite trap and syringe.[33] CO_2 was adsorbed to the trap on the interface; after heating of the trap, the CO_2 was transferred to a vacuum syringe using helium. A final CO_2/helium mixture of 6% was directed to the AMS ion source, at a pressure of 1 bar and a flow of 60 µL/minute. A 1-MV Tandetron AMS (High Voltage Engineering Europe B.V., Amersfoort, The Netherlands)[34] was used. The lower limit of quantification (LLOQ) of the liquid chromatography (LC)-AMS was 0.31 Bq/L, and the upper limit was 200 Bq/L.

Therapeutic midazolam plasma concentration quantification by LC–tandem mass spectrometry. Midazolam and the major metabolites were quantified by means of an LC–tandem mass spectrometry (LC-MS/MS) with electrospray ionization in the positive ionization mode (Waters) validated according to US Food and Drug Administration (FDA) guidance.[35] The LLOQ for midazolam was 2 µg/L, for OHM 3 µg/L, and for OHMG 10 µg/L. The upper limit of quantification for midazolam was 2,400 µg/L, for OHM 2,300 µg/L, and for OHMG 3,000 µg/L. The internal standard is midazolam-d4. During analysis three standards (covering the whole range of linearity) and four quality controls are used from different manufacturers, to obtain objectivity. A 100 µL sample is used. After sample preparation (e.g., adding internal standard), the supernatant (3 µL) is injected in the system. The run time is 7.6 minutes per sample.

Data collection

We collected data on the doses of therapeutic midazolam and [^{14}C]midazolam and the respective timings of administration and blood sampling. Patient characteristics and relevant clinical and laboratory measurements were prospectively recorded.

Pharmacokinetic analysis

Population pharmacokinetics to assess the oral bioavailability. The oral bioavailability of a drug was quantified by means of a population PK analysis. All [^{14}C]midazolam and midazolam concentration-time data were analyzed simultaneously using nonlinear mixed effects modeling with NONMEM version 7.4 (ICON; Globomax LLC, Ellicott, MD) after log transformation of the concentration data. [^{14}C]midazolam concentrations under the AMS detection limit (< LLOQ) were discarded.[36] Pirana (version 2.9.7, Certara, Princeton, NJ), R (version 3.4.1, Vienna, Austria), and R-studio (version 1.0.153, Boston, MA) were used to visualize the data. Model development was in four steps (see Methods S1 for detailed information): (i) selection of a structural model, (ii) selection of an error model, (iii) covariate analysis, and (iv) internal validation of the model. The absorption rate constant for midazolam was fixed at 4.16/hour, which yields peak concentrations to be reached in ~ 30 minutes, which is in agreement with the observed time to reach maximum peak plasma concentration in our data and with values reported for children in previous literature.[13]

Noncompartmental analysis to assess the systemic exposure to midazolam and its major metabolites after oral dosing. To calculate the systemic exposure of midazolam and its major metabolites, the concentration-time areas under the curve after oral dosing were determined

with noncompartmental analyses. The [^{14}C]midazolam and metabolite concentrations were measured in Bq/L. Values were converted from Bq to ng based on molecular weights ($9.6*10^{-4}$ mol/Bq), where [^{14}C]midazolam was 325.8 g/mol (0.31 ng/Bq), [^{14}C]OHM 341.8 g/mol (0.33 ng/Bq), and [^{14}C]OHMG 517.9 g/mol (0.50 ng/Bq). The area under the curve (AUC) from time zero to the last sampling time point ($AUC_{0\text{-tlast}}$) was calculated using the log-linear trapezoidal method; the AUC from time zero to infinite time ($AUC_{0\text{-inf}}$) was calculated by extrapolation beyond the last observation.[37] If $AUC_{tlast-inf}$ was larger than 20% of the actual $AUC_{0-tlast}$, then the AUC_{0-inf} was excluded from the analysis, as it would limit the accuracy of the results and hence would introduce unreliable estimation of the AUC_{0-inf}. The first sample below the LLOQ was set on 0.155 Bq/L (0.5 * LLOQ), and any following samples < LLOQ were discarded.

