Evolution in Pediatric Pharmacology

Microdosing, Metabolism, and Membrane Transporters

Miriam G. Mooij

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The studies described in this thesis were supported by:
Netherlands Organization for Health Research and Development (ZonMw) – research grant (113202007)
Novartis – investigator initiated grant
Travel grants from: Erasmus Trustfonds, Dutch Society of Clinical Pharmacology and Biopharmaceutics, F1000 review platform

Cover and chapter design: Inge J.S.M.L. Vanhooymissen

ISBN: 978-94-6169-823-0

Layout and printing: Optima Grafische Communicatie, Rotterdam, The Netherlands

Evolution in Pediatric Pharmacology

Microdosing, metabolism, and membrane transporters

Evolutie in de pediatrische farmacologie

Microdosing, metabolisme en membraan transporters

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
dinsdag 22 maart 2016 om 13:30 uur

door

Miriam Geerthe Mooij

geboren te Zwolle

Erasmus University Rotterdam

PROMOTIECOMMISSIE

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Voor mama

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Part I

Introduction

1 General introduction

SUITABLE DRUG TREATMENT FOR CHILDREN

A major part (40-70%) of drugs prescribed to children are used either unlicensed or off-label, leading to increased risk of drug toxicity or therapeutic failure [1, 2]. Despite increasing efforts by US and EU regulatory authorities who require that the pharmaceutic industry perform pediatric studies while developing drugs, most drug treatment is based on adult studies [3, 4]. Appropriate pediatric dose-selection solely based on adult data is not acceptable due to development causing changes in drug absorption, distribution, metabolism and excretion [5]. Simple size- or weight-based extrapolations from adult to pediatric doses is not enough, particularly not in neonates and young infants. Simulation models like physiologically-based pharmacokinetic models (PBPK) and population pharmacokinetic (popPK) are used to optimize pediatric dose predictions [6-8]. The limitation of these models is the limited ontogeny data that is available for input or to validate these models. Subsequently new data on the impact of age on the processes affecting drug disposition during childhood are needed.

ORAL DRUG TREATMENT FOR CHILDREN

Nowadays, most drugs prescribed to children in the community are taken orally [1]. Even critically ill neonates and children receive many oral drug formulations, due to the unavailability of intravenous formulations, intravenous access issues or cost and safety concerns or patients soon being discharged from the intensive care unit (ICU). Extrinsic factors, like food and drug formulation, and intrinsic factors of physiological nature affect the extent of absorption of orally administered drugs [9]. Intrinsic factors include many gastrointestinal processes such as gastric pH, gastric and intestinal motility, gastrointestinal fluids, the pH and buffer capacity of these fluids, digestive enzymes, intestinal membrane transporters and intestinal drug metabolism.

Important progress has been made to elucidate age-related changes in phase I hepatic drug metabolism and renal excretion [10, 11]. Meanwhile, our knowledge on developmental changes governing variation in oral bioavailability including intestinal and hepatic drug transport and phase II drug metabolism is far less developed. A major reason for a lack of studies in children is the ethical and practical limitations. Hence, the development of innovative methods and a thorough understanding of the ethical challenges and potential solutions are highly needed.

MEMBRANE TRANSPORTERS

Plasma membrane transporters are proteins that facilitate uptake and excretion of compounds in and out of the cell. More than 400 transporters are identified nowadays [12]. Located on, amongst others, the enterocyte, hepatocyte and renal cells, they play an essential role in mediating the uptake, distribution and excretion of many drugs [13, 14]. The classification of transporters is according to their properties and grouped in superfamilies. The disposition of drugs is most often associated with two superfamilies of transporters: the solute carrier (SLC) and ATP-binding cassette transporters (ABC) families. Their physiological function is to facilitate translocation of endogenous compounds, such as bile salts and steroids. Exogenous compounds, such as nutrients, drugs and metabolites, are also substrates for specific transporters [15]. The clinical relevance of transporters in drug disposition has been shown in drug-drug interaction and pharmacogenomics studies in adults [13, 16]. Little is known on the ontogeny of membrane transporters in pediatric organ tissues and their roles in pediatric pharmacotherapy. Growth and maturation are likely to impact on activity of transporters as has been shown in drug metabolizing enzymes, especially as transporters function in endogenous processes. Animal studies have indeed shown maturational changes in transporter expression. Human data in fetuses and children are very limited and definitive conclusions on their impact on drug disposition across the pediatric age range can hardly be drawn.

INNOVATIVE METHOD: EX VIVO TRANSPORTER STUDIES

To date, no solid substrate can be identified to study *in vivo* transporter activity in children and *in vitro* expression studies might serve as alternatives. For *in vitro* studies on transporters, human organ tissue is necessary and to study the ontogeny specifically, pediatric tissue is essential. Nevertheless, pediatric tissue is scarce and difficult to harvest. Tissues can be harvested from diagnostic biopsies, surgical waste material, postmortem or be collected within the framework of a biobank. Within the context of the postmortem biobank and surgical tissue waste collection for specific projects was available. This tissue availability has been shown to be unique, even worldwide.

This relative scarcity demands study techniques that rely on efficient use of minimal amounts of pediatric tissue. Protein expression methods, especially, require large amounts of samples, can only quantify single transporters and are very labor-intensive. Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been increasingly used to study protein expression, including membrane transporters in humans. It may also be an attractive method for pediatric studies, as it only requires very limited sample volume and enables the quantification of multiple transporters at once.

PHASE II DRUG METABOLISM

Drug metabolizing enzymes are abundant in the liver and gut and contribute to the first-pass metabolism of many orally administered drugs. Drugs are often metabolized in two phases. Phase I reactions involve formation of a new or modified group (oxidation, reduction, hydrolysis), whereas phase II reactions involve conjugation with an endogenous substance (e.g. glucuronic acid, sulfate, glycine) which serves the purpose of enhancing the water-solubility of the substrate and consequently its excretion by liver or kidneys. Uridine 5'-diphospho-glucuronosyltransferase (UGT) iso-enzymes and sulfotransferases (SULT) are families of drug metabolizing enzymes, important in the phase II drug metabolism. UGTs add glucuronic acid to a substrate, i.e., drugs, bilirubin, bile salts, and SULTs add a sulfo-group to a substrate.

In vitro, human hepatic UGTs show an enzyme-specific developmental pattern in early years of age of UGT1A9, UGT1A1, and UGT1A6 [17, 18]. A limitation is that liver samples of children younger than 2 years were not included. *In vivo* studies showed that postnatal age and postmenstrual age co-determine the interindividual variability in for instance tramadol glucuronidation in neonates after 10 days of age [19]. Morphine glucuronidation changes with age due to UGT2B7 maturation [20]. The ontogeny of other UGTs or SULTs are less well studied and especially not during the continuum of childhood age but in small populations of limited ages.

Paracetamol (acetaminophen, AAP) is a suitable probe drug to study UGT activity *in vivo* in children. Its main metabolism pathways are glucuronidation and sulfation; mainly via UGT1A1, 1A6, 1A9 and 2B15 and SULT1A1, 1A3, 1A4, and 2A1 [21]. Furthermore, AAP can be given orally and intravenously, is frequently prescribed to children, has dose-linear pharmacokinetics, and UGT metabolism can be detected by the ratio of glucuronide (AAP-glu) and sulfate (AAP-sul) metabolites in plasma and urine. Its metabolism has been characterized in children, but information gaps remain, especially in the first two years of age. Moreover, its metabolism after exclusive oral administration has not been very well characterized.

INNOVATIVE METHOD: MICRODOSING TO PHENOTYPE DRUG METABOLISM

Pediatric drug studies are hampered by practical and ethical limitations. To protect children as a vulnerable population often incapable of expressing themselves, stringent medical ethical criteria are justified for research trials. Nevertheless, to ensure safe and effective drug treatment, adequate and reliable research tools are necessary. Pharmacokinetic studies, according to a classic design, are done by giving a 'non-therapeutic' drug after which multiple blood samples are taken to determine the drug concentra-

tions. Ethical and practical arguments limit these studies in children, because it means 'non-therapeutic' drug doses with exposure to unnecessary effects and toxicity followed by extensive blood sampling, which is often a painful procedure. Blood sampling, for example, is especially limited in premature neonates in whom circulating blood volume is very small (e.g., blood sampling volume would be 0.4 mL per 24 h in a 500-gram-neonate with a circulating blood volume of around 40 mL).

To overcome the limitations of a drug dose only for non-therapeutic reasons, a microdose can be given. Microdosing promises to study the pharmacokinetics of drugs in children, without the risk of adverse events and with minimal burden [22, 23]. Microdosing is defined by the EMA and FDA as the lowest of i) one-hundredth of the No Observed Adverse Effect Level (NOAEL) or ii) one-hundredth of the predicted pharmacologic dose based on animal data or iii) as 100 micrograms of the new drug [4, 24]. Additionally, labeling of the drug enables oral bioavailability studies when the microdose is administered per one route and the (therapeutic) unlabeled dose is administered via another route. Even after simultaneously administering the doses, separation of drug concentration levels is possible and so limits blood sampling time points.

Dose linearity between the microdose and therapeutic dose is a prerequisite in obtaining pharmacokinetic data for clinically relevant dosing guidelines. For several drugs such dose-linearity has been shown in adults [22, 25]. Sensitive measurements are needed to detect the extremely low dose levels in plasma or urine. Accelerator mass spectrometry (AMS) measures low attomolar to zeptomolar isotope ratios ranges, and quantifies [^{14}C] labeled drug/metabolite levels [26]. The extremely low dose implies that a [^{14}C] labeled microdose can be administered simultaneously with a non-labeled therapeutic dose. And thus, the bioavailability can be studied. Radiation associated with [^{14}C] labeling in adults is less than 10 μSv which is negligible in the light of the yearly background exposure of 2.5 mSv in the Netherlands. In premature neonates, microdosing has been used once in a small pharmacokinetic study of ursodiol in the US [27].

We chose to use [^{14}C] labeled microdosing to delineate developmental changes in the intestinal and hepatic UGT metabolism pathway involved in paracetamol. The contribution of intestinal and hepatic drug metabolism of paracetamol during childhood needs to be elucidated. Dose linearity of paracetamol has been shown in adults under normal conditions and after probenecid glucuronidation inhibition [25].

AIMS AND OUTLINE OF THIS THESIS

The aims of this thesis are:

- To study the extent of oral drugs used in neonatal and pediatric intensive care.
- To review the current knowledge of age-related variation in processes that govern oral drug absorption.
- To review the current literature on human membrane transporters during childhood.
- To assess the ontogeny of relevant human membrane transporters gene and protein expression in pediatric intestinal and hepatic tissues.
- To study the feasibility of [^{14}C]-labeled microdosing studies in children.
- To investigate the effect of age on the combined intestinal and hepatic glucuronidation and sulfation in young children, using a paracetamol microdosing study.

Part I describes a study on the extent of the oral drugs used in neonatal and pediatric intensive care [chapter 2].The available data on age-related changes in gastro-intestinal processes that govern oral drug absorption is highlighted in chapter 3. In chapter 4 and 5 extensive up-to-date overviews are given on the ontogeny of membrane transporters in children. Chapter 4 is a complete history of available transporter ontogeny data. Chapter 5 reviews the literature on the role of transporter ontogeny on pediatric drug disposition and effect.

Part II focusses on the hepatic and intestinal membrane transporters. In chapter 6, the age-related changes in gene expression of MDR1, MRP2, OATP1B1, OATP1B3 and OATP2B1 are studied in intestinal and hepatic fetal and pediatric tissues. Intestinal PEPT1 mRNA expression and PEPT1, MDR1, MRP2 and OATP2B1 protein localizations are discussed in chapter 7. Chapter 8 evaluates the protein expression in hepatic tissues during fetal and childhood age.

Part III presents the results of a [^{14}C] labeled paracetamol microdosing study in children. Chapter 9 presents pilot data to describe the proof-of-concept of microdosing studies in children. Chapter 10 evaluates our study and a second study using [^{14}C]paracetamol in children of 0-2 years. The effect of age on intestinal and hepatic paracetamol glucuronidation and sulfation is described in chapter 11.

Part IV summarizes and discusses the results of these studies in the respect of available relevant literature, and speculates on areas of future research [chapter 12 and 13].

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2 Significant oral drug use in critically ill children: rational therapy or a black box?

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ABSTRACT

Introduction

The disposition of orally prescribed drugs in critically ill children may be affected by age and critical illness, resulting in erratic effects and safety. We aimed to study oral drug prescribing in the neonatal and pediatric intensive care unit (NICU and PICU).

Methods

A one-year retrospective cohort study of all drug prescriptions, including route of administration for all children admitted to the NICU and PICU of the Erasmus MC - Sophia's children's hospital.

Results

1723 children with 2091 unique admissions received per admission (median [IQR]) 5 (3-10) drugs; 1 (0-2) orally and 3 (1-7) intravenously (IV). During mechanical ventilation 15% and 75% of drugs were given orally and IV, respectively. In non-ventilated patients, 27% of drugs were given orally and 60% IV. The 5 most frequently orally prescribed drugs were: vitamin K, spironolactone, oral probiotics, amphotericin B (prophylaxis) and trimethoprim.

Conclusion

Critically ill infants receive a considerable proportion of drugs orally, which might expose them to an increased risk of ineffective or unsafe drug therapy. This may similarly apply to other patient populations with clinical conditions potentially affecting intestinal drug absorption.

INTRODUCTION

In the intensive care unit (ICU) it is assumed that patients typically receive considerably smaller proportions of oral drugs compared to patients in the community or at general hospital wards. Surprisingly, the extent to which ICU patients receive drugs orally has not been studied.

Most ICU patients have more than one intravenous (IV) line centrally or peripherally. Due to critical illness, oral drug absorption may be erratic, e.g., by changes in intestinal motility, gastric pH, intestinal wall permeability and venous portal flow. Moreover, in children the absorption of oral drugs may be affected by age-related changes in the processes involved in drug absorption leading to even more variation in oral drug absorption [1, 2]. Hence, in critically ill children, intravenous administration is generally preferred for life-saving drugs. Nevertheless, several reasons can be identified for oral drug administration: i.e., appropriate IV formulations may not be available, IV administration may be associated with more severe adverse events (e.g., infections associated with central lines and hypotension with sildenafil), or the site of action is the gastrointestinal tract (e.g., antifungal prophylaxis). Moreover, polypharmacy with incompatible drugs through the available intravenous access ports, inability to gain any venous access and higher costs of intravenous administration may all be reasons for oral drug administration.

Consequently despite the notion that oral drug administration may lead to erratic absorption, many drugs are prescribed orally to critically ill patients for legitimate reasons. As a first step towards improving the efficacy and safety of drug therapy in critically ill children, we aim to identify the prevalence of oral drug administration in this population, and to identify which types of drugs are most frequently administered orally. This study also serves as a proof of concept to further study oral drug use in other, non-critically ill patient populations with underlying disease also affecting drug absorption, such as oncology, heart failure and intestinal disease patients.

MATERIALS / METHODS

Data on drug administration were collected from all patients admitted to the neonatal or pediatric ICU of the Erasmus MC – Sophia children's hospital over a one-year period. The medical ethical review board of the Erasmus MC waived the need for ethics board approval for this study, according to the Dutch law on medical research with humans, as only patient chart data were collected.

In the electronic patient data management system (PDMS), the ICU nurse checks off the drug order as soon as the drug has been administered to a patient, and these verified orders were used for analysis. Other collected data from electronic medical records were:

gender, date of birth, primary diagnosis, intensive care (IC) admission and IC discharge date, invasive mechanical ventilation (yes/no), and all drugs administered to the patient with the associated route of administration. Oral route includes: oral, buccal, enteral, per feeding tube (gastric/duodenal), gastrostomy. Intravenous also includes per central line. Other routes included: rectal, nasal, eye, ear, tracheal, (sub)cutaneous, intramuscular local, topical, peritoneal, loco-regional, or epidural.

All data were collected per day of admission. Receiving a drug on a day was defined as a minimum of one dose (independent of actual dose). Parenteral nutrition was excluded. A patient was considered to have received invasive mechanical ventilation if the duration for a specific day was at least 6 hours. The primary outcome was the proportion of orally administered drugs in relation to drugs administered intravenously and by other routes. Secondary outcomes were the proportion of orally administered drugs, in ventilated and non-ventilated patients. Furthermore, we described the 10 most frequently prescribed oral drugs by age group.

The data were expressed as median values with ranges or numbers with percentages. The data have a multilevel structure, where the administration (including mode of administration) of each drug is measured for each admission each day. Each admission consists of one or more admission days, and multiple admissions are included for some patients. Descriptive data (age, admission days per patient, days with mechanical ventilation or enteral/tube feeding) are described on the patient level, whereas diagnosis, proportion drugs per route and most frequently prescribed drugs are described on the level of an admission. For the proportion of drugs per admission, the admission days are pooled, so that a patient was considered to have received a drug during an admission if the drug was administered on any day of the admission. For the secondary analysis, ventilated and non-ventilated days were pooled separately and per patient one or two proportions resulted (not ventilated at all and ventilated all days was one proportion, partially ventilated was two proportion: with and without ventilation). Data management and statistical analyses were performed using R and IBM SPSS Statistics software (SPSS Statistics for Windows, version 21.0; IBM, Armonk, NY).

RESULTS

From July 1, 2014 to June 30, 2015, 2091 admissions of 1723 unique patients were recorded, with 17256 days of admission with medication use. Patient demographics are summarized by age group in table 1. In total 499 unique drug/route combinations were given. The median (IQR) number of drugs patients received per admission was 5 (3-10), this was 1 (0-2) per oral route, and 3 (1-7) per IV route. In mechanically ventilated patients (578 admissions) 15% of drugs were given orally and 75% IV. When patients were

not ventilated either before or after ventilation episodes or not at all in an admission, 27% of drugs were given orally and 60% IV (Figure 1). The ten most prescribed oral drugs per age group are also shown in Table 1.

Table 1. Patient demographics and results

Legend: (Median [range]) MV: mechanical ventilation. Prednison/prednisolone, HCT: hydrocholothiazide

Characteristics	Newborns up to 1 month		1 month to 1 year		1 year to 12 years		12 years to 19 years	
Patients								
Number of patients	752		288		450		233	
Postnatal age at day of admission	0 (0-4) weeks		5 (1-12) months		4.6 (1-12) years		15 (12-19.5) years	
Gestational age at birth (days)	223 (167-296) (n=544, missing=208)		N/A		N/A		N/A	
Total number of admission days	10217		3107		2811		1121	
Median admission days per patient	6 (1-274) days		2 (1-188) days		2 (1-261) days		2 (1-135) days	
Patients receiving MV during admission	58% (n=334)		21% (n=122)		17% (n=96)		5% (n=26)	
Days MV per admission	4 (1-81)		4 (1-69)		3 (1-53)		3 (1-53)	
Days with enteral feeding	0 (0-50)		0 (0-33)		1 (1-259)		1 (0-64)	
Days with tube feeding	5 (0-255)		2 (0-163)		0 (1-224)		0 (0-129)	
Admissions								
Number of admissions	799		441		594		257	
Median number of days per admission	6 (1-274) days		3 (1-169) days		2 (1-261) days		2 (1-135) days	
Diagnosis:	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
1. Respiratory	108	14	40	9	86	15	21	8
2. Circulatory	36	5	44	10	43	7	23	9
3. Surgery	82	10	168	38	143	24	100	39
4. Other	240	30	189	43	322	54	113	44
5. Pre-/dysmaturity	333	42	N/A	–	N/A	–	N/A	–
Proportion drugs per:		*%*		*%*		*%*		*%*
Oral route		18		23		27		29
Intravenous route		70		62		62		62
Other route		12		15		11		9

Table 1. Patient demographics and results (continued)

Characteristics	Newborns up to 1 month		1 month to 1 year		1 year to 12 years		12 years to 19 years	
Oral drug		*n*		*n*		*n*		*n*
1	Vitamin K	299	Spironolacton	98	Trimethoprim	97	Paracetamol	54
2	Oral Probiotics	275	AmphotericinB	64	Macrogol	80	Macrogol	41
3	Spironolacton	112	Vitamin K	48	Spironolacton	64	Celecoxib	29
4	HCT	72	Lorazepam	44	Amphotericin B	56	Melatonine	22
5	AmphotericinB	51	HCT	39	Omeprazol	53	Omeprazol	19
6	Nystatin	49	Trimethoprim	34	Paracetamol	52	Diclofenac	19
7	Trimethoprim	29	Omeprazol	26	Prednison	41	Prednison	17
8	Omeprazol	23	Propranolol	24	Levetiracetam	36	Trimethoprim	15
9	Doxapram	22	Macrogol	22	Melatonine	35	AmphotericinB	15
10	Oxybutynin	16	Domperidon	20	Azithromycin	33	Azithromycin	12

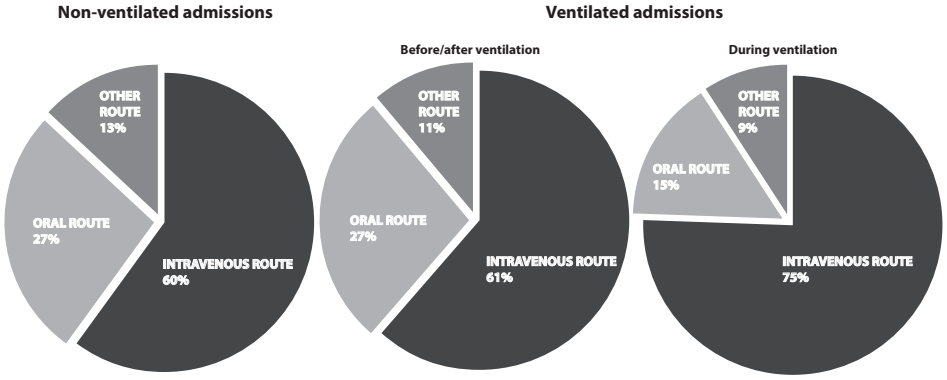

Figure 1. Proportion of drugs per route

Total number of drugs in the days per admission or per ventilated/non-ventilated days of admission. At least one day receiving a drug. Different routes of the same drugs are noted as different drugs.

DISCUSSION

In critically ill children, intravenous drug administration is preferable but for many reasons drugs are administered orally. The extent and nature of oral drug prescribing in this population is not known. Data in our level III NICU and PICU shows that 15 and 27% of drugs are given orally to ventilated and non-ventilated patients, respectively. As ventilated children are not able to take drugs by mouth and may not tolerate any oral food or drug, we also analyzed these groups separately. From a pharmacological point-of-view, this outcome is rather surprising as it shows a considerable extent of oral drug use and possible erratic drug absorption. The most often prescribed oral drugs

consisted of oral probiotics, amphotericin B, and macrogol, for which erratic oral absorption is less relevant as its target is the gut or the gut flora. In contrast, other frequently prescribed drugs for which erratic oral absorption may affect their efficacy and safety are: analgesics, diuretics, vitamin K, omeprazole, and lorazepam. To date, data on the efficacy and safety of for example omeprazole, hydrochlorothiazide, spironolactone and lorazepam in this population are very scarce or completely lacking [3]. Moreover, no licensed liquid formulations are available. Extratemporaneous formulations are used, including IV formulations and crushed tablets. This further illustrates the risks associated with oral drug use in this setting.

Information is available about drug utilization in pediatric pharmacotherapy; most studies, however, are aimed on the statistics of the use of off-label and unlicensed drugs, but did not specify differences in label status by administration route [4-6]. Interestingly very little data are available on the extent of drug prescription per route. One drug utilization study in a NICU in India found 92% of prescribed drugs in 6 months were intravenously used [7]. This is far more than the 70% of IV drugs in neonates of our study, although the non-western setting may be quite different.

Maat et al. looked at the rate of pharmacy interventions after electronic prescribing in a four-year study in a tertiary children's hospital (excluding the ICUs) [8]. Interestingly, of all medication-related characteristics, the oral dosage form and oral route of administration had relatively highest risk for interventions on prescriptions done by clinical pharmacists (OR=1.63 [95%CI 1.41-1.88] and OR=1.80 [95%CI 0.38-0.67]).

The strengths of this study include the large sample size, recent data and a reflection of current daily practice. Moreover, our data display actual administered drugs to the patient instead of prescriptions that might be changed before it arrives to the patient. To our knowledge, this is the first study specifically identifying oral drug use in the neonatal and pediatric ICU. There are some limitations. First, input mistakes on route of administration are rare but cannot be excluded. Second, in our institution, PDMS recordings continue while a patient is away from the ICU during surgery and drugs used during surgery are also recorded. This may have contributed to an underestimation of the proportion of oral drug use, as in this setting oral drug use is virtually absent. Third, drug dose and frequency of administration per day were not analyzed. Finally, this single-center study was performed in a tertiary Dutch referral hospital and data may not be completely translatable to other centers.

Oral prescriptions may not be all bad, as oral administration may reduce drug administration costs and IV line related infections. Physicians' choosing the oral route should nevertheless be aware of the uncertainties related to the oral disposition of drugs in critically ill children and should weigh these uncertainties against the apparent advantages. Our paper presents data on oral prescription in a specific patient population; i.e., critically ill children. The uncertainties in oral drug exposure may similarly apply to other

patient populations, including adults, where oral drug absorption may also be affected by underlying clinical conditions disease, e.g., intestinal disease, heart and liver failure, cancer and in palliative care.

In conclusion, orally administered drugs comprise up to 27% of the pharmacotherapy of patients in the ICU, possibly leaving children at risk of ineffective or unsafe drug therapy.

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3 Ontogeny of oral drug absorption processes in children

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Expert Opinion on Drug Metabolism & Toxicology 2012, Oct;8(10):1293-303

ABSTRACT

Introduction

A large proportion of prescribed drugs to children is administered orally. Age-related change in factors affecting oral absorption can have consequences for drug dosing.

Areas covered

For each process affecting oral drug absorption, a systematic search has been performed using Medline to identify relevant articles (from inception till February 2012) in humans. This review presents the findings on age-related changes of the following processes affecting oral drug absorption: gastric pH, gastrointestinal motility, bile salts, pancreatic function, intestinal pH, intestinal drug-metabolizing enzymes and transporter proteins.

Expert opinion

Clinicians should bear in mind the ontogeny of oral drug absorption processes when prescribing oral drugs to children. The authors' review shows large information gaps on almost all drug absorption processes. It is important that more knowledge is acquired on intestinal transit time, intestinal pH, and the ontogeny of intestinal drug-metabolizing enzymes and drug transporter proteins. Furthermore, the ultimate goal in this field should be to predict more precisely the oral disposition of drugs in children across the entire pediatric age range.

INTRODUCTION

Orally administered drugs in children

A large proportion of drugs prescribed to children is administered orally [1]. Absorption of orally administered drugs may be affected by extrinsic factors (food and formulation) and intrinsic factors of a physiological nature. The latter includes volume of gastrointestinal fluids, the pH and buffer capacity of these fluids, contraction patterns, gastrointestinal transit, digestive enzymes, intestinal cellular transporters, drug metabolism enzymes, and intestinal bacterial flora [2]. Solubility and intestinal permeability of the individual drug will influence the impact of gastrointestinal (GI) processes on its absorption. A theory-based oral drug classification based on solubility and permeability characteristics of drugs, such as the biopharmaceutics classification system (BCS), may serve to predict which extrinsic or intrinsic variables will alter oral drug absorption [2, 3].

As many of the GI processes change with age, oral drug absorption expectedly will change with age as well [4]. The current EU and US regulations aimed at stimulating the study of drugs across the pediatric age range, have given an impetus to promoting clinical trials in children [5, 6]. Age-specific information on the processes governing drug disposition in children is needed for modelling and simulation approaches. Important progress has been made to elucidate age-related changes in: hepatic drug metabolism and renal excretion [7, 8]. By contrast, our knowledge on developmental changes in the GI processes involved in oral drug absorption is far less developed [4, 9-11].

The aim of this review is to present the available data on age-related variation in GI processes that govern oral drug absorption processes. We performed a systematic search in the literature using Medline. Reference lists of relevant retrieved papers were screened for additional relevant articles. We discuss current information gaps and provide suggestions for future research that may lead to develop evidence-based dosing guidelines for oral drugs in children.

Age-related changes in oral drug absorption processes

Gastric pH

Gastric pH is an important factor determining the stability of a drug passing through the stomach. Studies on gastric pH across the pediatric age range used pH measurement of gastric fluid aspirates and 24-h intragastric pH monitoring [12-29]. Figure 1 displays the mean and median gastric pH values in healthy children in the first three months of life.

The mean gastric pH in newborns min after delivery is 7.05, and within a few hours it declines to a pH of 2.7 [13, 28]. A less acidic stomach environment in these newborns after delivery is most likely explained by swallowing of amnion fluids, which is supported by the decrease in pH within a few hours after birth [28]. More than seventy years ago,

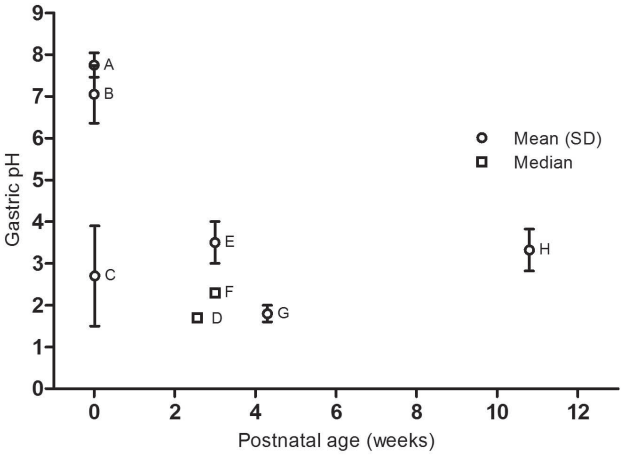

Figure 1. Gastric pH measured in neonates by 24-h monitoring or gastric aspirates.
References correspond with references in the text.
A: Gastric aspirates 3 minutes after delivery; vaginal delivery [12]
B: Gastric aspirates 3 minutes after delivery; caesarean section [12]
C: Less than 3 hours after delivery [13]
C, D, F, G: Continuous 24-h gastric pH monitoring [13, 18, 19, 25]
A, B, E, H: Gastric aspirates obtained by gentle suction [12, 12, 27, 26]

Miller observed a decrease in acidity over the first 10 days of life [30]. Many more recent studies report that the gastric pH declines already within a few hours after birth [13, 28]. Miller titrated gastric juice with NaOH and then determined the amount of HCl as a measure of acidity. However, Miller did not provide information on the acidity of the primary gastric content; therefore, we are unable to translate this outcome to pH. Many other studies subsequently showed that the gastric pH remains low at a pH around 2 and 3 in children of all ages [12, 14-27, 29].

More rarely, gastric pH can be described in terms of the proportion of time it peaks above 4 measured over 24 hours. In preterm infants, this proportion ranged from 46% to 70% over a 24-h period [31, 32]. In children up to 2 years of age it was around 51%; in older children it was 34% [33]. The higher proportion in younger children might be explained by the buffering effects of milk formula, older children are less frequently fed and receive more solid foods [23, 25, 33]. 24-h pH monitoring reflects the buffering effect as well; in preterm infants, the gastric pH first increased to 7 postprandially, but then immediately steadily declined to a pH of 2 [23]. Another study showed a similar pattern with a mean gastric pH returning to a value of 1.8 within 180 min postprandially [25]. Apparently, during the day, younger children tend to have more often a basic gastric environment than older children, although the mean gastric pH remains around 2 or 3 in children of all ages.

Interestingly, this overview gives reason to contradict the widespread notion that absorption of gastric pH-dependent drugs in both neonates and young infants is affected by high gastric pH [34, 35]. Gastric pH may be high due to continuous enteral feeds, but is comparable to adult values when oral feedings are given at longer than 3 hour intervals. Children older than one week of age will typically receive such intermittent feeding, especially during the night. We have not been able to identify studies that compared effect of different feeding regimes (continuous and intermittent) on drug absorption in neonates.

The consequences of changes in gastric pH are relevant for acid-labile drugs. These may be absorbed more efficiently in a higher gastric pH environment achieved by very frequent or continuous feeding regimens. Huang et al. found that serum penicillin levels in premature and term newborns were higher than those in infants and children. These age-related changes were hypothesized to be either due to higher gastric pH in the first 10 days of life or to altered renal function [36].

Gastrointestinal motility

Gastric emptying

Next to intestinal motility, gastric emptying is a primary determinant of the rate at which drugs are presented to the small intestinal mucosa for absorption. Gastric emptying is usually measured by the following methods: gastric emptying breath test, scintigraphic procedure by Technetium-99M liquid gastric emptying scan or the paracetamol absorption test. Gastric emptying time is reported in various ways: gastric emptying time, gastric half-emptying time or residual gastric activity at 1 h.

We identified three studies using the gastric emptying breath tests. Hoekstra et al. tested the effect of glucose and fructose on gastric emptying using an L-glycine-1-^{13}C breath test in four healthy children (mean age 14.3 years; age range 12.1-16.0 years). Gastric half-emptying time after fructose intake was 45.5 min (SD 4.9); after glucose intake 64.3 min (SD 2.4). Gastric half-emptying time was significantly longer when fructose and glucose were administered together, that is, 85.3 min (SD 7.0) [37]. The authors considered all values to be in the normal ranges established with other methods. Using the ^{13}C-octanoic acid breath test, Perri et al. found a gastric half-emptying time of mean 121 min (SD 42) in nine healthy control patients (mean age 9 years; age range 4-16 years). These children had eaten a standard test meal (bread, ham, juice, egg), however, which hardly compares with a smaller fructose and/or glucose administration [38]. Hoffman et al. subjected 22 patients with gastroesophageal reflux symptoms (mean age 13.2 years) to the ^{13}C-octanoic acid breath test and compared half-emptying results between patients with or without pathologic acid exposure (84 (SD 24) vs 86 (SD 26) min) and with or without duodenogastroesophageal reflux (105 (SD 47) vs 76 (SD 24) min) were

compared. Results were not statistically significantly different [39]. In conclusion, breath test measurements for gastric emptying rate are highly variable and, probably for practical reasons, that tests have only been performed in relatively older children.

Scintigraphic imaging makes it possible to measure the gastric emptying time or gastric half-emptying time as well as the residual gastric activity at 1 h. We identified seven useful scintigraphy studies. In 10 preterm infants (median gestational age 28.9 weeks; range 26-33), the median gastric half-emptying time at a postnatal median age of 9 days (range 6-37) was 60 min (30-180 min) [40]. Patients were all hourly fed although not receiving a standard meal size. The residual gastric activity at 1 h was 37.5% (range 19-100%) [40]. Di Lorenzo and colleagues conducted a study in 477 patients across a wide age span; 291 patients with and 186 patients without gastroesophageal reflux disease (GERD) (based on pH and/or scintigraphy investigations) [41]. In children without bolus or acidic gastroesophageal reflux, gastric residual activity at 1 h was around 65% in those up to 3 years of age; it decreased to 51% in the age group 4-6 years; and to 45% in children over 6 years of age [41]. Seibert et al. reported an opposite outcome in children being evaluated for gastroesophageal reflux (GER): the percentage emptied at 1 h instead of the percentage residual activity [42]. The percentage emptied at 1 h was 48% (SD 16) in 44 infants (mean age 5.7 months, range 1-23) and 51% (SD 7) in 8 children (mean age 9.1 years, range 2-14.5 years). When converted to residual gastric activity, values are still comparable (respectively 52% and 49%). Note that in the study reported by Di Lorenzo et al., a delay in gastric emptying was not related to GER symptoms until the age of 6 years [41]. Cucchiara et al. studied a poorly described control group suffering from diarrhoea and failure to thrive not related to gastrointestinal symptoms. The gastric emptying activity at 1 h was 38.1% (SD 6.5) [43]. Miele et al. reported a 43.3% (SD 8.7) gastric emptying activity at 1 h in a control group of 11 children without gastrointestinal or neurologic disorders (mean age 5.6 years; SD 3.9 years; range 2-12 years) [44]. Describing gastric emptying alternatively as a mean emptying half time, Yahav et al., reported 87.8 min (SD 22.9) mean gastric emptying time in a control group with a mean age of 10.4 months for which no other details are reported [45]. Demirbilek et al. found an average gastric emptying time of 51.6 min (SD 8.04) in a selected group of children with GERD (mean age 3.2 years; SD 1.1); the selection might have resulted in bias [46].

To relate these results to adults, reported healthy adult gastric emptying times range between 56 (32-85) and 104 (49-126) min, for liquid and solid markers respectively [47, 48].

Finally, the paracetamol absorption test was used in two small cohorts. In 15 critically ill patients (median age 5.3 years; interquartile range 1.2-6.5) who were food tolerant, it revealed a median 1.5 (interquartile range 0.7-2.2) ratio of time to reach paracetamol peak to the maximum paracetamol concentration (Tmax/Cmax) [49]. In seven adolescents (mean age 16.4 years; SD 0.7 years; range 15.5-17.5), it revealed a paracetamol

absorption ratio of 1.4 for high-fat meals and 0.5 for low-fat meals [50]. The evidence of these two studies is too limited to conclude on age-related changes.

However, population pharmacokinetic analysis applied in another study yielded a significantly lower oral paracetamol absorption rate in the first days of life before stabilizing after 1 week [51]. The lag time reflects the time to reach and permeate the absorbing surface of the intestine [2]. Considering that a lag time was observed after oral paracetamol administration only and not after rectal administration, it suggests that gastric emptying may be the primary determinant of a lag time for oral absorption of paracetamol.

Antroduodenal contractions

Antroduodenal motor activity plays a role in the gastric emptying next to fundic contraction, pyloric sphincter relaxation and intestinal motor activity. It can be determined by antroduodenal manometry, which measures intraluminal pressures of the distal stomach and the proximal small bowel.

Fasting antral motor activity and antral motor activity in response to intraduodenal feeding did not significantly differ between term and preterm infants [52]. By contrast, the proportion of antral clusters temporally associated with duodenal activity in preterm infants was significantly lower than that in term infants. Moreover, the degree of association of antral and duodenal activity increased significantly with gestational age [53]. In preterm infants 29 to 32 weeks of gestational age, the frequency of contractions, the number of contractions per burst and the intraluminal peak pressure of duodenal motility during contractions all increased with postgestational age, resulting in a more efficient motility [54]. Similarly Bisset et al. reported that both the magnitude and organization of motor activity increased with increasing gestational age [55]. Berseth et al. reported shorter lasting individual duodenal cluster activity during fasting periods in preterm than in term infants, but duodenal motor activity in response to feeding increased similarly in both groups [52]. The timing of introducing food seems to influence the preterm neonates' (28-32 weeks of gestational age) duodenal motor activity; introducing formula early (day 3-5 postnatally) resulted in more mature motor complexes than introducing formula late (day 10-14 postnatally) [56]. Preterm infants showed more immature duodenal motor activity response to bolus feeding then did term infants [57].

In conclusion, proximal intestinal (duodenal) motor activity in contrast to antral motor activity matures throughout the first weeks of life, with increasing frequency, amplitude, and duration of propagating contractions. Regrettable, there are no such studies in healthy children beyond the newborn period.

Intestinal transit time

Overall, gastrointestinal motility can be expressed as orocecal transit time (OCTT). This can be measured by different techniques: hydrogen breath test, ^{13}C Ureide breath test, radiotransmitting capsule, red carmine marker test or scintigraphy. Most common is the hydrogen breath test with lactulose as nonabsorbable carbohydrate substrate. This breath test has limited use in the general population, which may include hydrogen-non-responders. Also lactulose may accelerate transit time by its osmotic laxative effect. Accordingly, Vajro et al. reported in 11 control patients that the mean OCTT after a meal was significantly longer than that after lactulose [58]. Although this method can be used to compare groups in standardized studies, it is merely an approach to the physiological situation of intestinal motility.

We identified four studies using the hydrogen breath test to measure OCTT in different pediatric age groups [58-62]. The populations were quite heterogeneous, but there does not appear to be an age-related difference in OCTT. In the whole age range from 1 to 17 years, the mean OCTT was roughly between 60 and 110 min, as in adults [62]. The mean OCTT measured by the lactose-^{13}C-ureide breath test was 255 min (range 165-390) in children from 3 to 17 years of age [59]. This method cannot be used in infants below 6 months of age as they lack the intestinal bacterial enzymatic activity. In adults, the latter test was validated in respect to scintigraphy [63]. The lactulose-H2 breath test yielded a significant shorter OCTT than did the labeled ureide test, which may be due to the effect of lactulose [64]. Fallingborg et al. distinguished small intestinal and colonic transit times with the use of a radiotransmitting capsule in a small population of 12 healthy children (8 to 14 years) [65]. Small intestinal transit time was 7.5 h and colonic transit time was 17.2 h. Interestingly, from the number of observations in each segment they estimated that the capsule resided in the duodenum for 8% of the small intestinal passage, in the proximal part of the small intestines for 5%, in the mid part for 12% and in the distal part for 75%. The small intestinal transit time of 7.1 h is considerably longer than that established by the breath tests. The fact that the capsule, which was larger than 2 mm, was located in the distal part of the terminal ileum for 75% of the small intestinal transit time suggests a longer ileo-cecal transit for large particles. By means of scintigraphy, Bodé et al. measured a mean OCTT of 3.1 h (range 1.3-6.1 h) in nine premature infants (mean gestational age 28.9 weeks) [40].

Bile acids

Bile is a complex secretory product produced by the liver. It eliminates waste products from the body and it promotes digestion and absorption of lipids by the intestines. In preterm neonates, the concentration of the bile acids was found to be 4.55 mmol/l in the first few weeks postnatally [66]. In 65 healthy preterm newborns, the total bile acid concentration was consistently higher in those fed with human milk in comparison with

those fed with formula. Concentrations did not significantly increase over a 3-week follow-up period [67]. Concentration did not differ between small- and appropriate-for-gestational-age premature infants [66]. Challacombe et al. compared three age groups, that is, 2 days postnatal (n=12), 2 to 7 days (n=8), and 10 days to 7 months (n=14). Gestational ages were not documented. The total bile acid concentration in the oldest group was much higher than that in both other groups and at a value comparable to those in adults [68].

Changes in biliary function can influence solubilization and consequently absorption of lipophilic drugs [3].

Pancreatic function

The exocrine pancreas is a specialized secretory gland, which secretes juice rich in HCO3- and digestive enzymes that neutralizes the acidic gastric contents and helps digest food. Functioning of the exocrine pancreas is typically measured by the fecal elastase-1 (E-1) concentration. The E-1 enzyme is highly specific for the pancreas and is not degraded during the intestinal passage. Age-related differences in E-1 concentrations were absent in a large cohort of healthy subjects (mean age 11.2 years (SD 0.5); age range 2 months to 52 years) [69]. Even as many as 96.8% of preterm and term infants up to the age of 12 months without known bowel or pancreatic disorders had adult E-1 values after 2 weeks of life, independent of gestational age [70]. However, up to 48 hours after birth, none of the preterm infants had a fecal E-1 concentration of greater than 30 µg/g meconium, whereas 43% of the term infants had normal adult values. This discrepancy may be due to either immaturity or insufficiency of the exocrine pancreatic function in premature neonates. However, the small sample size did not allow differentiating between these two possible causes. Deficient exocrine pancreas function as seen in cystic fibrosis patients was associated with lower oral bioavailability of mycophenolate mofetil [71]. This suggests an effect on oral drug absorption in neonates with immature pancreas function, but this has not been studied to date to our knowledge.

Intestinal pH

In comparison with gastric pH, remarkably little is known about the intestinal pH in children. Fallingborg et al. measured gastrointestinal pH with a radiotransmitting pH-sensitive capsule in 12 healthy children aged 8-14 years. The mean value of pH rose from 1.5 in the stomach to 6.4 in the duodenum; in the distal part of the small intestine, it reached an alkaline peak value of 7.4. The pH profile was almost identical to that in healthy adults. A broad conclusion on the development of the intestinal pH cannot be drawn as his small population consisted merely of older children. It would be worthwhile to repeat the experiment in other age groups [65].

Intestinal drug metabolism

Many developmental changes in hepatic drug metabolism and renal clearance have been well documented. Data on the ontogeny of intestinal metabolism remain scarce. What is known is that enzymes of the cytochrome P450, especially the 3A (CYP3A) subfamily, are abundant in liver and gut and contribute to the first-pass metabolism of many orally administered drugs in adults [72]. Hepatic CYP3A forms present a developmental expression in fetal and pediatric samples; CYP3A4 and CYP3A7 expression levels show to be age dependent with respectively increasing and decreasing levels of total CYP3A expression levels [73].

CYP3A ontogeny can be reported as changes in mRNA, protein or activity levels. We identified two *in vitro* studies on CYP3A ontogeny in the intestine. One studied 59 histologically normal duodenal biopsies from children aged 1 month to 17 years for CYP3A mRNA by quantification and CYP3A proteins localization by immunohistochemistry [74]. The other studied duodenal biopsies and surgical sections from 104 children aged 2 weeks to 17 years and 11 fetuses for CYP3A protein expression by immunohistochemistry and activity by the formation of 6beta-hydroxytestosterone from testosterone [75]. CYP3A4 and CYP3A5 mRNA expression levels were to decrease with age, showing expression levels were high in the first year of life and decreased thereafter [74]. This is in contrast with protein expression levels reported in the second study showing CYP3A protein expression significantly increased with age [75]. The discrepancy of decreasing mRNA expression and increasing protein levels with age might reflect a posttranscriptional regulatory mechanism that is not elucidated to date according to the authors [74]. Dissociation between protein and mRNA levels during the maturation process was already reported for CYP2D6 liver enzymes [76]. The location of the CYP3A protein in enterocytes assumes a maturation profile occurs. In the duodenal biopsies of children less than 6 months of age, CYP3A protein was detected in only 50% of the enterocytes; in the older children, however, CYP3A protein was expressed in all cells [74]. Moreover, the increase in CYP3A protein levels with age is mirrored with increasing CYP3A4 activity. It changes from undetectable in fetal samples, low in neonates and adult levels in children older than 5 years of age, as reflected by 6beta-hydroxytestosterone formation [75].

Intestinal CYP3A4, CYP3A5 mRNA levels have been established in pediatric liver recipients (age 0.1-15 years) at the time of transplant surgery [77]. Unfortunately, the authors did not study the effect of age within their cohort. Adult data show similar CYP3A4 and CYP3A5 expression levels [78]. This suggests that intestinal CYP3A expression does not change beyond childhood. However, because the range of levels reported in children was very wide, age-related changes from 0.1 year of age onwards cannot be excluded [78]. Intestinal CYP3A5 mRNA levels were significantly higher in *CYP3A5*1* gene carriers (expressors) than *CYP3A5*3* homozygous patients (non-expressors) and observed in

both the children and adult study. In *CYP3A5*1* carriers, CYP3A5 mRNA accounted for 20-30% of all CYP3A mRNA detected [77, 78].

In vivo studies on oral bioavailability of CYP3A substrates in relation to age are scarce. Our own research showed that median midazolam oral bioavailability in preterm infants (28-32 weeks, <10 days of age) is significantly higher than in adults (50 vs. 30%) [79-81]. This probably reflects developmentally low intestinal and hepatic CYP3A activity, as midazolam is a validated probe drug for CYP3A4/5 activity.

Interestingly, the type of feeding (breast milk or formula) seems to impact the developmental pattern of combined intestinal and hepatic CYP3A in neonates. In children who received oral dextromethorphan six times between two weeks and 6 months of age, the urinary metabolite/dextromethorphan ratio as a measure of CYP3A4 activity clearly increased over this period. Moreover, this increase was faster for formula- than breastmilk-fed children [82]. This finding suggests a differential effect of components of these milk formulations on the induction of intestinal and hepatic CYP3A activity in the first months of life.

The ontogeny of other drug metabolizing enzymes in the intestine remains to be elucidated.

Intestinal drug transporter

Multidrug resistance protein 1 (MDR1/P-glycoprotein) is a plasma membrane glycoprotein acting as an efflux system. Based on *in vitro* studies, it is currently considered the most prominent gut transporter [83]. It is located in many tissues and specifically within the brush border in the small intestine. Its expression is genetically controlled by the *ABCB1* gene [84]. MDR1 action in the enterocyte reduces the bioavailability of orally administered drugs as these are expelled into the intestinal lumen. MDR1 protein can be localized by immunohistochemistry and mRNA quantification in intestinal tissue. In the earlier mentioned study evaluating 59 duodenal biopsies of children aged from 1 month to 17 year, MDR1 mRNA expression was highly variable and not related to age [74]. MDR1 protein was detected in all the enterocytes and was located on the apical surface. In the biopsies in children younger than 3 years, additional staining was located on a limited upper part of the lateral surface.

A possible age effect in relation to of the *ABCB1* genotype was found for oral bioavailability of the MDR1 substrate cyclosporine. One hundred and four children with renal disease (age 0.36-16.3 years) were grouped by age and genotyped for *ABCB1* gene. The pre-hepatic extraction ratio of cyclosporine was *ABCB1* genotype dependent only in children older than 8 years, resulting in corresponding differences in oral bioavailability. No such association was found in younger patients, which suggests an interaction of age and genotype on MDR1 activity [85].

In the context of the previously mentioned study in pediatric liver recipients, MDR1 mRNA was determined as well and results were similar as for CYP3A; that is, the median MDR1 mRNA expression did not differ between children and adults, but widely ranged in the pediatric population [77, 78].

Interestingly, in noninflamed duodenal biopsies of children with Crohn's disease, MDR1 mRNA expression was significantly higher than that in normal biopsies. Expression of MDR1 was highly variable in both groups [86]. The effect of age was not examined in this study. The higher levels of MDR1 expression could have been induced by systemic inflammation present in Crohn's disease, which is likely to lead to an elevated first-pass metabolism of xenobiotics used in the treatment.

To our knowledge, the intestinal ontogeny of other members of the ATP-binding cassette transporters, such as multidrug resistance protein 2 (MRP2/ABCC2) or breast cancer resistance protein (BCRP/ABCG2), has not been studied to date [83, 87-89].

CONCLUSION

This literature review makes clear that GI processes that govern drug absorption change from the neonatal period up to adulthood. Consequently, these changes could have an impact on drug absorption depending on the drug characteristics [3, 4]. The review also brought to light important knowledge gaps regarding these processes and especially their impact on drug absorption.

Key findings in the research done so far are the following. Apart from a brief peak postnatally, the gastric pH is about 2-3 in children of all ages. Postprandial, its rise is due to the buffering effect of milk-based feeding. Especially in frequently fed neonates, the pH may, therefore, be higher for a longer period during a 24-h period, than in older children who eat less frequently. Gastric emptying time reported in the literature is highly variable. Standard gastric emptying tests do not reveal evident age-related changes. Population pharmacokinetic analysis shows a markedly paracetamol absorption decrease in the first few days of life, which suggests delayed gastric emptying. This delay could perhaps be explained by maturation of antroduodenal contractions.