The ratios $AUC_{0\text{-tlast}}$ [^{14}C]OHM/[^{14}C]midazolam (OHM/M) and ratio $AUC_{0\text{-tlast}}$ [^{14}C]OHM/[^{14}C]OHMG (OHM/OHMG) were calculated with $AUC_{0\text{-last}}$ in Bq/L/hour, and therefore correction of molecular weight was not necessary. All PK parameters derived from individual patients were estimated using the Excel PKsolver add-in software.[37]

The relationships between AUC and AUC ratios and postnatal age were described with nonparametric Spearman's rank correlation. All statistical tests were two-sided, and a significance level of $P = 0.05$ was used.

RESULTS

Population

Between October 2015 and March 2018, 96 of 454 screened patients were eligible to participate, and informed consent was obtained from parents of 46 of these children (median gestational age at birth of 39.0 (29.4–43.0) weeks and a median postnatal age of 9.8 weeks (2 days–5.3 years)) (see **Figure 1**). Three-quarters were 0–6 months old. **Table 1** provides the characteristics of these 46 children.

Data of 3 of these 46 children were excluded from further analysis. In one, extubated shortly after receiving the [^{14}C]midazolam microtracer, no [^{14}C]midazolam concentration could be detected in the plasma samples. The undetectable concentrations can be explained by clinical practice, because immediately before extubation the child's stomach is completely emptied to avoid aspiration. The two others had, in hindsight, received interacting comedication that induced CYP3A.