There are no studies done examining antroduodenal contractions beyond the neonatal period. Intestinal transit time (in terms of mean OCTT) does not appear to be subject to age-related changes; it is roughly between 60 and 110 min in the age range of 1-17 years, as measured by hydrogen breath test. The one study that used a capsule to measure OCTT showed a much longer transit time than any of the other studies using the lactulose breath test. This latter test probably rather measures intra-individual changes in OCTT or differences between cohorts. A developmental change in biliary function appears to be present, with bile acids concentration reaching adult values around the age

of 4 years. Pancreatic function appears to be sufficient in the large majority of healthy newborns, independent of gestational age. Intestinal pH has only been studied in a cohort of older children. Adult values were found in this cohort; therefore, possible age-related changes remain to be elucidated. For a large proportion of drugs, there seems to be a developmental pattern in CYP3A, which is the most important drug-metabolizing enzyme in the intestines. CYP3A protein and activity levels were found to increase with age. These *in vitro* data are in line with higher oral bioavailability of midazolam in premature children compared with adults. Evidence on possible age-related effect on MDR1 activity is contradictory and not elusive yet.

EXPERT OPINION

Information gaps

In summary, the main information gaps on the ontogeny of GI processes governing oral drug absorption have not yet been bridged. We need more knowledge on intestinal transit time, intestinal pH and the ontogeny of intestinal drug-metabolizing enzymes and drug transporter proteins. The ultimate goal of research efforts in this field should be to predict more precisely the oral disposition of drugs in children across the pediatric age range. Below we describe some research approaches, both *in vitro* and *in vivo*, which are promising for future research to provide a better understanding of oral drug absorption in children.

In vitro drug dissolution/solubility model (TIM)

The Dutch Institute of Innovative Research has developed the TNO Gastro-Intestinal Tract model (TIM), a computer-controlled dynamic system that mimics the physiological human conditions in stomach and intestines [90, 91]. Parameters such as pH, temperature, peristaltic movements, transit time, secretion of digestion enzymes, bile and pancreatic juices can be adjusted. Intraluminal processing of drug dosage forms, including transit, release and dissolution, can be simulated [92]. Removal of dissolved drug molecules from the intestinal compartments allows assessing the fraction of drug potentially available for small intestinal absorption [91]. This model has been extensively validated to simulate these processes in adults. It appears an interesting approach to test oral drug absorption in conditions with typical age-related physiological characteristics. Especially, the additional impact of existing oral formulations frequently given to children can be studied. It may also help to study the effect of drug manipulations to enhance drug ingestion by children (e.g., dissolving tablets in apple juice, apple sauce, 'hiding' in regular food, crushing). Representative drugs of the different BCS classes can also be studied systematically for dissolution and solubility.

In vitro drug metabolism and transporter studies

The extensive studies on *in vitro* hepatic drug metabolism, for example, by the group of Hines and colleagues could serve as an example [93]. Similarly, the ontogeny of drug-metabolizing enzymes and transporters should be studied in intestinal samples from the different parts of the intestine and from children across the pediatric age span. New methods are quickly becoming available, to study not only drug transporter expression (mRNA) but also protein content, using sensitive LC-MS-MS methods.

Modeling and simulation: PB-PK models and population PK

The available data on age-related changes in relevant GI processes as well as possibly those from the TIM simulations can be incorporated in population-based pharmacokinetics (PB-PK) software programs such as Simcyp®, PKsim® or GastroPlus®. These programs can then simulate fate of drugs given to children of different ages and provide guidance for age-appropriate dosing.

At this time, the usefulness of these programs is still hampered by the relative lack of physiological data across all age groups. Moreover, validation of the model is also still limited as we have little pharmacokinetic data to validate the model; especially in the neonatal and infant age groups, data are scarce [94]. It is to be expected that increasing use of these programs will generate sufficient data to further validate the models.

Mechanism-based approach for in vivo studies

Another approach to learn more about the ontogeny of specific (intestinal) drug-metabolizing enzyme and/or transporter pathways is a mechanism-based one [95]. The pharmacokinetics of drugs that represent a single pathway, studied in children of all ages, may provide valuable information on the ontogeny of that specific pathway. For example, determination of the plasma clearance of midazolam is a validated and widely used method to study interindividual variation in CYP3A activity in both adults and children [96].

To elucidate age-related changes in intestinal enzymes/transporters, independent of hepatic activity, we will need both oral and intravenous pharmacokinetic data, preferably from the same patients. At this time, these data are scarce in children, even for CYP3A/midazolam. Full PK studies to determine bioavailability for a probe drug using a multi-day cross-over design are hardly feasible in children for ethical and practical reasons. As a major reason, children will not benefit from the drug but rather will experience the drug effect and risk adverse events and have significant burden. Alternatively, a stable-labeled isotope or a (very weak) radioactive-labeled microdose can be used [97, 98]. In both, a labeled probe drug is added to an intravenous therapeutic dose. Parent compound and metabolites can, therefore, be traced in serum and urine. This enables simultaneous determination of the pharmacokinetics of therapeutic IV and the labeled

oral dose. It eliminates the risk of therapeutic effect/toxicity as the child already receives the drug for clinical reasons. A prerequisite for the use of microdosing in this context is that dose linearity exists across the dosing range. For a number of drugs, dose linearity for microdosing has been established, whereas others clearly do not qualify [99, 100].

Microdosing is a relatively novel technique used in adults. The microdose (one-hundredth of the predicted pharmacologic dose or 100 µg) contains a natural occurring radioactive carbon label (carbon 14, ^{14}C), which can be detected with highly sensitive methods as accelerator mass spectometry (AMS) [99]. Developmental changes in intestinal drug-metabolizing enzymes can be delineated by investigating multiple age groups. Microdosing has been used once in preterm infants in a small pharmacokinetic study of ursodiol in the US [101].

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4 Human ontogeny of drug transporters: review and recommendations of the pediatric transporter working group

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Clinical Pharmacology and Therapeutics 2015, Sep;98(3):266-87

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ABSTRACT

The critical importance of membrane-bound transporters in pharmacotherapy is widely recognized, but little is known about drug transporter activity in children. In this white paper, the Pediatric Transporter Working Group presents a systematic review of the ontogeny of clinically relevant membrane transporters (e.g., SLC, ABC superfamilies) in intestine, liver, and kidney. Different developmental patterns for individual transporters emerge, but much remains unknown. Recommendations to increase our understanding of membrane transporters in pediatric pharmacotherapy are presented.

OVERVIEW OF TRANSPORTERS IN PEDIATRICS

Transporters are membrane-bound proteins that are present in many tissues throughout the body, notably in the apical and basolateral membranes of organs involved in absorption and excretion such as the gastrointestinal tract, liver, and kidney (Figure 1).[1] The biological role of transporters is to facilitate movement of important compounds across membranes; in addition to their physiological substrates, many transporters also have the capacity to transport drugs. It is now clear that drug transporters are critical determinants of tissue and cellular drug disposition, not only for the organs noted above, but also for sanctuary sites such as the brain.[2] Moreover, the sodium taurocholate cotransporting polypeptide (NTCP), a hepatic bile acid transporter with affinity for some drug substrates, can act as a viral entry receptor for the hepatitis B virus.[3] A considerable body of *in vitro* and animal work, and a growing body of *in vivo* adult data, have demonstrated that variations in the activity of drug transporters—whether on the basis of genetic differences, drug–drug interactions, or environmental influences such as diet—impact the disposition of drugs and may influence drug efficacy and/or safety.[2, 4] One important example is the statins, which are among the most commonly used medications in developed countries. It is clear that variations in transporter activity (e.g., OATP1B1) are key clinical determinants of statin-related myopathy. OATP1B1 activity can be modulated by polymorphisms in the *SLCO1B1* gene. In fact, this has led to changes in the product label by drug regulatory agencies with respect to the maximum dosages of some statins in specific ethnic populations.[5, 6] This point is further highlighted by the Clinical Pharmacogenetics Implementation Consortium Guidelines, which recommend the use of haplotypes for dosing decisions of simvastatin.[7] While there are a growing number of examples of the importance of drug transporters in adult medicine, there has been relatively little work in this area with respect to children, or the potential impact of ontogeny of transport function on clinically relevant outcomes.

Recent data suggest that variability in drug transporter activity may be critically important for safe and effective antileukemia drug therapy in children. A high-dose methotrexate study performed in 1,883 acute lymphoblastic leukemia patients enrolled in a multicenter Children's Oncology Group clinical trial revealed a relationship between multiple single nucleotide polymorphism (SNP) variants in the OATP1B1 gene *SLCO1B1* and high-dose methotrexate toxicity.[8] The study demonstrated that methotrexate clearance was lower in 1,279 patients carrying one of several loss-of-function SNP variants of *SLCO1B1*. Significant correlations also were established between lower clearance rates after high-dose methotrexate administration in older children, girls, and in patients receiving delayed infusion of the drug.[8] Lower doses, increased hydration, and/or altered urine alkalinization were recommended, although the extent to which

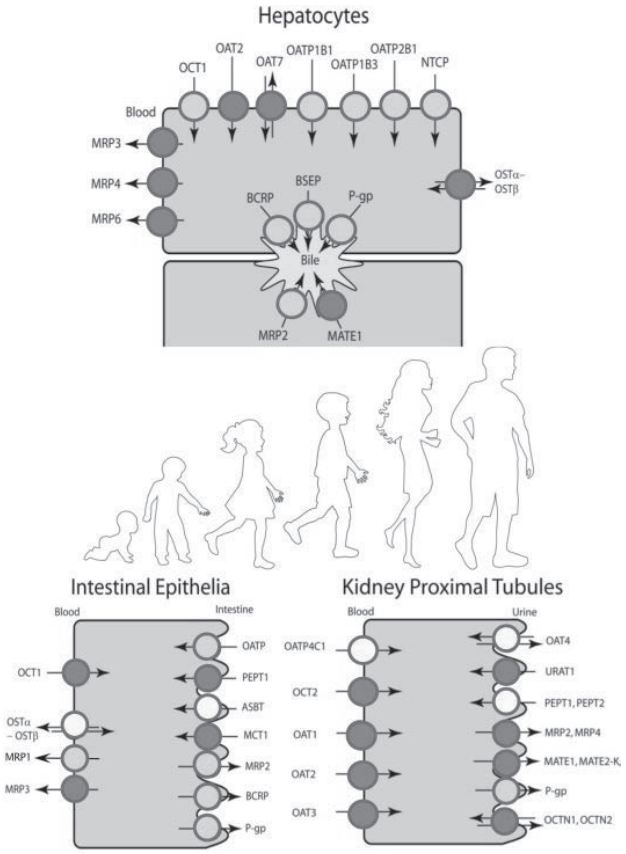

Figure 1. Human Transport Proteins for Drugs and Endogenous Substances.
Schemes depict localization of transporters [protein (*gene*) nomenclature] on the apical (luminal) and basolateral membrane of human intestinal epithelia, hepatocytes, and kidney proximal tubule cells. Developmental changes have been reported for some human transporters (green circles), but no information, or only limited data, are available for other transporters (purple circles). Yellow circles depict other drug and/or endogenous substrate transporters that were not included in the present literature search. Transporters recommended for evaluation in the 2012 FDA Draft Drug Interaction Guidance include: MDR1 P-glycoprotein (P-gp; *ABCB1*), breast cancer resistance protein (BCRP; *ABCG2*); two members of the organic anion transporting polypeptide (OATP) family (OATP1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*)); two members of the organic anion transporter (OAT) family (OAT1 (*SLC22A6*), OAT3 (*SLC22A8*)); and organic cation transporter 2 (OCT2 (*SLC22A2*)). Transporters proposed for prospective investigation in drug development include: multidrug and toxin extrusion protein 1 and 2 (MATE1 (*SLC47A1*) and MATE2-K (*SLC47A2*)). Transporters recommended for retrospective inhibition studies based on preclinical and clinical observations include: multidrug resistance-associated protein 2 (MRP2 (*ABCC2*)) and bile salt export pump (BSEP (*ABCB11*)). Other transporters that are of importance include: peptide transporter 1 and 2 (PEPT1 (*SLC15A1*) and PEPT2 (*SLC15A2*)); ileal apical sodium/bile acid cotransporter (ASBT (*SLC10A2*)); monocarboxylic acid transporter 1 (MCT1 (*SLC16A1*)); OCT1 (*SLC22A1*); heteromeric organic solute transporter (OSTα–OSTβ); sodium/taurocholate cotransporting polypeptide (NTCP (*SLC10A1*)); OATP2B1 (*SLCO2B1*); OAT2 (*SLC22A7*); OAT7 (*SLC22A9*); MRP3 (*ABCC3*); MRP4 (*ABCC4*); MRP6 (*ABCC6*); OAT4 (*SLC22A11*); urate transporter 1 (URAT1 (*SCL22A12*)); organic cation/ergothioneine transporter 1 and 2 (OCTN1 (*SLC22A4*)) and OCTN2 (*SLC22A5*)); OATP4C1 (*SLCO4C1*); and OAT3 (*SLC22A8*). Adapted from Giacomini et al.[1] and Zamek-Gliszczynski et al.[13]

these adjustments would alleviate methotrexate toxicity was not clear. The impact of these *SLCO1B1* variants on the clearance of other drug substrates in children remains to be determined. Polymorphisms in the MRP2 gene *ABCC2* also have been associated with variability in methotrexate pharmacokinetics and an increased risk for methotrexate toxicity including leukopenia, thrombocytopenia, anemia, oral mucositis, and vomiting in children with acute lymphoblastic leukemia.[9, 10]

The clinical relevance of transporter genetic variability and pediatric drug therapy extends beyond childhood leukemia. As an example, combined polymorphisms in *ABCC2* and the *UGT1A9* and *UGT2B7* genes can be important predictors of interindividual variability in mycophenolic acid exposure, and have been associated with higher area under the curve (AUC) in pediatric kidney transplant recipients.[11] Lower morphine clearance was reported in children with defective OCT1 variants undergoing outpatient adenotonsillectomy. These findings are consistent with the role of OCT1 in the hepatic uptake of morphine.[12] Racial differences in the allelic frequency of these variants may explain, in part, the higher incidence of morphine-related adverse effects in Caucasian compared with African-American children. Examples continue to emerge demonstrating the importance of transport proteins in determining drug efficacy and toxicity in the pediatric population.

METHODS TO STUDY DRUG TRANSPORTERS IN PEDIATRICS

Researchers have used primarily two approaches to characterize the ontogeny of transporters in the intestines, liver, and kidneys. The first approach is the quantification of transporter mRNA or protein expression in surgical or postmortem samples from humans at different ages. Typically, these studies are small in number and are not able to control for factors such as race/ethnicity, sex, environmental exposures, comorbidities, and other potentially important variables due to the limited sample size. The second approach is the assessment of transporter mRNA and/or protein levels, and to a lesser extent functional changes, in animal models such as rats and mice at various prenatal and postnatal ages.

Transport activity has been related to protein expression levels in adult tissue samples assuming that the transporter is localized predominantly in the plasma membrane, and that there is a correlation between the protein expression level and transporter function. Given that transporter expression at the mRNA and protein level do not always correlate well, it becomes important to understand transporter ontogeny at the protein level. Protein expression levels of membrane transporters have been estimated traditionally by western blot analysis using highly sensitive and specific, antibody-based methods. However, mass spectrometry-based targeted proteomics is being used increasingly to provide a quantitative assessment of protein expression levels.

ONTOGENY OF TRANSPORTERS

Key transport proteins relevant to drug disposition in the intestine, liver, and kidney were selected based on recent reviews by the International Transporter Consortium (see Figure 1 for protein and gene nomenclature and localization).[1, 13] A systematic search of the PubMed and EMBASE databases was performed to identify relevant published studies of drug transporters within these three organs. The search strategies were adapted to accommodate the unique searching features of each database, including database-specific MeSH and Emtree controlled vocabulary terms. Search queries for each transporter were combined with matching queries for the gastrointestinal tract (hereafter referred to as the intestine), the liver, or the kidney. Searches were limited to the pediatric population but were not limited by date, language, or publication status (see Appendix 1 for a detailed summary of the search strategies). The literature was reviewed, by organ, to evaluate changes in transporter expression with age, and the role that individual transporters may play from a developmental biology perspective. In addition, the age at which transporters reach adult levels and children "functionally" become adults with respect to transporter activity was evaluated, where possible, for each transporter. The following sections and accompanying tables summarize the relevant pediatric transporter data recovered from the literature. Transport proteins depicted in Figure 1 were excluded from discussion for a particular organ if no pediatric data were available, or if the data were so limited that a broader discussion of the temporal or spatial expression, or activity as a function of development, was not possible. In all cases, human proteins are denoted by upper case text and rodent proteins by lower case text, with gene nomenclature in italics.

Intestine

We searched for human and animal studies exploring the developmental changes of the following intestinal transporters: MDR1 P-glycoprotein (P-gp), BCRP, MRP1, MRP2, MRP3, OATP2B1, OATP1A2, OCT1, MCT1, and PEPT1. Only original, peer-reviewed research publications that explicitly presented fetal and/or pediatric data on transporter expression and/or localization were included. The focus was on human data, but in the absence of convincing human data relevant animal data are presented, as they may provide an indication of the expected developmental changes in humans (Table 1). When reported, the intestinal localization of the protein is denoted. For the fetal studies, no precise localization patterns are available due to intestinal immaturity.

P-gp

P-gp was the intestinal transport protein for which there is the most data relevant to pediatrics. Numerous studies were evaluated to determine developmental differences in

Table 1. Ontogeny data for human intestinal transport proteins

Protein	Human/animal	Age range and number of samples	Localization	Methods used	Major results	Reference First author
P-gp BCRP MRP1	Human Postmortem	*N* = 35homogenously distributed from the 5.5th – 28th week of intrauterine development (IUD)	Small intestine, not further specified	Immunohisto-chemistry (IHC)	A positive reaction for P-gp was not present in the early stages of gestation. From the 12th week of IUD onwards, P-gp was clearly present on the epithelial membrane. P-gp was localized on the apical membrane of enterocytes. BCRP was detected in both enterocyte membranes and in the cytoplasm of the epithelium at all gestational ages studied. MRP1 showed very weak staining around the 6th week of gestation. The staining of nuclei in enterocytes began to occur later and up to the 9th week of IUD, there was irregular localization of positive nuclei. Cell staining shifted to the apex of villi with the development of simple columnar epithelium.	[21] Konieczna
P-gp MRP2 OATP2B1	Human Surgical	Surgical small bowel samples (neonates *n* = 15, infants *n* = 3, adults *n* = 14)	Ileum and jejunum	mRNA (RT-PCR)	Neonatal intestinal expression of P-gp was comparable to adult. Intestinal OATP2B1 expression in neonates was significantly higher than in adults. Neonatal intestinal MRP2 mRNA expression was comparable to adults.	[20] Mooij
P-gp	Human Liver transplant recipients	*N* = 206 median age (range), 1.27 years (62 days–18.9 years)	Jejunum from part of the Roux-en Y limb for biliary reconstruction	mRNA (RT-PCR)	Intestinal P-gp mRNA reached adult levels shortly after birth. Large interindividual variability in intestinal P-gp expression was observed across all ages.	[14] Mizuno
P-gp	Human Healthy	*N* = 59: aged 1 month to 1 year (n = 19); 1 to 6 years (n = 16); patients > 6 years of age (*n* = 24).	Duodenum	mRNA (RT-PCR)	P-gp mRNA was detected in all the samples. Expression was highly variable between samples, with the P-gp/villin ratio ranging from 0.005 to 4, with nine patients having a ratio >1.3. No significant relation was found between P-gp mRNA expression and age ($p = 0.195$). 18S rRNA and villin mRNA levels were not different among the three groups.	[19] Fakhoury

Table 1. Ontogeny data for human intestinal transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Localization	Methods used	Major results	Reference First author
P-gp	Human Postmortem	*N* = 3 Prenatal: 1 fetus 15 wks and 1 fetus 27 wks Postnatal: 1 term 42 wks	Duodenum	mRNA (RT-PCR)	The P-gp:actin ratio increased from 0.00529 (15 wks) and 0.0335 (27 wks) to 0.0153 at term.	[16] Fakhoury
P-gp	Human Postmortem	Fetus, *n* = 8 Neonatal, *n* = 4 Young adults (15–38 yrs), *n* = 8 Middle age (45–65 y), *n* = 8 Elderly (67–85 y), *n* = 8	Small and large intestine	mRNA (RT-PCR)	P-gp expression was relatively low in small and large intestine from fetus to elderly with the exception of the small intestine in the young group.	[17] Miki
P-gp	Human Postmortem	*N* = 11 Prenatal: Fetal 7–20 wks, *n* = 9 Postnatal: Newborn preterm 25 wks gestation 4 month old, *n* = 1 Adult, *n* = 1	Not specified	IHCmRNA (RNAse protection assay)	In five fetal samples (after 11, 13, 14, 16, and 20 wks of gestation) no or only weak staining was detected. Strong heterogeneous staining was observed in the stomach and the colon of a 7-day-old premature born neonate, but evaluation of all samples was difficult as strong background staining of goblet cells interfered. No or very weak expression of P-gp mRNA was seen in intestinal specimens after 11, 13, 14 weeks of gestation, whereas a clear P-gp mRNA expression was observed in fetal intestinal specimens at a later stage of fetal development (16–20 weeks). Tissue distribution differed between fetus and adult.	[18] van Kalken
P-gp	Human Celiac disease: treated and untreated; Healthy controls	*N* = 25 Untreated: 6.7 (3.9–13.9) yrs Treated 6.7 (4.9–12.7) yrs Controls 8 (1.7–13) yrs	Duodenal biopsies	mRNA (RT-PCR)	The mRNA expression of P-gp was increased in children with treated celiac disease compared to controls.	[86][a] Vannay

Table 1. Ontogeny data for human intestinal transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Localization	Methods used	Major results	Reference First author
P-gp	Human Control and Crohn's disease biopsies	Controls $n = 19$ Crohn's disease $n = 19$ Postnatal age 1 month-17 yrs	Duodenum	mRNA (RT-PCR)	P-gp mRNA was detected in all of the samples and highly variable between samples, with P-gp/Villin ratio ranging from 0.009 to 1.421 in the control group (266-fold variation) and from 0.047 to 4.5 in the Crohn's disease group (121-fold variation). P-gp expression was significantly higher in the Crohn's disease group than in the control group ($P<0.0001$).	[15] Fakhoury
Mrp3	Rabbit Postmortem	Newborn: 5–8 day Weanling: 25–28 day Adult: >60 day	Distal ileum and colon	mRNA (RT-PCR) and western blotting	Mrp3 RNA and protein expression in colon highest in adult followed by weanling and then newborn animal. In adult animals, more Mrp3 RNA was observed in the colon than in the ileum; protein was almost unmeasurable in the ileum.	[22] Weihrauch
Oct1	Mouse	Embryos (embryonic days 11.5–16.5), Neonatal mice (day 5)	Small intestine and colon	*In situ* hybridization, Northern blot analysis	No obvious Oct1 transcripts were detected in fetal and newborn small intestine or colon (day embryonic 14 - day 5 postpartum).	[48] Pavlova
PepT1	Rat	Fetuses (embryonic day 18) Term neonates (21 days of gestation; 12h postnatal) Weaning (21 days old postnatal) Adult rat (3–4 months)	Duodenum: proximal small intestine immediately distal to the pyloric antrum	IHC	Duodenal PepT1 expression varies at different stages of pre- and postnatal development. At embryonic day 18, there is immunostaining for PepT1 at the epithelial brush border, but less prominent than in the adult and more variable from cell to cell. Directly after birth there is pronounced PepT1 immunoreactivity, consistently in the brush border but surprisingly also elsewhere in the epithelium. At day 21 postnatal (weaning) the distribution of PepT1 protein is similar to that seen in the adult: in the villus brush border.	[23] Hussain

Table 1. Ontogeny data for human intestinal transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Localization	Methods used	Major results	Reference First author
	Rat	Day 17, day 20 of gestation, day 1, 3, 5, 7, 14, 21, 24, 28, 60 and 75 (adult) after birth	Intestinal segments; duodenum, jejunum, ileum, colon	Northern blot (mRNA) and Southern blot (protein)	Rapid increase at birth in the duodenum, jejunum, and ileum, to a maximum by days 3–5, followed by a rapid decrease to 11–13% of maximal expression by day 14, and then an increase to 23–58% of maximal expression by day 24 (time of weaning). A similar pattern of expression was observed for PEPT1 protein. In the colon, significant expression was observed postpartum, but levels were undetectable from 1 week onwards.	[24] Shen
	Rat	Mothers, weanling and adult	Small intestine	mRNA (RT-PCR)	Level of intestinal PepTl mRNA was markedly increased in 10-day-old rats, and then decreased reaching adult levels by day 28 after birth.	[25] Miyamoto
	Turkey	Embryonic day 23 to birth	Not specified	mRNA (RT-PCR)	A quadratic increase ($P < 0.001$) in PepT1 mRNA abundance with age in turkey embryonic small intestinal tissue from embryonic day 23 to birth. PepT1 mRNA was barely detectable in turkey embryonic small intestine at embryonic day 23 but increased 3.2-fold prior to birth.	[26] Van
OCT3 OCTN1	No relevant human or animal ontogeny data					

expression or localization of P-gp in the intestine. Collectively samples from 55 fetuses, ranging from 5.5 to 28 weeks of gestation, and 302 additional samples across a pediatric age range, were investigated in this group of studies.[14-21] The majority of mRNA samples (upper jejunum) originated from one study in pediatric liver transplant patients, which may present confounding variation based on hepatic disorders.[14] In most studies, P-gp mRNA expression was determined; localization with immunohistochemistry was only conducted in a small number of samples. All of the studies demonstrated large interindividual variation. The overall developmental pattern of P-gp expression revealed a consistently emerging change from undetectable expression in the first trimester of fetal life to present and apparently stable P-gp expression from ~12 weeks of gestation onwards. Very limited data suggest that P-gp expression increased slowly to reach adult levels at or very shortly after birth. Interestingly, two studies reported higher intestinal P-gp expression in children with treated Crohn's and celiac disease compared to healthy controls.[15]

BCRP

The localization of BCRP staining appeared stable from 5.5 to 28 weeks of gestation in humans.[21]

MRP1

In 35 human fetal samples from 5.5 to 28 weeks of gestation, MRP1 staining was visualized in all samples using immunohistochemistry.[21] The intensity was weak during early gestation, but appeared to mature to an adult distribution pattern from 7 weeks of gestation onwards.

MRP2

Analogous to P-gp, the mRNA expression of MRP2 appeared stable from neonates to adults in ileal and jejunal surgical samples.[20]

OATP2B1

In contrast to MRP2, OATP2B1 mRNA expression was significantly higher in the samples from neonates compared to adults.[20]

Other intestinal transport proteins

Human data for MRP3 and PEPT1 appear to be missing, but animal data are available. In rabbits, Mrp3 mRNA expression was lowest in newborns and subsequently increased in weanlings until reaching the highest levels in adults.[22] The four available animal studies (three in rat; one in turkey) on PepT1 all support a similar developmental pattern of significant prenatal expression that reached maximal levels in the postnatal period, and

then decreased to adult levels from weaning onwards.[23-26] This finding seems consistent with the role of PEPT1 in the absorption of di- and tripeptides, which constitutes an important part of infant nutrition. Intestinal temporal and spatial expression data during development for OATP1A2, OCT1, and MCT1 appear to be lacking.

It is clear from the data in Table 1 that no single developmental pattern can be identified for all of the intestinal transporters. Different patterns are apparent based on the available, albeit limited human data: 1) low in the embryo and then stable from neonate to adult (e.g., P-gp, MRP2); 2) high at birth and decreasing in the first months of life (OATP2B1).

Liver

For the liver, all transport proteins designated by the International Transporter Consortium as important for disposition of drugs and endogenous substances were reviewed.[1, 13] These include the efflux transporters P-gp, BCRP, MRP2, MRP3, MRP4, MRP6, MATE1, BSEP, the uptake transporters NTCP, OATP1B1, OATP1B3, OATP2B1, OAT2, OCT1, and the bidirectional transporters OAT7 and OSTα/β. Similar to the other organs, this review on hepatic transporters exclusively focuses on human data, if available, but resorts to animal data in the absence of any relevant human information (Table 2). Similar to intestinal transporters, most published data on the ontogeny of hepatic transport proteins is available for efflux transporters, particularly P-gp.

P-gp

Hepatic P-gp was already detectable in the wall of bile canaliculi in early fetal life at 14 weeks by immunohistochemistry and at the mRNA level.[18] Although expression was low initially, it seemed to increase throughout fetal development and was considered moderate at the protein level by fetal week 19.[16, 27] mRNA expression for P-gp increased throughout childhood development. In a study with 61 liver specimens, mRNA expression levels were 20–30-fold lower in the fetal and neonatal age group compared to adults. mRNA expression, however, rapidly increased in the early months of life: in infants (1–12 months), mRNA was only 5-fold lower compared to adults, and in children and adolescents it was indistinguishable from adult expression.[20] These observations are supported by less extensive studies from others,[28] and also revealed a similarly high interindividual variability in expression in children compared to adults.[20, 29] The limited results on P-gp protein expression, however, do not corroborate the reported age-dependent P-gp mRNA expression. P-gp protein was detectable in samples as early as 1 month,[29] and relative protein expression was not significantly different in 65 liver specimens from age groups 0.3–0.7, 0.7–2, 2–5, and 5–12 years.[30] This observation may not be surprising in light of the observed lack of correlation between mRNA and protein levels for P-gp in adult liver.[31] P-gp protein expression quantified by liquid

Table 2. Ontogeny data for human hepatic transport proteins

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
P-gp	Human Postmortem	*N* = 35 Fetal: 14–28 weeks of gestation	Immunohisto-chemistry (IHC), mRNA (RNAse protection assay)	Staining for P-gp in bile canaliculi already at 14 weeks of gestation. No apparent difference in staining intensity in different fetal stages. Presence of mRNA confirmed in one specimen (16 weeks).	[18] Van Kalken
P-gp MRP2	Human Postmortem	*N* not determined Fetal: 14 and 19 weeks of gestation	IHC	Immunopositivity of P-gp and MRP2 was localized to the canalicular membrane of differentiating and mature hepatocytes. MPR2 was detectable in liver of 14-week-old fetuses, but had strong expression at 19 weeks. P-gp was detectable at very low levels at 14 weeks, and was moderately expressed at 19 weeks.	[27] Cizkova
P-gp	Human Postmortem	*N* = 3 Fetal: 15, 27 and 42 weeks of gestation	mRNA (RT-PCR)	Expression of P-gp was detectable in the 15 week samples, and gradually increased with gestational age until 42 weeks.	[16] Fakhoury
P-gp MRP2 OATP1B1 OATP1B3	Human Postmortem	*N* = 72 Fetal: *n* = 9 (8–23 wks) Pediatric: *n* = 52 (0–17 yrs) Adult: *n* = 11	mRNA (RT-PCR)	mRNA for P-gp expression in fetal and neonatal groups was 20–30 fold lower than in adults. Expression in infants was slightly lower than in adults, with no difference between children 1–7 years and adults. MRP2 mRNA was 30-fold lower in fetal, 200-fold lower in neonatal, and 100-fold lower in infant liver compared to adults. OATP1B1 expression was 20-fold lower in fetal, 500-fold lower in neonatal, and 90-fold lower in infant liver compared to adults. OATP1B3 mRNA was 30-fold lower in fetal, 600-fold lower in neonatal and 100-fold lower in infant liver compared to adults.	[20] Mooji

Table 2. Ontogeny data for human hepatic transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
P-gp BCRP BSEP MRP2 MRP3 MRP4 MRP6 MATE1 OATP1B1 OATP1B3 OAT2 OCT1	Human	Perinatal (0–30 days): *N* = 6 0–4 years: *N* = 8 >7 years: *N* = 6	mRNA	Gradual increase in mRNA expression from neonates to older children was observed for P-gp, MRP2, MRP3, MRP6, NTCP, OAT2, OATP1B1, OATP1B3, OCT1, BCRP, BSEP, and MATE1. MRP4 expression was high in neonates, but lower in older children.	[28] Klaassen
P-gp	Human Postmortem	*N* = 12 1–6 months	mRNA (Northern) Protein (Western)	P-gp expression is regulated developmentally. P-gp mRNA and protein were present at 1 month postnatal.	[29] Schuetz
P-gp MRP2	Human Living and Postmortem	*N* = 65 0.3–0.7 yrs: *n* = 6 0.7–2 yrs: *n* = 13 2–5 yrs: *n* = 13 5–12 yrs: *n* = 33	Protein (Western)	For P-gp, relative protein expression was not significantly different among the studied age groups. For MRP2, protein levels were significantly lower in infants under 8 months compared to older children.	[30] Tang
P-gp OATP1B1 OATP1B3	Human Living	*N* = 64 7–70 yrs	Protein (LC-MS/MS)	P-gp, OATP1B1 and OATP1B3 protein expression was not associated with age in the studied age range.	[32] Prasad

Table 2. Ontogeny data for human hepatic transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
P-gp BCRP MRP2 MRP3 MRP4 BSEP NTCP OATP1B1 OATP1B3	Human Postmortem	*N* = 20 Fetal: 18–23 weeks of gestation (*n* = 8) Adult (*n* = 12)	mRNA (RT-PCR)	All investigated transporters except for MRP4 were expressed, albeit at lower mRNA levels, in fetal hepatocytes compared to hepatocytes from adults.	[33] Sharma
BCRP	Human	*N* = 35 5.5 to 28 weeks of gestational age	IHC	BCRP was detected in all stages of intrauterine development under study.	[21] Konieczna
BCRP	Human Living	*N* = 50 7–70 yrs	Protein (LC-MS/MS)	BCRP protein expression was not associated with age in the studied age range.	[35] Prasad
BCRP MRP3 BSEP NTCP OATP1B1 OATP1B3	Human	*N* = 10 Neonates (*n* = 5) Adults (*n* = 5)	Protein (Western)	The relative protein expression was similar between neonates and adults for BCRP, MRP3, BSEP, NTCP, OATP1B1 and OATP1B3.	[34] Yanni
MRP2	Human Living	*N* = 51 7–63 yrs	Protein (LC-MS/MS)	MRP2 protein expression was not associated with age in the studied age range.	[37] Deo
MRP2 BSEP NTCP	Human Living and Postmortem	*N* = 15 Fetal: 14–20 weeks of gestation (*n* = 10) Adult (*n* = 5)	mRNA (RT-PCR)	NTCP mRNA was 1.8% of adult expression in fetal liver. BSEP and MRP2 were 30–50% of adult expression.	[36] Chen

Table 2. Ontogeny data for human hepatic transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
Ntcp	Rat	Fetal: 18, 20, 21 days of gestation Neonatal: day 1 Suckling: week 1, 2, 3, 4	mRNA (Northern) Protein (Western)	mRNA reached adult levels at day 7, but transport activity was only 25% of the adult value. Immunoreactive protein expression was near adult levels at birth, but molecular mass was substantially lower until 4 weeks of age due to incomplete glycosylation.	[38] Hardikar
OATP1B1 OATP1B3	Human Living and Postmortem	*N* = 78 0–0.7 yrs: *n* = 35 0.7–3 yrs: *n* = 8 3–6 yrs: *n* = 13 6–12 yrs: *n* = 22	Protein (Western)	OATP1B1 has low expression from birth until 6 years with increased expression thereafter. OATP1B3 exhibited high expression at birth, which declined over the first months of life, and then increased again in the pre-adolescent period.	[39] Thomson
OATP1B1 OATP1B3 OAT2 OCT1	Human	Hepatocytes from pediatric and adult donors	mRNA Transport function	No difference in gene expression for all four transporters. Uptake activity of OATP1B1 and OCT1 was significantly lower in pediatric relative to adult hepatocytes. OATP1B3 was significantly higher in pediatric hepatocytes.	[40] Hayashi
Mrp3	Rat	Embryonic days 15.5, 17, 19, 20, 22 and newborn (22 days gestational age)	mRNA (RT-PCR)	Mrp3 expression progressively increased from ~10% to over 30% from day 15 to day 20 of gestation, and increased to near 90% of maternal expression at day 21 and after birth.	[41] St-Pierre
Mrp4	Mouse	Postnatal days −2, 0, 1, 3, 5, 10, 15, 20, 30, 45	mRNA (bDNA assay)	mRNA expression for Mrp4 was consistent from 2 days before birth to 45 days of age, except for a moderate increase on day 1 of age.	[42] Cui
Mrp4	Mouse	Postnatal days −2, 0, 5, 10, 15, 23, 30, 35, 40, 45	mRNA (bDNA assay)	Mrp4 mRNA expression was maximal at birth and decreased ~70% by 2 weeks of age, but was relatively constant thereafter.	[43] Maher
Mrp6	Rat	Embryonic days 16 and 20; Postnatal days 0, 5, 12, 20, 29 and adult	mRNA (RT-PCR)	Mrp6 mRNA expression was detectable at low levels by embryonic day 16, increased to 40% of adult on postnatal day 0, and reached adult levels by day 29.	[44] Gao
Mate1	Mouse	Embryonic day 7.5 and adult	mRNA (RT-PCR)	Mate1 mRNA was barely detectable in embryos and was 200-fold less than that expressed in adult liver.	[45] Lee
OSTα/β	Human Living	*N* = 8 6–17 months	mRNA (RT-PCR) IHC	OSTα and OSTβ were expressed in the specimens investigated in this study.	[46] Chen
Ostα/β	Mouse	Postnatal days −2, 0, 1, 3, 5, 10, 15, 20, 25, 30, 45, 60	mRNA (bDNA assay)	Ostα mRNA was expressed at low levels throughout development. Ostβ mRNA expression increased rapidly after birth with peak expression at day 1 (4.5-fold increase from prenatal), and decreased to adult levels between days 5 and 10.	[47] Cui

chromatography/tandem mass spectrometry (LC-MS/MS) was not associated with age in 64 liver specimens in the age range of 7–70 years.[32] Thus, further studies are needed to clarify whether the age-dependent differences in mRNA expression translate into differences at the protein and ultimately the functional level for P-gp in the liver.

BCRP

BCRP was detectable by immunohistochemistry in fetal liver specimens from 5.5–28 weeks[21] and mRNA expression increased from 18–22 week fetal samples to adults.[33] A comparison of relative protein expression between five neonates and five adult livers by western blot analysis indicated no detectable difference,[34] although mRNA expression seemed to increase between neonates and older children.[28] In 50 livers from age 7–70 years, BCRP protein expression was correlated neither with age nor with mRNA expression.[35] These results together suggest that hepatic BCRP is expressed early during human development and does not undergo relevant developmental changes after term birth.

MRP2

Similar to P-gp, MRP2 has been detected by immunohistochemistry in the bile canalicular membranes of 14-week-old fetuses, with a tendency for higher expression levels in older fetal liver[27] and adults.[33, 36] The differences in age-dependent expression seem to be similar to P-gp in fetal liver (30-fold lower mRNA expression), but were substantially more pronounced in neonates (200-fold lower mRNA expression) and infants (1–12 months) (100-fold lower mRNA expression) compared to adults.[20, 28] These substantial differences at the transcriptional level seem to translate into developmental differences in MRP2 protein expression: MRP2 protein levels determined by western blot were significantly lower in liver specimens from children younger than 8 months ($n = 24$) compared to older children up to 12 years ($n = 59$).[30] Later in childhood development, however, MRP2 protein expression assessed by mass spectrometry was independent of age in 51 liver specimens in the age range of 7–63 years.[37]

BSEP

At gestational weeks 14–20, BSEP was detectable by immunohistochemistry (36), and mRNA expression increased from neonates to older children[28] and adults.[33] Functional studies in isolated sandwich-cultured fetal and adult hepatocytes suggest that the biliary excretion index for taurocholate is substantially higher in adults compared to fetal hepatocytes. This higher functional activity for the BSEP substrate taurocholate could be explained by a higher expression level of BSEP in adult cells, assuming that there is a correlation between BSEP mRNA and protein expression.[33]

NTCP

NTCP was detectable by immunohistochemistry in fetal liver specimens at 14–20 weeks of gestation,[36] and mRNA expression was reduced substantially to 4% of adult values in fetal livers of 18–22 weeks of gestational age.[33] At the protein level postpartum, NTCP expression in neonates was comparable to that in adults.[34] Thus, there also seems to be no developmental maturation of NTCP expression after birth in humans. Rodent data suggest, however, that acquisition of functional transporter activity lags behind the developmental trajectories of mRNA and immunoreactive protein, and is not present until glycosylation is mature.[38]

OATPs

mRNA for the OATP isoforms OATP1B1, OATP1B3, and OATP2B1 was detectable in fetal hepatocytes by gestational weeks 18–23, and was significantly higher in adults compared to fetal livers for OATP1B1 and OATP2B1.[33] In a limited number of neonatal and adult liver specimens (n = 5 each), no relevant difference was observed in OATP1B1 or OATP1B3 expression as determined by western blot analysis.[34] mRNA expression in 45 liver specimens, however, was found to be highly age-dependent. For OATP1B1, mRNA expression was 20-fold lower in fetal liver, 500-fold lower in neonates, and 90-fold lower in infants compared to adults.[20] For OATP1B3, mRNA expression was 30-fold lower in fetuses, 600-fold lower in neonates, and 100-fold lower in infants (1–12 months) than in adults.[20] These data are supported by western blot analyses based on relative protein quantification in 78 liver samples that suggest a low expression from birth to age 6 years with increased expression thereafter for OATP1B1, and high expression for OATP1B3 at birth, which declines over the first month of life, and then rises again by age 6 years.[39] In 64 livers from age 7–70 years, relative protein expression of OATP1B1, OATP1B3, and OATP2B1 as assessed by mass spectrometry did not correlate with age.[32]

OCT1

There are only very limited human data on the ontogeny of hepatic OCT1. In human hepatocytes from pediatric and adult livers, there was no significant difference in the mRNA expression of OCT1, but OCT1-mediated transport seemed lower in pediatric compared to adult hepatocytes.[40]

Other liver transport proteins

For MRP3, MRP4, MRP6, MATE1, OAT2, OAT7, and OSTα/β, there are no or only very limited data available on the human ontogeny of these transporters. Thus, observations from rodent species also are provided in the following section, although there is so far no indication that rodent protein, and especially mRNA expression profiles, are in any way predictive of human transporter ontogeny.

MRP3

In humans, MRP3 mRNA expression was significantly lower in fetal hepatocytes by gestational weeks 18–23 compared to adults.[33] Similarly, Mrp3 in fetal rat liver progressively increased from about 10% to over 30% of the maternal mRNA levels from day 15 of gestation to day 20, and increased to near 90% of the maternal level at day 21 and after birth.[41] This is consistent with increased mRNA expression observed from neonates to older children and adults in a small set of human specimens (perinatal $n = 6$, children $n = 8$, adult $n = 6$).[28]

MRP4

In humans, mRNA expression for MRP4 did not show any significant differences in fetal hepatocytes by gestational week 18–23 compared to adults[33] or when comparing neonates to older children and adults.[28] These observations are supported by mRNA expression in mice.[42, 43]

MRP6

Similar to MRP3, MRP6 mRNA expression increased in humans from neonates to older children and adults in a small set of subjects.[28] In rat liver, Mrp6 mRNA expression was detectable on embryonic day 16 at 5% of adult levels, and increased to 40% at birth, but did not reach adult levels until postnatal day 29.[44]

MATE1

mRNA expression for MATE1 increased in humans from neonates to older children and adults in a very small set of subjects.[28] Mate1 mRNA expression was absent on embryonic day 7.5 in mice.[45]

OAT2

Similar to MRP3 and MRP6, OAT2 mRNA expression increased from neonates to older children and adults in a small set of human subjects.[28]

OAT7

No information could be found on the ontogeny of OAT7 in humans or rodents.

OSTα/β

mRNA expression was detectable for OSTα and OSTβ in pediatric liver with an age around 1 year.[46] Although Ostα mRNA is expressed at low levels in liver throughout development from day –2 to day 45 in mice, Ostβ mRNA markedly increased to 4.5-fold of prenatal levels with a peak around 1 day after birth.[47]

Overall, there are limited data available on the human ontogeny of hepatic transport proteins. The emerging picture, however, suggests that there may be substantial differences between transporters in the time course of development and expression. Some transport proteins such as P-gp, BCRP, and NTCP are expressed early in childhood development, while others such as OATP1B1, BSEP, and MRP2 seem to exhibit delayed maturation and reduced expression levels compared to adults during at least the first months of life. In general, the differences seem to be absent between older children and adults. This conclusion is supported by the notion that localization of the canalicular transporters (BSEP, P-gp, MRP2) in pediatric liver (6–17 months) had reached a similar level and pattern as adult liver, indicating that the pediatric liver around 1 year of age has obtained a mature canalicular structure.[46]

Kidney

The drug transporters generally considered in the context of kidney development were: P-gp, BCRP, OAT1, OAT3, OCT2, and MATEs. Data on MRP2, MRP4, OATPs, OAT2, OCT1, OCTNs, and URAT1 (Rst) also were evaluated when presented in the literature regarding the aforementioned transporters. Currently, there is relatively little information regarding drug transporter expression, at either the mRNA or protein level, or function in the human developing kidney. Given the paucity of human data, the studies described in Table 3 and Appendix 2 also include the considerable data on the ontogeny of drug transporters in the developing rodent (rat, mouse) kidney. In a limited number of instances, there are also data on postnatal developmental function of particular transporters, for instance, in a knockout mouse model or a study on the developmental clearance of a drug (digoxin) or probe substrate (p-aminohippurate, PAH) in rodents. Although there are differences in individual transporter expression patterns (e.g., P-gp, Bcrp, Mrp2, Mrp4, Mate1, Oat1, Oat3, Oct2, Octn1, Octn2, Urat1), these rodent studies generally indicate low expression of various transporters in the late stages of kidney development, followed by a rapid rise in expression after birth, and a further increase in expression (and function) during postnatal maturation.[48-50] (Please see Table 3 and Appendix 2 for additional rodent information.)

P-gp

In humans, P-gp transcript and protein levels have been analyzed in fetal and adult kidney.[17, 18] P-gp expression is detectable as early as 11 weeks of gestation.[18] In the fetal kidney, reverse-transcription polymerase chain reaction (RT-PCR) of tissue obtained by laser capture microdissection revealed transcripts in the renal tubule.[17]

It is important to note that developmental biology studies suggest that there may be some significant differences in postnatal nephron development and maturation between mice, rats, and humans, as well as between sexes. Of note, recent mRNA data

Table 3. Ontogeny data for human kidney transport proteins

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
P-gp	Human	*N* ≥12 Fetal (weeks 7, 11, 13, 15, 16, 20, 25, 28, 38) to Adult	mRNA Protein (IHC)	P-gp was expressed as early as week 11 of gestation. Staining was observed in the developing renal tubule.	[18] van Kalken
P-gp	Human	*N* = 8 Fetal (mean: 18 weeks) *N* = 4 Neonatal (1–24 days after birth) *N* = 24 Adult	mRNA (PCR, RT-PCR of laser capture microdissected tissue)	P-gp was expressed in fetal, neonatal, young adult, middle age, and elderly kidney. Adult kidney tended to have higher expression. By RT-PCR, transcript was found in laser capture microdissected tubules.	[17] Miki
Mdr1a Mdr1b	Mouse	*N* = 56 Postnatal: 0, 7, 14, 21, 28, 45 days after birth	mRNA (RT-PCR)	Mdr1a and Mdr1b are marginally expressed in newborns and after 1 week of maturation. Mdr1b expression at day 21 was found to be higher than that seen on either day 14 or day 28.	[87][a] Pinto
Mdr1a Mdr1b Bcrp Mrp2 Mrp4 Mate1 Oat1 Oat3 Oct1 Oct2 Octn1 Octn2 Urat1	Rat Mouse	Rat: *N* = 45 Fetal: 13–21 days of gestation; Postnatal: days 1, 7, and 28 after birth Adult: 8 weeks	mRNA (micro-array)	An overall increase in the mRNA levels of various transporters was observed between the fetal, compared to the neonatal kidney. Large increases in transporter expression also were evident in the postnatal period.	[52] Sweeney

Table 3. Ontogeny data for human kidney transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
Mdr1 Bcrp Mrp1 Mrp2 Mrp3 Oatp1a4 Oat1 Oat2 Oat3 Oct1 Oct2	Rat	*N* = 80 Fetal: gestational day 21 Postnatal: 1, 4, 7, 11, 15, 18, 21, 26, and 42 days after birth	mRNA (RT-PCR)	Mrp1, Mrp3, and Oatp1a4 were most highly expressed at birth; Mrp2 and Bcrp showed constant expression during development, while Mdr1, Oct1, Oct2, Oat1, Oat2, and Oat3 expression was highest between postnatal days 11 and 26 with only limited expression at birth. There also were some differences in the expression of the transporters between males and females.	[49] de Zwart
Mate1	Mouse	*N* = 100 Fetal: Day 18 of gestation Postnatal: 0, 5, 10, 15, 23, 30, 35, 40, and 45 days after birth	mRNA (RT-PCR; bDNA)	Mate1 expression steadily increased during late prenatal and postnatal development with a gender difference becoming apparent at 30 days of age.	[88][a] Licktieg
Mate1 Mate2 Oct1 Oct2	Rat	*N* = 8 Fetal: 18 and 21 days of gestation	mRNA (RT-PCR)	Expression of Mate1, Mate2, Oct1, and Oct2 in the developing kidney significantly increased between 18 and 21 days of gestation.	[89][a] Ahmadi-moghaddam
Mrp2 Oatp1a1 Oatp3a1 Oat3 Oct1 Oct2	Mouse Rat	Fetal Adult	mRNA (microarray)	Focused analysis of previously published microarray data during kidney development revealed changes in many transporters.	[90][a] Martovetsky
OATPs	Human	Fetal and adult (ages not stated)	mRNA (RT-PCR)	OATP-B (OATP2B1) and OATP-D (OATP3A1) are expressed in the human fetal kidney.	[91][a] Tamai

Table 3. Ontogeny data for human kidney transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
Oatp1a1 Oatp1a6 Oatp3a1	Mouse	*N* = 100 Fetal: Day 18 of gestation Postnatal: 0, 5, 10, 15, 22, 30, 35, 40, and 45 days after birth	mRNA (RT-PCR; bDNA)	Oatp1a1, Oatp1a6, and Oatp3a1 display lower expression at birth compared to 6 weeks later, while other Oatps had similar levels of expression at both time points.	[92][a] Cheng
Oat1 Oat3 Mrp2 Mrp4	Rat	*N* = 63 Postnatal: 1, 2, 4, 6, 14, 21, and 28 days after birth (*N* = 5–8 mRNA; *N* = 4–6 protein)	mRNA (RT-PCR) Protein (western blot and immunohistochemistry (IHC))	Levels of Oat1 and Oat3 mRNA and protein increased significantly from postnatal days 1 to 28. Neither Mrp4 mRNA nor protein changed significantly over the same time frame. Mrp2 protein increased from postnatal days 14 to 21.	[93][a] Nomura
Oats	Rat	Engineered kidney tissue from embryonic rudiments	Protein function (functional uptake assay)	Differentiating tubules of engineered recombined embryonic kidney tissue are functionally capable of organic anion transport.	[94][a] Rosines
Oat1	Rat	Fetal: 17–20 days of gestation Postnatal: 0, 1, 2, and 6 days of age Adult: 8–10 weeks	mRNA (Northern blot, in situ hybridization) Protein (Western blot and IHC)	Oat1 mRNA is detectable at day 18 of gestation, and Oat1 protein is detectable at day 20 of gestation. The expression of both Oat1 mRNA and Oat1 protein increase markedly after birth.	[95][a] Nakajima
Oat1	Mouse	Fetal: 15–19 days of gestation Adult: 8–10 weeks	mRNA (Northern blot)	Oat1 mRNA expression by Northern blot was found at day 18 of gestation and continued to increase before birth.	[96][a] Lopez-Nieto
Oat1	Mouse	*N* = 44 Fetal: 13.5–18.5 days of gestation Postnatal: 1, 7, 21, 28 days after birth Adult: 8–10 weeks	mRNA (RT-PCR)	The expression of Oat1, which was initially observed at 15.5 days of gestation, progressively increased to adult levels.	[97][a] Parreira

Table 3. Ontogeny data for human kidney transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
Oat1 Oat3	Mouse	Cultured embryonic kidney, renal slices from knockout tissue	Protein function (functional uptake assay)	Embryonic kidney in organ culture transports antiviral drugs by Oat1 and Oat3. Uptake of fluorescent tracers in coronal slices of adult kidney from Oat1 and Oat3 knockout mice revealed functional localization of Oat1 and Oat3. Oat1 function appeared to be consistent with localization in the proximal tubule, while Oat3 function seemed to be found in both the proximal and distal tubule.	[98][a] Nagle
Oat1 Oat3	Mouse	*N* = 13 Fetal: 13–18 days of gestation Postnatal: days 1, 4, 7, 14, and 21 days after birth Adult: 8–10 weeks of age	Protein (IHC)	Oat1 was first detected on day 15 of gestation and localized to the proximal tubule of the inner cortex. Oat1 expression continued to increase, appearing in the outer cortex at 7 days after birth and by 3 weeks Oat1 distribution was comparable to that seen in adult kidneys. Oat3 was first detectable on day 14 of gestation in the distal tubule and was later localized to the S2 segment of the proximal tubule. Around the time of birth, Oat3 was localized to the S1 and S3 segments of the proximal tubule, and Oat3 expression continued to increase through postpartum day 21.	[99][a] Hwang
Oat1 Oat3	Mouse	Cultured embryonic kidney knockout tissue	Protein (functional uptake assay)	Cultured whole embryonic kidneys from Oat1 and Oat3 knockout mice were able to transport many antivirals ex vivo. Certain antivirals were found to have selective dependency on Oat1 or Oat3.	[100][a] Truong
Oat1 Oat2 Oat3	Mouse	*N* = 90 Postnatal: 0, 5, 10, 15, 20, 25, 30, 35, and 40 days after birth	mRNA (RT-PCR; bDNA)	Postnatal Oat1 and Oat2 levels of expression increased after 25 days of age, while Oat3 expression levels gradually increased from birth to 45 days of age. Overall Oat2 levels increased 30-fold, while Oat1 and Oat3 levels increased about 2-fold.	[101][a] Buist
Oat1 Oat3	Sheep	*N* = 28 Fetal: 80, 100, 120, 130, and 145 days of gestation Postnatal: 1 and 7 days after birth	mRNA (RT-PCR) Protein (IHC)	Oat1 was expressed at 80 days of gestation and was most highly expressed at 145 days of gestation; the postnatal kidney showed comparable levels of expression. Oat3 displayed a similar pattern of expression, although significant increases were evident in the postnatal kidney. Immunohistochemical analysis revealed localization of the transporters to tubular epithelia consistent with the proximal tubule.	[102][a] Wood

Table 3. Ontogeny data for human kidney transport proteins (continued)

Protein	Human/animal	Age range and number of samples	Methods used	Major results	Reference First author
Oat1 Oat3 Oct1 Oct2	Rat	Fetal: 13–18 days of gestation;cultured embryonic kidney; induced metanephric mesenchyme	mRNA (RT-PCR) Protein function (functional uptake assay)	Oat1, Oat3, and Oct2 are expressed early in kidney development, while Oct1 is expressed late in development. The ontogeny of the transporters in *ex vivo* cultures of embryonic kidney or its component tissues is comparable to that seen *in vivo*. Cultured embryonic kidneys and induced metanephric mesenchyme are functionally capable of organic anion transport.	[50] Sweet
Oat1 Oat2 Oat3 Oct1	Mouse	Fetal: 14–19 days of gestation Postnatal: 0 days of age Adult: 8–10 weeks	mRNA (Northern blot, *in situ* hybridization)	Oat1, Oat2, and Oat3 expression was seen as early as day 15 of gestation. The expression levels of the organic anion transporters continued to increase to adult levels.	[48] Pavlova

regarding the developmental expression of renal transporters in humans,[51] presented as an abstract, appears largely consistent with patterns reported in rats.[52]

In summary, information on the ontogeny of human drug transporters is scarce in the case of the kidney. A major knowledge gap exists regarding gene expression, protein abundance, and actual transporter activity in humans. This information is essential to understand how maturational changes impact the role that these transporters play in normal growth and development, and to accurately predict the impact of changes due to pathophysiological conditions on drug disposition, efficacy, and toxicity of medications. This knowledge is requisite to the development of personalized drug therapy in children.

COMPARATIVE DEVELOPMENT OF ORGAN FUNCTIONS

The majority of the data regarding transporter ontogeny has been obtained from developmental studies in rodents, although some literature does exist for larger species. The ability to extrapolate across species, namely rodents, to larger species (e.g., primates) and humans can be limited, however, by variation in the developmental timing of key anatomical, physiological, biochemical, and physicochemical events, as well as significant functional variance in isoforms. These differences arise from comparative differences in the gestational length and the timing of parturition between the different species and humans. The conventional approach is to compare developmental milestones relative to birth (prenatal vs. postnatal); however, this may not always be appropriate. For example, in contrast to humans and porcine models, newborn rodents exhibit relatively immature intestines with few villi and little evidence of crypt formation.[53, 54] The timing of tissue maturation between rodent species is also dependent on the organ. As an example, nephrogenesis is largely complete prior to birth in mice, which is similar to humans, but continues in rats during the postnatal period.[55] For this reason, the study of transporter ontogeny would be improved by reporting perinatal findings as days postconception, rather than days relative to parturition. By viewing development as a continuum instead of arbitrary categories (such as neonatal, infant, etc.), there will be greater potential to translate rodent and other mammalian studies to humans. One caveat to this approach is that some transporters increase in expression upon commencement of feeding, as has been observed for Ntcp, Bsep, and Mrp4 in the neonatal livers of mice.[42]

It should be noted that another considerable limitation occurs when contrasting mRNA expression data, because linearity in protein transcription and inferred function cannot be assumed. As mentioned in the Methods section, advances in quantitative proteomics using LC-MS/MS have led to a significant increase in our ability to quantify drug transporter protein abundance in adult samples. While a number of technical challenges

still exist with this methodology, the application of quantitative proteomic approaches to ontogenic studies with pediatric tissues will yield more useful data for establishing predictive developmental physiologically based pharmacokinetic (PBPK) models. One major limitation is that the availability of human pediatric tissue specimens is limited, and shared pediatric biobanks need to be established. The application of quantitative proteomics in animal tissue developmental studies would provide greater insight into the utility of scaling across species to predict the function of drug transporter activity in pediatric patients.

There is also the potential for significant confounding variables to limit the ability of cross-species comparisons with humans, particularly with respect to pediatric populations. Of considerable concern is the health of the patients in which the specimens were obtained. A significant portion of the human pediatric tissue biopsied specimens originate from patients who suffer from comorbidity or are collected postmortem. Collection procedures and timing also can impact tissue quality and subsequent expression data. Most animal studies are conducted under controlled conditions, whereas human tissue specimens are collected from patients who will have varying xenobiotic and dietary exposures. These confounding factors cannot be readily normalized for in many cases. Another emerging issue is that mRNA expression of housekeeper genes can vary significantly, thus calling into question quantitative data from qRT-PCR studies normalized to one control.[56, 57] These concerns may be alleviated by the use of RNA-sequencing to quantify human transporter ontogeny, as has been performed in rodents.[47]

There has been limited use of cell-based systems and mathematical models to describe the ontogeny of transporters. However, there may be the potential to complement *in vivo* rodent, other species, and human biopsy studies with human embryonic or induced pluripotent stem cells undergoing differentiation to hepatocytes, enterocytes, and renal tubule cells. While these cellular systems are artificial and lack the holistic development of an organism, they may be a mechanistic tool to probe the effects of exogenous factors such as hormones, drugs, and exposures on the sequence and timing of transporter expression. Initial studies have begun to profile the expression and activity of transporters in hepatocyte-like cells derived from human embryonic and induced pluripotent stem cells,[58] although comparisons with juvenile human livers are needed. In addition, there is a need to develop mathematical and statistical modeling approaches that integrate transporter ontogeny with the maturation of physiological processes, such as tubule reabsorption or intestinal secretion. This effort would provide researchers with the ability to "translate time" between species by developing algorithms that compare and predict development.[59, 60] Moreover, this approach can integrate the ontogeny of multiple tissues in parallel and provide a more global view on whole organism development. A computational and systems biology approach could be used to integrate genomic, epigenetic, proteomic, and pharmacokinetic endpoints (e.g., changes in pH,

plasma membrane composition, expression of drug metabolizing enzymes) to better assess the ontogeny of transport systems.[61] The utility of data-driven, PBPK models generated in this fashion would help to improve new pediatric drug translation from discovery to the clinical stages.[62]

EMERGING AREAS AND MAJOR CHALLENGES IN STUDYING PEDIATRIC DRUG TRANSPORT

Developmental programming and regulation of transporters

Little is known about the factors that govern the regulation of transporter expression and activity (e.g., induction and inhibition of transporters as a function of gestational age) during growth and development. The age-related variation in mRNA expression of the transcription factor PXR correlated with P-gp expression in a small number of fetal, neonatal, younger and older adult samples of human liver, kidney, and intestine.[17] This finding suggests a role for transcription factor-mediated regulation of age-related transporter expression. Moreover, the mechanism of age-related changes in transcription factors may be related to DNA methylation; in fetal liver, hypermethylation of important cytochrome P450 (CYP) 3A4 transcription factor binding sites has been observed, consistent with low CYP3A4 expression before birth.[63, 64] Endocrine changes in adolescents may impact drug transporter expression through hormonal and growth factor regulation of relevant transcription factors. To date, studies focused on the endocrine regulation of transporters have largely evaluated adult rodents[65, 66] and serve as a basis for future work that should be expanded to hormonal fluctuations during human development. Clearly, considerable work is needed to understand the factors that regulate drug transporters during human growth and development.

Pharmacogenomics

Genetic variation may add to the age-related variation in drug transporter expression and function. In contrast to adults, pharmacogenomic drug transporter studies in children are rare. The available studies have been performed primarily in age ranges at which full maturation of transporters can be expected, and the results in general are similar to adults.[12, 67] Further studies in children are needed to elucidate the interplay of age and genetic variation. Decreased transporter expression due to genetic variation may not become apparent until expression is at least at adult levels.[68]

Impact of disease, drug interactions, and/or environmental exposure

Other factors impacting drug transporter activity are disease, drug–gene interactions, drug–drug interactions (DDIs), food–drug interactions as well as exposures to environ-

mental chemicals. In adults, the impact of liver disease has been investigated, but the findings may not be applicable to children, as the underlying disease may be very different: e.g., alcohol-induced liver steatosis is not a pediatric disease, while biliary atresia is the most prevalent disease in children who receive a liver transplant. As underlying liver disease may impact transporter expression,[69, 70] these pathophysiological differences mandate studies in pediatric patients. In one study, MRP2, BSEP, and MDR3 expression in livers from patients with pediatric biliary atresia taken at postnatal age 1–2 months was much higher than both fetal and adult expression levels, but unfortunately no age-matched controls were studied.[36] Hence, it is unclear whether the observed differences can be attributed solely to the disease or whether age-related changes also played a role. DDIs also may be different in children due to developmental changes in drug disposition pathways; furthermore, the potential for a specific DDI may change during growth and development.[71] Finally, as nutrition changes during childhood, the impact of frequent milk or different types of formula feedings and fruit juice on drug transporter expression and function must be considered.

Development of human-relevant cell/in vitro and preclinical/in vivo transport models that are representative of the pediatric population in health and disease

Once transport protein expression and function have been characterized across the pediatric age spectrum in healthy and diseased tissue, an important next step will be to develop human-relevant *in vitro* models, such as cell lines, embryonic stem cells, or modified primary cells that mimic transporter expression and function at various ages. Such systems could be useful to predict drug disposition and DDIs in pediatrics. The utility of preclinical, *in vivo* transporter models as a predictive tool is less clear due to significant species differences in the expression and function of some transport proteins and regulatory machinery, and lack of established correlations between transporter data from animal models and humans.

Development of systems-based, mechanistic modeling approaches to integrate in vitro data and physiological processes to predict transporter-mediated changes in drug disposition as a function of age and disease

PBPK models link information about the anatomical and physiological structure of the body with the physicochemical and biopharmaceutical properties of the drug to predict drug disposition in the body. PBPK models are established tools for predicting human pharmacokinetics based on preclinical data from animals and *in vitro* studies.[72, 73] Successful applications include scaling the human pharmacokinetics from healthy volunteers to patient populations (e.g., liver cirrhosis) by accounting for changes in physiology.[74, 75] PBPK models are being applied increasingly in scaling adult pharmacokinetics to pediatric populations. This has been demonstrated for compounds elimi-

nated primarily by metabolism, where the ontogeny has been better characterized than for some transporters.[76, 77] The use of modeling and simulation methods in pediatric drug development was endorsed at a recent US Food and Drug Administration (FDA) Clinical Pharmacology Advisory Committee meeting,[78] and also is recognized by the agency as a potentially useful tool in the design of pediatric clinical trials and helping to expedite pediatric drug development.[79] Between the years 2008 and 2012, the FDA's Office of Clinical Pharmacology received 33 New Drug Application/Investigational New Drug submissions containing PBPK models; six of them were pediatric submissions.[80] The main applications of pediatric PBPK models in these submissions included dose selection, study design, informing enzyme ontogeny using benchmark drugs, and facilitating covariate analysis. The applications in pediatric drug development generally start with development and validation of an adult model (frequently utilizing *in vitro* characterization of the drug's interaction with enzymes and/or transporters) followed by scaling to pediatric populations by accounting for ontogeny of relevant physiological processes across the age continuum from neonates (including premature neonates) to adults. However, currently there is limited availability of transporter information for modeling and simulation (e.g., PBPK modeling) of drug disposition, tissue exposure, and pharmacodynamic response in pediatrics. A possible approach to circumvent this issue is to utilize available pediatric clinical data for a variety of drugs to estimate the ontogeny of relevant enzymes and transporters. In one example, the ontogeny of renal transport was estimated from age-dependent renal clearance of a model compound known to be a substrate for the same renal transporter as the investigated drug.[81] In another example, a pediatric population model for zidovudine was constructed by utilizing a previously derived pediatric covariate model for morphine glucuronidation.[82] Finally, it is worth noting that systems biology approaches, as well as methods for integration of "omics" data from multiple levels of analysis (e.g., genomics, transcriptomics, proteomics, metabolomics) are advancing rapidly, and there is an ongoing effort to apply these approaches to the adult and developmental contexts.[83] While distinct from the usual methods used in PBPK, it is anticipated that these approaches may begin to converge in the near future, providing a deeper understanding of pediatric drug disposition.

Limited availability of quality pediatric tissue (all age groups) for protein quantification and assessment of transporter function

One of the major roadblocks to drug transporter research during human growth and development is the dearth of quality pediatric tissue. Current tissue sources include left-over tissue from surgery and biopsies, as well as postmortem tissue from organ transplants and autopsies. Collaboration between clinicians with access to these tissue sources and researchers in need of tissue appears to be a major obstacle. In order to overcome these logistical challenges, a clear understanding and commitment on both sides

regarding the respective challenges and solutions may be the first step to increase the availability of quality pediatric tissue. For example, the logistics of collecting a sample, including asking for informed consent, retrieving a dry-ice container, transporting tissue to a storage facility, and collecting clinical data seem relatively straightforward, but are challenging when success depends on busy clinical staff to organize all these details. Also, a few inches of residual intestine may be very reasonable to obtain in adults, but cannot be considered "left-over" in neonates with a considerably shorter intestinal length. Timely handling of postmortem pediatric tissue is particularly challenging due to parents, who may need time to say goodbye when a child passes away, and the availability of autopsies only during the day. Furthermore, the availability of tissue may be limited compared to adults because the death rate among children is much lower than among adults, there may be reluctance on the part of parents to give consent for autopsy, and there are relatively few liver transplants from pediatric donors.

Ethical and practical challenges with performing nontherapeutic studies in minors

Research that does not potentially benefit the participating child ("nontherapeutic") is subject to several limitations,[84] most importantly, the restrictions of minimal risk (a slight increase over what is ordinarily encountered in daily life) and minimal burden. These ethical limitations, which are intended to protect the individual child, limit the possibility of performing nontherapeutic pharmacokinetics and/or DDI studies related to drug transporters in minors. Giving a child a therapeutic dose of a drug solely for these purposes will, in many places, not be considered minimal risk. However, in some centers this approach is acceptable when the child will receive the drug later for therapeutic reasons. One solution is to study the disposition and effect of the drug in the context of clinical drug treatment. This may introduce unwanted complexity to studies, such as variation due to underlying disease and/or comedication, and the need for more sophisticated pharmacokinetic analyses. Microdosing may overcome these limitations, but can only be used for drugs that exhibit dose-linearity.[85] Other challenges include the need for repeated blood sampling as well as limitations with blood volumes. These can be overcome by using indwelling catheters already in place for clinical care, low volume drug assays, and population pharmacokinetic analyses.

Lack of transporter-specific probes to assess in vivo function

In vivo probes for individual drug metabolizing enzymes (e.g., midazolam for CYP3A4, dextromethorphan for CYP2D6/CYP3A4, and caffeine for CYP1A2) have facilitated investigations about the impact of growth and development, and the effects of pharmacogenomics and disease, on drug metabolism. These critical tools have led to new knowledge. A similar approach is more challenging for drug transporters, as specific probes for individual transporters are lacking. Most drugs are substrates for multiple

transporters, which enable alternate transporter pathways to compensate in case the primary transporter is absent or has reduced activity. Nevertheless, pharmacogenomic studies on individual transporters have elucidated differences in drug disposition, efficacy, and safety in adults. This approach may aid in studying the developmental changes of these transporters *in vivo*. The use of microdosing in pediatric patients has been explored and may provide a basis for developing a better understanding of drug disposition and metabolomic profiling. While it is important to continue to analyze mRNA and protein expression levels of transporters in developing organs like the liver, kidney, and intestine, ultimately this must be related to physiological processes mediated by these particular transporters. Because of unique safety concerns about using exogenous compounds as functional probes in the pediatric population, continued exploration of endogenous metabolites and other markers that can serve as surrogates for assessing transporter function during organ development and maturation is needed. The relevant sets of endogenous metabolites may be specific to each organ, and to particular developmental points, reflecting unique patterns of transporter expression and aspects of organ-specific physiology.

Table 4. Recommendations

- Build multidisciplinary, international collaborative networks to facilitate collection and sharing of data on pediatric transporters, including expertise in preclinical studies (e.g., knockout and *in vitro* models), pediatrics, clinical pharmacology, pharmacogenomics, pharmacometrics, and pharmacovigilance
- Establish central (perhaps regional) tissue repositories where surgical and postmortem samples can be stored with clear guidelines for tissue collection and handling to preserve sample integrity
- Continue to support the training of scientists in pediatric clinical pharmacology with expertise in transporters, pharmacogenomics, pharmacometrics, and pharmacovigilance
- Increase the awareness of clinicians regarding the importance of transporters in pediatric drug disposition
- Identify examples relevant to pediatric pharmacotherapy where developmental differences in transporter expression or activity could translate into clinically relevant effects
- Work with professional groups to develop guidelines on how drug therapy may be altered due to variations in transporter expression or activity
- Identify selective and specific biomarkers for transporter activity in pediatric patients
- Investigate basic developmental mechanisms regulating transporter expression and activity in the different organs in pediatric health and disease
- Develop pediatric-relevant *in vitro/in silico* and systems biology models to predict transporter function in the context of overall drug disposition

CONCLUSION

Many fundamental and clinically relevant questions remain unanswered about the human ontogeny of drug transporters. As information highlighting the importance of drug transporters in adult medicine continues to emerge, this critical knowledge gap in the pediatric population becomes even more evident. In order to achieve safe and effective drug therapy for children, it is imperative that developmental patterns of transporter gene expression and protein abundance are elucidated, and that drug transporter function is defined across the age spectrum. Recommendations are provided (Table 4) to address some of the major challenges in obtaining this information. Fundamental and applied knowledge about the human ontogeny of drug transporters is absolutely essential to ultimately achieve personalized pharmacotherapy in pediatric patients.

Additional Supporting Information may be found in the online version of this article.
Appendix 1 Literature Search Strategy
Appendix 2 Ontogeny of Rodent Kidney Transport Proteins

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5 Development of human membrane transporters: drug disposition and pharmacogenetics

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Clinical Pharmacokinetics 2014, Sep; Epub ahead of print

ABSTRACT

Membrane transporters play an essential role in the transport of endogenous and exogenous compounds, and consequently they mediate the uptake, distribution, and excretion of many drugs. The clinical relevance of transporters in drug disposition and their effect in adults have been shown in drug-drug interaction and pharmacogenomic studies in adults. Little is known, however, about the ontogeny of human membrane transporters and their roles in pediatric pharmacotherapy. As they are involved in the transport of endogenous substrates, growth and development may be important determinants of their expression and activity.

This review presents an overview on our current knowledge on human membrane transporters in pediatric drug disposition and effect. Existing pharmacokinetic and pharmacogenetic data on membrane substrate drugs frequently used in children are presented and related, where possible, to existing ex vivo data, providing a basis for developmental patterns for individual human membrane transporters. As data for individual transporters are currently still scarce, there is a striking information gap regarding the role of human membrane transporters in drug therapy in children.

INTRODUCTION

Plasma membrane transporters play an essential role in the uptake of endogenous compounds into cells and their efflux from cells. They also mediate the absorption, distribution and excretion of a large number of drugs [1, 2]. In particular, two major transporter superfamilies are in the focus of pharmacological studies: the adenosine triphosphate (ATP)-binding cassette (ABC) transporters and the solute carrier (SLC) transporter superfamilies [3, 4]. The nomenclature is presented in Table 1. Numerous studies, mostly in adults, have investigated altered membrane transporter functions due to genetic variants or drug-drug interactions by co-medications [1, 5-9]. Studies on the role of membrane transporters in children are scarce, however. Still, growth and maturation are likely to have an impact on activity of these transporters in light of their role in endogenous processes. Animal studies have indeed shown developmental changes in membrane transporter expression [10]. The aim of this review is to present an up-to-date overview on our current knowledge on the role of human membrane transporters in pediatric drug disposition and effect. For this purpose, a short overview of ex vivo studies is presented after which results from pharmacokinetic and pharmacogenetic studies of relevant membrane transporters are reported that may broad our insight into developmental patterns for individual human membrane transporters.

EX VIVO STUDIES ON THE ONTOGENY OF HUMAN MEMBRANE TRANSPORTERS

Ex vivo data from pediatric samples may be used to extrapolate existing adult pharmacokinetic data to children, as is done using physiologically based pharmacokinetic (PBPK) modeling [11, 12]. Expression patterns of membrane transporters during human development have been studied in postmortem and surgical tissue samples with the use of different techniques such as immunohistochemistry to visualize tissue localization, reverse transcriptase polymerase chain reaction (RT-PCR) for messenger RNA (mRNA) expression, Western blotting and new liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques to quantify transporter protein abundance. To the best of our knowledge, transporter activity studies using human pediatric tissue are non-existent. Although animal data may provide valuable insight, potential developmental patterns of membrane transporters in animals are likely to differ from those in humans, as studies on drug metabolizing enzymes (DMEs) have shown [13-15]. Moreover, animal studies do not provide any information when there are no direct orthologs in rodents, as is the case, for example, for human organic anion-polypeptide (OATP) 1B1 and OATP1B3.

From the embryonic and fetal period, most transporter data result from immunohistochemistry and mRNA expression studies. These data, often covering a small age range

Table 1. Nomenclature of human membrane transporters: selection transporters discussed in this paper

Nomenclature of human membrane transporters: selection transporters discussed in this paper [source: NCBI Gene (http://www.ncbi.nlm.nih.gov/gene)]

ABC adenosine triphosphate (ATP)-binding cassette, *ADRB2* β_2-adrenergic receptor, *FAAH* fatty acid hydrolase, *SLC* solute carrier

Gene		Protein	
Name	Locus	Name	Synonyms
ABC transporters			
ABCB1	7q21.12	ABCB1	MDR1, P-glycoprotein (P-gp), CLCS, PGY1, ABC20, CD243, GP170
ABCC2	10q24	ABCC2	MRP2, CMOAT, DJS, cMRP, ABC30
ABCC3	17q22	ABCC3	MRP3, MOAT-D, cMOAT2, MLP2, ABC31, EST90757
ABCC4	13q32	ABCC4	MRP4, MOAT-B, MOATB
ABCG2	4q22	ABCG2	BRCP, MXR, MRX, ABCP, BMDP, MXR1, BCRP1, CD338, GOUT1, CDw338, UAQTL1, EST157481
SLC transporters			
SLCO1B1	12p	OATP1B1	OATP2, LST-1, OATP-C, HBLRR, LST1, SLC21A6
SLCO1B3	12p12	OATP1B3	OATP8, LST-2, LST3, HBLRR, SLC21A8, LST-3TM13
SLCO2B1	11q13	OATP2B1	OATP-B, SLC21A9
SLC3A2	11q13	4F2hc	4F2, CD98, MDU1, 4T2HC, NACAE, CD98HC
SLC22A1	6q25.3	OCT1	HOCT1, oct1_cds
SLC22A2	6q25.3	OCT2	
SLC22A6	11q12.3	OAT1	PAHT, HOAT1, ROAT1
SLC22A7	6q21.1	OAT2	NLT
SLC22A8	11q11	OAT3	
SLC15A1	13q32.3	PEPT1	HPEPT1, HPECT1
SLC47A1	17q11.2	MATE1	
SLC47A2	17q11.2	MATE2-K	MATE2, MATE2-B
Other			
FAAH	1p35-p34	FAAH	FAAH-1, PSAB
ADRB2	5q31-q32	ADRB2	BAR, B2AR, ADRBR, ADRB2R, BETA2AR
CDH17	8q22.1	HPT1	CDH16, LI cadherin

and/or small sample size, suggest transporter-specific maturation with a low fetal/neonatal or stable expression pattern, but quantification is lacking [16-19]. The ex vivo data from the first years of life consist mainly of hepatic and intestinal mRNA expression data, with the inherent limitation of a possible lack of correlation with protein expression [20-24]. In children from 7 years onwards, protein abundance data generated using LC-MS/MS have been recently published [25-27]. Although a large pediatric age range was covered by this project, the younger age range, where most developmental changes are expected, is lacking in protein abundance data.

The studies referenced above comprise the most significant studies investigating the maturation of human membrane transporters, with an emphasis on the clinically relevant transporters ABCB1, ABCC2, OATP1B1 and OATP1B3. The best-studied transporter during human development is ABCB1 (Fig. 1). Interestingly, its developmental pattern seems organ-specific. In fetal intestinal samples (16th to 20th week of gestation), ABCB1 could be visualized [16] and intestinal mRNA data suggests stable ABCB1 expression from the neonate up to the adult [22, 24]. In the liver, mRNA expression data suggest a pattern of low ABCB1 expression in fetuses, neonates, and infants until 12 months of age, after which it increases to adult levels [21, 22]. ABCB1 protein abundance measured using LC-MS/MS was quite variable (4.8-fold) in 64 subjects in the age range 7-70 years, but this variation could not be explained by either age or sex [25]. In fetal human brain samples ranging from 7 to 28 weeks of gestational age, ABCB1 immunostaining was detected in only one sample from a 28-week fetus [16]. In contrast, in postmortem central nervous system tissue from neonates (*n*= 28) of 22 - 42 weeks of gestational age and from adults (*n*=3), immunohistochemistry showed increasing ABCB1 staining with gestational age [28]. *ABCB1* gene expression was also detected in the brain of fetuses of 15, 27 and 42 weeks of gestational age [18]. Very recently, the ABCB1 protein was shown limited at birth and to increase postnatally to reach adult levels by 3-6 months of age [29]. Renal *ABCB1* mRNA expression appears to be related to maturity of nephrons. A trend towards lower expression in fetuses and neonates than in adults was observed. ABCB1 protein has been identified as early as 5.5th of gestation [16, 17, 21].

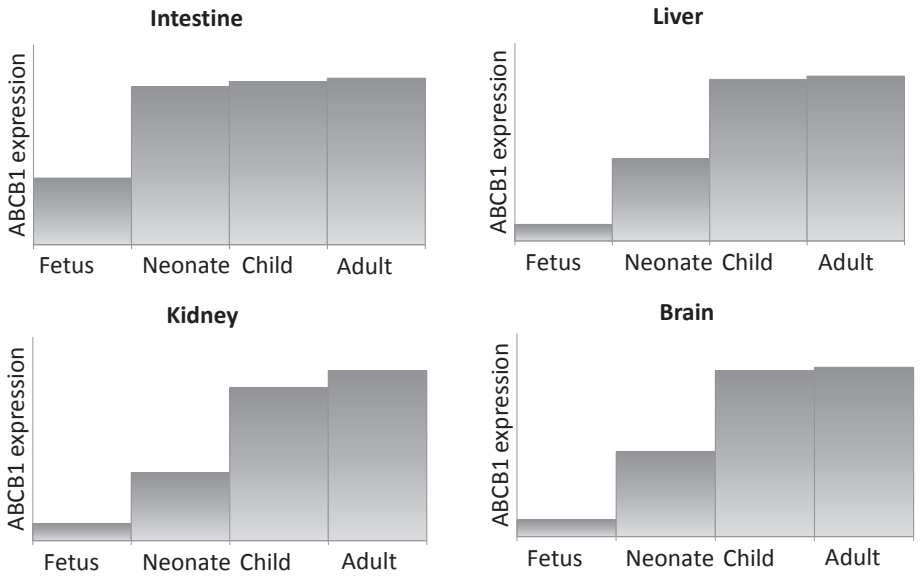

Figure 1. Suggested ontogeny of ABCB1 expression in intestine, liver, kidney and brain.
ABC adenosine triphosphate (ATP)-binding cassette

ABCC2 ontogeny shows similarities to ABCB1. Small intestine *ABCC2* mRNA expression is stable in neonatal surgical patients compared to adults. While, hepatic *ABCC2* mRNA expression is much lower in the fetus, neonate and young infant (up to 200-fold lower) than the adult [22, 27], in children from 7 years onwards its protein expression appears stable [27]. On the protein level, both the localization pattern and intensity of ABCC2 protein staining appear to change during fetal life, in concert with fetal liver maturation [19, 23].

Hepatic mRNA expression of *OATP1B1* and *OATP1B3* appears to show a different developmental pattern than *ABCB1* and *ABCC2*. Although fetal expression was 2- to 30-fold lower than adult expression, neonatal and infant expression appeared to be even lower (up to 600-fold) [19, 22]. This pattern appears to be supported by protein data (Western blotting) for OATP1B3 but not for OATP1B1. In one study, OATP1B1 protein expression already appears at adult levels in neonates, while in another OATP1B1 only increases after the age of 6 years [30, 31]. Again, for both OATP1B1 and OATP1B3, protein expression appears stable at adult levels from 7 years onwards [25].

PHARMACOKINETIC AND PHARMACOGENETIC STUDIES OF RELEVANT MEMBRANE TRANSPORTER SUBSTRATES

Pharmacokinetic and pharmacogenetic studies may provide insight into the impact of selected drug transporters in vivo. We identified 16 drugs frequently prescribed to children and that are known substrates of one or more specific membrane transporters (Table 2; see the Electronic Supplemental Material for the search strategy). Age-related differences in pharmacokinetic or pharmacogenetic studies may point to maturational changes in the transporter involved. On the other hand, concordance between adult and pediatric pharmacogenomic studies may support the presence and potentially similar expression of the involved transporters in children as in adults. To further support a potential developmental pattern, we compared the in vivo data with relevant ex vivo data of the individual transporters. As many drugs are also substrate of DMEs and/or multiple transporters, the presented data must be interpreted in the context of the interplay with metabolizing enzymes. It can be speculated that when a specific DME is developmentally low at a certain age, while the transporter is already mature, this may impact the disposition of a drug, by potentially altering the absorption, distribution, metabolism, and excretion (ADME) pathway from largely DME based to transporter based. Where possible, we have only included data that are highly supportive of a role for the transporter(s) involved. Table 2 provides a summary of the pharmacokinetic studies in children and the relationship with transporters. For detailed genetic information on individual transporters, the reader is referred to the pharmgkb.org database (http://pharmacogenetics.ucsf.edu/) and recent reviews [7, 8, 32-34]

Table 2. Summary PK and PGx studies of relevant membrane transporter substrates.
ABC adenosine triphosphate (ATP)-binding cassette, *AUC* area under the plasma concentration-time curve, *BRCP* breast cancer resistance protein, *GFR* glomerular filtration rate, *HPT* human oligopeptide transporter, *IV* intravenous, *M3G* morphine-3-glucuronide, *MATE* multidrug and toxin extrusion protein, *MPA* mycophenolic acid, *mRNA* messenger RNA, *MRP* multidrug resistance-associated protein, *OAT* organic anion transporter, *OATP* organic anion-transporting polypeptide, *OCT* organic cation transporter, *PGx* pharmacogenetics, *PK* pharmacokinetics, *UGT* uridine 5'-diphospho-glucuronosyltransferase

Drug	Relevant transporters involved in transport of drug	PK and PGx results in children
Digoxin	ABCB1	Higher bodyweight-corrected digoxin clearance in term neonates and young children {Yukawa, 2007 #274;Yukawa, 2011 #273;Hastreiter, 1985 #463}. Renal clearance of digoxin in young children may be more dependent on ABCB1 mediated tubular secretion than in adults {Ratnapalan, 2003 #285}.
Tacrolimus	ABCB1	PGx studies of *ABCB1* in relation to tacrolimus PK appear contradictory {Gijsen, 2011 #396;de Wildt, 2011 #286;Hawwa, 2009 #97;Guy-Viterbo, 2014 #397}. In pediatric liver transplant recipients, high intestinal ABCB1 mRNA expression was associated with a two-fold higher tacrolimus clearance {Fukudo, 2006 #7}.
Daptomycin	ABCB1	Higher body-size corrected renal daptomycin clearance in neonates and younger infants {Bradley, 2014 #458;Woodworth, 1992 #491;Cohen-Wolkowiez, 2012 #465}.
Fexofenadine	OATP2B1, ABCB1, MRP2	Apparent bodyweight corrected oral clearance was 1.5-fold lower in children 6 months to 6 years, than in children 6 to 12 years {Martinez, 2014 #461}.
Morphine	OCT1, ABCB1, ABCC2, ABCC3, OATP1B1	Neonates and infants have low morphine clearance in the first 10 days of life, increasing thereafter, largely due to immature UGT2B7 metabolism but transporters may contribute {Knibbe, 2009 #277;Krekels, 2014 #429}. Neonates are more prone to morphine-related respiratory depression. {Sadhasivam, 2014 #403}. *ABCB1* genotype was associated with respiratory depression in older children, in contrast to an adult study {Kharasch, 2003 #401}. Also *ABCB1* genotype affects the M3G-formation and *OCT1* genotype is associated with variation in morphine clearance and glucuronide-metabolites formation {Venkatasubramanian, 2014 #430}.
Pravastatin	OATP1B1, OATP2B1, OATP1B3, ABCB1, ABCC2	Children with hypercholesterolemia and the *SLCO1B1* -11187GA variant had lower mean pravastatin AUCs than those with the wild type, in contrast to an adult study where opposite effect was found {Hedman, 2006 #259;Niemi, 2004 #433}. No age-related variability in pravastatin PK from children age 5 years onwards {Hedman, 2003 #260}.
Atorvastatin	OATP1B1, BCRP	Atorvastatin PK in older children similar to adult PK {Knebel, 2013 #347}.
Bosentan	OATP1B1, OATP1B3, OATP2B1	In children, an exposure limit was found at a much lower dose than in adults, might be due to intestinal OATP2B1 saturation {Beghetti, 2009 #438}.

Table 2. Summary PK and PGx studies of relevant membrane transporter substrates (continued)

Drug	Relevant transporters involved in transport of drug	PK and PGx results in children
Ondansetron	OCT1	Ondansetron PK and clinical efficacy have been correlated with OCT1 genotypes in adults {Tzvetkov, 2012 #311}. Ondansetron clearance increased with age in children ages 1 to 48 months {Li, 2013 #310}.
Metformin	OCT, MATE1, MATE2K	Metformin PK in children from 9 years of age onwards was comparable with adult PK, suggesting stable OCT and MATE activity {Sanchez-Infantes, 2011 #409}.
Cimetidine	OCT2, MATE1, MATE2K, OAT2	In neonates and children cimetidine (and metabolites) renal clearance accounts for 80-90% of total clearance, whereas in adults it accounts for 60% of total clearance {Somogyi, 1985 #419;Ziemniak, 1984 #418;Lloyd, 1985 #416}. The relatively high renal clearance suggests mature OCT2 activity at birth in the presence of immature GFR {Kurata, 2010 #477}.
Tramadol	OCT1	In adults OCT1 genotype was related to metabolite plasma concentrations and prolonged miosis {Tzvetkov, 2011 #332}. Tramadol and metabolite PK show age-related changes in neonates {Allegaert, 2005 #335}
Methotrexate	OATP1B1, ABCC2	Increased renal toxicity in children 0-3 months compared to infants 7-12 months {Thompson, 2007 #481}. From 1 year of age onwards body-size corrected methotrexate clearance decreased linearly with age {Radtke, 2013 #492}. SLCO1B1 genotype was associated with increased AUC and was a predictor for toxicity {Radtke, 2013 #483}.
Mycophenolate mofetil	MRP2	In pediatric patients *ABCC2* rs717620 allele has been associated with reduced exposure to MPA, more side effects and rejection {Fukuda, 2012 #375}.
Acyclovir/ valacyclovir	4F2hc, HPT1, OAT1, OAT3	In neonates, the IV acyclovir bodyweight-corrected clearance showed a 2-fold increase from 25 to 41 weeks of gestational age {Sampson, 2014 #364}. In older children, 1 month to 5 years, apparent oral clearance of valacyclovir in children less than 3 months of age was 50% than that in older children {Kimberlin, 2010 #453}.
Adefovir	OAT1, MRP4	Adefovir is partly renally cleared (45%) {Maeda, 2014 #319;Imaoka, 2007 #455}. In 45 children (age range 2 years – 17 years) receiving oral adefovir dipivoxil, weight-corrected mean apparent clearance and renal clearance were higher in younger children {Sokal, 2008 #340}.

1. Digoxin – ABCB1

The cardiac glycoside digoxin is a well-known ABCB1 substrate (Fig. 2). Its US Food and Drug Administration (FDA) drug label warns about pharmacokinetic interactions with intestinal or renal ABCB1 inducers or inhibitors. Digoxin is mainly renally cleared as unchanged drug, 80 % by glomerular filtration and 20 % by tubular secretion [35]. Pharmacokinetic studies in children show clear age-related differences. In a population pharmacokinetic analysis in 71 neonates (age range 2-29 days), oral digoxin clearance

increased non-linearly with increasing bodyweight and gestational age [36]. The estimated clearance of digoxin in a term-born 3 kg newborn is 0.338 L/kg/h at a serum concentration of 1 ng/mL. A population pharmacokinetic study in older infants [*n*=117, mean age (range) 0.76 (0.08-4.43) years] also found increased oral digoxin clearance with increasing bodyweight [37]. In this study, the simulated apparent oral clearance (CL/*F*) of an 8 kg infant was 0.43 L/h/kg at a target concentration of 1 ng/mL. Interestingly, digoxin clearance normalized for bodyweight, appears much higher in term neonates

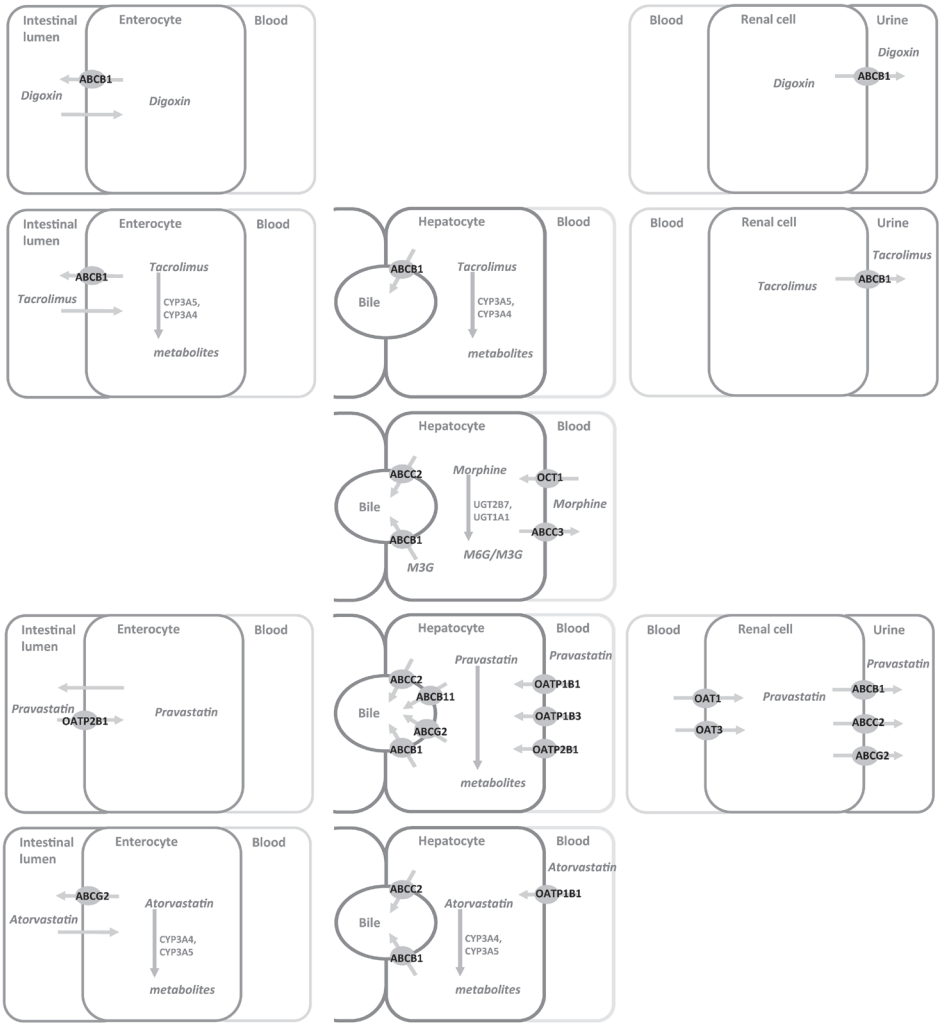

Figure 2. Membrane transporters in their relationship with common prescribed drugs to children: digoxin, tacrolimus, morphine, pravastatin and atorvastatin.

ABC adenosine triphosphate (ATP)-binding cassette, *CYP* cytochrome P450, *OATP* organic anion-transporting polypeptide, *OCT* organic cation transporter, *UGT* uridine 5'-diphospho-glucuronosyltransferase

and younger children than in adults (0.17 L/h/kg). This observation is also in line with higher (per kg) dosing recommendations in term neonates and infants. However, as the drug is mainly renally cleared, and glomerular filtration is still immature at birth, one would expect a lower body size-corrected clearance. This was indeed the case in preterm infants (<2.5 kg) whose digoxin bodyweight-corrected clearance was much lower than that of term infants (0.064 vs. 0.1 L/h/kg), in line with lower dosing recommendations for this age group [38]. Thus, in preterm newborns, both glomerular filtration rate (GFR) and ABCB1 may be immature at birth, while in term infants ABCB1 activity may already be more mature and compensate for developmentally low GFR.

Additionally, clearance decreases non-linearly with increasing concentrations in the range of 0.2 and 2 ng/mL in children up to 4.5 years of age, whereas non-linearity in adults is only found from a serum concentration of 7 ng/mL onwards. We can only speculate as to the underlying mechanism. Earlier transporter saturation due to immature ABCB1 activity in intestine and kidney contradicts the observation of higher bodyweight-corrected clearance in young children than in adults.

A clinically relevant interaction was found in eight children who were co-administered the ABCB1 inhibitor carvedilol. Digoxin clearance decreased twofold, while the digoxin clearance to GFR ratio decreased by 45 %, supporting intestinal and renal ABCB1 mediated inhibition [39]. In contrast, the same drug-drug interaction resulted in only a mild decrease in digoxin clearance in adults. These findings support our hypothesis that renal clearance of digoxin in young children may be more dependent on ABCB1-mediated tubular secretion than in adults.

Interestingly, the hypothesis of higher renal ABCB1 expression after birth is supported by a mouse study showing a relationship between renal Abcb1 expression and digoxin clearance in young mice, but not by the limited human data from neonates [21, 40].

2. Tacrolimus – ABCB1

The calcineurin inhibitor tacrolimus inhibits synthesis of cytotoxic lymphocytes and so prevents transplant rejection. Tacrolimus is a substrate for intestinal and hepatic ABCB1 (Fig. 2) [41]. Weight-normalized oral tacrolimus clearance, which is also dependent on cytochrome P450 (CYP) 3A4/5 metabolism, is higher in infants between 1 and 6 years of age than older children and adults [42, 43]. CYP3A4 activity matures in the first year of life, while CYP3A5 activity, when present, appears stable from fetus to adult [44].

Pharmacogenetic studies of *ABCB1* in relation to tacrolimus disposition appear contradictory [45]. In pediatric heart and kidney transplant recipients no relation was found between ABCB1 genotype and tacrolimus dosing requirements or concentration/dose ratio [42, 43]. In pediatric liver transplant patients, homozygous *ABCB1* 1236TT/2677TT/3435TT carriers needed higher tacrolimus doses than non-carriers both early and later after transplantation [42, 46]. In a population pharmacokinetic analysis

in 114 pediatric liver transplant recipients, the *ABCB1* 2677G>T allele was associated with a higher pre-dose and concentration/dose ratio at day 1 after transplantation [47]. Such associations were not found for recipient or donor *ABCB1* 1199G>A and 3435C>T variants. These findings can be understood from a combined ex-vivo/population pharmacokinetic study in 130 pediatric liver transplant recipients. High intestinal *ABCB1* mRNA expression was associated with an almost twofold higher tacrolimus clearance early after transplantation, indicative of a switch from primarily intestinal to hepatic tacrolimus clearance upon graft recovery [48].

The results from ex vivo studies suggesting stable *ABCB1* mRNA intestinal expression and lower hepatic *ABCB1* expression in young infants may explain the pharmacogenetic findings and the impact of the recipient intestinal ABCB1 on tacrolimus disposition [20, 22, 24, 48].

3. Daptomycin – ABCB1

The antibacterial daptomycin is used to treat infections caused by Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). Daptomycin is excreted primarily by the kidney and is an ABCB1 substrate. In 23 adult Caucasian patients, the *ABCB1* 3435T single nucleotide polymorphism (SNP) was associated with a higher intravenous daptomycin dose-normalized area under the plasma concentration-time curve (AUC) and lower steady state clearance [49, 50]. In 24 children (age range 3-24 months), intravenous daptomycin clearance in younger infants (approximately 20 mL/h/kg) was higher than previously reported for older children and adults (8-13 mL/h/kg) [51, 52]. Likewise, in 20 preterm and term infants (32-40 weeks of gestational age and 0-85 days' postnatal age, the mean clearance was approximately 20 mL/kg/h) [53]. No relationship with gestational or postnatal age was found, possibly due to the small sample size. The maturation pattern of daptomycin pharmacokinetics resembles that of digoxin pharmacokinetics, with higher clearance values early in life. This pattern is not consistent with immature GFR, but may reflect a compensatory role of ABCB1-mediated renal tubular secretion in young children. As discussed earlier, this may be the result of increased renal ABCB1 expression in young infants, but these findings need to be confirmed [16, 18, 21].

4. Fexofenadine – OATP2B1

The antihistamine fexofenadine is mainly excreted through bile as parent drug. Only a minor part is metabolized by intestinal microflora and CYP3A4. Its disposition appears to be subject to membrane transport by the uptake transporter OATP2B1, the efflux transporter ABCB1 and possibly ABCC2 [54-56]. In 14 healthy men, fexofenadine clearance was related to OATP2B1 polymorphisms and simultaneous apple juice ingestion [57]. This latter finding of a potential food-drug interaction concerning OATP2B1 substrates may be even more relevant for children, as heavy consumers of apple juice. In a population

pharmacokinetic study in 515 Japanese children (6 months to 16 years), CL/*F* was stable across the age groups (1 L/h/kg) with the exception of the 6- to 12-year olds, whose clearance was 1.5-fold higher (1.5 L/h/kg) [58]. In another population pharmacokinetic study including 46 Caucasian and 31 Non-Caucasian children (6 months to 12 years) and 138 adults, apparent bodyweight-normalized oral clearance was lower in children less than 1 year of age than in older children and adults [59]. Interestingly when comparing ethnicities, the CL/*F* was slightly higher in the 6- to 12-year-old Japanese children (1.5 L/kg/h) than in 14 non-Japanese children (0.8 L/h/kg) (13 Caucasian and one non-Caucasian), but this difference may be rather due to the small sample size than ethnicity [58].

Ex vivo data in these age groups are lacking, but *OATP2B1* gene expression in 15 neonatal intestinal samples obtained during surgery was nearly three times higher than in adult samples [22]. This may imply higher oral absorption of fenofexadine in neonates and young infants.

5. Morphine – ABCB1 and OCT1

The opioid morphine is almost completely metabolized by uridine 5'-diphospho-glucuronosyltransferase (UGT) 2B7 and UGT1A1 to morphine-3-glucuronide and morphine-6-glucuronide. The disposition of both morphine and/or its metabolites is subject to membrane transport by the uptake transporters organic cation transporter (OCT) 1 and OATP1B1 and by the efflux transporters ABCB1, ABCC2 and ABCC3 (Fig. 2) [60, 61]. Hepatic uptake of morphine appeared to be OCT1-mediated in an adult volunteer study and carriers of loss-of-function *SLC22A1* gene polymorphisms showed higher morphine AUCs [61]. In other healthy adult volunteers, co-administration of the ABCB1 inhibitor quinidine altered both morphine pharmacokinetics and its opioid effects after oral but not after intravenous morphine administration, suggesting a limited role for ABCB1 in the disposition of morphine at the liver and blood-brain barrier [62]. In adult cancer patients receiving oral morphine, pain relief was more prominent in homozygous carriers of the *ABCB1* 3435T/T SNP, probably due to higher intestinal uptake [63].