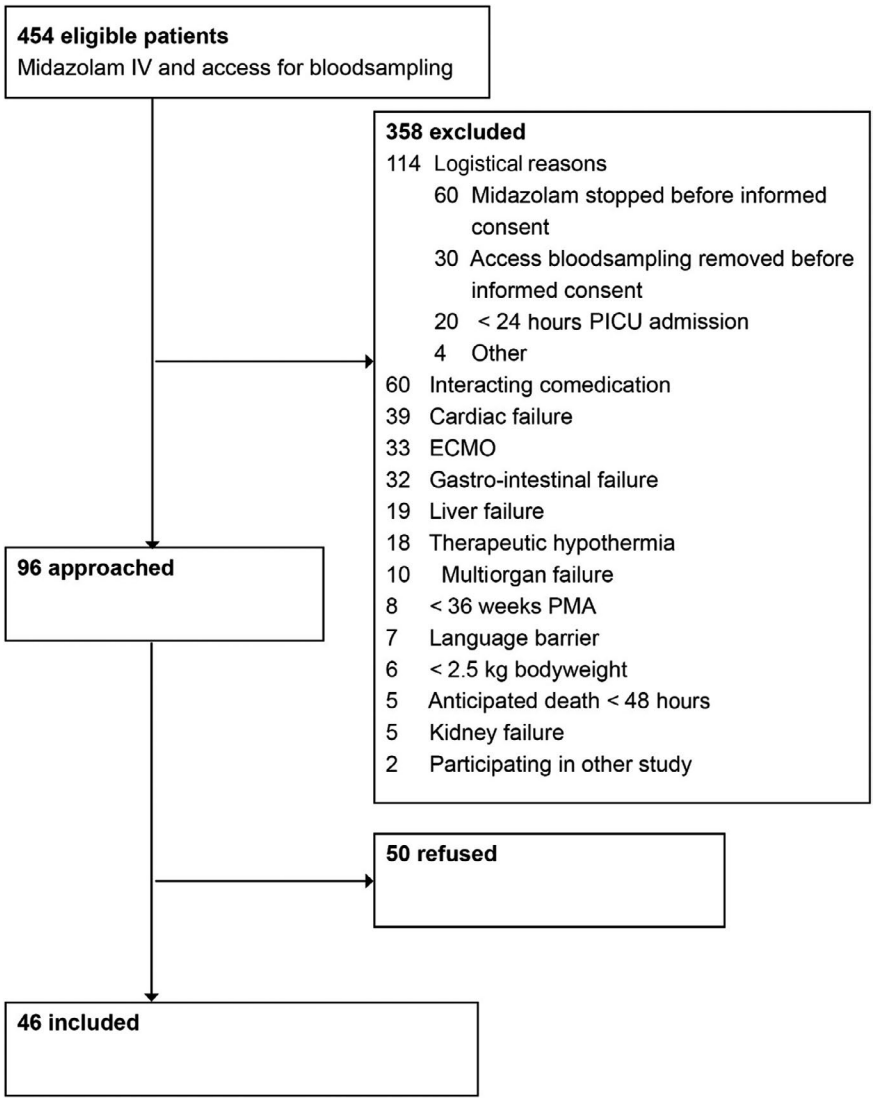

Figure 1 Flowchart of patient recruitment. ECMO, extra corporeal membrane oxygenation; IV, intravenous; PICU, pediatric intensive care unit; PMA, postmenstrual age.

Table 1 Characteristics of patients included in the analysis presented as median (range) or number

Patient characteristics		
Number of patients (*n*)	46	
Location (*n* Erasmus MC/*n* Radboudumc)	39/7	
Postnatal age (weeks)	9.8 (0.3–276.4)	
Postmenstrual age (weeks)	48.9 (38.9–316.4)	
Weight (kg)	4.7 (2.8–18.0)	
Z-score weight for age[a]	-0.9 (−3.0–2.5)	
Gender (M/F)	29/17	
Ethnicity (white/other)	41/5	
Reason for admission (*n*)	Respiratory failure	
	Pneumonia/bronchiolitis	18
	Congenital cardiac abnormality	7
	Pulmonary hypertension	2
	Traumatic injury to the airways	2
	Lobar emphysema	2
	Meconium aspiration	1
	Post cardiac surgery	12
	Status epilepticus	2
Disease severity scores		
PELOD	11 (0–21)	
Number of organs failing on study day	1 (0–2)	
PRISM	16 (3–32)	
PIM	−2.5 (−4.8 to −0.4)	
Laboratory values at day of administration [^{14}C]midazolam		
Plasma creatinine (μmol/L)	29 (11–63)	
AST (U/L)	42 (16–155)	
ALT (U/L)	18 (6–138)	
CRP (mg/L)	43 (2–298)	
Study medication		
Dose [^{14}C]midazolam (Bq)	282.7 (165.0–1080.0)	
Dose [^{14}C]midazolam (ng)	87.6 (51.15–334.8)	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; PELOD, pediatric logistic organ dysfunction; PIM, Pediatric Index of Mortality; PRISM, Pediatric Risk of Mortality.
[a]As determined by TNO growth curves

Oral bioavailability

In the final population PK model, the typical oral bioavailability in the population was 66% with a high intra-individual variability (IIV) of 0.86; individual bioavailability estimates ranged from 25% to 85%. See **Figure 2** for the variability in individual bioavailability. All PK parameter estimates of this model are presented in **Table 2**.

For this model, a total of 30 [^{14}C]midazolam concentrations under the AMS detection limit (< LLOQ) were discarded.[36] The complete data set included 326 and 245 radiolabeled and cold midazolam concentrations, respectively, from 43 patients. The final model entails a one-compartment model that best described the PK of oral and IV midazolam. Inclusion of IIV for clearance, volume of distribution, and oral bioavailability improved the model statistically significantly. Bodyweight was the most significant predictor for clearance (Δobjective function value (OFV) −11.11) and volume of distribution (ΔOFV −15.95) in exponential relationships (see **Table** 2). After this inclusion, both the variance of the IIV for clearance and volume of distribution decreased. Age and other tested covariates were found not statistically significant after inclusion of bodyweight.

All relative standard error values of the parameter estimates were below 50%, indicating that the estimates could be obtained from the data with good precision. The diagnostic plots for the final model are presented in **Figure S1** (oral data) and in **Figure S2** (IV data). Both figures indicate that the model describes the obtained data accurately, upon both oral and IV administration, even though for the oral data more random variability is observed. The robustness of the estimated model parameters was evaluated in a bootstrap analysis. The bootstrap analysis confirmed the precision of parameter estimates of the final model, as the parameter estimates were very similar to the bootstrap medians and within the 95% confidence interval (**Table 2**). The distribution of the normalized