Age-related morphine clearance in neonates and infants is very low in the first 10 days of life and increases thereafter [64]. Although this pattern was explained by UGT2B7 maturation, an impact of the maturation of the relevant transporters cannot be ruled out. Interestingly, in follow-up studies in which morphine doses were adjusted to the age-related clearance and similar exposure was reached across the first year of age, pain relief was adequate in neonates (<10 days of age), but the older children still needed high doses of rescue morphine [65]. Neonates are more prone to respiratory depression, which may be explained by increased exposure to morphine when doses are not adjusted to the age-related changes in its disposition. In addition, immature ABCB1 activity at the blood-brain barrier, as shown very recently, cannot be ruled out to contribute as well and deserves further study [29].

Sadhasivam and co-workers [66, 67] have extensively studied the impact of genetics of transporters in a large cohort of infants and children receiving intravenous morphine for tonsillectomy. In 220 infants, OCT1 homozygous genotypes (*SLC22A1* 1365GAT>del / 1498G>C) were associated with lower morphine clearance and lower morphine glucuronide formation [68]. *ABCC3* homozygous -211 CC carriers showed (approximately 40 %) higher metabolite transformation, indicating increased efflux of metabolites into plasma. *ABCB1* polymorphisms (3435 C>T) only affected morphine-3-glucuronide formation, not morphine or morphine-6-glucuronide pharmacokinetics. It should be noted that only pharmacokinetic data up to 45 min post-dosing were available and, hence, the full pharmacokinetic profile of morphine and metabolites could not be determined. In 263 children of the same cohort, the *ABCB1* G allele of rs9282564 polymorphism was associated with respiratory depression, resulting in prolonged hospital stay (odds ratio 4.7 (95 % confidence interval: 2.1-10.8, *P*=0.0002) [66]. This study contrasts the adult study in which ABCB1 inhibition did not influence the effect of morphine after intravenous administration [62]. In the extended cohort, now including 347 children, the interaction of genetic variants of *ABCB1* and two other genes, the fatty acid hydrolase [*FAAH*, which has been associated with opioid use and addiction acting via cannabinoid receptors type 1, and the β_2-adrenergic receptor (*ADRB2*, receptor blockade has been associated with pain and pain relief), helped discriminate low and high risk for morphine-related postoperative respiratory depression [67].

As hepatic *ABCB1* gene expression only appears to reach adult levels after the first year of life [20, 22, 24, 48], immature hepatic and possibly also blood-brain barrier ABCB1 expression may play a role in higher morphine plasma and brain exposure and the higher risk for respiratory depression in neonates.

6. Pravastatin – OATP1B1

Clearance of the cholesterol synthesis inhibitor pravastatin is mainly dependent on non-CYP450-mediated drug metabolism and several uptake and efflux transporters, such as OATP1B1, OATP2B1, OATP1B3, ABCB1 and ABCC2 (Fig. 2) [69]. In adults, *SLCO1B1*, but not *ABCC2*, *ABCB1*, or *ABCG2,* gene variants were associated with inter-individual variability in pravastatin pharmacokinetics, suggesting a major role of *SLCO1B1* in pravastatin disposition [70, 71].

Intriguingly, in one small pharmacogenetic study (*n*=20; mean ± standard deviation age 10.3 ± 2.9 years), children with familiar hypercholesterolemia with the *SLCO1B1* -11187GA genotype had lower mean pravastatin AUCs than children with the wild type [72]. The opposite effect was found in an adult study (*n*=41) [71]. These results, which should be interpreted with care in view of the small sample size, suggest age-related posttranslational differences in OATP1B1 expression. The pharmacokinetics of pravastatin in children (aged 5-16 years) waere similar to adults, which suggests no major

impact of age-related variability in OATP1B1 or other transporter activity from 5 years onwards [73]. These results appear to be in line with the ex vivo results of stable hepatic OATP1B1 expression in older children [25].

7. Atorvastatin – OATP1B1

Another cholesterol synthesis inhibitor increasingly used in children is atorvastatin. Like the other statins (HMG-CoA reductase inhibitors), atorvastatin is extensively metabolized, largely by CYP3A4, and is a substrate for OATP1B1 (hepatic uptake) and ABCG2 (oral absorption) (Fig. 2). In a population pharmacokinetic study in pediatric hypercholesterolemia patients aged 6-17 years, atorvastatin CL/*F* was described as a function of bodyweight. When scaled allometrically, CL/*F* was similar to values reported for adults [74]. Atorvastatin metabolism is probably mature from 6 years onwards. The stable clearance and the existing ex vivo data of OATP1B1 suggest similarly mature transporter activity, although an age-related change in the relative contribution of individual transporters cannot be ruled out [25, 26]. We could not identify pediatric pharmacogenetic atorvastatin studies, although a comprehensive study in adults clearly indicated the *SLCO1B1* variant 388A>G as a major determinant for atorvastatin PK [75].

8. Bosentan – OATP2B1

Bosentan is metabolized by CYP3A4 and CYP2C9, with both the parent compound and one metabolite being pharmacological active, but is also subject to hepatic uptake by OATP1B1, OATP1B3 and maybe OATP2B1 [76]. Next to the developmental pattern of CYP3A4, CYP2C9 already shows an increase prenatally, with stable, though variable, activity after the first week of postnatal age [77, 78]. Hence, the impact of the maturation of the transporters may be compounded by CYP3A4 and CYP2C9 maturation in the first year of life, but age-related variation as observed later in life may be more likely due to transporter maturation. At oral doses of approximately 2 mg/kg, bosentan plasma exposures for 19 children (aged 3-15 years) were similar to those for healthy adults [79]. In children [*n*=36, median (range) age 7.0 (2 - 11) years], plasma levels did not further increase at doses higher than 2 mg/kg (exposure limit), while in adults the exposure limit was 7 mg/kg, with no difference in the other pharmacokinetic parameters [80]. As bosentan appears an OATP2B1 substrate, intestinal saturation due to immature OATP2B1, and perhaps other anatomical or physiological age-related differences in oral absorption, may explain this observation. This result does not correspond with the *OATP2B1* mRNA expression results, suggesting higher expression in neonates than in adults [22]. In a pediatric population pharmacokinetic-pharmacogenetic study [*n*=46, mean (range) age 3.8 (25 days – 16.9) years] no relation between bosentan pharmacokinetics and genetic polymorphisms of *SLCO1B1*, *SLCO1B3*, *SLCO2B1* or *CYP3A5* was found [81].

9. Ondansetron – OCT1

The anti-emetic ondansetron is mainly metabolized by CYP2D6, which contributes to genetic variation in its disposition and effect. In addition to this CYP2D6 effect, the pharmacokinetics (*n*=45) and clinical efficacy (*n*=222) of ondansetron in adults (age range 18-83 years) have also been correlated with *SLC22A1* genotypes [82]. OCT1 deficiency potentially limits the hepatic uptake and increases plasma concentrations of ondansetron [82]. In a population pharmacokinetic analysis of 124 patients in the age range 1- 48 months, ondansetron bodyweight-normalized clearance was reduced by 76% in a 1-month-old patient and by 31% in a 6-month-old patient, compared with the older children [83]. This contrasts evidence from both in vitro and in vivo studies suggesting maturity of CYP2D6 activity as early as 1-12 weeks postnatal [84-86]. Thus, we hypothesize that lower ondansetron clearance in the first year of life may be related to immature OCT1 activity.

10. Metformin – OCT1 and MATE1

Several transporters have been implicated in metformin elimination, tissue distribution and response. OCT1 is a major determinant of the hepatic uptake of metformin, while multidrug and toxin extrusion protein (MATE) 1 /MATE2K determine the efflux of metformin [7, 34]. Recently, the transcription factor hepatocyte nuclear factor 1 was found to regulate OCT1 expression and was related to metformin pharmacokinetics and pharmacodynamics [87, 88]. The combination of *SLC22A1* (OCT1) and *SLC47A1* (MATE1) genotypes further explains variation in response to metformin in adults [8, 89]. In contrast, in 140 non-obese adolescent girls with androgen excess after precocious pubarche, *SLC22A1* genotype was not related to metabolic response at 1 year of metformin treatment [90]. In non-obese 9-year-old girls and diabetic patients aged 12-16 years, pharmacokinetics were comparable with those in adults [91, 92]. This suggests stable OCT1 or MATE activity from the age of 9 years onwards, but this needs further study, especially since data in younger children are lacking.

11. Cimetidine – OCT2

In adults, renal excretion of the histamine H_2-receptor antagonist cimetidine and its metabolites accounts for 60 % of total clearance, versus 80-90% in children and neonates [93-95]. Cimetidine is partially metabolized and is a substrate of the uptake transporters OCT2 and organic anion transporter (OAT) 2 and the efflux transporters MATE1 and MATE2-K [8, 34, 96, 97]. Cimetidine is a well-known OCT2 inhibitor and therefore could potentially counteract OCT2-driven cisplatin oto- and renal toxicity [98]. Although the drug is now rarely used in pediatric clinical care, this latter promise necessitates a full understanding of its disposition across the pediatric age range, as part of studies to confirm this new indication. The relatively high renal clearance in neonates and children suggests

an important role for renal tubular secretion and suggests mature activity already at birth. On the other hand, Ziemniak et al. [94] suggest that the unexplained gap between total and renal clearance in adults could be due to secondary metabolite formation in adults, which maybe missing in neonates due to immature metabolism. This is less likely, however, as the higher renal clearance was also observed in older children, whose drug metabolism is largely at adult levels. Moreover, in a rat study, bile duct ligation increased cimetidine renal tubular secretion by up-regulation of OCT2 (but not MATE1), supporting the hypothesis that the non-renal clearance in adults occurs through hepatic/bile excretion and is not related to unknown secondary metabolite formation [99]. Nevertheless, as analytical methods to measure drugs and metabolites have become more sensitive since these early studies in the mid-1980s, new studies in children of different ages could help elucidate why renal clearance of cimetidine differs between children and adults and give insight in the role of OCT2 in cimetidine disposition.

12. Tramadol – OCT1

Tramadol is a prodrug of the μ-opioid receptor agonist *O*-desmethyltramadol. It is metabolized mainly by CYP2D6 to its active *O*-desmethyltramadol metabolite [100]. The variation in tramadol pharmacokinetics cannot solely be explained by variation in CYP2D6, as was shown by Tzvetkov et al. [101] who showed an additive effect of OCT1 on tramadol disposition variation. Loss-of-function *SLC22A1* polymorphisms have been related to higher plasma concentrations of the active *O*-desmethyltramadol and prolonged miosis, as surrogate marker of the opioid effect. These effects are likely due to reduced OCT1-mediated hepatic uptake [101]. Allegaert and co-workers [102] showed that maturational clearance of tramadol, driven by CYP2D6 activity, is almost complete by 44 weeks post-menstrual age. In a pooled population pharmacogenetics-pharmacokinetic study covering the age range from preterm to elderly, only part of the variability in *O*-desmethyltramadol formation clearance could be explained by CYP2D6 genotype, further supporting a potential role for *SLC22A1* genetic variation [86]. A relationship between CYP2D6 genotype and tramadol metabolism was shown in young preterm infants, which is surprising as CYP2D6 is not fully mature at birth, especially not in preterm infants, and a genotype effect may have been obscured. It would be worthwhile, therefore, to study the impact of *SLC22A1* genotype in this young population [10]. In a population pharmacokinetic/pharmacodynamic analysis of 104 older children (2-8 years), age did not clearly contribute to variation in pharmacokinetics or the prediction of response [103].

13. Methotrexate – OATP1B1 and ABCC2

Methotrexate is a folic acid antagonist used to treat several forms of cancer and anti-inflammatory diseases. Methotrexate undergoes complex hepatic and intracel-

lular metabolism [104]. Many membrane transporters are responsible for its uptake and excretion and for its metabolism to active polyglutamine metabolites and inactive 7-hydroxy-methotrexate [104, 105]. Methotrexate is eliminated primarily by renal excretion through glomerular filtration and renal tubular reabsorption and secretion. Approximately 70–90 % of a dose is excreted unchanged in urine. A small pharmacokinetic study showed only marginally lower methotrexate steady-state clearance (body surface area corrected), but increased renal toxicity, in 0- to 3-month-old infants than in 7- to 12-month-old infants [106, 107]. From 1 year onwards methotrexate clearance (body surface area normalized) decreased linearly with age [107]. A 2014 review concluded that "although there is no pharmacogenetic marker for MTX [methotrexate] in use in the clinic at present, polymorphisms in *SLCO1B1* have an important role in MTX pharmacokinetics and toxicity in pediatric ALL [acute lymphoblastic leukemia] patients and show the most consistent and promising results" [105]. For example, in a cohort of almost 500 pediatric acute lymphoblastic leukemia (ALL) patients, methotrexate AUC from time zero to 48 h ($AUC_{0\text{-}48h}$) increased by 26 % ($P < 0.001$) per *SLCO1B1* rs4149056 *C* allele and was a significant predictor of overall toxic adverse events during methotrexate courses ($R^2 = 0.043$; $P < 0.001$), but no relationship was found for *ABCC2* [107]. This study confirmed the results of a genome-wide association study (GWAS) in 434 ALL children, the first to identify *SLCO1B1* as an important marker of methotrexate pharmacokinetics and clinical response, and recently validated by five different treatment regimens of high-dose methotrexate ALL treatment protocols at St Jude Children's Research Hospital (Memphis, TN, USA) [108]. Moreover, a deep sequencing approach for *SLCO1B1* demonstrated that rare damaging variants contributed significantly to methotrexate clearance and had larger effect sizes than common *SLCO1B1* variants [109, 110]. Other recent studies have detected a relationship between ABCC2 and methotrexate pharmacokinetics and toxicity. In 112 Han Chinese pediatric ALL patients, the *ABCC2* -24T allele (rs717620) was associated with significantly higher methotrexate plasma concentrations at 48 h and with significant hematological and non-hematological toxicities. These findings are partially supported by other studies in 127 Lebanese and 151 Spanish pediatric ALL patients [111, 112].

14. Mycophenolate mofetil – ABCC2

Mycophenolate mofetil is the prodrug of the active mycophenolic acid (MPA). It is metabolized by carboxylesterase 2 (CES2), after which MPA is further metabolized by several CYPs and UGTs [113]. MPA-glucuronide is excreted in the bile primarily by ABCC2 (encoded by *ABCC2*) and this transport is essential for enterohepatic circulation. The *ABCC2* rs717620 A allele has been associated with reduced exposure to MPA in pediatric renal transplant recipients [114]. In a large multi-center cohort of pediatric heart transplant recipients, *ABCC2* rs717620 A allele was also associated with more gastrointestinal

intolerance, but with fewer short- and long-term rejection episodes [114]. As ABCC2 is thought to excrete MPA-glucuronide in the bile, carriers of the active A allele, may have increased enterohepatic circulation with an increased concentration of free MPA in the intestine. This is potentially associated with more gastrointestinal intolerance, but simultaneously with higher exposure and efficacy. As SNPs in *UGT1A9*, *UGT2B7*, *SLCO1B3* and *IMPDH* have also been associated with altered MPA exposure, the combined effect of these SNPs and potentially interacting co-medication, may define high- and low- risk patients for MPA efficacy and toxicity. Full hepatic ABCC2 maturation appears to occur after infancy, suggesting a lower enterohepatic circulation of MPA, which may result in less gastrointestinal intolerance but potentially also with less efficacy in this age group [22]. This is merely a hypothesis without confirming pharmacokinetic data.

15. Acyclovir/Valacyclovir – OAT1 and OAT3

The oral bioavailability of the anti-viral agent acyclovir is poor, and therefore its prodrug valacyclovir was developed. A positive association was found between intestinal expression of 4F2hc (*SLC3A2*, amino acid transporter heavy chain, a membrane glycoprotein), and HPT1 (human oligopeptide transporter) and plasma levels of valacyclovir, but not peptide transporter 1 [PEPT1 (*SLC15A1*)] or any of the other investigated intestinal organic anion or cation transporters [115].

After hepatic metabolism, both drugs are mainly renally excreted by both glomerular filtration and renal tubular secretion, most likely by OAT1 and OAT3 [116, 117]. In preterm and term neonates (n=28, median age 30 weeks of gestation), the intravenous acyclovir bodyweight-corrected clearance showed a twofold increase from 25 to 41 weeks of gestational age [118]. In children 1 month to 5 yearsold with or at risk for herpes infection, CL/F of valacyclovir (mL/kg/min) in those younger than 3 months was 50 % of that in older children, in whom bodyweight-corrected clearance remained stable [119]. A recent study showed markedly increased acyclovir concentrations when co-administered with benzylpenicillin, which was shown to be due to OAT3 and possibly OAT1 inhibition [116]. This could be a very relevant interaction in septic newborns, who often receive both drugs, as increased acyclovir concentrations are associated with neurological adverse events as well as neutropenia.

16. Adefovir – OAT1 and ABCC4

Adefovir, the antiviral agent used for treatment of hepatitis B virus or HIV, is 45 % renally cleared through glomerular filtration and OAT1/ABCC4 renal tubular secretion [120, 121]. In 45 children in three age groups (2-6, 7-11, and 12-17 years) receiving oral treatment for hepatitis B virus with the prodrug adefovir dipivoxil bodyweight-corrected mean apparent clearance and renal clearance were decreasing with increasing age [122]. Similarly, in a phase I study in children (n=13, age range 6 months to 18 years) receiving oral

adefovir dipivoxil for HIV treatment, systemic exposure was lower in children younger than 5 years [123].

SUMMARY AND DISCUSSION

In summary, ex vivo, pharmacokinetic and pharmacogenetic studies suggest transporter-specific changes from the human fetus to the adult. At this time, data are very scarce and the impact of these changes on drug therapy in children is still largely unknown. However, despite data scarcity, our review may aid clinical pharmacologists and clinicians in rationale drug prescribing of the drugs presented, not only by showing how pharmacokinetics are probably similar in certain pediatric age groups compared to adults, but also by pointing out where potential age-related changes in individual transporters could impact the drug's efficacy and safety. It broadens our views on ontogeny of transporters by evaluation of the results of pharmacokinetic and pharmacogenetic studies on relevant transporters. Moreover, this review presents clear information gaps, which may guide future research efforts to elucidate the role of human membrane transporters in the developing child.

For most drugs, the in vivo data to support the ex vivo data in understanding the maturation of individual transporters are limited to older children, and, hence, their usefulness is limited. No clear transporter maturation pattern can be deducted from any of the available pharmacokinetic studies in children. This contrasts with our knowledge from individual DMEs. For example, using midazolam as phenotyping probe, the developmental expression of CYP3A4/5 from the preterm neonate to the adult has been extensively characterized [124, 125] or amikacin clearance to display the maturation of GFR in neonates [125]. Specific phenotyping probes to study individual membrane transporters are suboptimal and have only been validated in adults. For many drugs, multiple transporters are involved in their uptake and excretion, which in turn may also compensate for changes in individual transporter activity. In adults, knowledge has also been gained from pharmacogenetic and drug-drug interaction studies.

This review shows that pharmacogenetic variation in membrane transporter activity also impact drug disposition, effect and toxicity in children. Most pharmacogenetic studies in children are in line with adult data. However, these similar pharmacogenetic relationships should be interpreted with care, especially when it comes to translating these data across the whole pediatric age range. First, most pharmacogenetic study cohorts only contain older children, whereas pharmacokinetic studies in neonates and infants often show clear developmental changes up to 4-6 years of age. Hence, the impact of SNPs may be obscured by more prominent changes due to growth and development. Second, the relationship between *SLCO1B1* SNPs and pravastatin disposition in

adolescents was found to be the opposite of that in adults. If these results are validated in other studies, a potential impact of hormonal changes on individual transporters needs to be elucidated; this may also provide insight into the physiological role these transporters play during adolescence.

The limited data from ex vivo studies of postmortem or surgical samples support the notion of membrane transporter-specific maturational patterns. It is still difficult, however, to determine definitive patterns for the different transporters. One of the reasons for this is that most studies only used limited samples and limited age ranges. Most fetal and neonatal studies only applied immunohistochemistry or mRNA techniques, while the more quantitative protein expression data are mainly from older children, above the age range in which most developmental changes can be expected. In addition, the quality and interpretation is further challenged as the exact origin, handling, and storage of tissues, including detailed patient characteristics and exact procurement site from organ (e.g., where in the intestine?), is often unknown.

The mechanisms underlying maturational changes in transporters are largely unknown. Recent studies on the CYP3A4 show maturational changes in methylation patterns to mirror the maturational expression of CYP3A4, which may point towards similar mechanisms for the transporters [126].

Differences between ethnic groups in DME abundance or ethnicity have been described, even in newborn infants [77, 85, 127]. Like in DMEs, it is credible to believe that ethnicity might have an effect on transporter activity or abundance. Nevertheless, for 27 drug transporters in 95 pathologically normal kidney samples, the expression did not differ between European Americans or African Americans [128].

The interpretation of pharmacokinetic studies, to understand maturation of transporters, is complicated by the fact that these transporters are part of the larger system of the ADME processes involved in the disposition of drugs. In contrast to DMEs, where clearance to a specific metabolite can be estimated to understand the DME maturation, studying a single transporter is more difficult. One may not be able to pinpoint one specific membrane transporter involved, and if the dominant transporter is still immature early in life, other transporters may compensate, thereby obscuring individual transporter maturation.

FUTURE DIRECTIONS

Several approaches are needed to increase our understanding of membrane transporters in the fetus and child (Table 3). First, the impact of transporter maturation on efficacy and toxicity in daily clinical care needs to be elucidated.

Table 3. Approaches to future transporter studies

LC-MS liquid chromatography-mass spectrometry, *PD* pharmacodynamics, *PGx* pharmacogenetic, *PK* pharmacokinetic

Ex vivo research
- Build multidisciplinary research teams for tissue collection and study design, e.g., surgeons, pathologists, clinical study staff, basic researchers
- Use optimal age distribution, e.g., tissues samples from fetuses, neonates and young infants, where most developmental changes can be expected, as well as sample number to ensure adequate power to detect age-related changes
- Establish high-quality tissue collections with detailed tissue handling description and detailed description of patient characteristics
- Use protein quantification techniques (e.g., LC-MS) and develop tools to study activity with minimal human tissue amounts
PK studies
- Phenotyping studies with drugs that are clinically used, and consider microdosing studies
- Blood sampling at similar times as clinical blood draws (opportunistic sampling)
- Perform population PK analyses
- Design drug-drug interaction studies
PGx studies
- In pediatric populations, test genetic variants in transporters known to affect PKs or PDs in adults
- Take into account PGx variation in affected drug-metabolizing enzymes as added variants
- Include relevant age ranges: e.g., younger children and neonates
- Design adequately powered studies

Pharmacogenetic studies can be a powerful tool to this aim, provided they have adequate power and validation cohorts, the lack of which is a major limitation of currently published studies. Studies need to not only be powered to study the impact of a single SNP in one transporter gene, but at the least the interaction with age should be part of their designs. Preferably such studies are designed to study the disposition of a drug in the context of systems pharmacology, also including SNPs in other relevant pharmacokinetic and/or pharmacodynamic genes, as well as pharmacokinetic sampling enabling the separation of different excretion pathways (e.g., GFR vs. tubular secretion vs. bile secretion vs. metabolism). In addition to pharmacogenetic studies, well-designed studies reflecting clinical drug-drug interaction scenarios may improve our understanding of transporter maturation, such as pharmacokinetic studies in which patients' samples are taken before/during/after co-medication with a potentially interacting drug. For example, the carvedilol-digoxin study [39] or the older cimetidine studies in neonates in which renal clearance by glomerular filtration could be separated from renal tubular secretion [94], have, by their design, provided support for age-related differences in specific renal transporters. We also noted a specific lack of in vivo data from younger children.

Ethical challenges have limited studies in infants and neonates. However, many drugs in our overview are regularly prescribed, even for these young children. Therefore, opportunistic sampling or biobanking of left-over samples from children who take these drugs for therapeutic reasons may aid to overcome these ethical barriers.

At this time, endogenous markers to phenotype the activity of individual transporters are lacking. With the increased availability of metabolomics, specific metabolites or metabolite ratios may be identified to reflect transporter activity in vivo. A recent GWAS-metabolomics study detected specific metabolites/metabolite ratios for selected transporters such as OCT1. [129-133]. The feasibility of this approach was recently shown for CY2D6 phenotyping in children [129]. To design drug-dosing regimens in children, increasingly, population PBPK models are being used [134]. Modeling and simulation can be used in different ways to increase our understanding and to design dosing regiments. Using a systems approach, modeling of the disposition of a substrate model drug, can result in a mathematical description of maturation of the specific transporter. This maturational description can then be used to simulate dosing guidelines for other transporter substrates. The feasibility of this approach has been used to describe maturation of selected DMEs and GFR clearance [133, 135]. Secondly, PBPK modeling, which incorporates available drug property and physiological information, could be used to simulate the impact of maturation of specific transporters, preferably with actual ex vivo data on transporter expression/activity [134]. An example is a mechanistic PBPK model to predict morphine levels in breast-fed neonates of codeine treated mothers [136]. A major limitation of these models is the lack of high quality ex vivo data on transporter activity across the pediatric age range and the lack of in vivo validation of these models.

In the design of new studies the following issues should be considered. The collection of these data can only be achieved by an international effort to collect high-quality tissue in collaboration with surgeons, pathologists, ethicists, clinical researchers, and experts in drug transporter research. The limitations of current tissue collections have been described here and good protocols for tissue collection, preferably in the context of internationally accessible biobanks, should be developed. Newer laboratory techniques should be strongly considered to minimize tissue amounts needed, for example, for laser capture and LC-MS/MS to determine protein abundance. Moreover, a multi-omics approach, including not only genomics but also transcriptomics, proteomics, metabolomics, and microbiomics, may provide greater power to predict drug efficacy and adverse drug reactions [137, 138]. Also, with the fast developments in tissue engineering, the current ethical and practical issues regarding tissue sampling and storage could be overcome using pediatric-engineered tissues. This may even enable transporter activity studies, which are now not available in pediatric tissue.

Additional Supporting Information may be found in the online version of this article
Appendix 1 Literature search strategy

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Part II

Membrane transporters

6 Ontogeny of human hepatic and intestinal transporter gene expression during childhood: age matters

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Drug Metabolism and Disposition 2014, 42:1268-1274

ABSTRACT

Introduction

Many drugs prescribed to children are drug transporter substrates. Drug transporters are membrane-bound proteins that mediate the cellular uptake or efflux of drugs, and are important to drug absorption and elimination. Very limited data are available on the effect of age on transporter expression. Our study assessed age-related gene expression of hepatic and intestinal drug transporters.

Methods

Multidrug resistance protein 2 (MRP2), organic anion transporting polypeptide 1B1 (OATP1B1), and OATP1B3 expression was determined in postmortem liver samples (fetal *n*=6, neonatal *n*=19, infant *n*=7, child *n*=2, adult *n*=11) and multidrug resistance 1 (MDR1) expression in 61 pediatric liver samples. Intestinal expression of MDR1, MRP2, and OATP2B1 was determined in surgical small bowel samples (neonates *n*=15, infants *n*=3, adults *n*=14). Using real time reverse-transcritption polymerase chain reaction, we measured fetal and pediatric gene expression relative to 18S rRNA (liver) and villin (intestines), and we compared it to adults using the $2^{-\Delta\Delta Ct}$ method.

Results

Hepatic expression of MRP2, OATP1B1, and OATP1B3 in fetuses, neonates and infants was significantly lower than in adults. Hepatic MDR1 mRNA expression in fetuses, neonates and infants was significantly lower than in adults. Neonatal intestinal expressions of MDR1 and MRP2 were comparable to those in adults. Intestinal OATP2B1 expression in neonates was significantly higher than in adults.

Conclusion

We provide new data that show organ- and transporter-dependent differences in hepatic and intestinal drug transporter expression in an age-dependent fashion. This suggests that substrate drug absorption mediated by these transporters may be subject to age-related variation in a transporter dependent pattern.

INTRODUCTION

Drug transporters are membrane-bound proteins whose primary function is to facilitate the trafficking of drugs and their metabolites across a biologic membrane. Expressed in structural cells that compose the organs of importance to drug disposition, such as enterocytes and hepatocytes, they are involved in the active uptake and elimination of orally administered drugs. In adults, drug transporters are recognized as key determinants of variation in the pharmacokinetics of many drugs, as shown in studies using primary cell and ex vivo organ cultures as well as clinical studies [1]. In contrast, such data in children are scarce and clinical studies are absent [2-7].

Pharmacokinetics in children is known to be affected by developmental changes with age [8]. Drug metabolizing enzyme activity, for example, changes significantly from fetal to adolescent age associated with alterations in metabolic clearance of many drugs [9]. Notably, hepatic and intestinal expression of cytochrome P450 subfamily CYP3A4 shows a clear developmental pattern, and systemic clearance of midazolam, a known substrate of CYP3A, changes with age [10-12]. Similarly, compared with adults, differences in body composition and renal function in children affect drug tissue distribution and renal drug excretion, respectively.

As a consequence, it has become apparent that simply extrapolating drug doses from adults to children based on body weight is likely inaccurate owing to our lack of our understanding of age-dependent differences in the maturation of the drug absorption and elimination processes involved in drug disposition. As the expression of drug metabolizing enzymes appears to be age-related, it is also likely that this is the case for drug transporter expression. Data from animal studies report occurrence of transporter-specific maturation [13]. In humans, several efflux transporters that belong to the ATP-binding cassette family (ABC), including the multidrug resistance protein 1 (MDR1/ABCB1), multidrug resistance-associated protein 2 (MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2), were found to be expressed in fetal liver or intestine, but otherwise little is known of the developmental patterns of individual drug transporters [6, 14, 15]. Systematic characterization of age-related differences in transporter expression will aid the selection of the most appropriate dose for substrate drugs in children.

In a first step towards elucidating the developmental changes, this study determined the gene expression of clinically relevant hepatic and intestinal drug transporters across the pediatric age range. We focused on transporters with well-defined roles in drug pharmacokinetics in adults [1] (Fig. 1). These included the efflux carriers MDR1 and MRP2 in liver and intestine, and uptake carriers of the organic anion transporting polypeptide (OATP/solute carrier organic anion [SLCO]) family, including the liver-specific transporters OATP1B1 and OATP1B3, and OATP2B1 in the intestine.

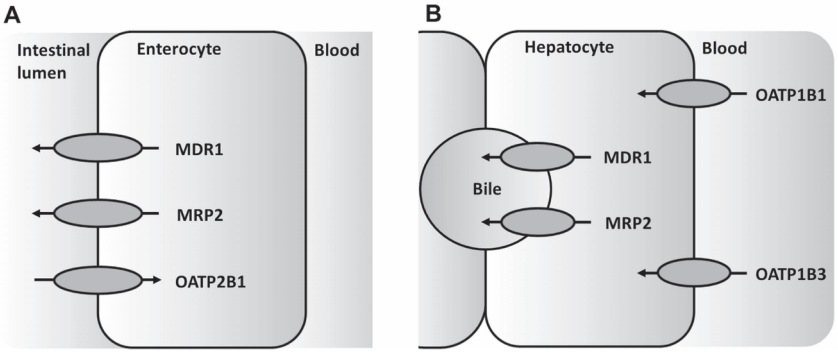

Figure 1. Transporters in enterocyte and hepatocyte.
Only transporters in enterocyte (A) and (B) hepatocytes were selected for this study.

MATERIALS AND METHODS

1. Tissue samples

Autopsy liver tissue samples from fetuses and from children up to 18 years of age were obtained from the Erasmus MC Tissue Bank. An opt-out clause was in place for use of tissue from the tissue bank. The Erasmus MC research ethics board provided a waiver for ethics approval according to the Dutch Law on research in humans. Fresh intestinal tissue was collected during surgery at the time of resection. Intestinal samples from children were derived from other research projects, and informed consent was previously obtained for the use of this tissue in the context of these studies approved by Erasmus MC research ethics beoard. After resection, all liver and intestinal tissues were immediately snap frozen in liquid nitrogen, stored at −80°C and processed on ice for mRNA isolation.

Healthy human adult liver samples were obtained from the Liver Tissue Cell Distribution System at the University of Minnesota under National Institues of Health (NIH) contract N01-DK-7-0004/HHSN267200700004C. Adult intestinal samples were obtained from the University of Western Ontario, and the use of these samples was approved by the research ethics board.

Additional pediatric tissue specimens for MDR1 analyses were obtained from the NIH-supported tissue programs: the Liver Tissue Cell Distribution System (LTCDS) from the Minnesota and Pittsburgh collection centers; the University of Maryland Brain and Tissue bank for Developmental Disorders (Baltimore, MD); and the Laboratory of Developmental Biology at the University of Washington (Seattle, WA). Additional samples were from Xenotech, LLC (Lenexa, KS). The use of these tissues was declared nonhuman subjects research by the Children's Mercy Hospitals and Clinics pediatric institutional review board.

The sources of the obtained samples are listed in Table 1.

2. mRNA Isolation and cDNA synthesis

Frozen human tissue samples were mechanically homogenized on ice. Homogenate was applied to a QiaShredder column (Qiagen) and RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the instructions provided by the manufacturer. RNA was treated with DNase to digest genomic DNA remnants. The quantity and integrity of RNA were determined by microfluidics-based analyses using the 2100 BioAnalyzer (Agilent, Santa Clara, CA). Samples with an RNA integrity number (RIN) of <5 were excluded from this study. Complementary DNA (cDNA) was synthesized using the High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and random priming.

3. Real Time Reverse Transcription Polymerase Chain Reaction

The mRNA expression was measured by SYBR green quantitative real-time polymerase chain reaction with the ABI 7500 sequence detection system (Applied Biosystems). Previously optimized primers were used (Supplemental Material) [16]. Modified primers were used for villin and OATP2B1 (reverse primer only), and primer sequences were obtained via qPrimer Depot (http://primerdepot.nci.nih.gov/). After each polymerase chain reaction, a melting curve analysis was performed to confirm product specificity. Eukaryotic 18S rRNA was assessed as the endogenous control with the use of a TaqMan VIC/MGB probe (4319413E, Applied Biosystems). Transporter transcript levels were normalized to18s rRNA, and relative expression was determined using the $2^{-\Delta\Delta Ct}$ method comparing pediatric samples to adult liver or intestine (calibrator samples) [17]. The adult target gene value was determined by using the median of the adult threshold cycle (Ct) values.

For comparison, all 96-well plates analyzing liver samples included a specific liver control sample to normalize expression for the gene of interest and 18S rRNA, if appropriate. Similarly, a specific intestinal sample was added to plates to normalize for the genes of interest, 18S rRNA, and villin if appropriate.

4. Statistics

All data were analyzed by Kruskal-Wallis analysis, followed by group comparisons using a nonparametric Mann-Whitney test and post-hoc Bonferroni multiple comparison test. All statistical analyses were performed using GraphPad Prism version 5.00.2 and IBM SPSS Statistics software (SPSS Statistics for Windows, Version 21.0; IBM, Armonk, NY). $P<0.05$ was considered statistically significant. Data are presented as median and range, unless indicated otherwise.

RESULTS

Sample characteristics

Characteristics of all samples are provided in Table 1. Forty-one liver samples from anonymous donors were collected from the Erasmus MC Tissue Bank. Seven samples were excluded because of poor mRNA quality, with 34 samples remaining for analysis. The causes of death were the following: congenital disorders (*n*=9), [including hydrops fetalis (*n*=2), ruptured giant omphalocele, hemangioendothelioma, extended congenital abnormalities, trisomy 7 or 8, cardiac, osteogensis imperfecta], cardiac failure (*n*=6), respiratory disorders (*n*=6), sepsis (*n*=4), gastrointestinal disorders (*n*=4), neurological disorders (*n*=3), and unknown causes (*n*=2). Adult liver samples (*n*=11) were derived from histologically normal parts of transplanted livers. Death was related to anoxia (*n*=5), trauma (*n*=3), neurologic disorders (*n*=2), or unknown cause (*n*=1), and ages were unknown. The RIN median of fetal, pediatric, and adult liver samples was 7 (range 5 - 10). The MDR1 mRNA expression was also measured in additional pediatric liver samples: three fetuses, 1 neonate, 4 children age 1 month to 12 months, and 17 children aged 12 months to 17 years. Thus, the total set of liver samples in which MDR1 mRNA expression was measured consisted of 9 fetal samples and 52 neonatal/pediatric samples. Cause of death of the additional pediatric donors was unknown.

Twenty-eight intestinal resection samples were obtained, of which 10 were excluded due to poor mRNA quality. The main reasons for resection were necrotizing enterocolitis and intestinal atresia (*n*=11); other reasons (*n*=7) were volvulus, persistent ductus omphaloentericus, Meckel's diverticulum, meconium peritonitis and ileostomy closure. Resection areas were aimed at the most proximal part of the intestine. Adult intestinal samples (*n*=14) were endoscopic biopsy samples and negative for any pathology. RIN median of pediatric intestinal samples was 7 (range 5 - 9). The RIN values of samples from adult intestinal donors were not measured. In pediatric and adult intestinal samples, 18S was significantly correlated with villin mRNA (*n*=32, r=0.6110, p<0.05). We also determined CYP3A4 gene expression. The mRNA expression of CYP3A4 is well reported in the literature. Patterns of intestinal and hepatic CYP3A4 mRNA expression were the same as those reported in the literature (data not shown) [3].

Information about medication use by the donors was not available.

Transporter-specific Ontogenetic Profile of MDR1, MRP2, OATP1B1 and OATP1B3 mRNA Expression in Liver

Hepatic transporter expression was significantly associated with age: MDR1 $H(4) = 35.3$, $P<0.05$; MRP2 $H(4) = 18.0$, $P<0.05$; OATP1B1 $H(4) = 27.4$, $P<0.05$; OATP1B3 $H(4) = 28.1$, $P<0.05$. Fetal, neonatal, and infant (up to 12 months of age) gene expression of all hepatic transporters was lower than in adults. In the age group 1 to 7 years, only two

Table 1. Tissue sources and age distribution

Tissue	Collection	Transporter examined	Number of samples	Sex	Gestational age at birth[a]	Postnatal age[b]	Source
Fetal and pediatric liver	Postmortem Autopsy	MRP2, OATP1B1, OATP1B3	Fetal: 6 Pediatric: 28 *Total: 34*	Male: 19 Female: 15	Fetuses: 22 (22-23) Pediatric: 36 (25-41)	Pediatric 1 wk (0 – 7 years)	Erasmus MC Tissue Biobank
Fetal and pediatric liver	Postmortem Autopsy	MDR1	Fetal: 9 Pediatric: 52 *Total: 61*	Male: 38 Female: 22 Unknown: 1	Fetuses: 22 (8-23)	Pediatric 11 wk (0-17 years)	1) Erasmus MC Tissue Biobank 2) The Liver Tissue Cell Distribution System; from the Minnesota and Pittsburgh collection centers; the University of Maryland Brain and Tissue bank for Developmental Disorders (Baltimore, MD); and the Laboratory of Developmental Biology at the University of Washington (Seattle, WA). Additional samples were from Xenotech, LLC (Lenexa, KS).
Adult liver	Biopsy	MDR1, MRP2, OATP1B1, OATP1B3	11	Male: 6 Female: 2 Unknown: 1	–	Unknown	The Liver Tissue Cell Distribution System; University of Minnesota under NIH Contract N01-DK-7-0004/HHSN267200700004C.
Pediatric intestine	Surgical jejunum 7 ileum 9 cecum 2	MDR1, MRP2, OATP2B1	18	Male: 12 Female: 6	Neonates: 32 (25-40)	1 (0 – 14) wks	Erasmus MC
Adult intestine	Biopsy ileum 14	MDR1, MRP2, OATP2B1	14	Male: 3 Female: 11	–	Unknown	University of Western Ontario

[a]median(range) in weeks; [b] median(range)

samples were assessed for the hepatic transporters MRP2, OATP1B1, and OATP1B3, as a result of which median mRNA expression could not be determined and comparison with adult expression was not possible.

MDR1 mRNA expression in fetal [0.061 (0.027 - 0.792)] and neonatal [0.036 (0.011 - 0.364)] age groups was 20- to 30-fold lower than in adults (Fig. 2). MDR1 mRNA expression in infants was slightly lower [0.248 (0.010 - 1.46)] than in adults (Fig. 2). Median MDR1 gene expression in children aged 1 to 7 years was not different from adults [0.777 (0.012 - 8.928); P=0.663]. Interindividual variability in all age groups was large.

MRP2 mRNA expression was about 30-fold lower in fetal liver samples [0.074 (0.004 - 0.162)], 200-fold lower in neonates [0.012 (0.001 - 0.238)], and 100-fold lower in infants [0.022 (0.008 - 0.357)] than in adult liver samples (Fig. 2). Compared with adult liver, OATP1B1 mRNA expression was 20-fold lower in fetal liver samples [0.042 (0.0004 - 0.683)], 500-fold lower in neonates [0.002 (0.0003 - 0.018)], and 90-fold lower in infants [0.010 (0.001 - 0.086)] (Fig. 2). Expression profiles of OATP1B3 in the liver samples were similar to those of OATP1B1. Expression of hepatic OATP1B3 was 30-fold lower in fetuses [0.046 (0.001 - 1.910)], 600-fold lower in neonates [0.002 (0.0004 - 0.0187)], and 100-fold lower in infants [0.010 (0.001 - 0.021)] than in adults (Fig. 2).

Similar MDR1 and MRP2 mRNA Expression in Neonatal and Adult Intestines, and Higher OATP2B1 in Neonatal Intestines

Intestinal transporter expression was statistically significantly associated with age for MRP2 $H(2) = 9.1$, $P<0.05$ and OATP2B1 $H(2) = 15.8$, $P<0.05$, but not for MDR1, $H(2) = 1.3$, P=0.5. MDR1 expression in intestinal samples of neonates was 0.838 (0.370 - 97.0; P=0.395) and of infants was 1.454 (0.409 - 3.174; P=0.413) and hence similar to adults (Fig. 3). Expression of MRP2 mRNA in the intestinal samples of neonates [1.262 (0.338 - 9.266); P=0.248] was similar to the adult expression (Fig. 3). In infant intestinal samples, however, MRP2 mRNA expression was significantly lower [0.038 (0.023 - 0.074); $P<0.05$] than in adult samples. Interestingly, the uptake transporter OATP2B1 showed a higher mRNA expression in neonates [2.86 (1.212 - 34.45); $P<0.0001$]. In infants up to 12 months of age, OATP2B1 was expressed at the same level as in adult intestinal samples [1.21 (0.328 – 2.288); P=0.147] (Fig. 3).

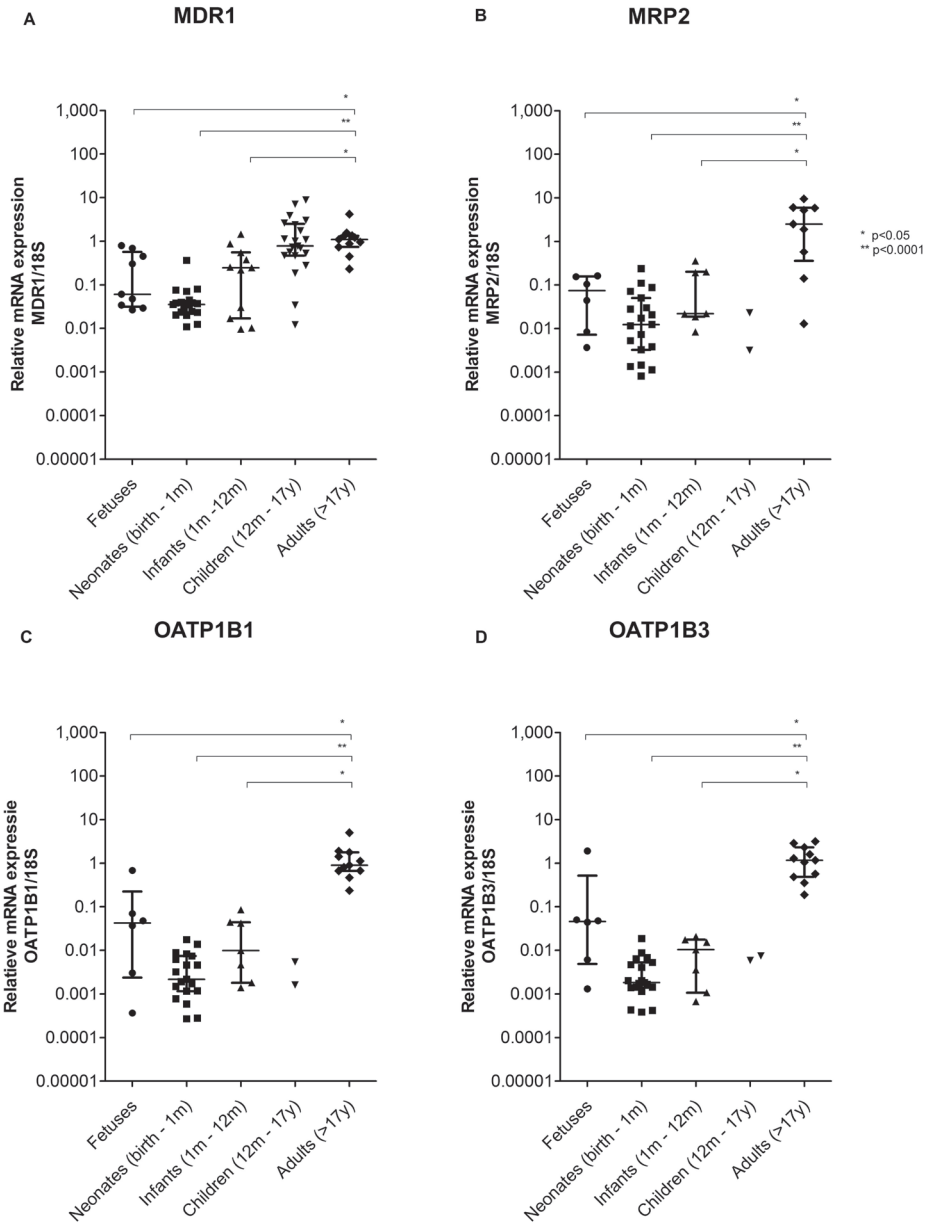

Figure 2. Transporter gene expression in liver.

Relative mRNA expression of MDR1 (A) MRP2 (B) OATP1B1 (C) OATP1B3 (D) from fetal, pediatric and adult liver samples, normalized to 18S mRNA expression and adult expression using the $2^{-\Delta\Delta Ct}$ method. Lines represent median and interquartile range.

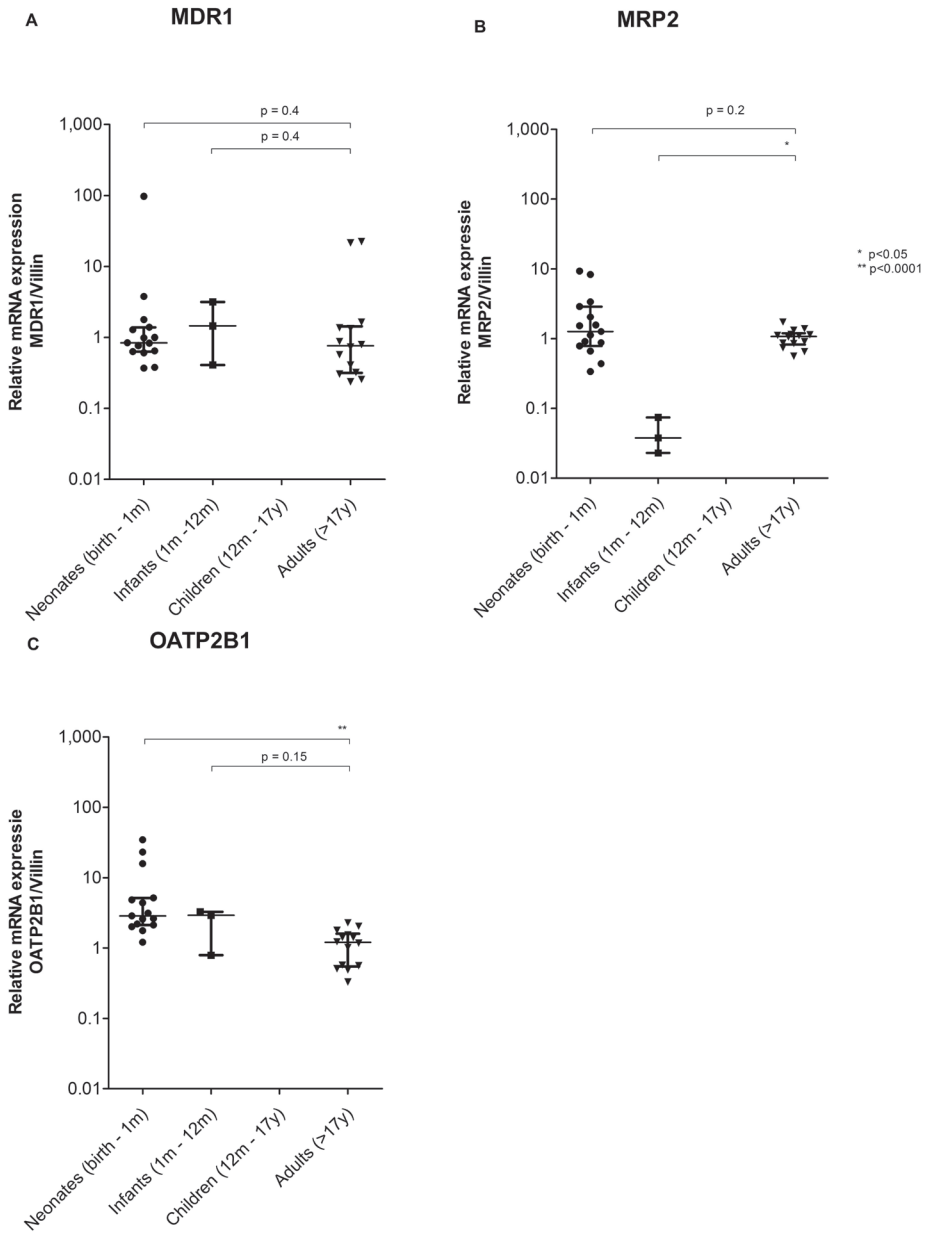

Figure 3. Transporter gene expression in intestine.
Relative mRNA expression of MDR1 (A) MRP2 (B) OATP2B1 (C) from pediatric and adult intestine samples, normalized to villin mRNA expression and adult expression using the $2^{-\Delta\Delta Ct}$ method. Lines represent median and interquartile range.

DISCUSSION

Little is known about transporter gene expression in children. Our study used samples from fetal to adult age, to examine the gene expression of hepatic transporters MDR1, MRP2, OATP1B1, and OATP1B3; they were found to be age-related, as their expression was lowest in fetal and pediatric liver samples. In contrast, intestinal OATP2B1 expression in neonates was higher than in adults. Intestinal MDR1 and MRP2 expression was similar between neonates and adults.

Ontogeny in hepatic transporters

Hepatic MDR1 expression is already detectable in early human fetal life. The maturation pattern found in our study is consistent with previous data on hepatic MDR1 mRNA expression [3, 15, 18]. Our findings are also supported by immunohistochemical staining showing that MDR1 expression followed the architectural changes during fetal development [18]. We show that MDR1 expression changes after birth, with low mRNA expression until 12 months of age whereupon it increases to adult levels. In a recent study that used a novel liquid chromatography with tandem mass spectrometry method to estimate protein expression, from 7 years onwards, MDR1 was stable up to 70 years of age in a cohort of more than 50 patients [19].

Low fetal MRP2 expression in humans has been reported earlier in two small studies (*n*=10, 14-20 weeks' GA; and *n*=3, 20-21 weeks' GA) [5, 14]. In the latter, however, two of five control adult liver samples were from normal tissue in patients with hepatocellular carcinoma. Because organic cation transporter 1 (OCT1) expression has been shown to be lower in nontumor hepatocellular carcinoma liver than healthy liver, a similar phenomenon with other transporters cannot be excluded [20]. Using immunohistochemical staining, distinct canalicular expression of MRP2 in adult liver was observed but a fuzzier canalicular occurrence in fetal liver, which may suggest immaturity of the localization pattern [5]. In addition, fetal human liver tissues obtained at 19 weeks' gestational age showed higher MRP2 signal intensity than tissues obtained at 14 weeks [21] In our study, neonatal MRP2 gene expression was lower than in adults. From 7 years onwards, MRP2 protein expression was stable. [22].

The observed lower fetal OATP1B1 and OATP1B3 mRNA expression levels in the present study are also in line with human data derived of three fetal (21-23 weeks' gestational age) and three adult livers [14]. In our study, OATP1B1 and OATB1B3 expression was also low in neonates. In contrast, the reported neonatal OATP1B1 and OATP1B3 protein expression (*n*=5), was similar in adults [23]. In 80 human pediatric liver samples ranging from age 2 months to 12 years, OATP1B1 was low until 6 years of age, whereas OATP1B3 protein levels were high at birth then rapidly decreased in the first year to then increase again from 8 years of age onwards to adult levels [24]. From 7 years onwards, OATP1B1

and OATP1B3 protein expression was stable, with only genotype related to OATP1B1 abundance [19].

Ontogeny in Intestinal Transporters

We observed stable intestinal MDR1 expression from neonatal to adult age, a finding that fills a gap between currently available fetal and pediatric data. Previously, fetal intestinal tissue obtained after induced abortion, showed no or minimal MDR1 mRNA expression in samples after 11, 13, and 14 weeks, but clear expression at 16 and 20 weeks' gestational age [18]. Another study reported similar small intestinal MDR1 mRNA expression in fetuses (14- 20 weeks' gestational age), neonates, and adults [3]. However, a limitation is the small sample size [*n*=5, n=12 (fetus plus neonates)] [3, 18]. Stable MDR1 mRNA expression in children from 1 month to adulthood was shown in nondiseased duodenal biopsy and jejunal tissue from liver donor recipients [2, 4]. We also found stable intestinal MRP2 expression but higher expression levels of OATP2B1 in neonates compared to adults.

The transporter maturation we observed likely reflects physiological roles of these transporters in organ development. Epigenetic mechanisms such as DNA methylation are thought to be involved in the maturation of drug metabolizing enzymes. For example, DNA methylation seems to partly explain a developmental switch from expression of CYP3A7 to CYP3A4 in human fetal and adult liver [25]. Epigenetic mechanisms may be also involved in the developmental regulation of drug transporter expression, but studies are currently lacking.

Due to mediation by transcription factors (which in their turn can be induced by chemicals or pathological conditions), some transporters show relatively high whereas others show low expression [13]. For example, expression of the steroid- and xenobiotic-sensing pregnane X receptor (PXR) was shown to correlate with intestinal breast cancer resistance protein (BCRP), MRP2 and MDR1 mRNA in many different human tissues (e.g., intestine and liver) [3, 26, 27]. We may further speculate that the ontogeny of transporters is a combined function of developmental changes, genetic heterogeneity, and exposure to substrates (drug or environmental) that induce or inhibit expression and/or activity. For example, CYP1A2 and CYP3A4 activity, assessed by phenotyping caffeine and dextromethorphan, respectively, was shown to be affected by the type of infant feeding (breast milk or formula) [28]. Dietary supplements in infant formula decreased MDR1 expression in colon cell cultures [29]. Moreover, the effect of feeding is more variable when a child's diet is expanded, as specific nutrients can influence MDR1 expression [30, 31].

This study is one of the first exploratory studies characterizing developmental changes in hepatic and intestinal transporters. Some limitations of the data should be addressed. First, most fetal liver samples were obtained in the second trimester of gestation, thus

results might not be representative for the entire range of gestational age. Second, the variation in fetal transporter mRNA expression may be partly due to the small number of samples representing ages more distant from the median age. Given the small number per age group, the range of expression might not be representative for the entire population.

Third, all liver samples were collected within 24 hours after death, but the exact time is unknown. Still, RNA stability was minimized by RNA integrity determination.

Also, our samples may display disease-dependent variability in transporter expression. Inflammation has been shown to affect drug metabolism and transport. Decreased midazolam clearance in critically ill children is likely a result of reduced CYP3A activity [32]. Inconsistency has been reported from studies assessing the effect of proinflammatory cytokines on intestinal MDR1 expression in vitro and in animal models, but they suggest a trend towards decreased expression [33]. In children with Crohn disease, MDR1 expression was higher in both inflamed and nonnflamed duodenal biopsies compared with healthy age-matched controls [34]. Inflammation was also shown to affect the expression of other transporters in primary human hepatocytes (i.e., OATP1B1, OATP1B3, MDR1, MRP2), resulting in reduced mRNA expression, protein, and activity [35]. Thus, evidence exists that the potential effects of underlying disease should be considered when interpreting age-related changes in transporter expression.

Finally, transporter genetic polymorphisms likely contribute to the observed variability in gene expression [1]. Recently, Nies et al. and Prasad et al. showed that hepatic expression of OATP1B1, but not OATP1B3, is significantly affected by genetic variants [19, 36].

The observed age-dependent maturation and large interindividual variability in drug transporter expression may have implications for oral drug absorption and hepatic drug excretion. The question whether posttranscriptional modifications occur and if expressed mRNA level can be extrapolated to protein expression and ultimately in vivo activity remains to be answered [4, 11]. Human hepatic OATP1B1 and OATP1B3 protein expression showed a trend with high interindividual variability in the first year of life followed by lower variability until the age of 8 years, when variability increases again [24]. This typical age-related change in variability at younger age is also reflected by our findings on OATP1B1 and OATP1B3 mRNA expression. In contrast, others could not correlate protein levels with OATP1B1 or MDR1 mRNA expression [37]. Further protein expression and transporter activity studies are needed to confirm these mRNA expression results.

In conclusion, hepatic MDR1, MRP2, OATP1B1, OATP1B3 and intestinal MDR1, MRP2, OATP2B1 drug transporter expression show organ- and transporter-specific maturation patterns during childhood. Therefore, the disposition of drugs substrates for these transporters may be subject to age-related changes other than that in body size alone.

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7 Intestinal peptide transporter PEPT1 expression and tissue distribution across the pediatric age range

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Submitted for publication

ABSTRACT

Introduction

The intestinal influx oligopeptide transporter PEPT1 (*SLC15A1*) is best known for nutrient-derived di- and tripeptide transport. Its role in drug absorption is increasingly recognized. To better understand the disposition of PEPT1 substrate drugs in young infants, we studied intestinal PEPT1 mRNA expression and tissue localization across the pediatric age range.

Methods

PEPT1 mRNA expression was determined using real time RT-PCR in small intestinal tissues collected from surgical procedures (neonates, infants) or biopsies (older children, adolescents). PEPT1 mRNA relative to villin mRNA expression were compared between neonates/infants and older children and adolescents. PEPT1 was visualized in infant tissue using immunohistochemical staining. Other transporters (MDR1, MRP2 and OATP2B1) were also stained to describe the localization in relation to PEPT1.

Results

22 intestinal samples (n=20 neonates/infants, n=2 pediatric, n=4 adolescents) were analyzed. The young infant samples were collected at a median (range) gestational age at birth of 29.2 weeks (24.7 - 40) and postnatal age of 2.4 weeks (0 – 16.6). The PEPT1 mRNA expression of the neonates/infants was only marginally lower (0.8-fold) than the older children ($p<0.05$). Similar and clear apical PEPT1 and MRP2 staining, apical and lateral MDR1 staining, and intra-epithelial OATP2B1 staining at the basolateral membrane of the enterocyte was detected in 12 infant and 2 adolescent samples.

Conclusion

Although small intestinal PEPT1 expression tended to be lower in neonates than in older children, this difference is small and tissue distribution is similar. This finding suggests similar oral absorption of PEPT1 substrates across the pediatric age range.

INTRODUCTION

The influx oligopeptide transporter PEPT1 (*SLC15A1*) is a member of the solute carrier (SLC) superfamily and is situated on the apical membrane of the enterocyte. Its expression in the human adult jejunum is at least more than 2 to 10 times higher than that of other transporters such as from the ABC-transporter family (MDR1, MRP2, or BCRP) or from the solute carrier (SLC) family (OATP's) [1]. In histologically normal intestinal biopsies from 10 adults, PEPT1 was expressed most abundantly in duodenum and ileum with a mean relative mRNA expression of 4 compared to <0.5 in colon [2]. In 6 adult intestine organ donors the PEPT1 protein expression accounted for approximately 50% of the total expression of all transporter proteins in the small intestine. In colon, PEPT1 represents 5% of all transporter proteins [3].

PEPT1 is best known for its function as a nutrient-derived di- and tripeptide transporter, but may also have a role in (pro)drug transport because it is the most abundant peptide transporter in the gut and drug properties mimicking di- and tripeptides may allow uptake by the peptide transporter [4]. Its role as intestinal transporter has been demonstrated for several (pro)drugs. In PepT1 (orthologue of human PEPT1) knock-out mice, the small intestinal uptake of the pro-drug valacyclovir was attributed for 90% to the PepT1 transporter [5]. Consequently these mice had a delayed T_{max} and decreased C_{max} of acyclovir (active metabolite of valacyclovir) relative to wild type mice [6].In human adults, however, PEPT1 mRNA expression was not correlated with valacyclovir pharmacokinetic parameters, even though, in vitro, valacyclovir was a PEPT1 substrate [7]. This suggests that valacyclovir may be not be a PEPT1 substrate in human adults, or that PEPT1 mRNA expression may not correlate to PEPT1 activity. The role of PEPT1 in valacyclovir pharmacokinetics in humans may differ from that in animals and remains to be elucidated. Several β-lactam antibiotics appeared to be PEPT1 substrates in Caco-2 cells with varying affinities: e.g. ceftibuten, cyclacillin, cefadroxil, cefaclor, benzylpenicillin, and cephalexin. Their structure resembles the tripeptide structure with additional groups [8]. The ACE-inhibitor fosinopril is a PEPT1 substrate; other ACE-inhibitors might be substrates, although this needs to be confirmed [8].

Most drugs prescribed to children are administered orally. Some PEPT1 substrates are dosed to children even very early in life. Considering the wide age-related variation in the processes affecting oral drug absorption, including gastric pH, gastric motility and drug metabolizing enzyme activity, age-related changes in membrane transporters are also very likely [9]. Earlier we showed a transporter-dependent maturation in gene expression in young infants for MDR1, MRP2 and OATP2B1 [10], but overall data on the intestinal expression of membrane transporters during growth and development are very scarce [11].

To the best of our knowledge, studies on the development of intestinal PEPT1 in humans are even lacking. From a pharmacological point a view, it is important to elucidate

the development of PEPT1 expression for known substrates. But this may also be for the development of new drugs in which PEPT1 could enhance oral absorption.

PEPT1 developmental changes have been studied in several animal species. A developmental pattern of PepT1 mRNA and protein expression has been shown in the duodenum, jejunum, ileum and colon of rats [12]. In the small intestine of newborn rats the expression peaked 3-5 days after birth, after which it rapidly decreased and increased again by the time animals were weaning. The authors ascribed the increase postpartum to suckling. in another study in rats, PepT1 small intestinal mRNA expression was stable from postnatal day 4 till day 21, and then decreased from postnatal day 50 onwards [13]. In neonatal miniature pigs, PepT1 mediated dipeptide (^{3}H-glycylsarcosine) disappearance in the ileal segment was highest in the youngest age group (1 week) , but PepT1 expression in post-weaned pigs was higher than in sucklings [14]. These data suggest that in the first weeks of life, intestinal PEPT1 is important for nutritional intake and later for diet transition following weaning

Comparative mRNA expression of various peptide transporters in mice, rats and human adults shows a PEPT1 expression in all species, but expression levels varied in relation to that of other peptide transporters (HPT1, PEPT2) [15]. This suggests that animal data cannot be directly extrapolated to humans and that human studies are needed.

To our knowledge expression or activity of PEPT1 in human fetal or pediatric population has not been described thus far, let alone a developmental expression pattern. To better understand the disposition of PEPT1 substrate drugs in neonates and young infants, we aimed to compare intestinal PEPT1 mRNA expression and tissue localization in these age groups with those in older children and adolescents. To describe PEPT1 protein staining in relation to other transporters with known mRNA expression data, we also aimed to detect MDR1, MRP2 and OATP2B1 protein in intestine.

MATERIAL AND METHODS

1. Tissue samples

Intestinal tissue samples were obtained surgically at time of resection (neonates/infants/adolescents) or as biopsies during duodenoscopies (older children/adolescents). For mRNA isolation, post-resection, tissue was snap frozen in liquid nitrogen and stored at −80°C. For immunohistochemical analysis, tissue was immediately put in 4% formaldehyde in PBS and processed to paraffin cubes.

Collection of neonatal/infant intestinal tissue and the use of left-over material was approved by the "Central Committee of Research involving Human Subjects" (the Hague, Netherlands) [16]. The Erasmus MC research ethics board in two other protocols ap-

proved collection of intestinal residual tissue from adolescent patients and endoscopy biopsies of older children and adolescents.

Informed consent was obtained from all parents/care-givers and children older than 12 years of age for use of left-over tissue and clinical data.

2. Real-Time Reverse-Transcription Polymerase Chain Reaction (real time RT-PCR)

Isolation and cDNA synthesis has been previously described [10]. In brief, frozen tissue samples were mechanically homogenized on ice. RNA was extracted using the RNeasy Mini Kit (Qiagen). To digest genomic DNA remnants, RNA was treated with DNase. The RNA integrity numbers (RIN) of the samples were analyzed using the 2100 BioAnalyzer (Agilent, Santa Clara, CA), and a value <5 was considered poor quality and reason to discard the sample. The mRNA expression was measured by SYBR green quantitative real time RT-PCR with 7900 Sequence Detector (Applied Biosystems, ABI prism).

Primers were used for PEPT1, villin and GAPDH, and sequences were designed using Oligo 6.22 software. Primer sequences were: GAPDH forward 5′-GTCGGAGTCAACG-GATT-3′, GAPDH reverse 5′-AAGCTTCCCGTTCTCAG-3′; villin forward 5′-TGCCAACAC-CAAGAGACT-3′, villin reverse 5′-TCCCAATCCAGAAGAAGAC-3′; PEPT1 forward 5′-TTG-GCCCAATGTCTCA-3′, PEPT1 reverse 5′-GGCCCTGCTTGAAGTC-3′. The melting curve was analyzed after every PCR to confirm product specificity. GAPDH and villin mRNA expression were used as the endogenous control. PEPT1 transcript levels were normalized to villin transcript levels (ratio PEPT1/villin), and relative expression was compared across the age groups (neonates/young infants vs children/adolescents)

3. Immunohistochemistry

Intestinal sections were dewaxed for immunohistochemistry (IHC), and endogenous peroxidases were quenched with 3% H_2O_2 in methanol for 20 min. Antigens were retrieved using Pepsin (0.1% in 0.01N HCL) pretreatment for 7 min at 37°C stove. The sections were blocked for 1h in 10% normal human serum and 10% normal rabbit serum diluted in Teng-T (10 mMTris (pH 8), 5 mM EDTA (pH 8), 0.15 M SodiumCl, 0.25% gelatin and 0.05% Tween-20). Primary antibodies goat anti-PEPT1 C-20 (Santa Cruz Biotechnology Inc., Heidelberg, Germany) were incubated over night at 4°C in 2% human serum. Immunoreactive sites were detected with biotinylated secondary rabbit anti-goat serum using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA), and 3,3 diaminobenzidine tetrahydrochloride (DAB) solution (Sigma-Aldrich, Zwijndrecht, Netherlands). The nuclei were counterstained with hematoxylin (Vector Laboratories). A negative control staining lacking the primary antibody was performed for every slide. A matched goat-antibody negative control was performed to assess background staining. Images were acquired and analyzed with Leica microscope and LAS-AF image acquisition software.

IHC was also performed, on the same tissue samples for three other membrane transporters with known intestinal mRNA expression data, MDR1, MRP2 and OATP2B1, to compare tissue distribution of PEPT1 with these transporters. In case of MDR1 and OATP2B1 staining, microwave pretreatment in citrate buffer (10 mM, pH6.0) was used to retrieve antigens. In case of MRP2, pepsin pretreatment, similar as for PEPT1, was used. Primary antibodies mouse anti-MDR1 and mouse anti-MRP2 were obtained from EMD Millipore, and rabbit anti-OATP2B1 from Abnova.

4. Statistical analysis

Data are presented as median and range, unless indicated otherwise. Group comparison (neonates/young infants – older children/adolescents) was made using nonparametric Mann-Whitney U test. All statistical analyses were performed using GraphPad Prism version 5.00.2 and IBM SPSS Statistics software (SPSS Statistics for Windows, version 21.0; IBM, Armonk, NY). The level of significance was set at $P<0.05$.

RESULTS

Descriptive results

Twenty-six samples (n=20 neonates/infants, n=2 children, n=4 adolescents) were collected (Table 1). The ages of the young infants ranged from gestational age at birth [median (range) GA] 29.2 weeks (24.7 - 40) and to postnatal age (PNA) 2.4 weeks (0 – 16.6). The main reasons for resection were stoma closure (in patients with history of necrotizing enterocolitis (NEC) (n=5), current NEC (n=6) and intestinal atresia (n=5). Other reasons were intestinal volvulus (=3), and Meckel's diverticulum (n=1). Samples of two children (9 and 10 years old) and two adolescents (16 and 17 years old) who underwent endoscopy for suspicion of inflammatory bowel disease, were histologically normal. The two samples for immunohistochemistry were collected from two children who underwent an ileocecal resection (age 15 years, history of Crohns disease, active disease at time of surgery) and ileoanal pouch surgery (age 17 years, history of ulcerative colitis, active disease at time of surgery), respectively.

mRNA was analyzed on samples of which snap frozen tissue was available; i.e. 17 young infant, 2 pediatric, and 2 adolescent samples. Immunohistochemistry was performed on samples of which paraffin-embedded tissue was available; i.e. 12 young infant and two adolescent samples (Table 1).

GAPDH mRNA strongly correlated with villin mRNA in all intestine samples (n=21, $r=0.6182$, $p<0.01$). One sample was excluded due to low mRNA expression of villin and GAPDH suggesting loss of enterocytes. Twenty samples remained for mRNA expression analysis.

No information on concomitant medications or nutritional intake was available.

Table 1. Patient characteristics

	IHC	mRNA	Gender	Ethnicity	Diagnosis	Gestational age at birth (weeks)	Postnatal age (weeks)	Pathology report	Resection area
1	*		Male	Caucasian	NEC And stoma closure	25.3	0.2 and 6.9	Necrotizing enterocolitis and stoma closure	Jejunum
2	*		Male	Caucasian	Stoma closure (history of NEC)	30.3	5.7	–	Ileum
3		*	Male	Caucasian	volvulus jejunum, malrotation	39.3	3.9	–	Jejunum
4	*		Male	Caucasian	Stoma closure (history of NEC)	25.6	16.6	–	Ileum
5	*	*	Male	Unknown	NEC	30.9	8.9	Resection stenosis with necrotizing enterocolitis	Cecum
6	*	*	Male	Unknown	jejunum atresia	35.7	0	stenosis	Jejunum
7		*	Male	Unknown	NEC in patient with complex cor vitium Death - Enterobacter-sepsis	33.0	0.9	NEC	Ileum
8	*	*	Female	Caucasian	NEC	26.9	2.7	NEC	Ileum
9	*	*	Male	Unknown	CHD complicated by volvulus and intestinal necrosis after repair hernia	36.9	2.0	ischemia	Jejunum
10	*	*	Female	Caucasian	NEC	26.4	2.0	NEC	Ileum
11		*	Male	Unknown	NEC	25.3	3.1	Ischemic enteritis	Jejunum
12		*	Female	Caucasian	Stoma closure (history of NEC)	24.7	9.57	–	Ileum
13		*	Male	Caucasian	midgut volvulus	28.1	3.86	Infarction and peritonitis	Ileum
14	*	*	Male	Unknown	Meckel's diverticulum	40.0	3.1	Meckel's diverticulum with ulcerations and normal small intestine	Ileum
15	*	*	Male	Caucasian	jejunum atresia	38.3	0.1	stenosis	Jejunum
16	*	*	Male	Caucasian	jejunum atresia	38.0	0.1	Small reactive changes	Jejunum
17		*	Male	Caucasian	jejunum atresia	0	0	Atresia with extensive reactive changes	Jejunum
18	*	*	Male	Unknown	Ileum atresia	38.9	0.1	Ileumatresia, no ischemia	Ileum

Table 1. Patient characteristics (continued)

	IHC	mRNA	Gender	Ethnicity	Diagnosis	Gestational age at birth (weeks)	Postnatal age (weeks)	Pathology report	Resection area
19		*	Female	Caucasian	NEC	24.9	1.1	NEC	Ileum
20		*	Female	Unknown	Stoma closure (history of NEC)	25.6	13.9		Ileum
21	*		Male	Caucasian	M.Crohn: active	*Adolescent*	15 years	*Ileocaecal resection*	Ileum
22	*		Female	Caucasian	Ulcerative colitis: active	*Adolescent*	17years	*Ileoanal pouch surgery*	Ileum
23		*		Caucasian	Biopsy in case of abdominal complaints; non-IBD	*Adolescent*	9 years	*Normal*	Ileum
24		*		Caucasian	Biopsy in case of abdominal complaints; non-IBD	*Adolescent*	10 years	*Normal*	Ileum
25		*		Caucasian	Biopsy in case of abdominal complaints; non-IBD	*Adolescent*	16 years	*Normal*	Ileum
26		*		Caucasian	Biopsy in case of abdominal complaints; non-IBD	*Adolescent*	17 years	*Normal*	Ileum

PEPT1 gene expression

PEPT1 mRNA was detected in all samples. The relative intestinal PEPT1 mRNA expression (PEPT1/villin) in young infants slightly varied (0.15-fold) (Fig 1). In the neonatal/infant group the PEPT1 expression was 0.8 fold lower than in the older age group (p=0.01), with median relative mRNA expression of 0.80 (range 0.77 – 0.92) and 1.02 (1.01 – 1.04), respectively.

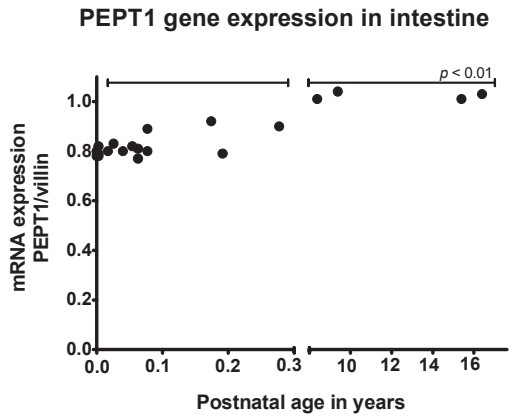

Figure 1. Relationship of age with intestinal PEPT1 gene expression
Relative mRNA expression of PEPT1 in relation to age normalized to villin mRNA expression.

Presence of PEPT1 protein in enterocytes

Clear PEPT1 staining was present at the apical membrane in the brush border of the enterocyte in all samples (Fig 2). Microscopically, PEPT1 staining intensity seemed to vary among samples, but we did not quantify staining. PEPT1 apical localization was similar in neonatal and adolescent intestinal samples. No PEPT1 staining was detected in Goblet cells, most likely due to the artificial effect of enlargement of Goblet cells during the process of paraffin embedding. No staining was observed at the basolateral membrane or at the tight junctions.

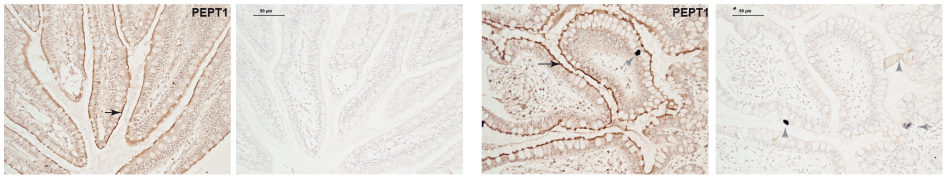

Figure 2. Age and PEPT1 protein localization in enterocyte.
Immunohistochemical detection of PEPT1 in paraffin-embedded intestinal sample. From left to right: pediatric intestinal sample, negative control; intestinal sample from adolescent; negative control. Apical staining of PEPT1 on enterocyte. Black arrows indicate PEPT1 detection, grey arrows indicate artifacts.

Presence of MDR1, MRP2 and OATP2B1 protein in enterocytes

MDR1 staining was visible at the apical and lateral surfaces of the enterocyte (Fig 3). The lateral MDR1 staining is clearly visible in figure 4. OATP2B1 staining was present intra-epithelial at the basolateral membrane (Fig 3). MRP2 was localized only in the brush border at the apical surface (Fig 3). Specific transporter staining was present in all neonatal, infantile and adolescent samples.

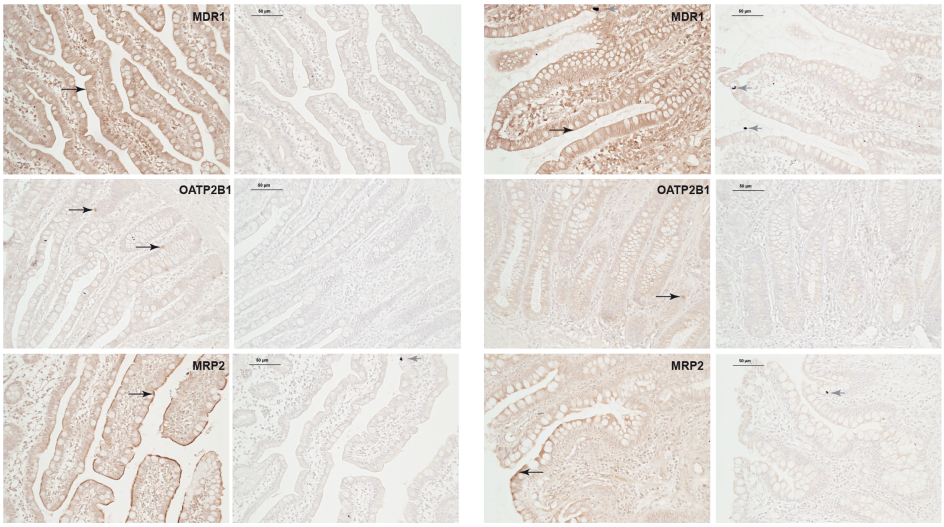

Figure 3. Age and MDR1, OATP2B1 and MRP2 protein localization in enterocyte.
Immunohistochemical detection of MDR1, OATP2B1, and MRP2 in paraffin-embedded intestinal samples. From left to right: pediatric intestinal sample, negative control; intestinal sample from adolescent; negative control. Apical and lateral MDR1 staining, intra-epithelial OATP2B1 staining at the basolateral membrane, and apical MRP2 staining in the enterocyte. Black arrows indicate transporter detection, grey arrows indicate artifacts.

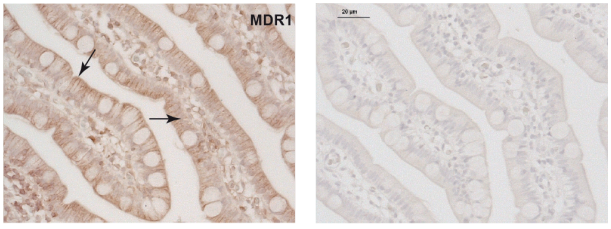

Figure 4. MDR1 protein localization in enterocyte.
Immunohistochemical detection of MDR1 in paraffin-embedded intestinal samples. From left to right: 40x magnification of pediatric intestinal sample. Clear apical and lateral MDR1 staining (black arrows). Grey arrows indicate non-specific signal.

DISCUSSION

PEPT1 mRNA expression and protein expression were found in neonatal and young infant intestinal tissues immediately postnatally. The PEPT1 mRNA expression in young children was slightly lower than in older children, although the clinical relevance of this difference is probably negligible. This study is the first to demonstrate intestinal gene expression of the PEPT1 transporter across the pediatric age range. The gene expression of PEPT1 in neonatal intestine samples was confirmed by immunohistochemical staining showing protein PEPT1 expression in the brush border of the enterocyte. Localization of PEPT1 in the apical part along the brush border of villus epithelial cells was comparable with staining in human adolescents (this study) and adults, rat and mice [17-20].

Based on the clear developmental expression patterns of intestinal drug metabolizing enzymes and hepatic transporters, with in general low expression at birth and increasing with postnatal age, we anticipated lower PEPT1 transporter expression in neonates [11]. However, our results suggest slightly lower PEPT1 expression and similar localization as in adolescents. Hence the uptake of PEPT1 substrates in neonates and young infants is likely not to be affected by growth and maturation and dose-adjustments for PEPT1 activity therefore do not seem necessary. Attention should lay at other factors that govern drug disposition in children, e.g. GFR or drug metabolism, do not seem necessary.

Stable mRNA expression of small intestinal MDR1 from neonatal age onwards was also found in other studies [10, 21-23]. In a previous study from our group, MRP2 mRNA expression in the small intestines was also stable during infant age, but OATP2B1 expression in neonates was about three times higher than in adults [10]. This suggests that intestinal membrane transporters show stable or higher expression during childhood and that a developmental expression might occur before birth. These findings are supported by our immunohistochemistry data, which show clear localization of PEPT1 and the other transporters studied. Immunohistochemical staining of MDR1, MRP2 and OATP2B1 was done to compare the localization of PEPT1 in relation to the other transporters. MDR1 was stained at the apical border of the enterocyte, similar to MDR1 staining in 59 duodenal biopsies from infants up to 7 years of age and from fetuses from a gestational age of 16 and 20 weeks [22, 24]. MRP2 apical staining matches to staining in human colorectal cancer tissue, as well as intestinal tissue from horse, rabbits and rats [25-27]. Intraepithelial OATP2B1 staining at the basolateral enterocyte border was similar to staining in human colonic biopsies from adults [28]. The localization corresponds with the function of MDR1, MRP2 and PEPT1 to facilitate uptake of substrates in the enterocyte, whereas OATP2B1 facilitates excretion from the enterocyte to blood [29].

Our data do not contradict previous data from juvenile animal studies. In rats, PepT1 expression was increased on days 3 to 5 after birth, after which it rapidly decreased

and then increased at the time animals were weaning [12]. If we would extrapolate this data to neonates, we would expect a PEPT1 elevation several days after birth, and time of weaning might be translated to infant age at the time of introducing food next to breastfeeding of formula. The slightly higher PEPT1 expression in older children and adolescents than in young infantss might be compared to weaned rats from the animal study. Still, a clinical impact of slightly lower infantile PEPT1 expression is questionable.

PEPT1 has been studied in relation to feeding and nutrition [30]. Interestingly, the PEPT1 transporter expression seems sensitive to nutritional status. In adult short-bowel syndrome patients, small intestinal and colonic mRNA expression of PEPT1 was upregulated compared to healthy controls [17]. Studies in several animal species have further explored the impact of nutrition on intestinal PEPT1 expression. After maternal overnutrition during pregnancy (but not after maternal undernutrition), PepT1 mRNA expression was significantly increased in jejunum of newborn or weaned piglets [31]. In contrast, maternal protein restriction in rats also led to higher duodenal PepT1 mRNA expression in 3- and 16-week-old offspring. Irrespective of feeding, PepT1 mRNA expression in 16-week-old rats was higher than in 3-week-old rats [32]. In another study in adult rats on a protein-rich diet, PepT1 mRNA and protein expressions, as well as transporter activity were increased [33]. In low-birth-weight piglets colonic PepT1 activity (measured by flux of cephalexin) was increased after high- instead of normal-protein formula feeding [34]. The effects of feeding and nutrition may in part explain the observed interindividual variability in PEPT1 gene expression in young infants in the present study. Unfortunately we have no detailed clinical nutritional data to prove this.

Several limitations of this study should be addressed. One, most samples were obtained during the first few weeks of life, and pediatric samples after the age of 4 months up to 9 years are lacking. Therefore the expression in this age range is still unknown. Second, variability in our samples might be due to co-medication, nutritional differences, disease or extent of inflammation. Still, most samples were obtained during surgery of stoma closure and therefore the intestinal tissue can be expected to be relatively normal. Third, variability might be due by genetic polymorphisms in *SLC15A1* gene causing a change in PEPT1 expression. Thus farno polymorphism has been proven to be clinically relevant, however, and therefore we did not genotype our samples [35].

In conclusion, although small intestinal PEPT1 expression was slightly lower in neonates than in older children, this difference is small and tissue distribution is similar. Therefore, this finding suggests similar oral absorption of PEPT1 substrates across the pediatric age range.

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8 The effect of age on human hepatic membrane transporter protein expression

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Submitted for publication

ABSTRACT

Introduction

Human hepatic membrane-embedded transporter proteins are involved in trafficking endogenous and exogenous substrates. Even though impact of transporters on pharmacokinetics is recognized, little is known on maturation of transporter protein expression levels, especially during early life. We aimed to study the protein expression of 10 transporters in liver tissue from fetuses, infants and adults.

Methods

Transporter protein expression levels (ABCB1, ABCG2, ABCC2, ABCC3, BSEP, GLUT1, MCT1, OATP1B1, OATP2B1, and OCTN2) were quantified using UPLC-MS/MS in snap-frozen post mortem fetal, infant and adult liver samples. Protein expression was quantified in isolated crude membrane fractions. The possible association between postnatal and postmenstrual age versus protein expression was studied.

Results

We studied 25 liver samples: 10 fetal [median gestational age 23.2 weeks (range 16.4-37.9)], 12 infantile [gestational age at birth 35.1 weeks (27.1-41.0), postnatal age 1 week (0-11.4)], and 3 adult. Four patterns emerged. ABCB1, OATP1B1, and OATP2B1 expression levels were stable from fetal to adult age. ABCC2, ABCC3, and BSEP were low in fetus and infant compared to adults, while ABCG2, GLUT1, OCTN2 protein levels were higher early in life than in adults. MCT1 expression levels were highest in infants compared to fetus and adult. These patterns were also found for postmenstrual age within the fetal/infant groups.

Conclusion

The impact of growth and development on human membrane transporter protein expression is transporter-dependent. The age-related differences in transporter protein expression aid our understanding of normal growth and development, and also may impact the disposition of substrate drugs in neonates and young infants.

INTRODUCTION

Membrane-embedded transporter proteins are involved in the transport of a myriad of endogenous and exogenous substances. These transporters can be found in various cell types, such as enterocytes, hepatocytes, brain and kidney cells. By trafficking exogenous substrates, they have an important impact on drug absorption, distribution and excretion of drugs and their metabolites, thereby determining drug efficacy and toxicity. In the hepatocyte, transporters have been identified on both basal and canalicular membranes regulating the uptake and excretion of drugs and their metabolites into and from the systemic circulation as well as excretion into bile [1].

The international transporter consortium has identified a large set of clinically relevant membrane transporters, subdivided in families with similar peptide sequences [2]. Transporters belonging to the ATP-binding cassette (ABC) family are multidrug resistance protein 1 (ABCB1/MDR1/P-gp), multidrug resistance-associated protein 2 and 3 (ABCC2/MRP2, ABCC3/MRP3), breast cancer resistance protein (ABCG2/BRCP), and bile salt export pump (ABCB11/BSEP). To the solute carrier organic anion (SLCO) family belong organic anion transporting polypeptide 1B1 and 2B1 (SLCO1B1/OATP1B1, SLCO2B1/OATP2B1), and are acknowledged to be the most clinically relevant. Other transporter proteins taken along in the current study are glucose transporter 1 (SLC2A1/GLUT1), monocarboxylate transporter 1 (SLC16A1/MCT1), and organic cation/carnitine transporter (SLC22A5/OCTN2) which belong to the solute carrier (SLC) family. This selection of transporters were chosen because, they have proven to be important in drug disposition in adults or have shown a large abundancy in human adult liver [3].

Children's growth and maturation importantly impact the processes involved in the disposition of drugs such as absorption, metabolism and renal excretion [4]. It is therefore likely that expression and activity of transporters are also subject to age-related changes, as has been convincingly shown for the drug metabolizing enzymes [5]. Indeed, animal studies have shown transporter-specific maturation profiles in transporter expression [1, 6]. For example, hepatic mRNA expression of mouse Oatps gradually increases after birth until adult levels at 6 weeks of age [7]. In spite of the maturation in animal studies, interspecies differences prevent us to translate animal maturation data to humans [8]. For humans, only few transporters have been studied and a clear information gap remains on the developmental trajectory of individual transporters in fetuses and young children and the related impact on drug disposition [6, 9]. Recently, we showed a transporter dependent maturation profile in mRNA expression of hepatic ABCB1, ABCC2, OATP1B1 and OATP1B3 [10]. To predict drug disposition on the basis of these results is a challenge since posttranscriptional variation may result in a discrepancy between mRNA and protein levels, as has been shown for some transporters in human adults [11, 12]. Protein expression data in children are limited. For example, a

selection of transporters (ABCC2, ABCG2, ABCB1, OATP1B1, OATP1B3 and OATP2B1) has been quantified in children using quantitative LC-MS methods, but the younger age range (<7 years of age) where most developmental changes occur was not included in these studies [12-14]. Moreover, other pediatric transporter protein data are derived from non-quantitative immunohistochemistry studies [6].

With the aim to further elucidate developmental changes in human membrane transporters, especially during early life, we conducted an exploratory study using quantitative LC-MS/MS to determine the hepatic protein abundance of a large selection of transporters in liver samples from fetuses, neonates and young infants and relate the findings to adults.

MATERIAL AND METHODS

1. Tissue samples

Post mortem liver tissue samples from autopsy of fetuses and infants were collected by the Erasmus MC Tissue Bank. Tissue was procured at the time of autopsy within 24 hours after death and snap-frozen at -80^0C for later research use. The Erasmus MC Research Ethics Board waived the need for ethics approval according to the Dutch Law on Medical Research in Humans. The tissue was collected after parental written informed consent was obtained for both autopsy and the explicit use of the tissue for research, in line with the Dutch guidelines on the secondary use of human tissue. Human adult liver tissue samples (n=3) were a kind gift from Prof. G.M.M. Groothuis, University of Groningen, The Netherlands. They were collected anonymously as surgical waste material from patients after partial hepatectomy because of liver metastasis.

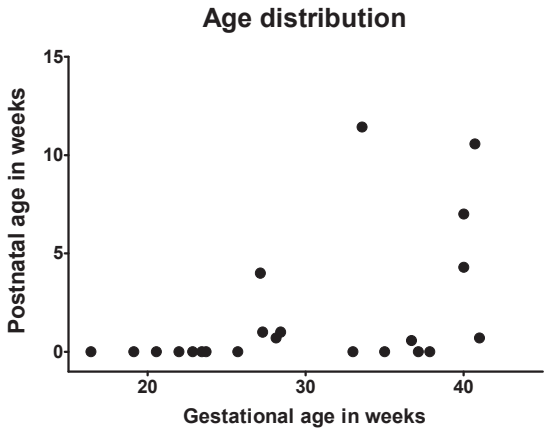

Figure 1. Age distribution in fetal and infantile samples.

The cohort of selected samples reflected a widespread gestational age, in second and third trimester of pregnancy, as well as postnatal age (up to three months) (age distribution is displayed in figure 1).

2. Membrane isolation and Liquid Chromatography-Tandem Mass Spectrometry

Isolation of crude membrane fractions from tissue samples was conducted as described by Wisniewski et al., with some minor adaptations [15]. In brief, approximately 10 mg of liver tissue was homogenized in a hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, and a cocktail of protease inhibitors containing 2 mM phenylmethylsulfonylfluoride, aprotinin, leupeptin, and pepstatin) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000*g* for 30 minutes at 4°C using a LE-80k Centrifuge with SW28 rotor (Beckman Counter, USA). The pellet, containing crude membrane protein fraction, was resuspended in 500 µL SDT-lysis buffer (0.1 M Tris pH 7.6, 0.1 M DTT, 4% SDS). Approximately 50 µL of cell lysate, corresponding to 1 mg tissue, was incubated at 95°C for 5 minutes and briefly sonicated on ice, mixed with 200 µL of 8 M urea and loaded onto a 30K Amicon Ultra-0.5 centrifugal filter device (Millipore, Darmstadt, Germany) and centrifuged at 14,000*g* for 15 minutes. After adding another 200 µL 8 M urea the tubes were centrifuged again at 14,000*g* for 15 minutes. Subsequently, 100 µL of 0.05 M iodoacetamide in 8 M urea in 0.1 M Tris-HCl, pH 8.5 was added and the samples were incubated for 20 minutes at room temperature, and centrifuged at 14,000*g* for 15 minutes. The resulting concentrate was diluted with 100 µL 8 M urea in 0.1 M Tris-HCl, pH 8.5 and centrifuged (14,000*g* for 15 minutes) again. This step was repeated twice. Subsequently, 100 µL 0.05 M ammoniumbicarbonate was added to the concentrate and centrifuged (14,000*g* for 15 minutes). This step was also repeated twice. Proteins were digested into peptides by incubating the samples overnight at 37°C in a 0.05 M ammoniumbicarbonate solution containing 0.5 µg/µL trypsin. The digests were collected into a new collection tube by centrifugation (14,000*g* for 10 minutes,) and the filter device was rinsed with 50 µL 0.5 M NaCl and centrifuged (14,000*g* for 10 minutes). The combined filtrate was acidified with 25 µL 0.1% CF_3COOH, desalted on a 7 mm/ 3 mL extraction disk cartridge (3M Empore, Zoeterwoude, The Netherlands) and eluted with 70% acetonitrile. Subsequently, the samples were vacuum dried (SpeedVac) until the solution was vaporized and stored < −70°C until further analysis.

All samples were analyzed using a UPLC coupled to a 6500 QTrap mass spectrometer (AB Sciex). The crude membrane fractions were injected on an Acquity C18 BEH UPLC column and separated using gradient elution. During analysis, the column was maintained at 50°C and the samples were kept at 10°C. The mass spectroscope was operated in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 3.3 kV, a source temperature of 150°C, and a desolvation tempera-

Table 1. Multiple reaction monitoring (MRM) transitions of the used peptides and the corresponding internal standards (AQUA)

Name	Labelled	Peptide sequence[a]	Molecular weight	Q1	Q3-1	Q3-2	Q3-3	Q3-4
ABCB1	unlabeled	NTTGALTTR	934.0	467.7	719.4	216.1	618.4	
	AQUA	NTTGA**L**TTR	950.0	471.2	726.5			
ABCC2	unlabeled	VLGPNGLLK	910.1	455.8	698.5	185.3	213.3	
	AQUA	VLGPNGL**L**K	926.1	459.2	705.4			
ABCC3	unlabeled	ALVITNSVK	944.1	472.8	760.4	661.4	548.4	
	AQUA	ALVITNS**V**K	950.1	475.8	766.5			
ABCG2	unlabeled	SSLLDVLAAR	1.044.2	522.8	644.3	757.5	529.4	
	AQUA	SSLLDV**L**AAR	1.060.2	526.3	651.3			
BSEP	unlabeled	STALQLIQR	1.029.2	515.3	657.4	841.6	529.4	
	AQUA	STALQ**L**IQR	1.045.2	518.8	664.3			
GLUT1	unlabeled	VTILELFR	990.2	495.8	790.5	677.4	201.2	
	AQUA	VTILEL**F**R	1.000.2	500.8	800.5			
MCT1	unlabeled	SITVFFK	841.0	421.2	173.2	641.3	201.1	
	AQUA	SITVF**F**K	851.0	426.2	651.3			
OCTN2	unlabeled	WLISQGR	859.0	430.2	560.3	272.2	300.2	
	AQUA	WL**I**SQGR	875.0	433.7	567.3			
OATP1B1	unlabeled	LNTVGIAK	815.0	408.2	399.4	588.3	228.2	702.3
	AQUA	LNTVG**I**AK	831.0	411.7	402.9			
OATP2B1	unlabeled	SSISTVEK	849.9	425.7	563.3	676.3	175.1	
	AQUA	SSIST**V**EK	855.9	428.7	569.3			

*[a] AQUA: Amino acid presented in **bold** is labelled with ^{13}C and ^{15}N.*

ture of 600°C. Cone voltage and collision energy were optimized for each compound individually. The multiple reaction monitoring transitions were determined from tandem mass spectra obtained by direct infusion of 0.5 µg/mL. Per peptide three transitions were chosen (Q3-1, Q3-2 and Q3-3) for quantitation and confirmation. The transitions for the different proteins are listed in Table 1. A peptide labeled with ^{15}N and ^{13}C (AQUA peptide) was synthesized (Sigma Aldrich, Steinheim DE) and used as an internal standard for quantification (Table 1). Peak identification and quantification was performed using Analyst software version 1.6.

3. Statistics

Data are presented as median and range, unless indicated otherwise. To describe the variation in protein expression, the relative difference between the lowest and highest observed value was calculated per transporter. Median (range) protein expression per age group (fetuses, infants and adults) was calculated to describe the fold-change in

protein expression. The possible relation between transporter protein levels and age was assessed using scatter plots. As these plots suggested a monotonic relationship for almost all transporters, the association between transporter protein levels and age was assessed using Spearman's rank correlation coefficient. In addition, in the fetal and infant samples, the association between transporter protein expression and postmenstrual age was also assessed using Spearman's rank correlation.

All statistical analyses were performed using IBM SPSS Statistics software (SPSS Statistics for Windows, version 21.0; IBM, Armonk, NY) and a significance level of $P<0.05$ was used throughout the study.

RESULTS

Descriptive results

We studied 25 liver samples: 10 of fetuses [median gestational age (GA) 23.2 weeks (range 16.4-37.9)], 12 of infants [postnatal age 1 week (0-11.4), GA at birth 35.1 weeks (27.1-41.0)] and 3 of adults. Patient characteristics are listed in Table 2. The clinical diagnoses of the fetuses and infants were: therapeutic abortion for severe genetic disorders (non-metabolic diseases) (n=7), fetomaternal transfusion (n=1), hydrops fetalis (n=1), and intrauterine death for unknown reason (n=1). The clinical diagnoses of the infants were congenital malformations (n=5), viral/bacterial infections (n=4), necrotizing enterocolitis (without liver insufficiency) (n=2), and benign liver tumor (n=1). The adult liver tissue was histologically normal tissue. Information about diagnosis, reason for biopsy or medical history of the three adult patients concerned was not available.

Table 2. Hepatic protein expressions

		Fetuses N = 10	**Infants** N = 12	**Adults** N = 3
Profile I 'stable'	OATP1B1	17.6 (5.4-35.6)	22.8 (9.5-47.9)	34.6 (21.5-57.5)
	OATP2B1	42.2 (29.3-59.3)	38.5 (27.2-46.3)	44.9 (30.1-48.9)
	ABCB1	79.2 (23.2-117.7)	107.9 (60.6-150.6)	96.9 (93.5-103.4)
Profile II 'low to high'	ABCC2	8.8 (5.5-9.9)	12.6 (5.3-43.9)	19.8 (15.5-22.2)
	ABCC3	5.4 (1.6-9.9)	21.9 (3.6-32.8)	23.1 (15.9-23.7)
	BSEP	18.4 (11.4-33.7)	35.9 (10.6-49.8)	47.7 (40.3-67.0)
Profile III 'High to low'	ABCG2	4.0 (2.2-7.0)	2.7 (1.5-4.2)	1.7 (0.8-2.2)
	GLUT1	434.2 (49.1-1413.3)	108.7 (39.0-255.4)	5.2 (5.1-7.7)
	OCTN2	6.3 (4.1-8.9)	4.4 (1.9-6.6)	3.0 (2.2-4.8)
Profile IV 'Non-linear'	MCT1	12.9 (10.0-20.0)	20.3 (10.8-39.0)	9.8 (8.9-12.4)

Data are expressed as median (range) protein expressions in pmol/g tissue

Following the Dutch guideline on secondary use of human tissue, the Tissue Bank only provided the following clinical data: gestational age, postnatal age, gender and main clinical diagnoses. Consequently, data on nutrition or medication use prior to death were unavailable.

Transporter protein expression from fetus to adult

The selected hepatic transporter proteins could be detected in all samples. There was a 10 to 300-fold relative difference in transporter protein abundance between the patients with the lowest and the highest protein expression (Fig. 2). The highest variability was seen in GLUT1 protein expression with a nearly 300-fold relative difference. Table 3 shows the median abundance of studied transporters in fetal, infant and adult samples. With exploration of the relationship between age and protein expression the following four developmental patterns emerged. Table 4 shows the four developmental profiles in relation to previous results from other studies on transporter mRNA and protein expression.

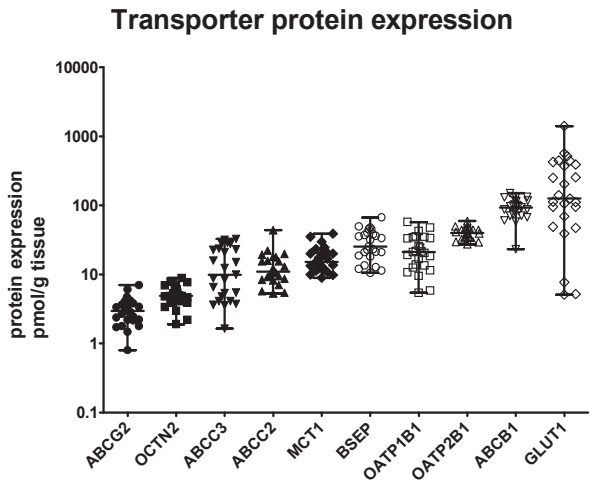

Figure 2. Variability in protein expression
Each dot represents an individual liver sample, the bars present median and range

First, in profile I ('stable'), ABCB1, OATP1B1, and OATP2B1 protein expression levels were similar across age groups (postnatal age) (ρ=0.33, p=0.11; ρ=0.18, p=0.39; and ρ=−0.11, p=0.59 respectively), but also within the fetal and infant groups (postmenstrual age) (ρ=0.18, p=0.43; ρ=0.23, p=0.31; and ρ=0.05, p=0.84 respectively) (Fig. 3 and 4).