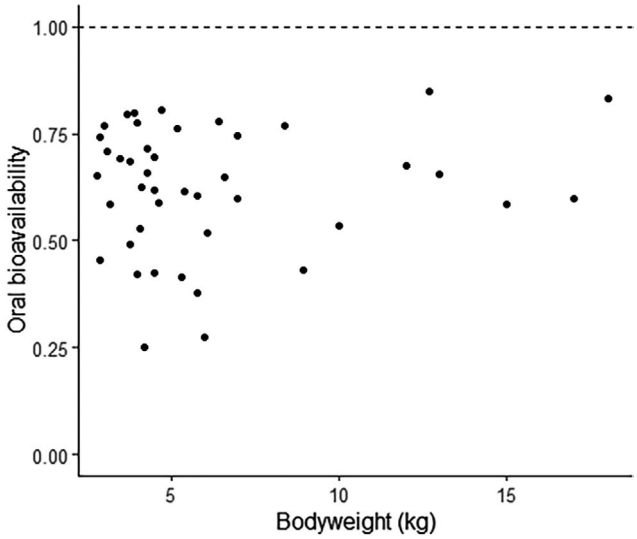

Figure 2 Oral bioavailability of midazolam and its variability vs. bodyweight. Bodyweight did not explain the variability in oral bioavailability.

prediction distribution errors (NPDEs) indicates that the model can adequately predict both the median trend and the variability in the observed concentrations. This is further supported by the absence of visible trends in NPDE vs. time and NPDE vs. predictions (see **Figure S3** and **Figure S4**).

Systemic exposure to midazolam and its major metabolites after oral dosing

The systemic exposures as reflected by the AUCs of midazolam and its major metabolites after administration of the oral [^{14}C] midazolam microtracer are presented in **Table 3**. The complete data set included data on 335 plasma samples from 43 patients. A total of 21 (6%), 41 (12%), and 14 (4%) samples were set on 0.5*LLOQ for respectively [^{14}C]midazolam, [^{14}C]OHM, and [^{14}C]OHMG. A total of 9 (3%), 93 (28%), and 2 (0.6%) samples were discarded for respectively [^{14}C]midazolam, [^{14}C]OHM, and [^{14}C]OHMG. Eight (19%), 9 (21%), and 21 (49%) patients were excluded from $AUC_{0\text{-}inf}$ analyses of respectively [^{14}C]midazolam, [^{14}C]OHM, and [^{14}C]OHMG as the $AUC_{tlast-inf}$ was larger than 20% of the actual $AUC_{0-tlast}$. For another three patients (7%) the $AUC_{0\text{-}inf}$ of [^{14}C]midazolam could not be calculated. For two patients, only one plasma sample taken after the absorption phase was available due to loss of the arterial catheter, and in one patient the plasma concentration-time profile had no apparent log-linear slope, for which no explanation was found. For five patients (12%) the $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}inf}$ of OHM could not be calculated as most plasma concentrations were < LLOQ.

Figure 3 shows the $AUC_{0-tlast}$ of [^{14}C]midazolam, [^{14}C] OHM, and [^{14}C]OHMG vs. postnatal age after administration of the oral [^{14}C]midazolam microtracer. The AUC of [^{14}C] OHM and [^{14}C]OHMG were the highest for the youngest age groups, even though the AUC of [^{14}C]midazolam was similar across age. Analysis of the data revealed a statistically significant

Table 2 Parameter estimates of a one-compartmental model

Parameters	Model parameters estimates (RSE%)	Bootstrap median (2.5th to 97.5th bootstrap percentile
Oral bioavailability		
	$F_i = e^{log(TVF/(1-TVF))}/(1 + e^{log(TVF/(1-TVF))})$	
TVF	0.66 (8%)	0.66 (0.56–0.78)
Absorption rate constant		
ka (h^{-1})	4.16 FIXED	-
Clearance		
	$CL_i = CL_{5kg} * (WT/5)^{k1}$	
CL_{5kg} (L/h)	0.98 (13%)	0.99 (0.78–1.28)
k1	0.92 (31%)	0.93 (0.44–1.59)
Volume of distribution		
	$V_i = V_{5kg} * (WT/5)^{k2}$	
V_{5kg} (L)	8.70 (11%)	8.68 (6.94–10.78)
k2	1.16 (21%)	1.17 (0.79–1.85)
Interindividual variability		
ω^2 CL	0.65 (19%)	0.61 (0.39–0.87)
ω^2 V	0.40 (24%)	0.37 (0.18–0.58)
ω^2 TVF	0.86 (49%)	0.78 (0.17–1.78)
Residual error		
Additive error oral [^{14}C] midazolam data	0.08 (29%)	0.07 (0.04–0.13)
Additive error intravenous midazolam data	0.47 (30%)	0.47 (0.25–0.77)

ω^2, variance for the interindividual variability of the indicated parameter; CL, clearance; CL_i, predicted clearance of individual i; CL_{5kg}, population-predicted clearance for a subject with a median weight of 5 kg; CV, coefficient of variation; F, absolute oral bioavailability; F_i, predicted absolute oral bioavailability of individual i; IV, intravenous; k1, exponent to relate body weight to clearance; k2, exponent to relate body weight to volume of distribution; ka, absorption rate constant; RSE, relative standard error; TVF, population parameter in the logit equation for oral bioavailability; V, volume of distribution; V_i, individual predicted volume of distribution for individual i; V_{5kg}, population-predicted volume for a subject with a median weight of 5 kg; WT, body weight.

negative correlation for both [^{14}C]OHM $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}inf}$ and [^{14}C]OHMG $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}inf}$ with postnatal age (see **Figure 3b,c** for $AUC_{0\text{-}tlast}$, results for $AUC_{0\text{-}inf}$ not shown). No significant relationship was identified between postnatal age and [^{14}C]midazolam $AUC_{0\text{-}tlast}$ (see **Figure 3**), [^{14}C]midazolam AUC_{0-inf}, OHM/M AUC ratio, and OHM/OHMG ratio (data not shown).

DISCUSSION

To study the oral bioavailability of midazolam and the systemic exposure to midazolam and its major metabolites in children, we designed a prospective oral [^{14}C]midazolam microtracer population PK study in children receiving midazolam for clinical purposes. Our main observations were that (i) the median oral bioavailability of midazolam was 66% and varied greatly with a range of 25–85% and (ii) the systemic exposure (AUC) of the

Table 3 Area under the curves of midazolam and its major metabolites and their ratios after administration of an oral [^{14}C] midazolam microtracer (20.3 (14.1–23.6) ng/kg; 58 (40–67) Bq/kg)

	Midazolam	OHM	OHMG
$AUC_{0\text{-tlast}}$			
Bq/L/h	162.6 (10.4–898.4) (*n* = 43)	12.0 (1.1–77.0) (*n* = 38)	254.4 (62.6–821.6) (*n* = 43)
ng/L/h	50.4 (3.2–278.5) (*n* = 43)	4.0 (0.4–25.4) (*n* = 38)	127.2 (31.3–410.8) (*n* = 43)
$AUC_{0\text{-inf}}$			
Bq/L/h	160.9 (10.6–753.3) (*n* = 32)	17.9 (3.0–81.7) (*n* = 29)	272.2 (71.6–921.8) (*n* = 22)
ng/L/h	49.9 (3.3–233.5) (*n* = 32)	5.9 (1.0–27.0) (*n* = 29)	136.1 (35.8–460.9) (*n* = 22)
$AUC_{0\text{-tlast}}$ ratio OHM/M		0.1 (<0.1–1.5) (*n* = 38)	
$AUC_{0\text{-tlast}}$ ratio OHM/OHMG		0.05 (<0.01–0.20) (*n* = 38)	

Data is presented as median (range). See paragraph 'Systemic exposure to midazolam and its major metabolites after oral dosing' for explanation on the patient numbers. AUC, area under the curve; $AUC_{0\text{-inf}}$, AUC from time zero to infinite time; $AUC_{0\text{-tlast}}$, AUC from time zero to the last sampling time point; M, midazolam; *n*, number of patients; OHM, 1-OH-midazolam; OHMG, 1-OH-midazolam-glucuronide.