Next, in profile II ('low to high'), ABCC2, ABCC3, and BSEP protein expression levels were low early in life and high in adulthood (ρ=0.56, p<0.01; ρ=0.59, p<0.01; and ρ=0.60, p<0.01 respectively) (Fig. 3). Median protein levels of ABCC2, ABCC3, and BSEP

Table 3. Summary table of results in relation to literature data

Family	Transporter	Protein expression profile group	Literature data: Age vs mRNA expression	Literature data: Age vs protein expression	Current data: postnatal age vs protein expression	Current data: postmenstrual age vs protein expression	Current data: Inter-transporter correlation of protein expression
Solute carrier organic anion (SLCO) family	OATP1B1	Profile I 'Stable'	Low-high: with low expression in fetuses and infants up to 1 year of age (n=32) [10], and low expression in fetal (n=30) versus pediatric and adult samples (n=30 each)[24], and again lower mRNA expression in 3 fetuses than 3 adults [27].	Stable: In neonates and adults (n=10, western blotting) expression was similar [26]. In 7-70 years of age, OATP1B1 (n=64, LC-MS/MS) protein expression was stable [13]. Low-high: In children ageing 0-12 years (n=78, western blotting), OATP1B1 protein expression was low from birth until 6 years of age and increased thereafter (abstract only) [25].	Similar: PNA <3 months vs adults	Stable from PMA 16.43 to 51.29 weeks	ABCB1*, ABCC2*, BSEP**, ABCG2**, GLUT1*
	OATP2B1		Low-high: OATP2B1 mRNA expression was lower in 3 fetuses from second trimester of pregnancy than in 3 adults [27].	Stable: In 7-70 years of age, OATP2B1 (n=64, LC-MS/MS) protein expressions was stable [13].	Similar: PNA <3 months vs adults	Stable from PMA 16.43 to 51.29 weeks	
ATP-binding cassette (ABC) family	ABCB1		Low-high: Increase during fetal life, continues postnatal to reach adult levels around 1 year of age [10, 21-24].	Stable: Stable protein expression from 0.3-12 years of age (n=65, western blotting, abstract only) [20]	Similar: PNA <3 months vs adults	Stable from PMA 16.43 to 51.29 weeks	MCT1*

Table 3. Summary table of results in relation to literature data (continued)

Family	Transporter	Protein expression profile group	Literature data: Age vs mRNA expression	Literature data: Age vs protein expression	Current data: postnatal age vs protein expression	Current data: postmenstrual age vs protein expression	Current data: Inter-transporter correlation of protein expression
	ABCC2	Profile II 'Low to high'	Low-high: 4-fold higher in pediatric and adult than fetal liver samples [24]. Low ABCC2 mRNA expression up to 1 year of age where it reaches adult levels [10].	Low-high: Increasing protein expression in 0.3-12 years of age (n=65, western blotting [20]. From 7 years of age onwards stable expression (n=51, LC-MS/MS) [14].	Low-high	Stable from PMA 16.43 to 51.29 weeks	ABCB1**, GLUT1**
	ABCC3		Low-high: ABCC3 mRNA expression was significantly lower in livers of 3 fetuses than 3 adults [27] and in perinatal/neonatal (prenatal to postnatal day 30, n=6; and 0-4 years, n=8) compared to older than 7 years liver samples [1].	Stable: In liver samples of neonates and adults (n=10, western blotting), ABCC3 protein expression was stable [26].	Low-high	Low-high	ABCB1*, ABCC2**, GLUT1**
	BSEP		Low-high: BSEP mRNA expression increased from fetal (n=3) to approximately 3-fold in adult (n=3) livers, in another study, expression was lower in neonates than in children older than 7 years [1, 27].	BSEP protein was immunohistochemically detected in second trimester fetuses [28].	Low-high	Low-high	ABCB1**, ABCC2**, ABCC3**, GLUT1**

Table 3. Summary table of results in relation to literature data (continued)

Family	Transporter	Protein expression profile group	Literature data: Age vs mRNA expression	Literature data: Age vs protein expression	Current data: postnatal age vs protein expression	Current data: postmenstrual age vs protein expression	Current data: Inter-transporter correlation of protein expression
	ABCG2	Profile III 'High to low'	Stable: stable ABCG2 mRNA expression was found; in fetal (n=30, second trimester of pregnancy), pediatric (n=30, age 1-17 years) and adult (age 28-80 years) liver samples and in a study comparing 3 fetal and 3 adult livers [24, 27].	Stable: ABCG2 protein expression was stable in neonates and adult livers (n=10, western blotting) [26]. And in 7-70 years of age, ABCG2 (n=56, LC-MS/MS), protein expressions was stable [12]. ABCG2 protein immunohistochemistry staining showed weak coloring in first trimester fetal hepatocytes, while adult liver showed strong ABCG staining [29].	High-low	High-low	OATP1B1**, OCTN2**
Solute carrier (SLC) family	GLUT1				High-low	High-low	OATP1B1*, ABCC2*, ABCC3**, BSEP**, ABCG2**, OCTN2**
	OCTN2				High-low	High-low	GLUT1**, ABCG2**
	MCT1	Profile IV 'Non-linear'			Non-linear	Low-high	ABCB1*

* $P<0.05$, ** $P<0.01$

Table 4. Correlations of transporter protein expressions

		OATP1B1	OATP2B1	ABCB1	ABCC2	ABCC3	BSEP	ABCG2	GLUT1	OCTN2	MCT1
Profile I 'stable'	OATP1B1	1	–	0.43*	0.46*	–	0.52**	−0.56**	−0.49**	–	–
	OATP2B1		1	–	–	–	–	–	–	–	–
	ABCB1			1	0.69***	0.47*	0.55**	–	–	–	0.47*
Profile II 'low to high'	ABCC2				1	0.63**	0.62**	–	−0.65***	–	–
	ABCC3					1	0.58**	–	−0.61**	–	–
	BSEP						1	–	−0.58**	–	–
Profile III 'High to low'	ABCG2							1	0.70***	0.67***	–
	GLUT1								1	0.59**	–
	OCTN2									1	–
Profile IV 'non-linear'	MCT1										1

*P<0.05, ** P<0.01, *** P<0.001

were approximately 2-fold, 4-fold, and 3-fold lower in fetuses and infants than in adults respectively. ABCC3, and BSEP, but not ABCC2 protein expression, increased with postmenstrual age (ρ=0.53, p<0.05; ρ=0.55, p<0.01; and ρ=0.34, p=0.12, respectively Fig. 4).

The opposite pattern holds in group III ('high to low'), for ABCG2, GLUT1, and OCTN2, higher protein abundance early in life and low at adult age was observed (ρ=−0.48, p<0.01; ρ=−0.59, p<0.01; and ρ=−0.78, p<0.001 respectively) (Fig. 3). Median protein levels were approximately 2-fold, 86-fold and 2-fold higher in fetuses and infants than adults, respectively (Table 3). Similarly, ABCG2, GLUT1, and OCTN2 protein expression decreased with increasing postmenstrual age within the fetal and infant groups (ρ=−4.55, p<0.05; ρ=−0.72, p<0.001; and ρ=−0.58, p<0.01 respectively) (Fig. 4).

Lastly, in profile IV ('non-linear') the MCT1 protein expression had a curvilinear relationship with postnatal age, with a 2-fold higher expression in infants than in both fetuses and adults (Fig. 3). Within the fetal and infant groups, the MCT1 protein expression increased with postmenstrual age (ρ =0.52, p<0.05, Fig. 4).

Correlation analysis of transporter protein abundances

To explore potentially shared expression regulation, Spearman correlations of transporter protein expression levels between transporters were performed. In total, the expression levels of 16 transporter-pairs were significantly correlated to each other (Table 5). The strongest correlations were found for the pairs: GLUT1/ABCG2, GLUT1/ABCC2, ABCB1/ABCC2, and ABCG2/OCTN2 (ρ=*0.70*, p<0.0001; ρ=*−0.65*, p<0.001; ρ=*0.69*, p<0.001; and ρ=*0.67*, p<0.001)(Table 4 and fig. 5).

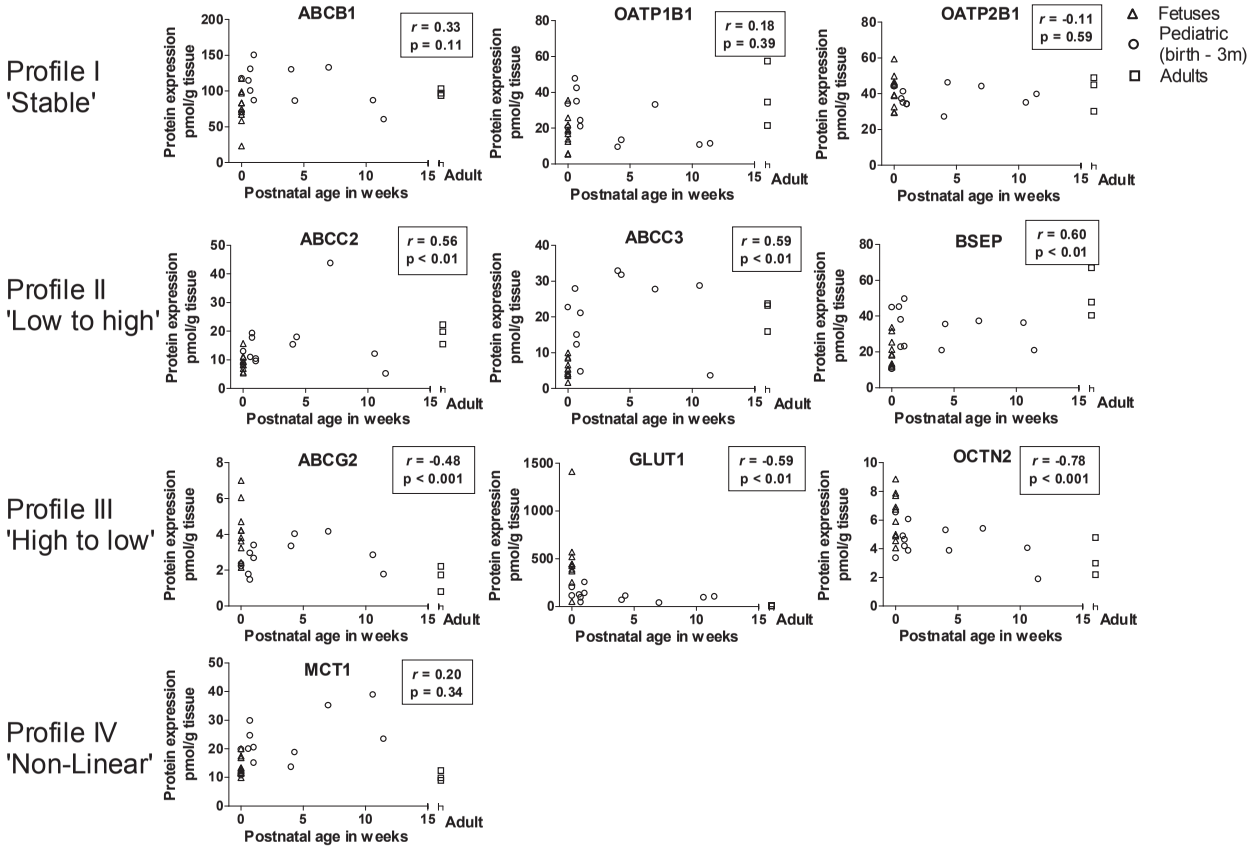

Figure 3. Transporter-specific postnatal maturation of protein expression

Triangles represent individual fetal samples, circles represent pediatric samples, and squares represent adult liver samples. Corresponding Spearman's rank correlation (r) are shown in each graph. Organized by developmental profile groups (I to IV).

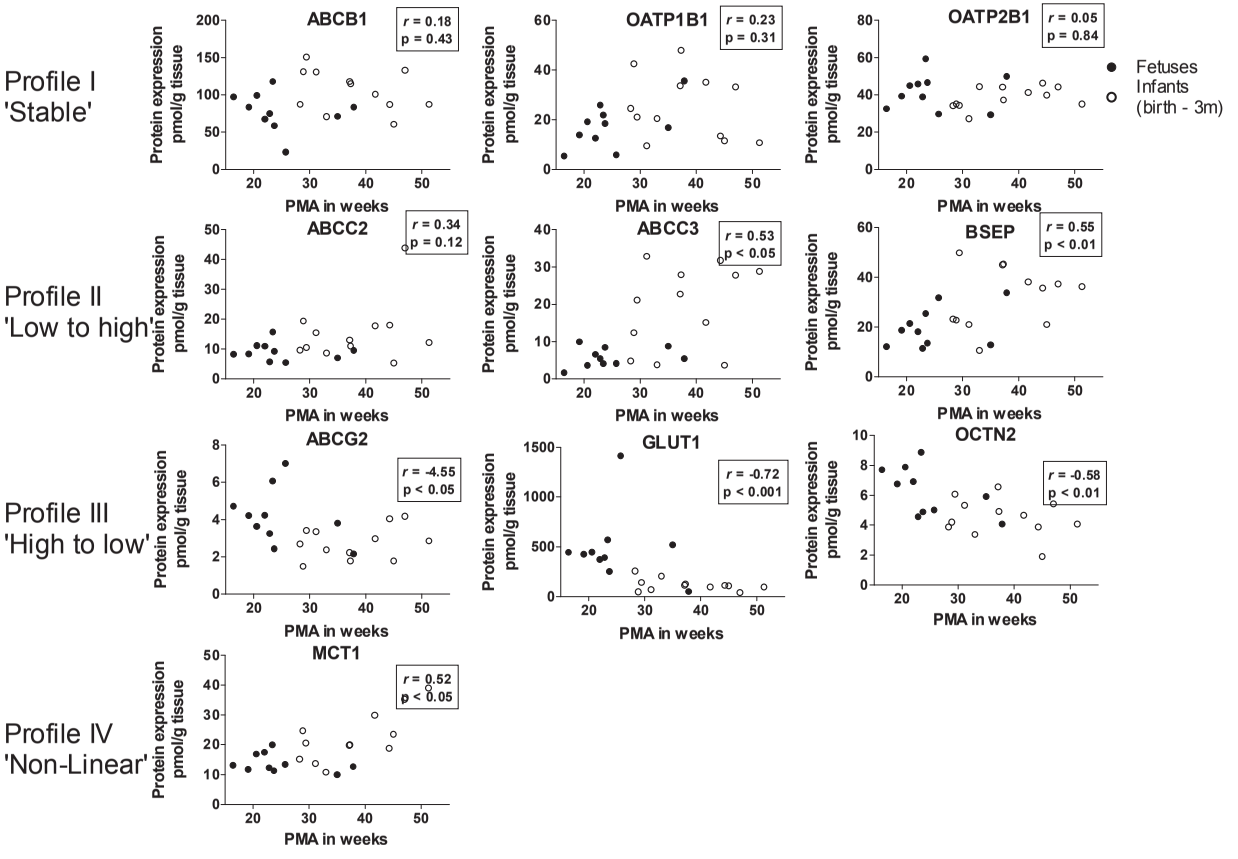

Figure 4. Transporter-specific postmenstrual maturation of protein expression

Closed circles represent individual fetal samples and open circles represent pediatric liver samples. Corresponding Spearman's rank correlation (r) are shown in each graph. Organized by developmental profile groups (I to IV).

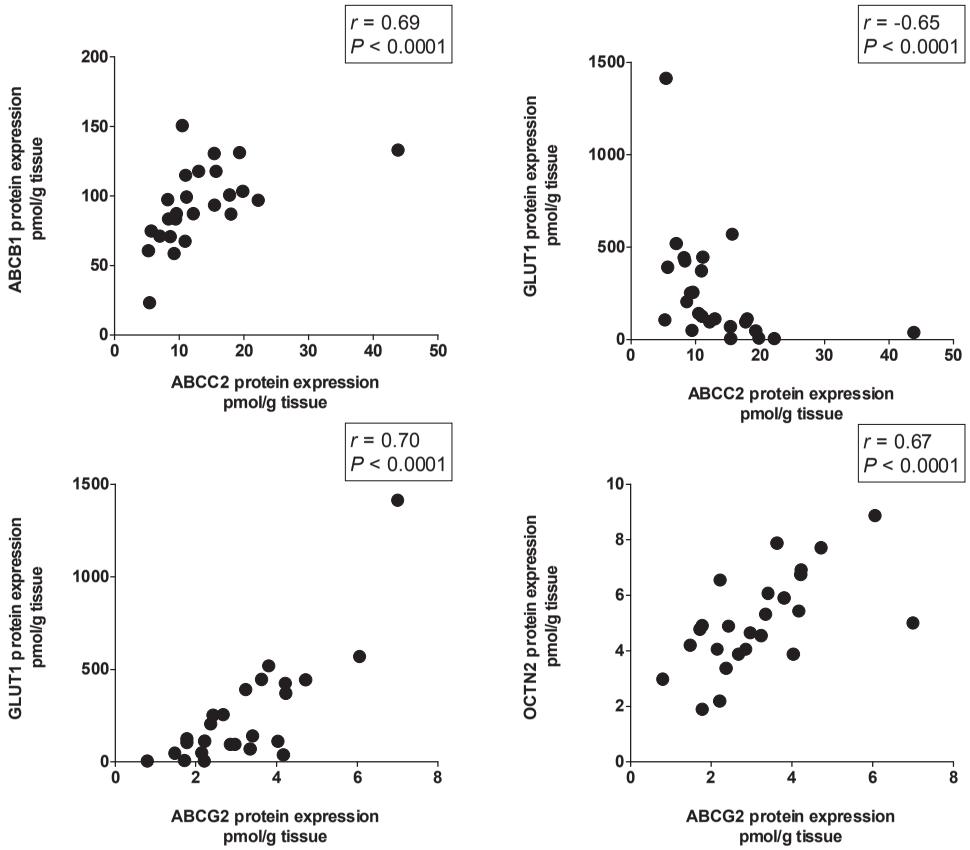

Figure 5. Examples of inter-transporter relationship of protein expression

Each circle represents an individual liver sample. These four pairs represent the strongest inter-transporter correlation. Corresponding Spearman's rank correlation (r) are shown in each graph.

DISCUSSION

This study reveals important age-dependent differences in protein abundance of ten clinically relevant hepatic membrane transporters. Four different developmental profiles of protein abundancy could be distinguished in liver samples from fetuses, young infants and adults. These results and existing data from the literature are summarized in Table 4.

Stable expression of hepatic transporters: Profile I

Protein expression of ABCB1, OATP1B1, and OATP2B1 transporters was similar in fetuses, infants and adults. This adds to current LC-MS/MS data on patients between 7-70 years of age (n=64), in which ABCB1, OATP1B1, and OATP2B1 protein expressions were also stable [13].

ABCB1 is the most studied transporter in fetuses and children. Examples of ABCB1 substrate drugs are digoxin, dexamethasone, tacrolimus, and morphine [16-19]. Interestingly, our finding that ABCB1 protein expression data is consistent in the investigated age-ranges is in line with other protein studies using immunoblotting in pediatric postmortem and living donors, in which stable protein expression was found from 0.3-12 years of age (abstract only) [20]. This apparent stable ABCB1 protein expression differs from the previously reported immature ABCB1 mRNA expression in the first year of life [10, 21-24]. In a study in adults absence of correlation between ABCB1 mRNA and protein expressions was also found [11].

In contrast to our finding, stable OATP1B1 protein expression during childhood was not found in 78 liver samples from children aged 0-12 years, in which the OATP1B1 protein expression by Western blotting was low from birth until 6 years of age and increased thereafter (abstract only) [25]. Yet, a smaller protein study using Western blotting on liver samples from neonates (n=5) and adults (n=5) confirms our finding regarding OATP1B1 [26]. OATP1B1 mRNA expression data from several other cohorts do not correspond to our protein data, with low expression in fetuses and infants up to 1 year of age (n=32) [10, 24, 27].

Stable OATP2B1 protein expression in infants and adults contrasts to lower OATP2B1 mRNA expression in samples of fetuses (n=3) from the second trimester of pregnancy than in samples from adults (n=3) [27].

Low to high expression of hepatic transporters: Profile II

The ABCC2, ABCC3, and BSEP transporters showed increasing protein expression from fetus to infant and highest expression in adults. We found increasing ABCC2 protein expression with age in the first months of life, confirming the results of protein expression using Western blotting in children from 0-12 years of age (abstract only) [20]. In addition,

other studies found lower ABCC2 mRNA expression in samples from fetuses and infants up to 1 year of age than in samples from adults [10, 24]. From 7 years onwards, ABCC2 absolute protein expression levels, also quantified with LC-MS/MS, was stable [14].

The BSEP protein expression profile confirms results from the few available mRNA expression studies. One showed approximately 3-fold increasing expression from fetal (n=3) to adult (n=3) liver samples; a second study, found lower expression in neonates than in children older than 7 years of age [1, 27]. BSEP protein was immunohistochemically detected in second trimester fetuses [28].

Lower expression of ABCC3 early in life is not seen in a small study using liver samples of 5 neonates and 5 adults, in which relative ABCC3 protein expression using Western blotting was similar in both groups [26]. In contrast, mRNA expression of ABCC3 in 3 fetal liver samples was significantly lower than that in 3 adult liver samples [27] and in perinatal/neonatal liver samples (prenatal to postnatal day 30, n=6; and 0-4 years, n=8) compared to liver samples from children older than 7 years [1].

High to low expression of hepatic transporters during childhood: Profile III

The ABCG2, GLUT1, and OCTN2 transporters showed high protein expression early in life and lower expression at adult age. This adds to current data of stable protein expression from 7 years onwards [12].

The higher ABCG2 protein expression early in life does not correspond to the stable ABCG2 protein expression found using Western blotting in 5 neonatal livers and 5 adult livers [26]. ABCG2 protein visualized by immunohistochemistry staining showed weak coloring in first trimester fetal hepatocytes, while adult liver showed strong ABCG2 staining [29]. On the other hand, stable ABCG2 mRNA expression with age was found in two studies; in fetal, pediatric, and adult (n=90) liver samples and a study comparing 3 fetal and 3 adult livers [24, 27].

To the best of our knowledge, there are no human developmental expression data available for GLUT1 and OCTN2. The same holds for MCT1, which forms the fourth developmental profile group with non-linear expression profile amongst the three age categories.

Variability in transporter expression

This exploratory study confirms earlier reports of transporter-specific discrepancy between mRNA and protein expression. Our ABCB1, ABCG2, OATP1B1, OATP2B1 protein developmental patterns do not seem to correspond to earlier mRNA expression data [10, 26, 27]. This discrepancy shows the relative weakness of relying solely on mRNA expression data to understand transporter maturation. An explanation could be post-transcriptional changes – for example mediated by microRNAs – on account of which mRNA does not correspond to protein expression [30]. Recently, developmental changes

in hepatic miRNA expression have been shown, and for some, miRNA expression was correlated to mRNA expression of drug disposition genes [24]. The interpretation of protein data should also be done with care, as transporter protein expression itself may not necessarily translate to transporter activity. Glycosylation, phosphorylation, and ubiquination, might alter proteins into active or non-active forms [1].

It can be speculated that the maturation of transporter expression is in line with the physiological transporter function with respect to growth and development. For example, transporters that are involved in energy supply in fetal life may be up regulated in fetuses and early in life. Glucose transporter GLUT1 and L-carnitine (molecule that shuttles long-chain fatty acids to the mitochondria for oxidation) transporter OCTN2, are probably both essential for fetal energy supply, which seems to tally with our findings of high protein expression in fetuses and early in life. On the other hand, transporters involved in the uptake and efflux of steroids and bilirubin (i.e. OATPs, ABCC2), may show less variability throughout life.

We hypothesized that pairwise-correlation in transporter expression might reveal a common developmental origin and point towards interlinked physiological processes. We found strong correlations for the following pairs: GLUT1/ABCG2, GLUT1/ABCC2, ABCB1/ABCC2, and ABCG2/OCTN2. Similarly, in adult liver samples correlations between multiple CYP and UGT protein expression were demonstrated [31-33]. To our knowledge, these inter-transporter correlations at the protein level have not been described previously. Many drugs have been shown to be substrates for multiple transporters, which may result in compensatory pathways in case a specific transporter is inactive due to genetic variation or drug-drug interactions. In addition, hepatocyte-hopping of substrates using different transporters may enhance efficient hepatic drug clearance [34]. Therefore, when using transporter expression data to predict drug disposition it is recommended to consider drug transport, and also drug metabolism as an interrelated biological system.

Variability in transporter expression may also be explained by other factors than age, such as disease, genetic heterogeneity, gender, drug-drug and drug-food interactions. This may explain some of the discrepancy between studies, as the sample origin and clinical characteristics are often only sparsely described. In human hepatocytes, inflammation via interferon-γ resulted in reduced mRNA expression of ABCB1, ABCC2, OATP1B1 and OATP2B1 [35]. More specifically in pediatric patients (1-2 months of age) with biliary atresia, hepatic ABCC2, BSEP, ABCB4, OATP1B1, OATP1B3, but not ABCC3 mRNA expression differed significantly from that in both fetuses and adults [36]. Genetic polymorphisms are well known to govern drug efficacy and toxicity; variants in OATP1B1 and ABCG2 encoding genes, for example, were found to influence statin efficacy/toxicity [37]. Gender did not impact protein expressions of ABCG2, ABCB1, ABCC2, OATP1B1,

OATP1B3 and OATP2B1 [12-14]. Due to the limited sample size and the lack of detailed clinical data, we could not incorporate other co-variates than age in our study.

While this explorative study adds importantly to existing pediatric transporter data by studying protein levels in the age group where most developmental changes are expected for 10 transporters, some limitations are present. The sample size was small and most samples are from young infants up to 3 months, so that possible effects of maturation later in pediatric life could not be studied. On the other hand, most developmental changes are expected in the first year of life. Second, all liver samples were collected postmortem, within 24h, but the exact time was unknown, the time gap might have influenced the quality of tissue. Further studies should be aimed to extend the pediatric age range, extend the selection of transporters, preferably involve larger sample size, and combining transporter expression with regulator proteins and genotyping.

Eventually, transporter maturation profiles and their interrelation may serve as input for physiologically-based pharmacokinetic (PBPK) models, which can be applied to use existing juvenile infant and human adult pharmacokinetic information to predict pharmacokinetics and drug response in children [38, 39].

In conclusion, we revealed age-dependent differences in absolute protein expression levels of ten different hepatic transporter proteins. Namely, profile I 'stable' (OATP1B1, OATP2B1, ABCB1), profile II 'low-to-high' (ABCC2, ABCC3, BSEP), profile III 'high-to-low' (ABCG2, GLUT1, OCTN2) and profile IV 'non-linear' (MCT1). These findings are important as they strongly suggest that disposition of drugs and endogenous transporter substrates will be subject to age-related changes, that could impact the efficacy and safety of drugs in the first months of life.

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Part III

Phase II drug metabolism

9 Pediatric microdose study of [^{14}C]paracetamol to study drug metabolism using accelerated mass spectrometry: proof of concept

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Clinical Pharmacokinetics 2014 Nov;53(11):0176-8

ABSTRACT

Rationale

Pediatric drug development is hampered by practical, ethical, and scientific challenges. Microdosing is a promising new method to obtain pharmacokinetic data in children with minimal burden and minimal risk. The use of a labeled oral microdose offers the added benefit to study intestinal and hepatic drug disposition in children already receiving an intravenous therapeutic drug dose for clinical reasons.

Objective

The objective of this study was to present pilot data of an oral [^{14}C]paracetamol [acetaminophen (AAP)] microdosing study as proof of concept to study developmental pharmacokinetics in children.

Methods

In an open-label microdose pharmacokinetic pilot study, infants (0-6 years of age) received a single oral [^{14}C]AAP microdose (3.3 ng/kg, 60 Bq/kg) in addition to intravenous therapeutic doses of AAP (15 mg/kg intravenous every 6 h). Blood samples were taken from an indwelling catheter. AAP blood concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and [^{14}C]AAP and metabolites ([^{14}C]AAP-Glu and [^{14}C]AAP-4Sul) were measured by accelerator mass spectrometry.

Results

Ten infants (age 0.1 to 83.1 months) were included; one was excluded as he vomited shortly after administration. In nine patients, [^{14}C]AAP and metabolites in blood samples were detectable at expected concentrations: median (range) maximum concentration (C_{max}) [^{14}C]AAP 1.68 (0.75-4.76) ng/L, [^{14}C]AAP-Glu 0.88 (0.34-1.55) ng/L, and [^{14}C]AAP-4Sul 0.81 (0.29-2.10) ng/L. Dose-normalized oral [^{14}C]AAP C_{max} approached median intravenous average concentrations (C_{av}): 8.41 mg/L (3.75-23.78 mg/L) and 8.87 mg/L (3.45-12.9 mg/L), respectively.

Conclusions

We demonstrate the feasibility of using a [^{14}C]labeled microdose to study AAP pharmacokinetics, including metabolite disposition, in young children.

INTRODUCTION

Up to 70% of drugs prescribed to children are unlicensed or off-label, which brings risk of drug toxicity or therapeutic failure [1, 2]. However, pediatric drug studies face important ethical, practical, and scientific challenges [3]. A major challenge – against the background of developmental changes in drug absorption, distribution, metabolism, and excretion – is appropriate dose selection [4]. Simple size- or weight-based extrapolations from adult to pediatric doses do not suffice, particularly in neonates and infants. Current strategies include simulations using population pharmacokinetic (popPK) and physiologically based pharmacokinetic (PBPK) models [5-9]. The usefulness of these models may be limited, as relative little pediatric pharmacokinetic and physiological data are available to validate them. Hence, new data are needed to support these models, as well as alternative methods to collect pharmacokinetic data without the inherent risks of toxicity when a therapeutic dose is given for the first time in a specific age group.

Microdosing is an interesting alternative. The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) define a microdose as one-hundredth of the No Observed Adverse Effect Level (NOAEL) or predicted pharmacologic dose based on animal data or as 100 µg of the new drug, whichever dose is lower [10, 11]. Dose linearity between the microdose and therapeutic dose is a prerequisite to extrapolate pharmacokinetic data to dosing guidelines [12]. The extremely low dose concentrations call for highly sensitive measurements. Accelerator mass spectrometry (AMS) can measure low attomolar to zeptomolar isotope ratio ranges, to quantify [^{14}C]labeled drug or metabolite levels in urine or plasma samples, even after at least five half-lives following a microdose [13]. [^{14}C] Labeling of a microdose is associated with very low radiation exposure, i.e., less than 10 µSv in adults, when compared to the yearly background exposure of 2.5 mSv/year in The Netherlands [14]. Hence, microdosing is safe to use to study pharmacokinetics in children. In clinical care, [^{14}C]urea has been safely used to test for *Helicobacter pylori* infection [15]. A [^{14}C]ursodiol microdose study in preterm infants was briefly described in an excellent review on the potential for use of AMS in children [16]. Administering a labeled oral microdose to children already receiving a therapeutic drug dose for clinical reasons intravenously offers the added possibility to study oral bioavailability [12, 17]. This approach may also serve to delineate developmental changes in the drug-metabolizing enzymes involved in intestinal and hepatic drug metabolism [17, 18].

Paracetamol (acetaminophen; AAP), is much used in children and is an interesting study drug for several reasons. First, its metabolism shifts from primarily sulphation (AAP-sulphate [AAP-Sul]) to glucuronidation (AAP-glucuronide [AAP-Glu]) in the first year of life, as reflected by urinary metabolite kinetics [19, 20]. The relative contribution of intestinal and hepatic drug metabolism has not been studied to date. Second, dose linearity under normal conditions and after probenecid glucuronidation inhibition was

shown in adults [18]. We therefore selected oral [^{14}C]AAP to study the developmental changes in AAP glucuronidation and sulphation in children already receiving the non-labeled or 'cold' drug intravenously for analgesia [14]. This paper presents our first pilot data as proof of concept of this promising, safe method to study developmental pharmacokinetics in children.

METHODS

Study Design

This proof-of-concept study is part of a larger phenotyping study in 60 children delineating developmental changes in AAP to metabolite clearance (EudraCT 2011-005497-28). The Dutch Central Committee on Research Involving Human Subjects (The Hague, The Netherlands) approved the larger study. Parents or legal guardians gave informed consent. Radiation exposure was explained during the informed consent procedure in relation to background exposure and exposure from medical imaging. The Dutch Nuclear Research and Service Group estimated individual exposures at approximately 1 μSv for a 0-2-year-old child, well below the minimal risk category 1 (100 μSv) of the International Commission of Radiological Protection and the yearly background exposure (2.5 mSv). Category 1 risk studies are considered minimal risk and are allowed when they provide new scientific knowledge [11].

Subjects

Patients admitted to the Intensive Care of the Erasmus MC-Sophia Children's Hospital (Rotterdam, The Netherlands) were considered for inclusion. Inclusion criteria were age between 0 and 6 years, gestational age >36 weeks, medical need for intravenous AAP, and an indwelling central venous or arterial line in place. To reduce pharmacokinetic variability due to underlying disease, patients with kidney and liver injury or the use of more than one vasopressor drug were excluded, as well as children intolerant to enteral nutrition.

Study Procedures

A single oral [^{14}C]AAP microdose (3.3 ng/kg, 60 Bq/kg, 0.25 ml/kg) followed by 1 ml of saline (to ensure rinsing of the enteral feeding tube) was administered in addition to the intravenous therapeutic dose of AAP (15 mg/kg intravenously every 6 h) prescribed by the treating physician to provide analgesia. The [^{14}C]AAP oral dose also contained 1.7 μg/kg non-labeled AAP, but this amount was negligible in relation to the therapeutic intravenous dose and thus considered irrelevant for the pharmacokinetic estimations. The microdose was based on a previous adult [^{14}C]AAP microdose study using 100

µg/7.1 kBq/individual and was normalized for weight [21]. Blood samples (0.5 mL) were taken from the indwelling catheter before and at 10-30 min, 1, 2, 4, 6, 12, and 24 h after dosing. Blood samples were centrifuged and plasma was stored at –80 ^{0}C until analysis.

Medicinal Products

The AAP formulation (10 mg/mL) was purchased from Fresenius Kabi, Schelle, Belgium. [^{14}C]AAP was purchased from Moravek Biochemicals (Brea, CA, USA).

Radiopharmaceutical Preparation

The formulation for oral administration [^{14}C]AAP was prepared by adding [^{14}C]AAP to an AAP formulation for intravenous use at the good manufacturing practice (GMP) radiopharmaceutical production laboratories of the Department of Radiology and Nuclear Medicine at the VU University Medical Center (Amsterdam, The Netherlands) (GMP license no. NL/H 11/0005) at final concentrations of 13 ng/mL for [^{14}C]AAP and 6.7 µg/mL for non-labeled AAP. The mixed formulation was passed over a Millex - GV 0.22 µm filter and dispended in 20 mL sterile vials. Radiochemical purity was > 99%; chemical purity >98%. The radiopharmaceutical was heat sterilized and was shown to be stable for 2 months.

Non-specific binding was tested by running the [^{14}C]AAP formulation followed by 1 mL of saline through enteral feeding tubes. Radioactive recovery measured by liquid scintillation was greater than 95%.

Paracetamol (Acetominophen; AAP) analysis

AAP concentrations were measured in the Hospital Pharmacy laboratory of Erasmus MC with a clinically used enzyme multiplied immunoassay technique (EMIT, Abbott Laboratories®) with a lower limit of quantification (LLOQ) of 2.8 mg/l.

[^{14}C]AAP and Metabolite Analysis

Plasma Sample Extraction and Ultra Performance Liquid Chromatography Separation

Using 175 µL 100% v/v methanol containing 6.6 µg/mL APAP in 96-well protein precipitation plates, 45 µL of plasma was extracted. The pellet was washed with 100 µL 0.9% NaCl:100% methanol (1:4 v/v). Resulting filtrates were evaporated to dryness, and re-dissolved in 30 µL 10 mM ammonium phosphate pH3.4 (Eluent A) of which 25 µL was used for ultra- performance liquid chromatography (UPLC) analysis. An AAP solution with a specific radioactivity of 4,100 Bq [^{14}C]AAP /100 µg AAP in blank pooled plasma was used to prepare eight calibrators levels and three quality control (QC) sample levels from 0.4 to 180 mBq/mL, and from 1.7 to 131 mBq/mL, respectively. Calibrators (duplicate), QCs (triplicate), and sample extracts were injected onto a UPLC coupled to a photodiode

Table 1. UPLC conditions AAP

Eluent A	10 mM ammonium phosphate pH 3.4
Eluent B	100 % v/v methanol
UPLC column (Waters Acquity)	BEH C18 1.7 µm 2.1x100 mm column
Flowrate	0.3 mL/minute
Column temperature	30°C
Pressure	700 Bar
Chromatography conditions	0-1 min 100% A and 0% B
	1-10 min linear gradient from 100% A and 0% B to 95% A and 5% B
	10-12 min 95% A and 5% B
	12-15 min linear gradient from 95% A and 5% B to 0% A and 100% B
	15-20 min 0% A and 100% B
	20-20.10 min linear gradient from 0% A and 100% B to 100% A and 0% B
	20.10-20.50 min 100 %A and 0% B
	20.50-28 min 100% A and 0% B at a flowrate of 0.4 ml/min
	28-29 min 100% A and 0% B
Collected fractions	[^{14}C]AAP-Glu (3.8-5.3 min)
	[^{14}C]AAP-4Sul (6.1-7.9 min)
	[^{14}C]AAP (8.1-9.8 min)

array (PDA). Chromatographic conditions can be found in Table 1. AAP in 100% methanol was added to each collected fraction to increase the carbon-12 content to 25 µg.

Accelerator Mass Spectrometry Analysis

[^{14}C]AAP and metabolites were determined as described recently [22]. A novel AMS sample introduction method was used in this study to allow the routine analysis of biomedical samples. The method consists of an automated carbon diaoxide (CO_2) combustion device online coupled to an AMS. Briefly, dried fractionated samples are placed in a tin foil cup and subsequently combusted using an elemental analyzer (EA) (Vario Micro, Elementar, Germany). The resulting CO_2 was captured on a zeolyte trap at the interface, connecting the EA to the AMS. CO_2 was released by heating of the trap and transferred to a vacuum syringe using helium. The resulting 6% v/v gas mixture of CO_2 with helium was infused at a pressure of 1 bar at 60 µL/min into the titanium target in the SO110 ion source of a 1 MV Tandetron AMS (High Voltage Engineering Europe B.V., Amersfoort, The Netherlands) [23]. Within the source, CO_2 is converted into negative carbon ions. The validation for the liquid chromatography (LC) + AMS analysis was based on the recommendation of the European Bioanalytical Forum. Three QC concentration

levels were included, QC High 145 mBq/ml, QC Medium 14.5 mBq/ml and QC Low 2.4 mBq/ml. The accuracy of QC High, Medium, and Low analysis corresponded to 104, 103 and 90%, respectively. The precisions, defined by a coefficient of variation, were 9.1%, 6.7% and 6.9% respectively. All values are well within the requirements for LC + AMS analysis [24]. The LLOQ of the method was 0.58 mBq/mL.

Data analysis

Data were summarized as median (range), unless noted otherwise. The plasma [^{14}C]AAP and metabolite concentrations were calculated by converting measured Bq/L to ng/L based on the dose given (3.3 ng/kg contained 60 Bq/kg) and for the metabolites corrected for molecular weight ([^{14}C]AAP 151 g/mol, [^{14}C]AAP-Glu 237 g/mol and [^{14}C]AAP-4Sul 231 g/mol). To compare the disposition of oral [^{14}C]AAP microdose to the intravenous therapeutic doses, the microdose concentrations were dose normalized to 15 mg/kg by multiplying with 5 x 10^{E}6.

RESULTS

Patients

Between 13 January and 31 May 2014, 32 patients were eligible. Nine patients were excluded for logistical reasons and 13 for refusal of informed consent. Parents' informed consent was received for ten patients who were subsequently dosed according to the protocol. One patient vomited within 15 min post-dose and was excluded from pharmacokinetic analysis. Table 2 provides a summary of patient characteristics.

Detection of [^{14}C]AAP and Metabolites in Plasma

Time profiles of [^{14}C]AAP, [^{14}C]AAP-Glu and [^{14}C]AAP-4Sul metabolite plasma concentrations of two representative patients are shown in Fig. 1. These patients were chosen as

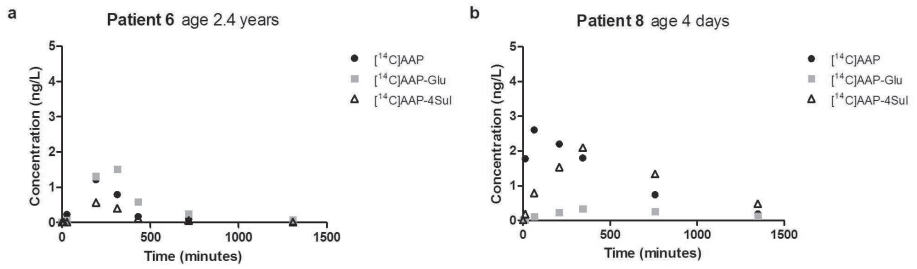

Figure 1. [^{14}C]AAP and metabolite plasma concentrations
[^{14}C]AAP and metabolite plasma concentrations after oral 3.3 ng/kg [^{14}C]AAP dose (LLOQ 0.03 ng/L).

Table 2. Patient characteristics

Patient	Postnatal age (months)	Gender (male/female)	Primary diagnosis	Intervention
1	3.6	Male	Post necrotizing enterocolitis sigmoïd stenosis	Postoperative partial proximal colon resection
2	10.6	Female	Scaphocephaly	Postoperative craniofacial correction
3	1.7	Female	Congenital cystic adenomatoid malformation of the lung	Postoperative partial lung resection
4	0.3	Male	Congenital diaphragmatic hernia	Postoperative hernia correction
5	53.8	Male	Scaphocephaly	Postoperative craniofacial correction
6	28.9	Male	Germ cell tumor	Respiratory insufficiency due to mediastinal pressure
*7**	*83.1*	*Male*	*Increased intracranial pressure*	*Postoperative craniofacial correction*
8	0.1	Male	Congenital cardiac disease	Monitoring respiratory insufficiency
9	6.2	Male	Scaphocephaly	Postoperative craniofacial correction
10	5.6	Male	Duodenal web	Postoperative duodenoduodenostomy and placing gastrostomy tube

** Patient 7 excluded from results after vomiting within 15 minutes after microdose intake.*

they represent the youngest and the oldest age group. The Electronic Supplementary Material shows graphs of all patients. The median time to maximum concentration (t_{max}) and maximum concentration (C_{max}) median (range) for [^{14}C]AAP were, respectively, 153 min (10-245 min) and 1.68 ng/L (0.75-4.76 ng/L), for [^{14}C]AAP-Glu were 248 min (161-382 min) and 0.88 ng/L (0.34-1.55 ng/L), and for [^{14}C]AAP-4Sul were respectively 193 min (115-343 min) and 0.81 ng/L (0.29-2.10 ng/L). Sample collections were not complete for all patients as the arterial line was prematurely removed or access to the line was restricted for clinical reasons.

Dose-Normalized [^{14}C]AAP and AAP Disposition

Semilog plots of the same two patients of dose-normalized 3.3 ng/kg oral [^{14}C]AAP and 15 mg/kg every 6 h intravenous AAP concentrations are shown in Fig. 2. Individual graphs of all patients are displayed in the Electronic Supplemental Material. Dose-normalized median [^{14}C]AAP C_{max} concentrations approached median intravenous average concentrations (C_{av}) [median (range)]: 8.41 mg/L (3.75 to 23.78 mg/L) and 8.87 mg/L (3.45 to 12.9 mg/L), respectively.

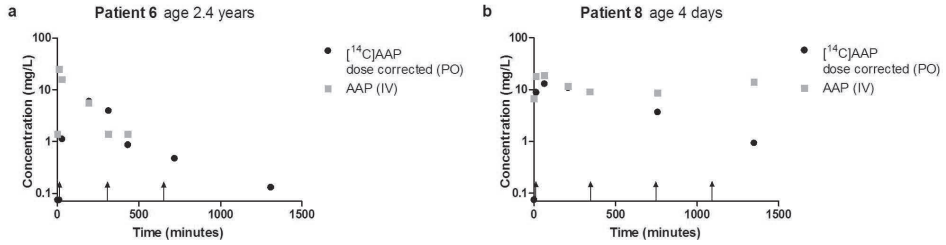

Figure 2. Dose-normalized plasma concentrations

Dose-normalized plasma concentrations after oral [^{14}C]AAP (3.3 ng/kg) and intravenous AAP (15 mg/kg/q6h) dose (dose-normalized LLOQ AMS 0.03 mg/L; LLOQ LC-MS/MS 2.8 mg/L). AAP IV administration time points are indicated with black arrows. Patient 6: at latter two timpoints insufficient blood collected to also analyze AAP levels in plasma.

DISCUSSION

This proof-of-concept study shows the practical and ethical feasibility of a [^{14}C]microdose study in children to study developmental pharmacokinetics.

The concentrations of [^{14}C]AAP are in the expected range, when compared to dose-normalized concentrations previously reported in neonates and children [25-27]. The average [^{14}C]AAP and metabolite C_{max} values are also similar to those in a previous adult study: 1.64 vs 1.68 ng/ml for [^{14}C]AAP, 0.88 versus 0.92 ng/ml for [^{14}C]AAP-Glu, and 0.62 versus 0.88 ng/ml for [^{14}C]AAP-4Sul [18]. The apparent lack of an age-related difference in metabolite disposition is easily explained by our small sample size and the even smaller number of neonates in our cohort. This is supported by our observation that in the 4-day-old neonate [^{14}C]AAP-4Sul concentrations are much higher than the [^{14}C]AAP-Glu concentrations, while the opposite is observed in the 2.4-year-old, in line with developmental changes in AAP metabolism. In the follow-up study up to 50 additional patients will be included to study developmental changes in AAP disposition with enough statistical power. Interestingly, the average t_{max} values in our patients are much later than in the adult study: 0.25 versus 2.6 h for [^{14}C]AAP, 0.25 versus 4 h [^{14}C]AAP-Glu, and 0.5 versus 3.2 h for [^{14}C]AAP-4Sul. A possible explanation for this finding may be slower oral absorption due to maturation and the underlying critical illness or post-operative state in our patients [26, 28].

The major barrier to a [^{14}C]microdosing study in children has been the perceived risk of radiation in the context of a non-therapeutic trial [29, 30]. Still, radiation exposure in this study was extremely low (<1 μSv), much lower than yearly background exposure (2.5 mSv/year in The Netherlands), a continental flight (>4 μSv), exposure from chest X-ray (10 μSv) or computed tomography scans (100 μSv) [7, 14]. To overcome parental ethical barriers to the study, we added a letter from the Dutch collaborative patients'

organization for rare and genetic diseases (VSOP) to the patient information leaflet, explaining the need for pediatric drug research and the minimal risk involved in this study. Surprisingly, from the informed consent conversations it appeared that most parents perceived there was minimal risk involved. Fear for harmful radiation exposure was not the main reason to deny informed consent. Parents of the other children refused informed consent for reasons relating to the burden of additional procedures and/or blood sampling.

Very sensitive LC-tandem mass spectrometry (LC-MS/MS) techniques to measure pharmacokinetics of an unlabeled microdose may be an alternative to using a radiolabeled microdose in general [31]. Nevertheless, while low drug concentrations can be measured with LC-MS/MS, this analytical method has not reached the very low limits of detection of AMS. Hence, a larger unlabeled dose may be needed, which may increase the risk of a therapeutic or toxic effect [13]. Moreover, the use of an unlabeled microdose would also prohibit the separation of the disposition of an oral and intravenous dose given at the same time. This could be overcome by using a stable isotope-labeled probe drugs, which has successfully been used to study oral bioavailability using $^{15}N_3$-midazolam in adults [32]. This method also has the important disadvantage that a much higher labeled drug dose is needed with similar or even higher risk of therapeutic or toxic effect.

CONCLUSION

We have shown proof of concept for the practical and ethical feasibility of a [^{14}C]labeled microdose to study pharmacokinetics in young children. This approach offers innovative possibilities to perform phase I first-in-child studies, especially for drugs with a small therapeutic window and high toxicity. In addition, it enables studies in vulnerable populations such as critically ill neonates and studies on developmental pharmacokinetics using probe drugs for specific elimination pathways such as drug-metabolizing enzymes and renal excretion.

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SUPPLEMENTAL FIGURES

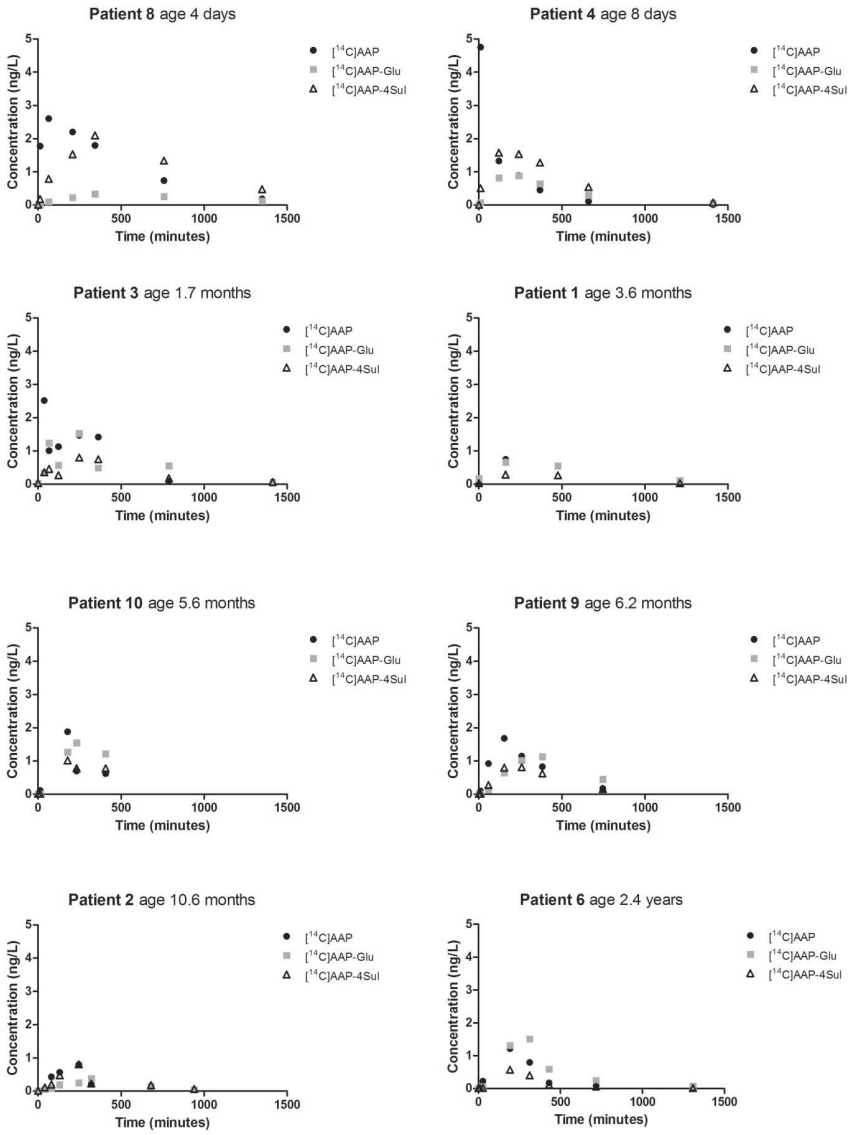

Figure 1. [^{14}C]AAP and metabolite concentrations in plasma

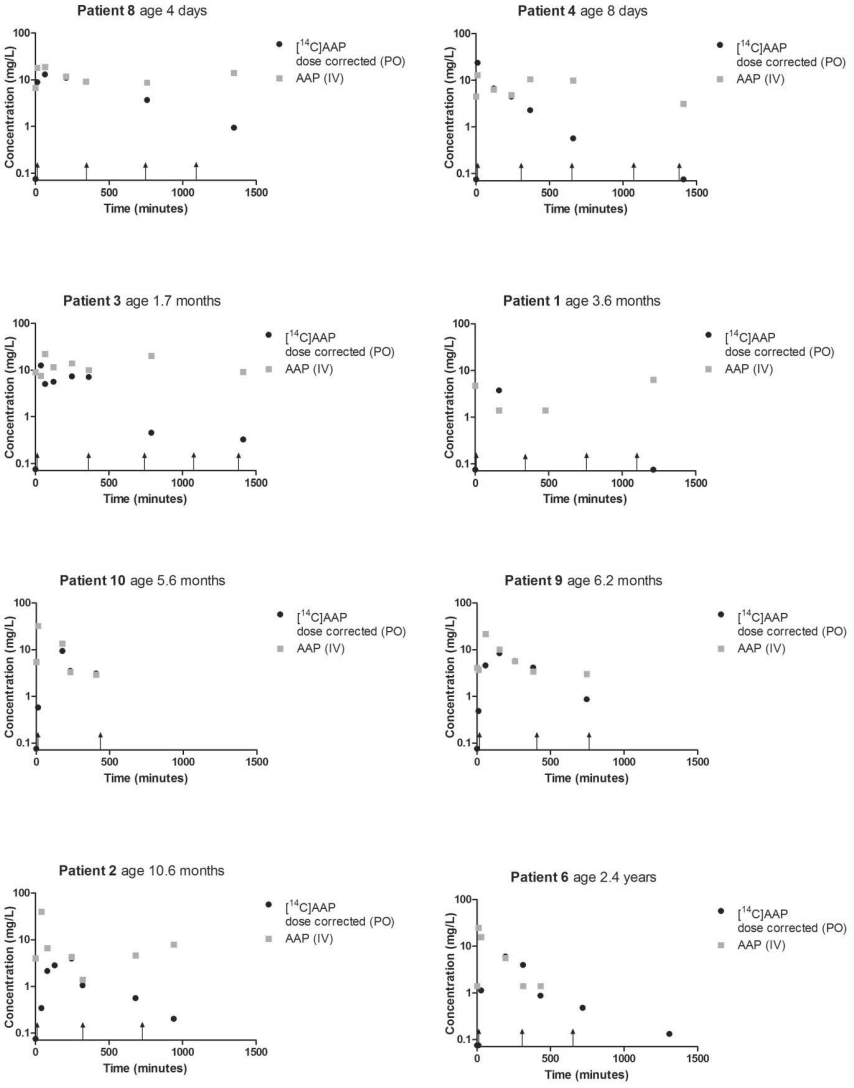

Figure 2. AAP concentrations following IV therapeutic dose and [^{14}C]AAP dose normalized concentrations following an oral microdose

10 Pediatric microdose and microtracer studies using ^{14}C in Europe

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Clinical Pharmacology and Therapeutics 2015, Sep;89(3):234-7

INTRODUCTION

Important information gaps remain on efficacy and safety of drugs in children. Pediatric drug development encounters several ethical, practical and scientific challenges. One barrier to the evaluation of medicines for children is a lack of innovative methodologies that have been adapted to the needs of children. This article presents our successful experience of pediatric microdose and microtracer studies using ^{14}C-labeled probes in Europe to illustrate the strengths and limitations of these approaches.

Microtracer / microdose studies in pediatric drug development

Dose selection for pediatric phase I/II studies is usually based on extrapolation from human adult and juvenile animal data, increasingly using population pharmacokinetic (PK) and physiology-based pharmacokinetic modeling to simulate dosing regimens. These methods have important limitations. Critical information gaps remain on the developmental changes in the processes involved in the disposition of drugs [1]. Especially for drugs with complex metabolism and/or poor understanding of ontogeny, a useful model is lacking. PK studies with a ^{14}C-labeled drug at a subtherapeutic dose may aid to close these information gaps while overcoming many limitations associated with regular pharmacokinetic studies in children. This can contribute to systems-level understanding (e.g., information about ontogeny) and drug-level understanding (e.g., population PK studies during drug development).

When a radiolabeled drug is administered concomitantly with a therapeutic dose of the same unlabeled drug via either the same (for example, for mass balance studies) or another route (for example, for absolute bioavailability studies), this is referred to as a microtracer dose [2]. For example, a radiolabeled probe administered enterally can generate information about enteric and hepatic metabolism distinguishing between the two routes. A microdose is a subtherapeutic dose in the absence of other dosing with the drug of interest. Following a microdose the disposition will reflect the microdose *per se*. The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) defines a microdose as 1/100th of the No Observed Adverse Effect Level (NOAEL) or predicted pharmacologic dose based on animal data, with a maximum of 100 µg of the new drug. Microdosing has been used in adult drug development.

A prerequisite of both microtracer and microdosing is the availability of sensitive analytical techniques to measure the low plasma concentrations. Very sensitive liquid chromatography/tandem mass spectrometry (LC-MS/MS) techniques have been used to measure PK of an unlabeled subtherapeutic dose. However, these techniques are not sensitive enough to detect the low concentrations of metabolites seen during the elimination phase following the administration of a microdose. LC-accelerator mass spectrometry (AMS) can measure low attomolar to zeptomolar range and quantify ^{14}C-

labeled drug or metabolite levels in urine or plasma samples [3]. The exquisite sensitivity of AMS allows extremely low doses of drugs to be given, which minimizes the amount of ^{14}C administered. The administration of doses that have no pharmacological effect and thus no concerns about toxicity would allow a microdose study to be conducted early in pediatric drug development. In addition, AMS uses very small samples of plasma (<20 µL) that are compatible with sampling limits during research involving children.

As a result of the 2006 EU [4] regulation on medicinal products for pediatric use, both European (Era-NET) and national (ZonMW Priority Medicines for Kinderen) funding opportunities encouraged drug studies in children. Two projects: PAMPERS (Paediatric Accelerator Mass Spectrometry Evaluation Research Study) and PEDMIC (Pediatric microdosing: elucidating age-related changes in oral absorption to guide dosing of new formulations) were funded to study the use of ^{14}C-labeled probes in children to elucidate PK. The first results of these studies were published recently, showing the feasibility of the approach, including acceptable dose linearity [5, 6].

PAMPERS and PEDMIC project study aims

The PAMPERS project aims to show the feasibility of microdose and microtracer studies for drug development in children. ^{14}C-Paracetamol and ^{14}C-midazolam, frequently prescribed to children and probes for UGT and CYP3A metabolism, were chosen to show feasibility [6].

The PEDMIC study was designed on the premise of dose-linearity of oral absorption, as shown in adults, and aimed to study developmental pharmacology. By administering an oral microtracer (^{14}C-paracetamol or ^{14}C-midazolam) while the patient received the drug for therapeutic reasons i.v., in children from 0 to 6 years of age, the developmental change in intestinal and hepatic drug metabolizing enzymes, specific for these drugs, can be elucidated [5].

Details of both projects can be found in Table 1.

Perceived risks of radioactivity

In discussions with colleagues, we found that the major concern about ^{14}C-labeled microtracer and microdose PK studies in children lay in the perceived risk of adverse effects related to radioactive exposure from a ^{14}C-labeled drug. While this hesitancy is understandable, it is unfounded. Radioactive dose calculations were performed using a worst-case half-life scenario of 40 days, which for an 111Bq/kg ^{14}C-dose gave a radioactive exposure between 0.33 and 0.8 microSV. This is much lower than yearly background exposure (2.5 mSv/year in the Netherlands), a regional European flight (1-15 microSv), a computed tomography (CT)-scan of the head (1200 microSv) or chest x-ray (12 microSv) (Figure 1). The International Committee on Radioactivity Protection (ICRP) considers an effective radioactive dose of <100 microSv for a healthy adult volunteer as risk

Table 1. Study details of PAMPERS and PEDMIC projects

	PAMPER	PEDMIC
Study design	Microdosing and Microtracer studies 1) i.v. microtracer mixed with i.v. therapeutic dose 2) i.v. microtracer while patient concomitantly receives enteral therapeutic dose 3) Enteral microdose in the absence of therapeutic dose 4) i.v. microdose in the absence of therapeutic dose	Microtracer study: Enteral microtracer while patient concomitantly receives i.v. therapeutic dose for clinical reasons
Compound	^{14}C-APAP and ^{14}C-midazolam	^{14}C-APAP and ^{14}C-midazolam
Objective	To study feasibility of microdosing and microtracer studies in children	To study ontogeny of intestinal and hepatic drug metabolism
Study partners	UK, Estonia, Poland, The Netherlands	The Netherlands
Clinical study sites	UK, Estonia	The Netherlands
Grant awarded	Priomedchild	ZonMw
Drug purchase	^{14}C-APAP acquired from Moravek Biochemical Inc, USA, purified to >99% radiochemical and chemical purity at the Pharmaceutical Research Institute, Warsaw, Poland (2 months once specification was agreed)	^{14}C-APAP acquired from ARC (St. Louis, MO, USA)
Drug development	In MHRA GMP registered Radiopharmacy, Addenbrookes Hospital, Cambridge, UK (MHRA Licence Holder MS 12854). Here the high specific radioactivity ^{14}C-APAP was diluted in 5% w/v dextrose, filter sterilized and released to the paediatric clinics by a Qualified Person and QC work (1 month once specification was agreed)	In GMP registered Department of Radiology & Nuclear Medicine, VUmc, Amsterdam, NL: 300ng (3700 Bq) of ^{14}C-APAP was mixed with APAP I.V. solution and added to saline (0.3% w/v of ^{14}C-APAP and 100 µg/mL of paracetamol solution in saline). The drug product was heat-sterilized. Quality control showed purity and that the product was sterile and pyrogen free. (one month once specification was agreed)
Awarded REB consent	September 2012	September 2013
Radiation Safety Protocol	No	Yes
CTIMP	No	Yes
Assay development	Method transfer, development and implementation based on extant metabolite profile Validation according to EBF recommendation (parent drug) (2 months once specification was agreed)	
Age range studied	36 weeks to 127 weeks corrected gestational age	0.1-83.1 months postnatal age
Dose of labeled drug	4.5 ng/kg (111 Bq / kg)	2 ng/kg (60 Bq/kg)

Table 1. Study details of PAMPERS and PEDMIC projects (continued)

	PAMPER	PEDMIC
Included patients	^{14}C-APAP: n= 34. Oral microdose (n=4); i.v. microdose (n=6); oral therapeutic microtracer (n=6) and i.v. therapeutic microtracer (n=18).	^{14}C-APAP: n=10 (pilot data)
PK sampling schedule	Maximum of 6 blood samples including a pre-dose baseline sample and at least two from a series of optimal time points between 5 min and 8 hrs postdose.	8 blood samples (1mL each) over 24 hrs, 24-hr urine collection from urinary catheter
Handling biomaterials	Normal handling of blood / urine	Normal handling of blood/urine. Protocol regarding handling was needed for radiation safety office.
AMS Sample analysis	TNO, Zeist, NL AMS	
Cost ratio of LC-AMS compared to LC-MS/MS analysis	Method transfer development and implementation (assuming a metabolite profiling method is available) 1; Validation 1; Sample analysis (only parent drug, price per sample) 3; Each additional metabolite (price per sample) 3.	
Preliminary findings	Feasibility of microdosing/microtracer studies Acceptable dose linearity	Feasibility of microtracer studie and appaerent dose linearity

Published ^{14}C-APAP (^{14}C-Paracetamol) study details are provided, ^{14}C-midazolam studies are ongoing, more data can be provided upon request.

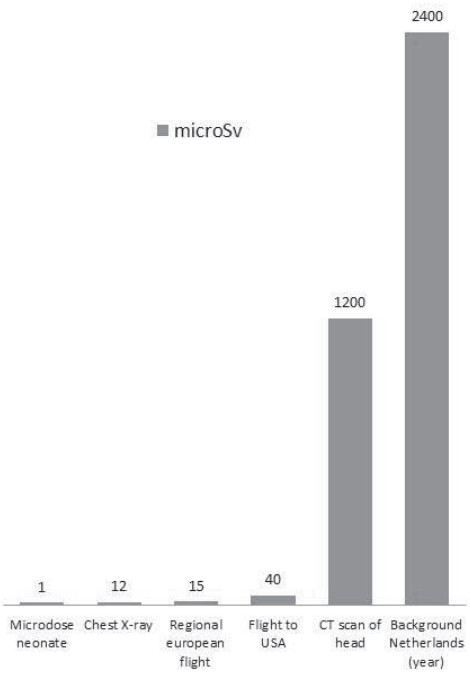

Figure 1. A comparison of radiation exposure

category 1. Thus, the doses of radiation that can be administered during studies that use AMS pose no increase above minimal risk.

Ethics

With this explanation about the dose of radioactivity, approval by Ethics Committees (equivalent to Independent Review Boards (IRBs)) was unproblematic. The study designs in PAMPERS and PEDMIC for the microdosing arms involve administering a medicine without a direct therapeutic benefit to the individual child. These studies administered a background dose of radioactivity with a medicine in common use and took blood samples through available vascular access. Ethics Committees in three countries deemed this compatible with minimal risk and burden for children receiving pediatric intensive care. Similarly, a neonatal ^{14}C-ursodiol microdose study was approved in the U.S. [7]. All these studies met the criteria of minimal risk and burden in the specific patient population studied. But for each study an evaluation of the balance between risks and benefits in the specific context needs to be made. For example, a microdose study with urine sampling only may be evaluated as minimal risk and burden in healthy children, while repeated blood sampling may only be allowed in children with available vascular access. Hospital staff also understood the concept of microdosing and supported the studies at all three sites.

Consent

The concept and safety of these studies appeared easy to explain to parents. The recruitment rate in both projects was around 50% of eligible patients. This is comparable to other interventional studies in the Intensive Care Units at Erasmus MC Sophia, Alder Hey Children's Hospital and Tartu Children's Hospital. For most parents who did not consent, information overload during critical illness or the perceived burden of repeated blood sampling were the main reasons not to let their child participate [8]. Fear of radiation was cited as a reason not to consent by a small minority of parents in all three centers.

Dose linearity

A major prerequisite for a microdose to study pharmacokinetics is a linear relationship in PK from the microdose to the therapeutic dose range. ^{14}C-paracetamol and ^{14}C-midazolam show dose-linear pharmacokinetics in the dose range from the microdose to a therapeutic range in healthy adults [9, 10]. For other drugs, microdosing studies in children should preferably be preceded by adult microdosing studies to show dose-linearity, as otherwise extrapolation of data from microdosing studies to pediatric therapeutic doses may be incorrect.

Regulatory issues

A major challenge was the preparation from the commercially available chemical compound ^{14}C-paracetamol into a formulation suited for human use according to Good Manufacturing Practice (GMP) guidelines. This challenge was overcome by the addition of a very small amount of ^{14}C-paracetamol acquired from a commercial supplier to saline or glucose with (PEDMIC) or without (PAMPERS) unlabeled i.v. solution paracetamol. Solubility in itself was not an issue considering the extremely low concentrations. Extensive GMP quality control showed the drug product to be pure, sterile, and pyrogen free.

In The Netherlands, a single microtracer to study pharmacokinetics was considered a drug so that the study was a clinical trial of an investigational medicinal product (CTIMP). This was not the case in the UK and Estonia, where it was considered a well-characterized probe in a physiological experiment (a study involving a less well-characterized probe would be a CTIMP in the UK). In The Netherlands, the hospital's Radiation Safety Officer considered the formulation radioactive and requested a Radiation Safety training by the junior researcher, as well as a study-specific radiation handling protocol, in contrast to the UK and Estonia, were it was not considered radioactive. Body fluids and blood/urine samples were considered radioactive only in The Netherlands. The radiation dose, however, was such a low dose that no special handling was required in all three countries.

Costs

^{14}C AMS studies may be costly. First, the ^{14}C-labeled probe needs to be manufactured and formulated according to GMP, without loss of its unique chemical property. The costs are probe-specific. Second, the AMS analyses are usually more costly than LC-MS/MS analyses. Finally, additional adult studies may be needed to show dose-linearity across the full dose range and possibly also in inhibition and/or induction scenarios. Nevertheless, timely information about disposition will accelerate the development of safe and effective pediatric products, outweighing the financial cost of such studies.

CONCLUSION

We have shown the ethical, practical and scientific feasibility of microtracer and microdose studies in children. Microtracer studies could be a valuable tool in pediatric drug research. Taking the challenges such as dose-linearity and costs into account, we believe that microdosing/microtracer trials are of added value for pediatric drug development. Microdosing may be of specific value during the early evaluation of new drugs when metabolism is likely to be complex and the timely determination of the pharmacokinetics of a new drug is critical to the risk-benefit analysis of a new medicine in the pediatric population.

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11 Microdosing to study ontogeny of drug metabolism: the case of [^{14}C]paracetamol

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Submitted for publication

ABSTRACT

Background

We aim to study the feasibility for [^{14}C]microdosing to study the impact of age on drug disposition pathways, more specifically, glucuronidation and sulfation, using [^{14}C] paracetamol (AAP).

Methods

Infants admitted to the pediatric intensive care received a single oral [^{14}C]AAP microdose while receiving intravenous therapeutic AAP q6h. The plasma $AUC_{0\text{-}inf}$ and urinary recovery ratios ([^{14}C]AAP and metabolites measured with accelerator mass spectrometry) were related to age as surrogate markers of metabolism.

Results

50 children [median age 6 months (range 3 days - 6.9 years)] received a microdose (3.3 [2.0-3.5] ng/kg; 64 [41-71] Bq/kg). Plasma [^{14}C]AAP CL/*F* was 0.4 (0.1-2.6) L/h/kg, V_{ss}/F was 1.7 (0.9-8.2) L/kg, $T_{1/2}$ was 2.8 (1-7) h. With age, plasma and urinary AAP-glu/AAP and AAP-glu/AAP-sul ratios significantly increased by 4-fold, while the AAP-sul/AAP ratio significantly decreased.

Conclusion

Using [^{14}C]labeled microdosing, the effect of age on orally administered AAP metabolism was successfully elucidated in both plasma and urine.

INTRODUCTION

Drug disposition in children may differ largely from that in adults due to age-related changes in drug absorption, distribution, metabolism and excretion [1, 2]. Drug metabolism is one of the main determinants of variation in drug disposition. While several knowledge gaps on the ontogeny of drug metabolizing enzymes (DMEs) have already been elucidated (e.g. cytochrome (CYP) 3A family) much is still unknown, especially on the 'phase II' drug metabolizing enzymes [3, 4].

Phenotyping studies can be used to elucidate *in vivo* drug metabolism pathways but in the pediatric population these studies pose several challenges in children. For one, when a validated phenotype probe for the pathway of interest is available [5], it is often a marketed drug with potentially therapeutic and toxic effects, which may not be acceptable to ethics committees, physicians, parents and their children. Second, repeated blood sampling for pharmacokinetic analyses may be difficult in light of blood volume limits (<5% of estimated circulating volume) and repeated painful punctures. The alternative method, urinary sampling, is technically challenging in young children especially without urinary catheter and results often in unreliable collections.

To overcome some of these challenges, microdosing with carbon-14 labeled drugs may be an interesting alternative to classic pharmacokinetic phenotyping studies in children [6-8]. A very low drug dose is not expected to be toxic or result in any therapeutic effect. Also, only very small blood volumes are needed for highly sensitive analytical methods, such as accelerator mass spectrometry (AMS) [9]. A microdose is defined as a maximum of 100 micrograms or 1/100th of the No Observed Adverse Effect Level (NOAEL) or animal-based predicted pharmacologic dose [10, 11]. Microdosing has been accepted by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) as a tool to study pharmacokinetics in adults [9]. If pharmacokinetics shows dose linear behavior, pharmacokinetic data can be extrapolated to therapeutic exposure [12-14]. Moreover, when an labeled oral dose is given simultaneously with a therapeutic intravenous dose (or routes are reversed), bioavailability can easily be determined. The microdose is then referred to as a microtracer. Two recent proof-of-concept studies in a limited number of patients, have shown the ethical and practical feasibility of pediatric microdosing/microtracer studies using [^{14}C]paracetamol (AAP) [13, 14]. Dose linearity was shown in adults for paracetamol from microdose to therapeutic dose under standard and inhibition conditions [15, 16]. Our own proof-of-concept study supported this dose linearity of pharmacokinetics in children [13].

Paracetamol, also known as acetaminophen or N-acetyl-p-aminophenol, is a common analgesic and antipyretic agent. Its main metabolism pathways are glucuronidation and sulfation. Glucuronidation occurs mainly via uridine 5'-diphospho-glucuronosyltransferase (UGT) 1A1, 1A6, 1A9 and 2B15 and sulfation is catalyzed by sulfotransferase (SULT)

1A1, 1A3, 1A4, and 2A1 [17]. Although not specific for individual enzymes, paracetamol pharmacokinetic studies have provided valuable insight in the relative contributions of glucuronidation and sulfation to the disposition of paracetamol. Paracetamol metabolism in young children differs from that in adults. In neonates, a higher proportion of urinary paracetamol sulfate than glucuronide suggested immaturity of the involved UGTs, compensated for by more mature sulfotransferases [18]. Other studies showed a significant increase in urinary AAP-glu/AAP-sul ratio from 0.27 in 23-week-old preterm neonates to 0.75 in 3 to 9-year-olds and 2 in adults [19]. Despite these studies, gaps remain in the knowledge on the ontogeny of paracetamol metabolism, especially for children with bodyweights between 3 and 7 kg as well as between 25 and 50 kg [20, 21]. Most developmental changes in drug metabolizing enzymes occur in the first months of life, a better insight in the exact pattern of the age-related change paracetamol metabolism may provide valuable novel information. Moreover, studies on exclusive oral paracetamol and metabolite disposition are scarce, while population pharmacokinetic studies using multiple study cohorts have often combined administration routes [20, 21] [22]. Bioavailability of oral and rectal administration differs, hence phenotyping of combined small intestinal and hepatic drug metabolism may be less precise, through by-pass of the liver after rectal administration [23]. Even though the oral bioavailability of paracetamol approaches 90% in adults, data in children are lacking and therefore a contribution of intestinal metabolism on oral paracetamol disposition cannot be excluded (Tylenol ® product information). Finally, most studies used the urinary metabolite ratio as surrogate marker for paracetamol metabolism. This method has limitations due to the difficult nature of complete and prolonged urine collections in young children, with incomplete or short collection intervals. Hence, these data may have unexplained large inter-individual variation reducing the power to detect more subtle age-related variation.

Previously, we showed that microdosing is practically and ethically feasible to study AAP pharmacokinetics in children. Now, using this innovative [^{14}C] microdosing method, we aim to study the impact of age on paracetamol disposition exclusively after oral administration, with plasma and urine analyses including metabolites (AAP-glu, AAP-sul), over the whole age range from 0 to 6 years. In this way, we aimed to further elucidate the exact developmental pattern of glucuronidation and sulphation in young children and to ultimately narrow the information gap on phase II drug metabolism.

METHODS

1. Subjects

Patients admitted to the pediatric intensive care unit of the Erasmus MC-Sophia Children's Hospital were considered for inclusion. Inclusion criteria were age from 0 through

6 years, medical need for intravenous paracetamol, an indwelling arterial or central venous line in place and written informed consent from parents or legal guardians. To reduce the impact of critical illness or bowel disease on the pharmacokinetics of paracetamol, patients with kidney and liver failure, more than one vasopressor drug, inflammatory bowel disease, or intolerant to enteral nutrition, or concomitantly receiving co-medication known to interact with paracetamol (according to Micromedex ® www.micromedexsolutions.com) were excluded.

2. Study design

The study design has been previously described in detail [13]. The Dutch Central Committee on Research Involving Human Subjects (The Hague) approved the study (EudraCT 2011-005497-28). Purchase of [^{14}C]AAP formulation and radiopharmaceutical preparation have been previously described [13]. A single [^{14}C]AAP microdose (3.3 ng/kg, 60 Bq/kg, 0.25 ml/kg) was given by mouth or enteral feeding tube. The [^{14}C]AAP oral dose also contained 1.7 µg/kg non-labeled AAP, which is negligible in relation to the therapeutic intravenous dose and considered irrelevant for pharmacokinetic estimations. Enteral feeding tubes were flushed with 2 ml saline to ensure complete rinsing. Patients would simultaneously receive therapeutic AAP as part of clinical care, dosed according to the Dutch Pediatric Handbook (www.kinderformularium.nl): 20 mg/kg loading dose, followed by 10 mg/kg every 6 h (<1 month of age) or 15 mg/kg q6h (≥ one month of age).

Blood samples (1.0 ml) were taken from the indwelling catheter immediately before administration; at around 10, and 30 min; and at 1, 2, 4, 6, 12, 24 hrs after dosing or until the catheter was removed. After centrifuging, plasma was stored at −80 ^{0}C until analysis. Urine was collected in patients who needed a urinary catheter for clinical reasons. It was collected for a maximum 24 hrs or until the urinary catheter was removed. Urine volume was entered by the nurses in the clinical electronic patient record. One sample (2 mL) was taken from the 24h-urine collection and stored at −80 ^{0}C until analysis.

3. Analytical procedures

Previously a method has been qualified to quantify [^{14}C]AAP, [^{14}C]AAP-glu and [^{14}C]AAP-sul levels in human plasma, using liquid chromatography (LC) in combination with AMS [13, 24]. The LC+AMS qualification was performed in accordance with the recommendation of the European Bioanalytical Forum [25], qualification results are summarized in Mooij et al. [13].

In short the experimental outline was as follows; a 45 µL aliquot of plasma was extracted using 175 µL100% v/v methanol containing 6.6 µg/ml AAP, 8.10 µg/mL AAP-glu, 10.0 µg/mL AAP-sul and 6.6 µg/mL 3-acetamidophenol in 96-well protein precipitation plates. The pellet was washed with 100 µL 0.9% NaCl:100% methanol (1:4 v/v). The filtrates were combined and after evaporation, redissolved in 30 µL 10 mM ammonium

phosphate pH3.4 (Eluent A). For each sample 25 µL is injected on the UPLC. Calibration standards at 8 different levels, ranging from 0.4-180 Bq/L were prepared by spiking an [^{14}C]AAP solution (SA 4100 Bq [^{14}C]AAP /100 µg AAP) to blank pooled plasma. Similarly quality standards were prepared at 3 levels, from 1.7 to 131 Bq/L respectively. For the calibration and quality standards only 15 µL of sample was used for extraction. Chromatographic conditions can be found in Table 1 of Mooij et al.[13]. The extraction recovery per sample is determined by the AUC of spiked AAP, AAP-glu and AAP-sul versus the AUC of the corresponding compounds after a direct injection of the extraction solution. The collected fractions of AAP, AAP-glu and AAP-sul were supplemented with 25 µg carbon-12, by adding 25 µL 1.97 mg/mL AAP. Fractions were transferred to a tin foil cup and evaporated to dryness prior to AMS analysis.

Urine samples were processed similarly, with a few exceptions. Urine samples were diluted 20 fold with MilliQ prior to extraction. Like for plasma samples, 45 µL of the 20 fold diluted urine samples was extracted. The extraction solution was spiked with 6.6 µg/mL 3-acetamidophenol only, which was used as an internal standard to determine the recovery of the extraction. As relatively high concentrations of cold AAP, AAP-glu and AAP-sul were present in urine samples due to the therapeutic dose the patients received, recoveries could not be determined from spiked AAP, AAP-glu and AAP-sul. Each analytical run included the duplicate analysis of the calibrators and triplicate analysis of quality standards, and passed based on the acceptance criteria [25].

Tin foil cups were combusted on an elemental analyzer (Vario Micro, Elementar, Germany). Generated CO_2 was transferred to a home-built gas-interface, composed of a zeolite trap and syringe [24]. CO_2 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/min. A 1 MV Tandetron AMS (High Voltage Engineering Europe B.V., The Netherlands) [26] was used.

4. Pharmacokinetic analysis

The maximal concentration of [^{14}C]AAP in plasma (C_{max}) and time to reach C_{max} (T_{max}) were extracted from the plasma concentration versus time curve. Area under the concentration-time curve from time zero to the last sampling time point ($AUC_{0\text{-}t}$) was calculated using the log-linear trapezoidal method. The apparent volume of distribution (V_{ss}) and total plasma clearance (Cl) were calculated by using standard non-compartmental techniques. Pharmacokinetic parameters of [^{14}C]AAP-glu and [^{14}C]AAP-sul were determined as described above for [^{14}C]AAP. All pharmacokinetic parameters were estimated using the Excel PKsolver add-in software [27].

The plasma $AUC_{0\text{-}inf}$ and urinary recovery ratios of [^{14}C]AAP/[^{14}C]AAP-glu, [^{14}C]AAP/[^{14}C]AAP-sul, and [^{14}C]AAP-glu/[^{14}C]-AAP-sul were calculated to serve as 'surrogate'

markers for glucuronidation and sulfation and their relative activity, respectively. Since AUC-ratios were used for the primary outcomes, individual AUC's were not corrected for individual dose. For clarity, the $AUC_{0\text{-}inf}$ ratios of [^{14}C]AAP/[^{14}C]AAP-glu are denoted as 'AAP/AAP-glu' etc., and the urinary recovery ratios are denoted as 'U-AAP/AAP-glu' etc.

Criteria for excluding study patients from all pharmacokinetic analyses were inadequate intake or too few blood samples (less than 4). If Cl/F, V_{ss}/F or $t_{1/2}$ were not predicted due to non-negative estimated λ_z, they were excluded for respectively Cl/F, V_{ss}/F or $t_{1/2}$ analyses. If the part of $AUC_{0\text{-}inf}$ that was extrapolated beyond the last observation was larger than 20% of the actual $AUC_{0\text{-}t}$, then the $AUC_{0\text{-}inf}$ was excluded from the analyses as it would introduce unreliable overestimation of the $AUC_{0\text{-}inf}$.

5. Statistics

The [^{14}C]AAP and metabolite concentrations were measured using Bq/L and converted to ng/L based on the exact dose given (3.3 ng/kg contained 60 Bq/kg). For the metabolites, C_{max} was corrected for molecular weight (AAP-Glu/AAP 2,165 and AAP-Sul/AAP 1,530). To compare the disposition of oral [^{14}C]AAP microdose to literature data of therapeutically dosed AAP, the microdose concentrations were dose-normalized to 15 mg/kg (assuming bioavailability is dose-linear) by multiplying the plasma [^{14}C]AAP and metabolite concentrations (in ng/L) by $5*10^6$.

Descriptive statistics of all variables are presented as medians and ranges. Nonparametric Spearman's rank correlation was used to describe the relationship between plasma and urinary metabolite ratios. Univariable linear regression analyses were performed with postnatal age as independent variable and the pharmacokinetic parameters (C_{max}, T_{max}, $t_{1/2}$, $AUC_{0\text{-}inf}$, weight-corrected Cl/F, and weight-corrected V_{ss}/F), including the AUC ratios as dependent variables. If necessary, dependent and independent variables were log-transformed to ensure normality of the residuals and an adequate model fit. The C_{max} was compared between the different modes of oral administration (oral, gastric/duodenal tube, or gastrostomy) using the Mann-Whitney test. All statistical tests were two-sided and used a significance level of P=0.05.

RESULTS

Patients

From January 13 2014 till July 1 2015, 50 patients (41 boys) with a median gestational age at birth of 40 (30-42) weeks, and a median postnatal age of 6 months (3 days till 6 years and 11 months) participated in the study (see flowchart Figure 1 and Table 1). The main reason for admission was postsurgical care (38 out of 50 patients). One patient needed mechanical ventilation. Patients received one oral [^{14}C]AAP microdose (median[range]

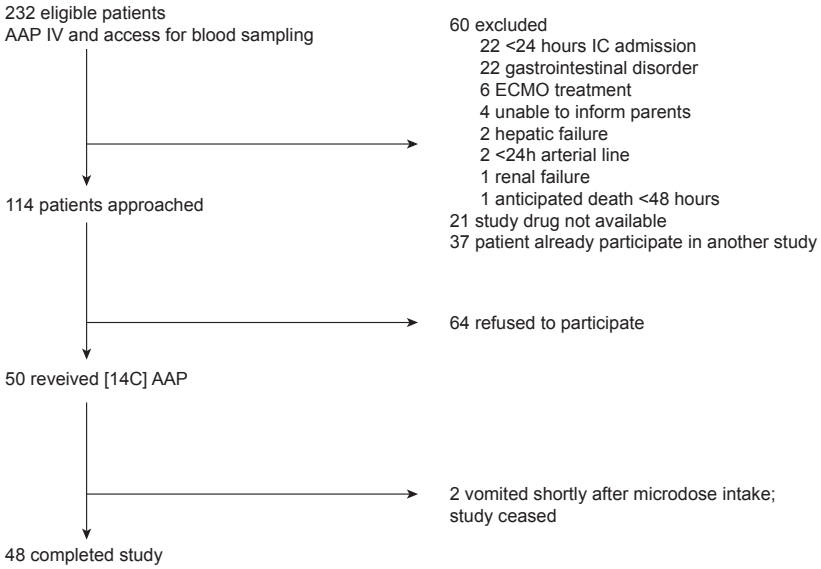

Figure 1. Flowchart

3.3 [2.0-3.5] ng/kg; 64 [41-71] Bq/kg) and up to four intravenous AAP doses during the 24 hours study period as per clinical protocol. Variation in dose was introduced by round-off errors and in one case a bodyweight-error was found in the health record and rectified after microdose administration leading to a dose of 2.0 ng/kg, 41 Bq/kg. In some cases the route of administration was switched from IV to rectal for clinical reasons after the microdose was given. In two cases, the blood sampling was discontinued the patient vomited shortly after administration of the microdose, potentially resulting in inadequate [^{14}C]AAP intake. Three patients received potentially interacting co-medication (phenytoin, phenobarbital and pentobarbital) after administration of the study drug for clinical reasons. For nine patients the number of blood samples taken was too small (less than 4) to accurately estimate all pharmacokinetic parameters and these patients were excluded from the pharmacokinetics analysis. Of the 39 patients for [^{14}C]AAP pharmacokinetics analysis, nineteen received the microdose [^{14}C] AAP via a gastric tube, 10 via oral administration (in buccal cavity) 8 via duodenal tube, and 2 via gastrostomy. For an additional patient, $AUC_{0\text{-}t}$, C_{max}, and T_{max} could be estimated but not CL/F, Vss/F, $t_{1/2}$.

Pharmacokinetics

The exemplary plasma concentration – time curves for [^{14}C]AAP, [^{14}C]AAP-glu, and [^{14}C] AAP-sul of two individual patients are depicted in Figure 2, and the pharmacokinetics are summarized in Table 2. Apparent paracetamol CL/*F* was 0.4 (0.1-2.6) L/h/kg, V_{ss}/F was 1.7 (0.9-8.2) L/kg, and $T_{1/2}$ was 2.8 (1.0-7.0) h. C_{max} for [^{14}C]AAP, [^{14}C]AAP-glu, and [^{14}C] AAP-sul equivalent for the parent compound were respectively 34.9 (3.8-89.6) Bq/L, 8.6

Table 1. Patient demographics

	Age group (birth – 1 month)	Age group (1 month – 6 months)	Age group 3 (6 months – 2 years)	Age group 4 (2 years- 6 years)
Number	9	15	14	12
Postnatal age in weeks	0 (0-1)	23 (7-25)	35 (26-98)	197 (112 – 361)
Gestational age at birth	38.1 (34.9 – 40.7)	39.8 (33.1-42)	39.9 (30-41.7)	40 (36-40)
Weight (kg)	3 (2 – 3.5)	7 (3.5-9.2)	7.5 (6.1-12.8)	16.1 (12.9-25)
Weight (z-score)	−1.12 (−2.1 - 0.1)	−0.63 (−2.5-1.5)	−1.3 (−2.5-0.9)	−0.02 (−2.9-2.1)
Gender (M/F)	8/1	14/1	10/4	9/3
Ethnicity (Caucasian/ African /Asian/Other)	5/1/2/1	11/1/1/2	12/0/0/2	11/0/0/1
Reason for admission (surgical/medical)	8/1	12/3	12/2	6/6
Mechanical ventilation at start study (yes/no)	1/8	0/15	0/14	0/12
Enteral/tube feeding (yes/ no)	3/6	10/5	9/5	6/6
PELOD (study day)	11 (1-11)	10 (0-30)	6.5 (0-30)	10 (0-20)
Number of failing organs (study day)	2 (1-3)	2 (1-2)	2 (1-3)	2 (1-2)
PRISM	16 (0-31)	18 (4-41)	17 (0-34)	6 (0-44)
PIM (%)	4 (0-20)	1 (0-10)	0 (0-9)	2 (0-33)
PIM II (%)	5 (1-15)	0 (0-17)	0 (0-6)	1 (0-29)
Paracetamol dosing				
[^{14}C]AAP dose (ng)	9.8 (6.5 – 11.7)	22.8 (11.7-29.9)	23.4 (15.6-41.6)	52 (41.6-81.2)
[^{14}C]AAP dose (Bq)	209 (125 – 228)	472 (235-639)	477 (313-890)	1035 (833-1594)
[^{14}C]AAP dose normalized (mg/kg) (dose*5E6)/weight)	15.6 (16.3-17.3)	16.2 (16.07-17)	16.3 (10-16.7)	16.3 (16.1-16.7)
Microdose administration via (oral/gastric tube/ duodenal tube/ gastrostomy)	0/6/1/0	6/4/2/0	3/6/1/0	1/3/4/2

(0.8-38.1) Bq/L, and 10.0 (1.0-41.0) Bq/L. T_{max} for [^{14}C]AAP, [^{14}C]AAP-glu, and [^{14}C]AAP-sul were respectively 1.0 (0.2-6.2)h, 4.1 (0.8-12.6)h, and 3.0 (0.3-12.6)h. T_{max} for [^{14}C]AAP was similar for all routes of administration, but with large variability (Table 2).

Dose-normalized (mg/kg dose) C_{max} for [^{14}C]AAP, [^{14}C]AAP-glu, and [^{14}C]AAP-sul were respectively 8.4 (0.9-23.0) mg/L, 3.8 (0.4-16.0) mg/L, and 4.6 (0.5-20.5) mg/L.

One outlier could be identified, but was kept in the analysis; this patient had very low plasma [^{14}C]AAP and metabolites concentrations, even though no abnormalities were noticed during oral dose administration. The patient may have partially spit out the buccally given microdose.

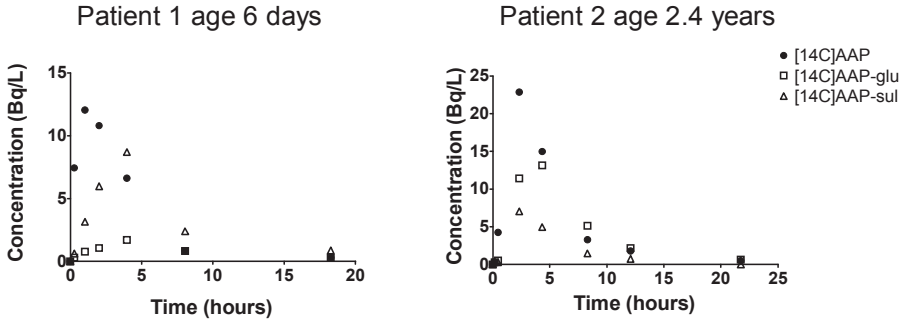

Figure 2. Two individual plots: concentration-time curve AAP, AAP-glu en AAP-sul (Bq/L)

Table 2. [^{14}C]AAP pharmacokinetics

	[^{14}C]AAP	[^{14}C]AAP-Glu	[^{14}C]AAP-Sul
Plasma			
AUC (0,t) (Bq/L*h)	152 (24-462) (n=39)	70 (9-339) (n=39)	69 (8-576) (n=39)
AUC (0,inf) (Bq/L*h)	152 (25-489) (n=38*)	75 (11-367) (n=35*)	75 (9-597) (n=37*)
CL/F (L/h)	3.1 (0.4-26.6) (n=38)	–	–
CL/F (L/h/kg)	0.4 (0.1-2.6) (n=38)	–	–
V_{ss}/F (L)	11.1 (3.0-82.2) (n=38)	–	–
V_{ss}/F (L/kg)	1.7 (0.9-8.2) (n=38)	–	–
$t_{1/2}$ (h)	2.8 (1.0-7.0) (n=38)	–	–
C_{max} (Bq/L)	34.9 (3.8-89.6) (n=39)	8.6 (0.8-38.1) (n=39)	10.0 (1.1-41.0) (n=39)
C_{max} (ng/L)	1.7 (0.2-4.6) (n=39)	0.9 (0.1-4.1) (n=39)	0.8 (0.1-3.2) (n=39)
T_{max} (h)	1.0 (0.2-6.2) (n=39)	4.1 (0.8-12.6) (n=39)	3.0 (0.3-12.6) (n=39)
Oral (n=10)	2.0 (0.2-4.5)	–	–
Gastric tube (n=19)	1.1 (0.2-7)	–	–
Duodenal tube (n=8)	0.5 (0.3-2.4)	–	–
Gastrostomy (n=2)	1.5 (0.3-2.7)	–	–
AUC(0,inf) ratio *AAP-glu/AAP*	0.4 (0.1-1.6) (n=35)	–	–
AUC(0,inf) ratio *AAP-sul/AAP*	0.5 (0.2-1.4) (n=36)	–	–
AUC(0,inf) ratio *AAP-glu/AAP-sul*	1.1 (0.2-2.8) (n=34)	–	–
Urine			
Urinary recovery as percentage of [^{14}C] AAP dose (%)	10 (4-17)%,	29 (4-46)%,	34 (12-74)%.
Total urinary recovery as percentage of [^{14}C]AAP dose (%)	75% (28-99%)		

Legend: Medians and ranges.
(*) Another 1, 4, and 2 patients were excluded from $AUC_{0\text{-}inf}$ analyses of respectively [^{14}C]AAP, [^{14}C]AAP-glu, and [^{14}C]AAP-sul, as the part of the $AUC_{0\text{-}inf}$ that was extrapolated was larger than 20% of the $AUC_{0\text{-}t}$.

Effect of age on [^{14}C]AAP disposition

No significant relationship was detected between postnatal age (which was log-transformed) and paracetamol C_{max}, T_{max}, $t_{1/2}$, $AUC_{0\text{-}inf}$, weight-corrected Cl/F or weight-corrected V_{ss}/F.

Effect of age on plasma [^{14}C]AAP metabolite ratios

The AAP-glu/AAP ratio increased with postnatal age (n=35, R^2=0.224, P<0.01) (Figure 3a), whereas the AAP-sul/AAP ratio decreased with postnatal age (n=36, R^2=0.255, P<0.01) (Figure 3b). Moreover, the AAP-glu/AAP-sul ratio increased with age from 0.32 at birth to 1.23 at the age of 6 years (n=34, R^2=0.439, P<0.001) (Figure 3c). In these analyses, postnatal age and all plasma and urine ratio-outcomes were both log-transformed.

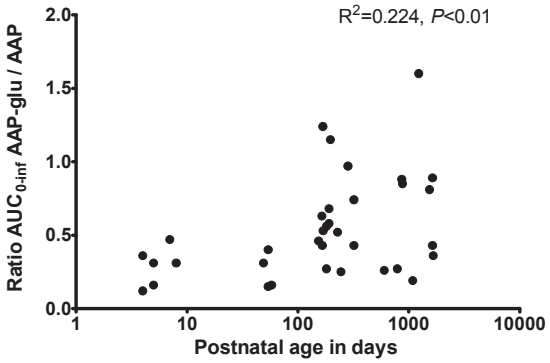

Figure 3A. Increase of serum AAP-glu/AAP ratio with postnatal age.
For linear regression analysis: plasma ratio and age were both log-transformed

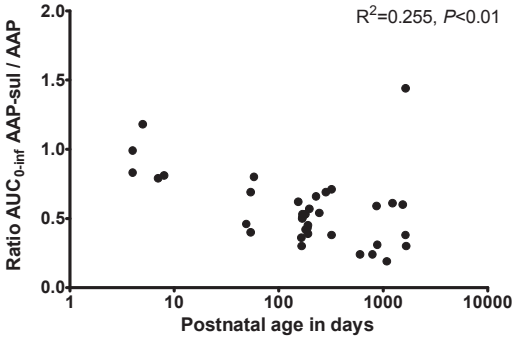

Figure 3B. Decrease of serum AAP-sul/AAP ratio with increasing age
For linear regression analysis: plasma ratio and age were both log-transformed

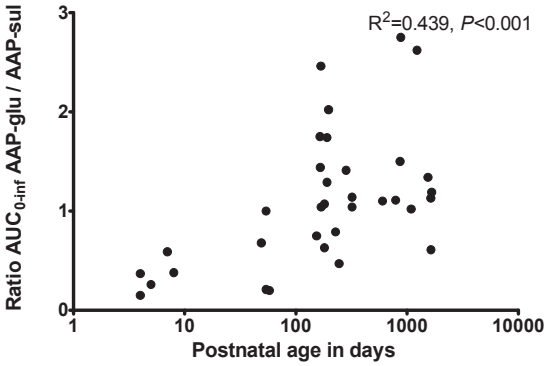

Figure 3C. Increase of serum AAP-glu/AAP-sul ratio with postnatal age
For linear regression analysis: plasma ratio and age were both log-transformed

Urinary disposition and effect of age

Urine was collected reliably for at least 12 h (20.4 h [11.8-28.5]) in 24 patients. Total median (range) urinary recovery of [^{14}C]AAP plus [^{14}C]AAP-glu and [^{14}C]AAP-sul as percentage of dose was 75% (28-99%). Percentage recovery of [^{14}C]AAP, [^{14}C]AAP-glu, and [^{14}C]AAP-sul was respectively 10 (4-17)%, 29 (4-46)%, and 34 (12-74)%. Percentage recovery of [^{14}C]AAP-glu increased with postnatal age (n=24, R^2=0.306, P<0.01); [^{14}C] AAP-sul recovery decreased (n=24, R^2=0.175, P<0.05); while [^{14}C]AAP recovery remained stable. The percentage of total urinary [^{14}C] recovery ([^{14}C]AAP + [^{14}C]AAP-glu + [^{14}C] AAP-sul) did not change with age.

The U-AAP-glu/AAP ratio significantly increased with postnatal age (n=24, R^2=0.456, P<0.001) (Figure 4a), whereas the U-AAP-sul/AAP ratio showed a borderline non-significant decrease with age (n=24, R^2=0.150, P=0.06) (Figure 4b). The U-AAP-glu/AAP-sul ratio increased linearly with postnatal age (n=24, R^2=0.575, p<0.001) (Figure 4c).

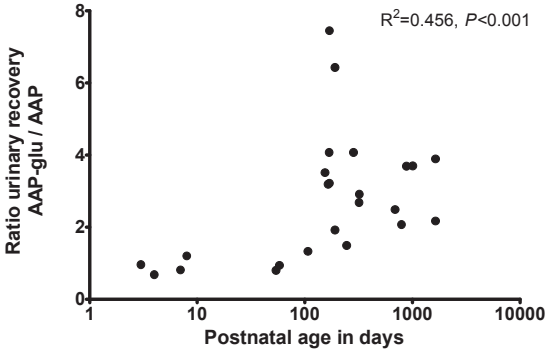

Figure 4A. Urinary recovery U-AAP-glu /AAP ratio vs. postnatal age
For linear regression analysis: urine ratio and age were both log-transformed

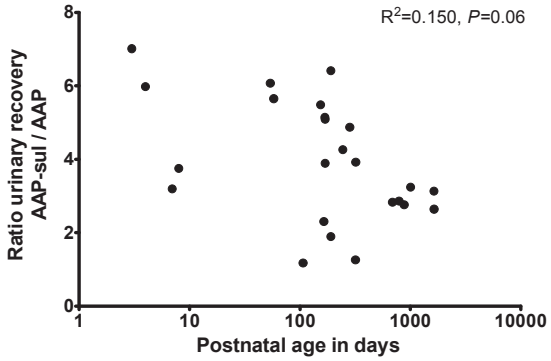

Figure 4B. Urinary recovery U-AAP-sul /AAP ratio vs. postnatal age
For linear regression analysis: urine ratio and age were both log-transformed

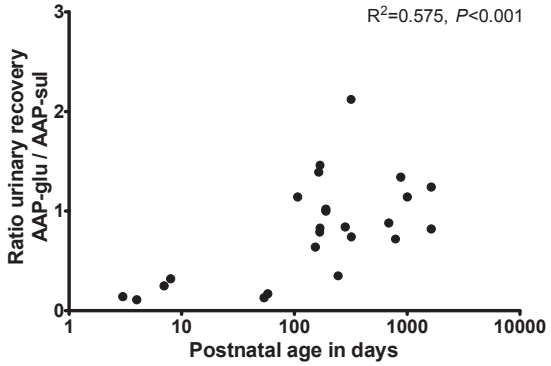

Figure 4C. Urinary recovery U-AAP-glu/AAP-sul ratio vs. postnatal age
For linear regression analysis: urine ratio and age were both log-transformed

A strong positive correlation was found between AAP-glu/AAP-sul and U-AAP-glu/AAP-sul ratio (n=19, r=0.89, $P< 0.001$).