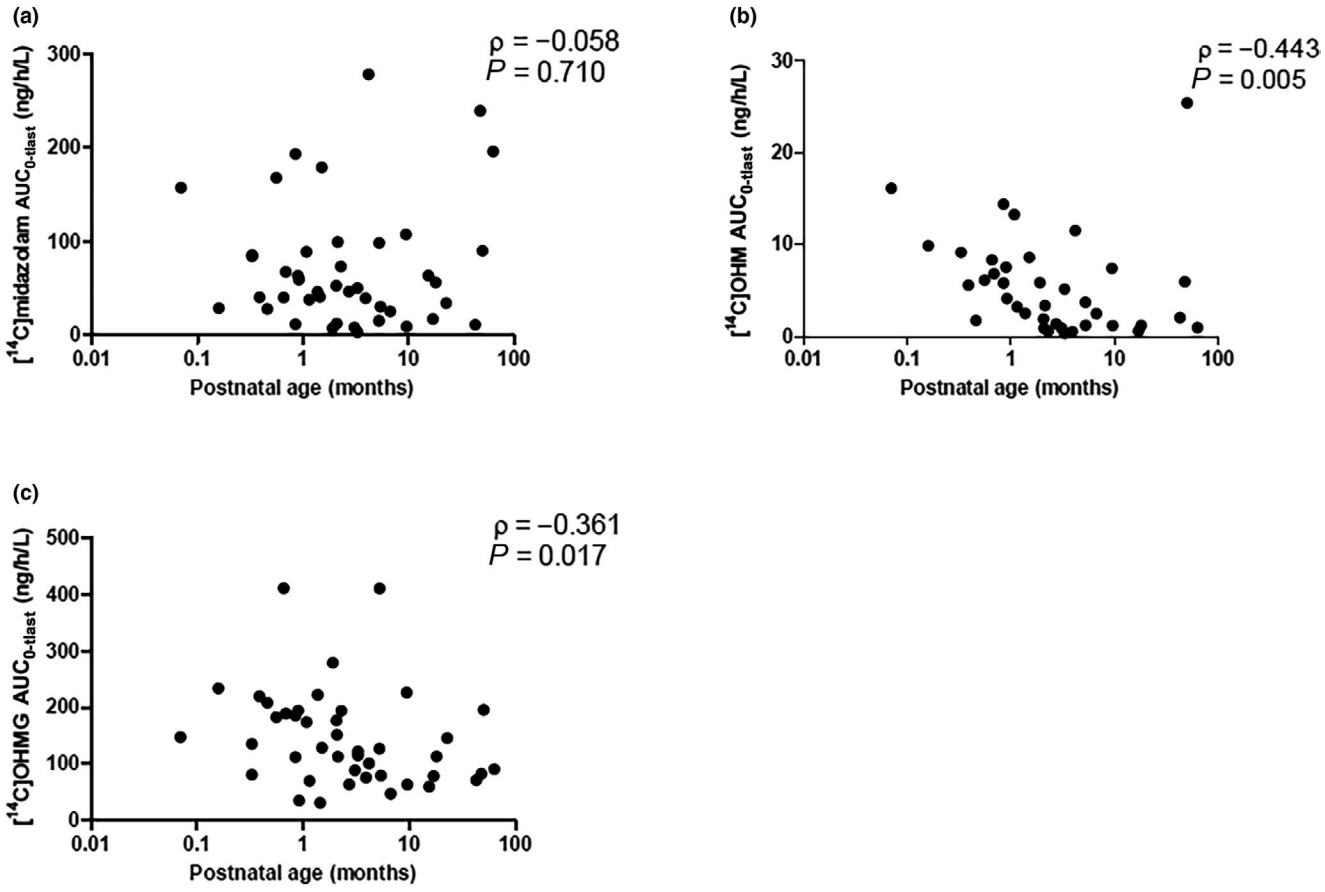

Figure 3 Area under the curve from time zero to the last sampling time point ($AUC_{0-tlast}$) after administration of an oral [^{14}C]midazolam microtracer (20.3 (14.1–23.6) ng/kg; 58 (40–67) Bq/kg) of (**a**) midazolam, (**b**) 1-OH-midazolam, and (**c**) 1-OH-midazolam-glucuronide vs. postnatal age (log scale). ρ, Spearman's rank correlation; OHM, 1-OH-midazolam (*n* = 38); OHMG, 1-OH-midazolam-glucuronide (*n* = 43); *P*, *P* value where $P < 0.05$ is statistically significant.

major metabolites 1-OHM formed by CYP3A and 1-OHMG formed out of 1-OHM by UGT2B4, UGT2B7, and UGT1A4 were highest for the youngest age ranges, despite weight-normalized midazolam doses.

Our study design has previously been applied to investigate the oral bioavailability of paracetamol and the systemic exposure to its metabolites in children[15,17] and has now shown to be successful for midazolam. The informed consent rate of 50% was in agreement with the consent rate of other nontherapeutic studies in pediatric intensive care.[38] Moreover, our population PK model results were in agreement with reported values on oral PK parameters for midazolam, confirming the feasibility of the [^{14}C]midazolam microtracer approach. The median CL of 0.20 L/hour/kg in our study was in line with literature values

of 0.26 L/hour/kg in children 0–18 year in the intensive care unit.[39] Our median distribution volume of 1.7 L/kg lies in the range of 0.2–3.5 L/kg as found in critically ill children with an age between 8 days and 16 years.[40]

More specifically, for the oral bioavailability in our patients, of whom three-quarters were 0–6 months old, the median of 66% is lower than the reported median value of 92% (range 67–95%) in 37 preterm neonates with a gestational age of 26–34 weeks[11] and higher than the reported median value of 21% (range 2–78%) in 264 older children of 1–18 years.[12] Also the reported mean ± SD of 28 ± 7% in adults is lower than in our population.[41] These latter findings can be explained by the expected CYP3A ontogeny, as older children and adults are thought to have a higher CYP3A activity in both the gut wall and liver, resulting in a lower oral bioavailability[3] than found in our patients, and *vice versa* for preterm neonates. Statistically significant covariate relationships that could explain part of the interindividual variability in oral bioavailability were not found. However, a highly variable oral bioavailability was also found in previous pediatric PK studies,[10-12] particularly in preterm neonates,[10,11] and we can conclude it is independent of the microtracer design. The higher variability in oral bioavailability in our study may be due to the nature of the studied population: stable, critically ill patients instead of healthier patients. A previous study from Vet *et al*. found a significant impact of organ failure on midazolam clearance,[39] with the greatest impact on midazolam clearance in the presence of ≥ 3 failing organs and inflammation as reflected by C-reactive protein (CRP).[39] We were not able to identify these covariates in our data, likely because children with severe circulatory, kidney, or liver failure were excluded, and the number of failing organs in our study ranged from 0 to 2 per child. CRP values in our study were comparable to those in the previous study from Vet *et al*.; i.e., (median (range) 43 (2–298) vs. 32 (0.3–385) mg/L, respectively). However, we only included six patients with a CRP > 100 mg/L, whereas the previously reported cohort consisted of more patients with CRP > 100 mg/L.

The variability in oral bioavailability as observed in our study leads to unpredictable systemic exposure to midazolam, and potentially also other CYP3A substrates, after oral dosing in (critically ill) children. These children may be at risk of subtherapeutic or toxic exposure after oral dosing of other CYP3A substrates.

In addition, the systemic exposures of the main metabolites OHM and OHMG were highest in the youngest age ranges at similar exposure of midazolam across age. This observation can most likely be attributed not solely to CYP3A ontogeny, but to other developmental changes as well, as the exposure of each metabolite is dependent on various factors. First, the CYP3A activity drives the formation of 1-OHM. Second, the reported age-related changes in UGTs may increase the glucuronidation of 1-OHM over age.[42] The age-related decrease in AUC of 1-OHMG can be partly explained by the fact that the OHMG metabolite is excreted renally and considering that children's renal function increases over age.[43-45] This explanation is supported by a reported postconceptional age-related increase in urinary excretion of OHMG in preterm neonates.[46] A metabolic shift, as seen for paracetamol, where a switch from mainly sulfation to glucuronidation is seen in the first years of life, is less likely. As in the case of midazolam, this would mean decreased formation of another metabolite than 1-OHMG in the younger age group compared with older children than adults. But as no other major metabolite for midazolam, in addition to the minor metabolites 4-OHM(G) and midazolam-glucuronide that have been identified in adults, this seems unlikely.[47,48] Third, the distribution volume of the metabolites may change with age, impacting the total systemic exposure of the metabolites.[49] Considering the case reports on the association between OHMG accumulation and prolonged sedation,[25] clinicians should be aware that the systemic exposure to OHMG may be higher in neonates than in older children, potentially also contributing to its sedative effect.

The following limitations of the study need to be addressed. First, our innovative study design limits the inclusion of pharmacodynamic (PD) data as the extremely low dose of the microtracer midazolam is not expected to have pharmacological effects. Hence, we can speculate that the variability in oral availability of midazolam and the higher systemic exposure to OHMG may lead to subtherapeutic or toxic exposure. The real impact of this variability on PD parameters should be assessed in future studies. Second, data of 21 patients were excluded from $AUC_{0\text{-}inf}$ analyses of OHMG because the elimination of this metabolite was not complete after 24 hours. In retrospect, longer sampling time would have benefited this analysis. Third, the absorption of midazolam may be influenced by food intake as food in the gastrointestinal tract may alter the gastrointestinal physiology, including the motility patterns, intestinal transit time, and the local blood flow.[50] However, information on food was not collected and the study was not powered to detect an effect of food on midazolam absorption but may have contributed to the variability in our data. Also, dose linearity of the PK of an oral microdose to those of a therapeutic dose of midazolam has been established in adults.[51-53] We made the assumption that this also accounts for children, further supported by dose-linearity of IV midazolam in children,[54] but has not been formally established.

This study presents some future opportunities. Recently, a framework was published for between-drug extrapolation of covariate models[55] which was used by Brussee *et al*. to study whether scaling with a pediatric covariate function from midazolam will lead to accurate clearance values of other CYP3A substrates.[56] Clearances of drugs were accurately scaled when they were mainly eliminated by CYP3A-mediated metabolism with, for example, high protein binding to albumin (> 90%) and a low-to-intermediate extraction ratio of < 0.55 in adults. However, the covariate relationship for clearance was based on data from children > 1 year of age. As our population consists of infants mainly < 1 year of age, our data now present a unique opportunity to test the proposed framework for this younger age group. Also, this study design is promising for drugs under development. When there is interest to, besides an IV administration, study the drug as an oral administration, an oral [^{14}C] labeled microtracer can be added without setting up a new pediatric cohort, or *vice versa*. Lastly, a microdose pediatric study can be used to obtain information on the PK for drugs with high toxicity, like oncology agents, and a clear PK/PD relationship followed by the determination of an effective dose based on the PK profile.

In conclusion, the results of this population PK study added data on oral bioavailability of midazolam as a marker for CYP3A in an age range where data were missing. It shows that children

may be at an increased risk of subtherapeutic or toxic exposure of midazolam and potentially also of other CYP3A substrates when dosed orally. The study design with an oral [^{14}C]microtracer was shown successful for safely studying the oral bioavailability of midazolam in children. To ultimately improve the safety and efficacy of pediatric drug therapy, we recommend considering study designs with microdoses for minimal risk PK studies and [^{14}C] microtracer studies to elucidate oral bioavailability.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

B.D.v.G., E.H.J.K., M.G.M., E.v.D., W.H.J.V., A.D.W., J.v.R., S.J.F.H., N.H.H., B.C.P.K., K.A., D.T., C.A.J.K., and S.N.d.W. wrote the manuscript. M.G.M., W.H.J.V., D.T., C.A.J.K., and S.N.d.W. designed the research. B.D.v.G., E.v.D., W.H.J.V., S.J.F.H., and S.N.d.W. performed the research. B.D.v.G., E.H.J.K., E.v.D., J.R., C.A.J.K., and S.N.d.W. analyzed the data. E.v.D., W.H.J.V., A.D.W., N.H.H., and B.C.P.K. contributed new reagents/analytical tools.

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