DISCUSSION

Our study shows that [^{14}C]-microdosing allows the study of age-related changes in drug metabolism in children, using both plasma and urine as matrix. Our data present new data covering the infant age range from 0 to 6 years. Both the plasma and the urine [^{14}C]AAP glucuronide/sulfate ratio increased approximately four-fold with age, which reflects the maturation of combined intestinal and hepatic paracetamol glucuronidation and sulfation.

Few pediatric studies have reported the paracetamol pharmacokinetics after oral administration [18, 21, 22, 28-30]. Our data, with average Cl/F 0.4 (0.1-2.6) L/h/kg and V/F 1.7 (0.9-8.2 L/kg) are largely in line with these studies. In a population pharmacokinetic study combining data of these studies, concerning more than 250 patients receiving oral and/or rectal paracetamol, the mean CL/F_{oral} was 0.2 l/h/kg [95%CI 0.16-0.23] (14.1 L/h/70kg) and Vd/F was 1.12 l/kg [95%CI 1.02-1.21] (78.7 L/70kg) [21]. Cl/F was 0.07 L/h/kg and V/F was 1.088 L/kg in a 'post-hoc' NONMEM analysis of a selection of 30 neonates[22]. In contrast to this finding, we did not find an effect of age on weight-corrected Cl/F, but is very likely because the number of neonates in our cohort was relatively small.

In our study, both the plasma and the urine AAP-glu/AAP-sul ratio were related to age; they increased from 0.32 in neonates to 1.23 in 6-year-olds and a similar increase in urine. The AAP-glu/AAP-sul ration served as a surrogate marker for the relative contribution of glucuronidation and sulfation to AAP metabolism as in previous studies [18, 19, 21, 31]. Previous studies reported changes in the urinary metabolites ratio after oral paracetamol administration from 0.27 in 2-3-day-old neonates, to 0.69 in 12-month-olds, 0.81 in 2-years-olds, 0.75 in 3- to 9-year-olds, 1.61 in 12-year-olds to 1.8 in adults [18, 21, 28]. Limitations of existing studies were: only presenting neonatal data [28], or few patients less than 9 years of age [18] and a combined cohort of oral and rectal administration, with urinary data on AAP and metabolites (instead of plasma) of 15 patients with a small age range (11.8±2.5 months) [21]. To the latter study an additional cohort of >200 children receiving oral and/or rectal AAP was added in the AAP-clearance analysis (without metabolites concentrations). However, as rectal paracetamol completely bypasses the small intestine and partially bypasses the liver by draining in the lower haemorrhoidal circulation its metabolism not optimally represent combined intestinal and hepatic glucuronidation and sulfation activity.

Allegaert et al. showed in 23 preterm and term newborns a significant increase of the urinary glucuronide/total drug ratio with both increasing postnatal and postconception age after IV administration of the pro-drug propacetamol [32]. In 75 children (3 neonates, 25 infants, 25 children, and 22 adolescents) who received repeated intravenous paracetamol, glucuronidation was the primary pathway of elimination in the older, but not the neonatal age group, where sulfation dominated [33]. The urinary AAP-glu/AAP-sul ratio collected over 4 h after the last dose, was low in neonates and higher at older age; 0.6 in neonates, 1.0 in infants, 1.4 in children, and 1.2 in adolescents. First-pass drug metabolism after oral drug administration may occur at both the intestine and the liver. Here we see similar ratio AAP-glu/AAP-sul data after oral (our study) and IV administration (Zuppa et al., Allegaert et al.), suggesting that the intestinal contribution to paracetamol metabolism and its ontogeny either equals hepatic metabolism or is negligible.

Exploring the possibility to model the maturation half-life of AAP-glu and AAP-sul activities as depicted in figure 3, we were able to model a sigmoidal E_{max} curve for the ratio of AAP-glu to AAP-sul (results not shown). Nevertheless, the confidence intervals of the estimated parameters were wide, suggesting a limited amount of evidence for a sigmoidal relationship. Visual inspection of the maturation curves suggests a quite sudden surge in glucuronidation after 200 days of age, but a more gradual decrease in AAP sulfation from birth to 6 years of age.

This study has several strengths. First, we show the feasibility to perform a drug metabolism phenotyping study using [^{14}C]AAP with only 0.2 millionth of the therapeutic dose. Although such a design has been used in healthy adults previously, we now show the feasibility to include a considerable number of pediatric patients in the intensive care unit with a rich blood sampling design and reliable urine collections. In addition, this study provides longitudinal data on both the plasma and the urinary [^{14}C]AAP- metabolism after exclusive oral administration in a continuous cohort from 3 days to 6 years of age. These data add to our understanding on the combined intestinal and hepatic glucuronidation and sulfation activities, which is of importance given that most drugs are given orally. Another strength is the combination of urine and plasma collection, showing the feasibility of [^{14}C]microdosing to use either matrix for drug metabolism phenotyping. The very strong correlation between plasma and urinary AAP-glu/AAP-sul ratios, as well as the similar developmental changes in plasma (our study) and urine [28] suggest that both matrices can be used to study paracetamol disposition. Plasma calculations were more reliable (n=39 complete plasma collections, versus n=24 complete urine collections) with less variation compared to the urinary recovery, but blood sampling are more invasive. Hence, dependent on the study population (with/without indwelling arterial/venous access), age range, or renal clearance-dependent drug, both plasma and urine could be considered.

A possible barrier for investigators to use a [^{14}C]-microdosing study in children is the perceived risk of radiation [7]. To our own surprise, in this non-therapeutic study with radioactive labelled probe, fear of radiation was hardly a reason for parents to withhold consent. Similar to other drug studies on our intensive care unit, almost half of all approached parents agreed to participate. Refusal of consent was rather for reasons relating to the burden of blood sampling or additional procedures. The PAMPER group, also using [^{14}C]AAP, experienced the same inclusion rate [14].

A limitation of our study was that study patients did not receive a single microdose exclusively and therefore one could argue that it is not a 'true' microdosing study but a microtracer study. We choose this design with the microdose in addition to the same therapeutic drug, as this was one of the first microdosing studies in children, and it was likely more acceptable to Ethics Board and parents. We are now convinced that this is not a major issue for parents, who understood the concept of microdosing clearly and

realized it was associated with minimal burden for the patient. Another limitation is the relatively low number of neonates meeting inclusion criteria.

In conclusion, we show the feasibility of using a [^{14}C]drug microdose to phenotype the ontogeny of drug metabolism in children. The previously reported developmental change of paracetamol metabolism from mainly sulfation to glucuronidation was comparable, while plasma metabolite data were added on the combined intestinal and hepatic pathways in a large pediatric age range. The success of this study design is promising for future studies on the development of drug disposition.

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Part IV

Discussion and summary

12 General discussion

Pharmacotherapy is the key ingredient of many treatment approaches for diseases in children. Yet, the effects of pharmacotherapy change as children evolve during life; consequently therapy strategies need to be adjusted to growth and development. Several information gaps remain on the impact of age on the processes involved in the disposition of drugs. More specifically, important factors governing oral bioavailability in children, including intestinal metabolism as well membrane transport, are understudied so far. The extent of oral drug use in critically ill children, the current state of knowledge on oral drug absorption in children, and the ontogeny of membrane transporters and phase II drug metabolism are the subjects presented in this thesis. The lack of pediatric data can partly be ascribed to difficulties in collecting pediatric tissue and performing pharmacokinetic studies. Using two innovative techniques primarily developed for adult studies, novel data were generated on drug metabolism and drug transport in young children.

To end with, future research perspectives, building on the data in this thesis, are presented that ultimately may have the potential to improve efficacy and safety of drug therapy in children.

ORAL DRUG USE

Oral drug prescribing in the NICU and PICU

Oral drug absorption in critically ill children may be erratic and the use of the oral route for life-saving drugs is therefore discouraged. In the intensive care setting intravenous (IV) drugs are preferred and most patients will have an indwelling arterial or venous line. Nevertheless, for several reasons the oral route may still be preferable: i.e., appropriate intravenous formulations may not be available (e.g., spironolactone, bosentan); IV administration may be associated with more severe adverse events (e.g., sildenafil and hypotension, infections associated with central lines); or the site of action is the gastrointestinal tract (e.g., antifungal prophylaxis). Moreover, polypharmacy with incompatible drugs through the available intravenous access ports, inability to gain any venous access and higher costs of intravenous administration may all be reason to resort to the oral route.

Very few reports are available on the use of oral drugs in the neonatal and/or pediatric intensive care units (ICUs). In one drug utilization study in an Indian NICU 92% of prescribed drugs over 6 months were given intravenously, of which the remainder were prescribed via other routes, including oral [1]. In contrast, we showed that up to 27% of drugs were prescribed orally to children in neonatal and pediatric intensive care units (NICU and PICU) [chapter 5]. The dissimilarity may be explained by differences in age ranges and setting (developing vs. western country).

The potential erratic oral absorption may lead to unwanted variation in drug efficacy and safety. When looking at the 10 most frequently prescribed oral drugs, the target of probiotics, amphotericin B, and macrogol is the gut or gut flora; and therefore erratic oral intestinal absorption is less relevant. Nevertheless, when gut motility is severely impaired in critical illness, the drug may not even reach the relevant intestinal compartment (e.g., colon for macrogol). In contrast, for other frequently prescribed oral drugs, erratic oral absorption may be more problematic: e.g., analgesics, diuretics, vitamin K, omeprazole, and lorazepam. Moreover, data from critically ill patients are often lacking. For example, the efficacy and safety of omeprazole for the prevention of ICU related gastric bleeding have not been shown in children. Moreover, no suitable oral formulation is available to be given to young children or by gastric tube. And while oral diuretics for neonatal chronic lung disease seem effective, very limited data on their efficacy for treating heart failure or edema are available for older critically ill children [2]. Moreover, no licensed liquid drug formulations suitable for pediatric use are available for the oral diuretics hydrochlorothiazide and spironolactone. Also, for lorazepam no oral formulation is available and instead the intravenous formulation or crushed tablets are given, very erratic drug levels and sedative effect could be expected.

Additionally, in a study on the risk of intervention on prescriptions by a clinical pharmacist the oral dosage form and oral route of administration had the highest risk for interventions. This was monitored in electronic dose prescribing for four years in a tertiary children's hospital (excluding the ICUs) [3]. An intervention was defined as any action taken on a drug prescription to correct or complete the prescription. 81% of all interventions concerned a correction of a prescription that otherwise might have had adverse clinical consequences. This result emphasizes the importance of awareness on oral drug use in the NICU and PICU.

In summary, oral drug use is frequent in critically children and exposes them to potentially ineffective and unsafe drug therapy.

Age-related changes in oral drug absorption processes

To better understand the impact of age on the gastrointestinal processes that govern the intestinal absorption of drugs, we reviewed the existing literature. Important age related variation was found for gastric pH. Apart from a brief peak postnatally, the gastric pH is about 2-3 in children of all ages. It rises postprandially due to a buffering effect of milk-based feeding [4]. In frequently fed neonates (such as extreme low birth weight infants or in case of continuous nasogastric tube feeding) the pH may therefore peak longer than in older children who eat less frequently. This questions the general idea that pH is basic in the first days to weeks of life, reported more than seventy years ago by Miller et al. [5]. Other, more recent studies report that the gastric pH declines already within a few hours after birth [6, 7]. Many other studies subsequently showed that the

gastric pH remains low at a pH around 2 and 3 in children of all ages [4, 8-22]. Nevertheless, large information gaps still exist on the impact of age on almost all drug absorption processes: gastric pH, gastrointestinal motility, bile salts, pancreatic function, intestinal pH, intestinal drug metabolizing enzymes (DMEs) and transporter proteins. Hence, as even in critically ill children of all ages many drugs are prescribed orally, it is important to better understand the impact of age on these processes, as well as their interplay, so as to be able to individualize dosing.

The TNO Gastro-Intestinal Tract model (TIM) is an interesting *in vitro* model to study the passage of a drug across the intestinal tract. TIM is a computer-controlled dynamic system that mimics the physiological conditions in stomach and intestines [23, 24]. Gastrointestinal parameters can be adjusted and intraluminal processing of drug dosage forms can be simulated [25]. The effects of drug manipulations to enhance drug ingestion by children can be studied using TIM (e.g., dissolving tablets in apple juice, apple sauce, crushing). In a pilot study, the feasibility to study the influence of different dosage forms on pediatric oral drug disposition was shown using the pediatric TIM model [26].

Once a drug has reached the small intestine it needs to cross the intestinal wall and pass the liver to reach the systemic circulation. Important processes involved in this step of oral drug absorption are drug metabolism and membrane transporters, which will be discussed below.

Conclusion

Oral drug absorption in critically ill children may be erratic, leading to inadequate pharmacotherapy, nevertheless up to 27% of drugs are administered via oral route. To date, large information gaps exist regarding the effect of age on gastrointestinal processes involved in oral drug absorption.

MEMBRANE TRANSPORTERS

New insights on ontogeny of transporters

In vitro studies, adult pharmacogenetics and drug-drug interaction studies have shown that human membrane transporters play a clinically relevant role in drug disposition [27]. It seems not unreasonable to assume that they are also important in the developing child. Moreover, as these transporters are also implicated in physiological processes, age-related variation subsequent to their role in normal growth and development can be expected. A quite recent broader interest in pediatric membrane transporters activity has resulted in a white paper on this subject from the International Transporter Working Group [chapter 3]. Not surprisingly, data in fetuses and children about transporter expression and activity are very scarce.

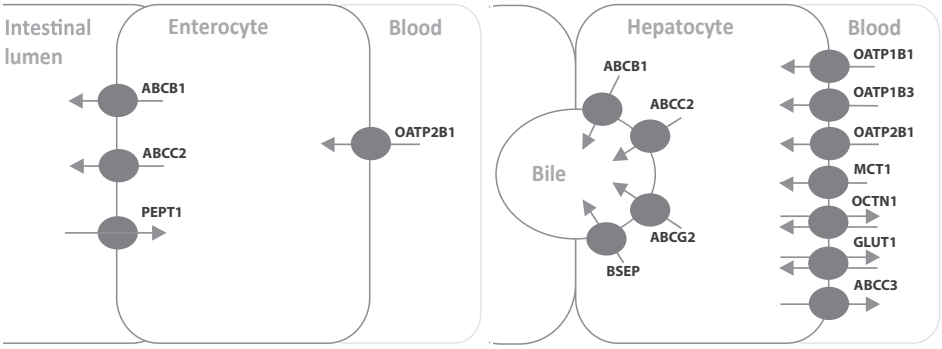

Figure 1. Transporters in enterocyte and hepatocyte
OATP2B1: immunohistochemistry shows basolateral coloring; literature suggests apical localization. MCT1: unknown

The research presented in this thesis has generated novel data on the ontogeny of hepatic and intestinal transporters in fetuses and children. We studied a selection of efflux transporters from the ATP-binding cassette (ABC) family (ABCB1, ABCC2, ABCC3, ABCG2, ABCB11) and uptake transporters from the solute carrier organic anion (SLCO) family (OATP1B1, OATP1B3 and OATP2B1), and from the solute carrier (SLC) family (SLC2A1/GLUT1, SLC15A1/PEPT1, SLC16A1/MCT1, SLC22A5/OCTN2) (figure 1).

At gene and protein expression level, we have shown that hepatic and intestinal membrane transporters change with age in a transporter-specific and organ-dependent manner. At hepatic protein level, four different expression patterns emerged 1) 'Stable' through childhood; 2) 'Low to high'; 3) 'High to low'; 4) 'Non-linear' with highest expression in infants.

Interestingly, age-related changes in gene expression were not reflected in the same changes in protein expression for ABCB1 and OATP1B1, but were found for ABCC2. This discrepancy shows the relative weakness of relying solely on gene expression data to understand transporter maturation. Posttranscriptional changes – for example mediated by microRNAs – could explain that mRNA does not correspond to protein expression [28]. Also in hepatic miRNA expression developmental changes have been shown, and for some, miRNA expression was correlated to mRNA expression of drug disposition genes [29]. Aside from the gene expression data, also the protein data should be interpreted carefully. Transporter protein expression itself may not necessarily translate to transporter activity. Glycosylation, phosphorylation, and ubiquination might alter proteins into active or non-active forms [30, 31]. Hence, posttranslational regulation in light of ontogeny of transporters needs further elucidation.

The finding of age-dependent variation in transporter activity may also increase our understanding of the physiological transporter function during growth and development. For example, transporters involved in energy supply in fetal life may be up regu-

lated in fetuses and newborns. Glucose transporter GLUT1 and L-carnitine (molecule that shuttles long-chain fatty acids to the mitochondria for oxidation) transporter OCTN2, are probably both essential for fetal energy supply, which seems to agree with our findings of high protein expression in fetuses and newborns [32-34]. On the other hand, transporters involved in the uptake and efflux of steroids and bilirubin (i.e., OATPs) may show less variability throughout life.

The research field on membrane transporters in pediatrics remains with numerous gaps and the following research efforts are recommended:

I) *Expanding the pediatric age range*. Even though most changes are to be expected in the first months of life, no conclusions can be made for the entire pediatric age range due to the lack of data. More specifically, data on hepatic protein expression between the ages of 3 months and 7 years is completely lacking. Given the functional role of transporters to translocate hormones, of particular interest are children reaching puberty, in whom hormonal changes might influence transporter function and gender differences might appear [30].

II) Given the role of transporters in translocating drug substrates over membranes and mediating pharmacokinetics, *the ontogeny of transporters in other organs than liver and intestine* should be explored. Human gene and protein expression studies suggest ontogeny of transporters in the kidneys (influencing renal clearance of drugs); in the brain (crossing blood-brain barrier causing effect or side-effects) lower ABCB1 protein expression was shown in fetuses or neonates [35-38]. Hence, these and other sites are of particular interest.

III) *Including clinical information in biobank tissue collections* to supplement data cohorts. From drug-drug interaction studies we know that drugs may inhibit or induce transporter activity. Also, disease state may affect the expression and activity of membrane transporters as has been shown in children with inflammatory bowel disease [39]. Including more patient details asks for more efforts in data collection and storage. While this may provide useful additional information to explain interindividual variation, its benefit should be balanced against the potential decrease in the number of samples collected because of more logistical, practical and ethical hurdles [40, 41].

IV) *Expanding the selection of studied transporters*. To date more than 400 transporters have been identified [42]. The disposition of drugs is most often associated with two superfamilies: the solute carrier (SLC) and ATP-binding cassette transporters (ABC) families. We have studied a selection of 11 hepatic transporters and 4 intestinal transporters. Even though this selection included the most relevant transporters for mediating drug pharmacokinetics based on adult pharmacogenetic studies or high abundance in liver or intestine, this selection is merely a start. For example, we did not study OCT1, which nevertheless seems important in the disposition of opioids

such as morphine and tramadol [43, 44]. Other transporters as well may appear to play an important role in pediatric drug disposition.

In vivo membrane transporter research

In vivo validation is the next step in transporter research. Gene and protein expression might change during childhood, but does this also results in differences in transporter activity? The *in vivo* activity of transporters remains a black box in view of the ambiguous nature of substrates to multiple transporters. *In vitro* activity tests in cell lines or tissue cultures do not account for age-related differences. To study transporter activity *in vivo*, preferably the disposition of a probe drug or endogenous substrate should be exclusively altered by changes in the activity of an individual transporter. For example, changes in clearance may serve as surrogate marker of transporter activity. Due to the broad membrane specificity of most drugs, very few relevant probe drugs have been identified. In addition to drugs, endogenous substrates may serve as phenotyping probes, such as in the case of CYP3A4 and CYP2D6 [45, 46]. This approach avoids the ethical challenges associated with the administration of a non-therapeutic probe drug to children.

The clinical relevance of single transporters in children is mainly based on the occurrence of unexpected drug-drug interactions. An interesting example of altered transporter activity in children is the drug-drug interaction in a study with 8 children (age 2 weeks – 3.7 years) treated for dilated cardiomyopathy with digoxin and carvedilol [47]. Digoxin renal clearance relies on ABCB1; due to the interaction with carvedilol, less digoxin was excreted in the urine, and serum digoxin concentrations increased. This effect was also observed in adult patients although the digoxin clearance decreased less. This supports the hypothesis that renal ABCB1 is present from the age of 2 years and it suggests that renal digoxin clearance in young children might be more dependent on ABCB1-mediatred tubular secretion than in adults.

The use of pharmacogenetics is another way of studying transporter activity and resulting effect in pharmacokinetics. As an example, in childhood leukemia patients, multiple single nucleotide polymorphism (SNP) loss-of-function variants in the *SCLO1B1* gene (encoding for OATP1B1) are related to lower methotrexate clearance leading to toxicity [48]. This points to an important role of OATP1B1 activity in methotrexate pharmacokinetics, as well as maturity of the transporter in the studied age group.

These transporter-specific, organ-dependent ontogeny profiles might already have consequences for a patient's individual pharmacotherapy. Still it is too early to justify dose adjustments of substrates based on lesser transporter expression in the first month of life. Nevertheless we can argue that closer monitoring of drug levels by therapeutic drug monitoring (TDM) is justified for substrates of transporters with age-related changes in expression (ABCC2, ABCC3, ABCG2, GLUT1, MCT1, OCTN2, BSEP, OATP1B3), with a small therapeutic window and important safety problems.

Conclusion

Current data shows maturation profiles in human membrane transporters in an organ-specific and transporter-dependent manner. These differential patterns are likely to affect the disposition of drugs and their metabolites in an age-dependent matter. Further research should cover the entire pediatric age range, and include clinical and genetic co-variates in expression and activity studies. Clinical studies should include observational drug-drug interactions, pharmacogenetic and potential probe-based phenotyping studies.

PHASE II DRUG METABOLISM

New insights on the ontogeny of phase II drug metabolizing enzymes

Drug metabolism is the biochemical modification of pharmaceutical substances by enzymes and is one of the main determinants of variation in drug disposition. Phenotyping studies can be used to elucidate age-related changes in *in vivo* drug metabolism pathways [49]. While phase I metabolism has been studied relatively well in children [50] by means of *in vitro* and phenotyping studies, phase II metabolism is understudied [51]. Moreover, most studies have focused on hepatic metabolism using intravenous substrates. Since drug metabolizing enzymes are also abundant in the intestine, the combined intestinal and hepatic metabolism forms a notably argument to investigate this aspect of pediatric drug metabolism.

To elucidate the combined intestinal and hepatic phase II metabolism, we chose to study the oral disposition of paracetamol [chapter 11]. Although multiple UGT and SULT enzymes metabolize paracetamol, its disposition has previously shown clear age-related changes [52-55]. However, exclusive oral paracetamol dosing has been sparsely used in these studies. Moreover, in larger pediatric population pharmacokinetic studies, multiple administration routes (oral, rectal and intravenous) have been analyzed together [53, 56]. This may have obscured age-related differences in oral paracetamol disposition, since paracetamol bypasses the gut after intravenous administration, or bypasses the gut and liver after rectal dosing. Furthermore, children with bodyweights 3-7 kg and 25-50 kg) were not included in these population pharmacokinetics studies. Hence, the exact developmental pattern of paracetamol metabolism after oral administration has not been fully elucidated. To bridge these gaps we studied the oral disposition of paracetamol in children in the age range of 0 to 6 years.

To overcome the ethical objections of therapeutic drug dosing solely for research, we used a single [^{14}C]paracetamol microdose in children already receiving intravenous paracetamol for clinical reasons. Since the oral bioavailability of paracetamol in adults is around 85-98% (Tylenol® product information), a major contribution of intestinal me-

tabolism might not be expected. However, bioavailability paracetamol studies in young children are lacking and therefore the role of intestinal paracetamol metabolism is unclear. In a later stage, this study design will enable to specifically study the hepatic and intestinal contributions to paracetamol metabolism, as well as the ontogeny of both.

Our most important findings agree with literature data [52-55]; we showed the converse developmental pattern of paracetamol with mainly sulfation in younger children to mainly glucuronidation in older children. Reported urinary metabolites ratio after oral paracetamol administration rise steady from 0.27 in 2-3 day-old-neonates, to around 1.8 in adolescents and adults [53, 57, 58]. Population pharmacokinetic studies propose a non-linear increase of glucuronidation with increasing age, reaching maturity at 3 years. We have tested a non-linear model, however, in which the confidence intervals were wide, suggesting little evidence for a reliable estimation and therefore we preferred a linear model.

At the *in vitro* level, the ontogeny of DMEs involved in paracetamol metabolism has been described. Hepatic UGT1A1, UGT1A6, and UGT1A9 activities were almost negligible in neonates [59, 60]. Despite the assumed absence of UGT1A1, UGT1A6 and UGT1A9, urinary AAP-glu is present in neonates, and therefore UGT2B15 is supposed to have substantial activity at birth [57, 58]. The observed maturation of paracetamol glucuronidation may therefore reflect the combined maturation pattern of these three other UGTs, from the UGT1 family, with relatively minor contribution of UGT2B15 from birth onwards.

The relative contributions of different SULT enzymes are different in fetal and pediatric liver samples, with mainly SULT1A3/4 and SULT1E1 in fetal or SULT1A1 in pediatric liver samples contributing to variability in AAP metabolism [61]. The observed slower decrease in paracetamol sulfation may be a reflection of a gradual switch from SULT1A3/4 and SULT1E1 to SULT1A1 activity. A similar switch is seen for fetal CYP3A7 to postnatal CYP3A4 metabolism, but this switch already occurs in the first days of life [62].

Based on urinary metabolite recoveries, it has been suggested that multiple dosing of paracetamol in neonates, infants, and adults up-regulates glucuronidation [63-65]. A recent population pharmacokinetic study, however, did not show up-regulation of glucuronidation after multiple paracetamol dosing, but only the time-related increase in bodyweight reflected the developmental changes in glucuronidation [66].

Paracetamol is a widely used analgesic agent in the ICU. Although critically ill patients may show altered drug pharmacokinetics, this is of minor relevance for paracetamol clearance. Paracetamol pharmacokinetic data in 38 adult patients, from medium care and intensive care units, showed comparable median and range paracetamol clearance [67]. Moreover, hepatic clearance in healthy adult persons and patients with medium liver-failure was comparable, suggesting no effect of critical illness on paracetamol metabolism [68]. Besides, plasma protein binding of paracetamol is low, so AAP me-

tabolism will not be affected in critical ill patients who often have low albumin levels [68]. Lastly, our outcome measure (AAP-glu/AAP-sul ratio) was the same as reported for healthy neonates and critically ill neonates (0.33) [chapter 11][64, 69]. This suggests only a minor effect of critical illness on paracetamol glucuronidation. Nevertheless it cannot be excluded that it has an effect on individual DMEs, as has been suggested for CYP3A4 [70].

A small portion of AAP is metabolized into other metabolites than glucuronide and sulfate metabolites: 3-hydroxy-paracetamol, 3-methoxy-paracetamol, 3-cysteinyl-paracetamol, paracetamol-mercaptopurate, which in their turn might be conjugated with glucuronide or sulfate (Tylenol ® product information) [71]. Some metabolites have been measured in children receiving therapeutic IV paracetamol dose, though possible age-related differences in the formation of these metabolites are unknown[71]. The highly reactive intermediate NAPQI (N-acetyl-p-benzo-quinone imine) is formed from a small portion of AAP CYP2E1 metabolism. When NAPQI is not detoxified by glutathione it can cause hepatotoxicity. It has been speculated that young children are more resistant to AAP-induced toxicity than adults [72] due to higher glutathione stores, metabolism differences, or relatively larger liver size. These metabolites have not been analyzed in this study due to financial constraints.

Conclusion

Paracetamol metabolism shows age-related changes with mainly sulfation in neonates to mainly glucuronidation in older children. Urinary and plasma AAP-glu/AAP-sul AUC ratio can serve as a surrogate marker for paracetamol metabolism. Remaining gap in the ontogeny of paracetamol is the knowledge on formation of the toxic metabolites.

INNOVATIVE METHODS TO STUDY PEDIATRIC PHARMACOLOGY

Children are a vulnerable population who are relatively incapable in understanding and expressing the protection of their own interest and therefore need to be protected against harm. The criteria for pediatric research trials are rather stringent, therefore, so that children are 'therapeutic orphans', also in view of the fact that many trials in pediatric populations fail. The widespread off-label or unlicensed use of drugs is a legitimate reason to generate new knowledge and improve care. The research question addressed in this thesis – to elucidate the ontogeny of membrane transporter and drug metabolism – cannot be studied outside the pediatric population however. Animal studies might reveal certain ontogeny, nevertheless the results cannot be simply extrapolated to humans.

Thus we need to explore new ways of performing drug studies within the boundaries of the law and the medical ethical reviews. This thesis describes two innovative methods, LC-MS/MS and microdosing to elucidate developmental changes in membrane transporters and metabolism pathways. Below, we discuss the background as well as the pros and cons of these techniques.

Microdosing

Microdosing is an interesting tool to study pharmacokinetics in children for the following reasons: **I)** it has minimal burden and carries minimal risk; **II)** it requires a small blood volume since plasma (or urine) samples can be as little as 30 µL due to the highly sensitive Accelerator Mass Spectrometry (AMS) technique; **III)** an oral labeled microdose enables bioavailability studies when a therapeutic IV dose is administered simultaneously, which reduces the amount of blood samples.

To date, three actual pediatric microdosing studies in both Europe and the US have been performed. Including position papers and reviews, seven reports were published on microdosing in children (table 1) [chapter 11][73-78]. Our experience using microdosing is predominantly positive, and rational designed microdosing studies can be of benefit to pediatric drug research.

A few critical notes are in order here. First, to predict pharmacokinetics, dose-linearity is essential. Some compounds show non-linearity in adults and are therefore unsuitable to study pharmacokinetics in children, e.g., erythromycin and warfarin [79]. It is recommended to test dose-linearity in an adult population first if possible. From an organizational perspective, pediatric microdosing studies are challenging. Radiopharmaceutical preparation requires extra licenses for pharmaceutical and radiochemical handling, which implies that involvement of a common pharmacy alone is insufficient. Extra permissions from institutional radiation offices may be needed [76]. As for the analysis stage, AMS facilities are scarce. Though prices are probe-specific, both radiopharmaceutical preparation and AMS analysis drive prices and in our study resulted in the main costs expenditures (Euro 500.000,-).

Regulatory issues were differently addressed in the published studies. In our case, a single microtracer was considered 'a drug in a pharmacokinetics study' therefore the study was considered as a clinical trial of an investigational medicinal product (CT-IMP). This was not the case in the UK and Estonia [75, 76], where it was considered 'a well-characterized probe in a physiological experiment' (a study involving a less well-characterized probe would be a CTIMP in the UK). In the Netherlands, the hospital's Radiation Safety Officer considered the formulation radioactive and requested Radiation Safety training by the junior researcher, as well as a study-specific radiation handling protocol. In the UK and Estonia it was not considered radioactive. Body fluids and blood/urine samples were considered radioactive only in the Netherlands. The radiation dose,

Table 1. Overview of pediatric microdosing reports

	Vuong et al. [73]	Gordi et al. [74]	Garner et al. [75]	Mooij et al. [78]	Turner et al. [76]	Roth-Clinic et al. [77]	Mooij et al. [chapter 11]
Year	2014	2014	2014	2014	2015	2015	2015
Article type	Review	Original data	Original data	Pilot study	Review	Commentary	Original data
Aims	To explore use of AMS for pediatrics	To show proof-of-concept [^{14}C] microdose and PK of ursodiol	[^{14}C]AAP PK after A) mixed in therapeutic dose or B) isolated microdose	To show proof-of-concept [^{14}C]AAP microdosing in children	Experience of pediatric microdose and microtracer studies	A US Regulatory perspective on pediatric micorodosing studies	To study ontogeny of glucuronidation and sulfation using [^{14}C]AAP
Study group	5 term neonates	Total 8 term neonates: A) 5 without cholestasis; B) 3 with cholestasis	34 infants 0-2 years	9 children 0-6 years	–	–	48 children 0-6 years
Inclusion criteria	indwelling venous or arterial catheter	indwelling venous or arterial catheter	IV line or tolerate to enteral dose [14C] AAP and access for blood sampling	arterial or central venous line and receiving therapeutic AAP	–	–	arterial or central venous line and receiving therapeutic AAP
Probe	[^{14}C]ursodiol	[^{14}C]ursodiol	[^{14}C]paracetamol	[^{14}C]paracetamol	[^{14}C]paracetamol	–	[^{14}C]paracetamol
Route	oral	oral	oral and IV	oral	–	–	oral
Dose	3 doses every 48h: 37 Bq (8 ng), 122 Bq (26 ng), 370 Bq (80 ng) consecutively	A) 3 doses every 48h; B) 370 Bq (80 ng) mixed with therapeutic dose of 40 mg/kg	single dose 111 Bq/kg (6 ng/kg)	single dose 3.3 ng/kg (60 Bq/kg)	–	–	single dose 3.3 ng/kg (60 Bq/kg)
Sampling methods	blood (250 µL) 7 samples (21 per patient in total)	blood (250 µL) 7 samples	blood (100-250 µL) 5 samples	blood (1 mL) max 8 samples	–	–	blood (1 mL) max 8 samples and urine (12-24 h)

Table 1. Overview of pediatric microdosing reports (continued)

	Vuong et al. [73]	Gordi et al. [74]	Garner et al. [75]	Mooij et al. [78]	Turner et al. [76]	Roth-Clinic et al. [77]	Mooij et al. [chapter 11]
Conclusion	Regulatory and practical feasible to design [^{14}C]labeled compounds and AMS study for use in pediatric population	Ursodiol exhibited linear pharmacokinetics with no dose or time-dependency at the tested doses. It was possible to differentiate between endogenous and exogenous compounds	The PK parameters of therapeutic dose and microdose in infants 35-127 weeks of postmenstrual age were within a two-fold range.	[^{14}C]microdosing is practical and ethical feasible in pediatrics	Taking the challenges such as dose-linearity and costs into account, microdosing/ microtracer trials are believed of added value for pediatric drug development.	Pediatric microdosing studies must first be scientifically necessary. Due to the absence of a therapeutic effect, it is not considered under 21 CFR 50,25 (prospect of benefit)	[^{14}C]microdose is feasible to phenotype the ontogeny of drug metabolism in children. Developmental change of metabolism form mainly sulfation to glucuronidation was shown.

however, was so low that in none of these three countries special handling was required in all. Vuong and Gordi et al. did not describe the regulatory issues concerning handling of [^{14}C]-materials within their hospitals [73, 74].

Regarding the use of microdosing studies in pediatrics, the U.S. Food and Drug Administration (FDA) requires they address a question which is a 'scientific necessity' and put minimal burden on the child [77]. Correspondingly, the Dutch legislation on non-therapeutic trials allows them only when there is minimal risk and minimal burden.

The major barrier to a [^{14}C]microdosing study in children has been the perceived risk of radiation [80, 81]. Still, doses in the published microdosing studies are low, ranging from 37 Bq (Gordi et al.) to a maximum of 1594 Bq (60 Bq/kg) (our study), and a dose of 115 Bq/kg in 0-2-year-olds (Garner et al.). This leads to very low radiation exposure of 1 μSv in neonates and less than 10 μSv in older children, which is far below the yearly background exposure of 2.5 mSv/year in the Netherlands. And the exposure from the microdose is low compared to the exposure during a European continental flight (>4 μSv), from a chest X-ray (100 μSv) or CT-scans (10 mSv). During informed consent conversations, most parents appeared to understand the minimal risk involved. Also, fear of harmful radiation exposure was not the main reason to deny informed consent. Consent was usually refused for other reasons: e.g., the burden of additional procedures such as blood sampling [chapters 9 and 10].

Alternatively, microdosing studies without a radioactive label are an option. Very sensitive LC-tandem mass spectrometry techniques measure very low concentrations [82]. Nevertheless, this method has not reached the very low limits of detection of AMS, and a larger unlabeled compound would be necessary. Also the use of an unlabeled microdose excludes the option to administer the dose simultaneously with an (unlabeled) therapeutic dose. Thus, the use of an unlabeled microdose might be suitable to study single dose pharmacokinetics in children or the effect of pharmacogenetics on pharmacokinetics. Blake et al. used a subtherapeutic dose of dextromethorphan to study the CYP2D6 genotype's correlation with pharmacokinetics [83].

Aside from using microdosing for phenotyping studies, other objectives can be considered. **I)** An early phase I study to predict the pharmacokinetics of a new drug specifically for the pediatric population, especially when metabolism is likely to be complex. **II)** A study in which it is important to distinguish between an exogenous and an endogenous compound, e.g., as in the ursodiol study [73]. And **III)** to study pharmacokinetics in special populations such premature neonates or critically ill children, where the PK may be different than the reference population (e.g. older, healthier children).

Tissue sampling and LC-MS/MS

Transporter protein abundance in our study was quantified with LC-MS/MS. Few studies so far have used LC-MS/MS to quantify transporter protein levels and to related results

Table 2. Overview of LC-MS/MS transporter studies with pediatric samples

	Prasad et al. [84]	Prasad et al. [85]	Deo et a. [86]	Mooij et al. [chapter 8]
Year	2014	2013	2012	this thesis
Transporters	ABCB1, OATP1B1, OATP1B3	ABCG2	ABCC2	ABCB1, ABCC2, ABCC3, ABCG, BSEP, OATP1B1, OATP2B1, OCTN2, GLUT1, MCT1
Organ	Liver	Liver	Liver	Liver
Sample size	n=64	n=50	n=51	n=25
Age range	7-70 years	7-70 years	7-63 years	10 fetuses, 12 infants 0-11 weeks, 3 adult
Method	Total membrane fraction	Total membrane fraction	Total membrane fraction	Crude membrane fraction
Results	Protein expression of transporters were not associated with age	ABCG2 protein expression was nog associated with age	ABCC2 protein expression was not associated with age	With postnatal age four patterns emerged from fetal to adult age: 1) ABCB1, OATP1B1, and OATP2B1 expression levels were stable 2) ABCC2, ABCC3, and BSEP were low to high expressed 3) ABCG2, GLUT1, OCTN2 protein levels were high to low 4) MCT1 was highest in infants These patterns were also found for postmenstrual age within the fetal/ infant groups.

to age (table 2) [chapter 8] [84-86]. Moreover, taken our study aside, only a few children from the age of 7 years onwards have been studied to date. This section reviews the use of LC-MS/MS to study transporters during childhood.

LC-MS/MS is an attractive new method to optimize the use of sparse samples with limited sizes pediatric tissue. Other quantitative protein determinations have used antibody-based immunoassays, including Western blotting of membrane preparations, fluorescence activated cell sorting (FACS) methods, and enzyme-linked immunosorbent assay (ELISA). A general drawback of these methods is the requirement to prepare specific antibodies. Although a large variety of antibodies are commercially available, the issue of cross-reactivity between protein isoforms or species remains of concern [87]. Other than these antibody-dependent methods, the LC-MS/MS technique relies on proteotypic peptides to detect and quantify the protein of interest. Cross-reaction might still occur, but this can be accounted for in the design of the peptide or by choosing two different peptides for the protein of interest, so that cross-reactivity remains only with unknown proteins. No more than 10 mg of human tissue is needed for LC-MS/MS [88]. In contrast to for example a single protein identification with a Western blotting experiment, multiple proteins can be quantified on a single LC-MS/MS run.

Functional membrane transporters are the ones present on the plasma cell membrane. Therefore determination of the total transporter protein amount per cell will not

suffice to relate to possible activity. Therefore, the abundance of transporter proteins in tissues is quantified after subcellular fractionation resulting in enriched membrane fractions. The preparation of the most relevant matrix (e.g., plasma cell membrane) is not uniform across laboratories. This may result in inter-study variability. To incorporate absolute protein abundance information into PBPK models, losses of target proteins, need to be estimated in a standardized manner [89].

An important limitation of this method is the need for a specialized lab and equipment, while Western blot is a standard laboratory technique, which most labs can use easily. This likely also results in a difference in costs between the methods.

Future challenges

Incorporating ontogeny data for pediatric drug dosing

Many processes involved in drug disposition mature and change with age, and the ontogeny of a single process is hardly meaningful without the context of other body processes and their maturation. Modeling and simulation can be used in different ways to design dosing regimens. Examples of evidence-based complex models based on physiologically processes or population pharmacokinetic data, are respectively, physiologically based pharmacokinetics (PBPK) or population pharmacokinetics (popPK) models.

These models are valuable tools in designing clinical trials. Simulations from PBPK models, which incorporate available drug property and physiological information, can predict pediatric dosing [90]. Importantly, high-quality ex vivo data must be incorporated and continuous updating of physiological ontogeny data is crucial. PBPK modeling can also be used to simulate the impact of maturation of specific transporters or enzymes, preferably with actual ex vivo data on expression/activity [91]. Ultimately, these models also must be validated with real-life pharmacokinetic data, which still asks for confirmatory pharmacokinetic sampling in clinical trials including the concept of opportunistic sampling, using doses derived from these simulations [92].

PopPK models of substrate drugs for specific drug disposition pathways have been used to describe the developmental patterns of these pathways, e.g., CYP3A4 and glomerular filtration rate (GFR) clearance [93, 94]. Validation of these models with the use of other substrate drugs has been quite challenging, as drugs may share one main disposition pathway, e.g. GFR clearance, but the contribution of membrane transporters may differ, thus affecting the age-related variation in pharmacokinetic parameters. Nevertheless, for individual drugs these models have proven useful to generate dosing guidelines. The final step of evidence-based pharmacotherapy is prospective validation of the popPK models. This approach has been successfully applied in case of morphine in postoperative neonates [95].

Nowadays PBPK and popPK models are used in fields such as in clinical drug development and investigator-initiated research. It would be worthwhile, though, to make these models more accessible and user-friendly for clinicians, for example as a bed-side tool in the form of an App- or other program. After entering details of the drug in question, age, kidney and liver function, and possible co-medication a pediatrician would receive a dosing suggestion based on substrate properties and *in vitro* ontogeny data of, among other things, transporter or metabolism. Considering that these models are currently far from ideal, this might still be the best option for a present patient who needs a drug for which no age-appropriate dosing guidelines are available.

The question remains at what point ontogeny data, PBPK or popPK models can be used to design dosing guidelines. Although validated evidence-based dosing guidelines are preferred, often no data is available on essential drugs even though these drugs are already given to children.

Conclusions

Although the ontogeny of membrane transporters and drug metabolism pathways remains to be further elucidated and clinically demonstrated, the current maturational profiles can be used in multifactorial dose-prediction models.

For the purpose of ontogeny studies, and taking its limitations into account, LC-MS/MS is suitable to quantify transporter abundance while optimally using sparse pediatric tissue samples; and [^{14}C]-labeled microdosing studies in children are a feasible tool to phenotype the ontogeny of drug metabolism, as has been shown for paracetamol metabolism.

The ultimate goal is to provide optimal age-appropriate drug dosing in children.

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13 Summary
Samenvatting

SUMMARY

Appropriate pediatric dosing needs to account for developmental changes in drug absorption, distribution, metabolism and excretion. There is a lack of knowledge on the age-related changes in drug transporters and metabolism pathways. This thesis addresses the ontogeny of intestinal and hepatic membrane transporters, as well as of glucuronidation and sulfation, which comprise important building blocks of drug disposition pathways in children. As pediatric drug studies are hampered with practical and ethical challenges, this thesis addresses the use of innovative clinical methods.

Part I Introduction

While oral drug absorption in critically ill children may be erratic and the use of this route therefore is discouraged for drugs. We show in **Chapter 2** that up to 27% of drugs are prescribed orally to children in neonatal and pediatric intensive care. This stresses the importance to study the processes involved in oral drug absorption in this population and the impact of age-related changes on these processes. The current state of knowledge is reviewed in **Chapter 3**. Large information gaps exist on the impact of growth and development of almost all drug absorption processes (gastric pH, gastrointestinal motility, bile salts, pancreatic function, intestinal pH, intestinal drug metabolizing enzymes, and transporter proteins). More in depth on the membrane transporters; **Chapters 4** and **5** present our current knowledge on developmental changes in clinically relevant transporters in intestine, liver, and kidney. In **Chapter 4**, based on *in vitro* studies, different developmental patterns for individual transporters emerge. Following on from this work, in **Chapter 5** drug disposition and pharmacogenetics studies in children are reviewed to add to our understanding of age-related changes in individual transporters. Overall, a striking information gap remains on the role of membrane transporters in pediatric drug therapy.

Part II Membrane transporters

The age-related changes in gene and protein expression of intestinal and hepatic drug transporters are presented in **Chapters 6**, **7**, and **8**. A selection of efflux transporters from ATP-binding cassette (ABC) family (ABCB1, ABCC2, ABCC3, ABCG2, ABCB11/BSEP) and uptake transporters from solute carrier organic anion (SLCO) family (OATP1B1, OATP1B3 and OATP2B1), and from the solute carrier (SLC) family (SLC2A1/GLUT1, SLC15A1/PEPT1, SLC16A1/MCT1, SLC22A5/OCTN2), are studied in one or more chapters.

Chapter 6 shows that hepatic ABCB1, ABCC2, OATP1B1 and OATP1B3 gene expressions in fetuses, neonates and infants are significantly lower than in adults. Neonatal intestinal expressions of ABCB1 and ABCC2 are comparable to those in adults. Intestinal OATP2B1 expression in neonates is significantly higher than in adults. **Chapter 7**

presents slightly lower, but clinically insignificant, intestinal PEPT1 gene expression in neonates and young infants than in adolescents. Furthermore, we found comparable localization of PEPT1, ABCB1, ABCC2 and OATP2B1 proteins in the neonates and the young infants compared to adolescents. They are visualized in the apical part of the brush border (PEPT1, ABCB1, ABCC2) and intraepithelial at the basolateral enterocyte border (OATP2B1). **Chapter 8** focuses on hepatic transporter protein expression, using innovative liquid chromatography tandem mass spectrometry (LC-MS/MS). Four patterns emerge with postnatal and postmenstrual age: ABCB1, OATP1B1, and OATP2B1 expression levels are stable from fetal to adult age. ABCC2, ABCC3, and BSEP are low in fetuses and infants and higher in adults, while ABCG2, GLUT1, OCTN2 protein levels are higher early in life to low in adults. MCT1 expression levels are highest in infants compared to fetuses and adults. Interestingly, age-related changes in gene expression are not reflected in the same changes in protein expression for ABCB1 and OATP1B1. In conclusion, these studies show transporter-specific maturation, with a discrepancy between gene and protein expression for selected transporters.

Part III Phase II Drug metabolism

Drug metabolism is one of the main determinants of variation in drug disposition. Phenotyping studies can be used to elucidate *in vivo* drug metabolism pathways in the pediatric population, but they are hampered by practical, ethical and scientific challenges. To overcome these challenges, an innovative approach of an open-label microdose study was performed using oral [^{14}C]paracetamol(AAP). The results of this project are presented in **Chapters 9**, **10**, and **11**. **Chapter 9** presents pilot data, showing that microdosing is a feasible and promising tool to study AAP pharmacokinetics. In collaboration with the European PAMPER consortium, **Chapter 10**, evaluates the experiences from two microtracer and -dosing studies, and concludes that if challenges in dose-linearity and costs are overcome this technique is of added value for pediatric drug development. **Chapter 11** presents the final results of this phenotyping microdosing study to investigate the age-related changes in glucuronidation and sulfation. Metabolite ratio of paracetamol-glucuronide (AAP-glu) and paracetamol-sulfate (AAP-sul) was used as a surrogate marker for paracetamol metabolism. In 50 children [median age 6 months (range 3 days - 6.9 years)] both plasma and urinary AAP-glu/AAP-sul ratios significantly increased by 4-fold. These data confirm literature data on the developmental pattern of paracetamol sulfation and glucuronidation, and add data on combined intestinal and hepatic pathways in a large pediatric age range. [^{14}C]labeled microdosing appears feasible to phenotype age-related differences in drug metabolism in children.

Part IV General discussion

Chapter 12 discusses the results of our research in light of the current literature and makes recommendations for future studies on ontogeny of transporters and drug metabolism. We conclude that

A) major gaps remain on the ontogeny of oral drug disposition processes, in particular of membrane transporters;

B) intestinal and hepatic transporter expressions change in transporters-specific patterns in an age-dependent manner;

C) AAP glucuronidation and sulfation are age-related, from mainly sulfation in neonates, changing to mainly glucuronidation in older children;

D) [^{14}C]labeled microdosing is a feasible and promising approach for phenotyping studies in children to elucidate age-related changes in drug disposition;

E) innovative methods in drug development i.e., microdosing and LC-MS/MS protein abundances offer excellent opportunities to overcome ethical and practical limitations in pediatric drug research.

This thesis shows new insights of the ontogeny of transporters, presenting one of the first transporter expressions (gene and protein) data in children. Due to the ambiguous nature of substrates for transporters no solid marker can yet be defined to study activity *in vivo*. Hence, at this moment we rely on expression studies. LC-MS/MS serves as an elected tool as it quantifies a palette of multiple transporters in a minimal amount of pediatric tissue. First, transporters in other organs, regulation factors (i.e., epigenetics, transcription factors) and influencing factors (i.e., disease, nutrients, substrate exposure) need to be identified. *In vivo* studies should aim to study amongst others; pharmacogenetics of transporters and drug-drug interactions of transporter substrates.

Our data adds, in a meaningful way, to the information about combined intestinal and hepatic metabolism of paracetamol using an oral probe, and about the important age gap (1-2 years). Furthermore, this was reached using an inventive minimal risk and minimal burden microdosing study, which serves as a stepping stone on the path to increasing pediatric drug study options. The use of this promising technique should be expanded into other substrates for phenotyping drug metabolism (if dose linearity is present), especially in vulnerable populations (i.e., prematures, the critically ill), but also in the early evaluation of new drugs when metabolism is likely to be complex and suited for a specific pediatric population.

Data from *in vitro* membrane transporter studies and *in vivo* pharmacokinetic phenotyping studies serve as input for complex multifactorial models (physiologically-based or population pharmacokinetic models), which predict pediatric drug dosing. The ultimate goal is to provide better age-appropriate drug dosing for children, taking into account the evolution of drug disposition pathways.

SAMENVATTING

Bij kinderen wordt de juiste dosering van een geneesmiddel bepaald door de mate van absorptie, distributie, metabolisme, en excretie van dat geneesmiddel op een bepaalde leeftijd. Over de leeftijdsafhankelijke veranderingen in de transport en omzetting van geneesmiddelen is nog weinig bekend. Dit proefschrift beschrijft de ontwikkeling van membraantransporters en geneesmiddel metaboliserende enzymen in de darmen en in de lever, als belangrijke processen voor de dispositie van geneesmiddelen bij kinderen. Geneesmiddelenstudies bij kinderen kennen praktische en ethische beperkingen. Innovatieve onderzoeksmethoden zijn toegepast om de onderzoeksvraag te kunnen beantwoorden met minimale belasting en risico voor het individuele kind.

Deel I Introductie

Bij kritiek zieke kinderen kan de absorptie van orale geneesmiddelen sterk wisselen, en daarom wordt de orale toediening vaak ontmoedigd. In **hoofdstuk 2** laten wij zien dat tot 27% van alle geneesmiddelen in onze neonatale en pediatrische intensive care units oraal wordt gegeven. Dit benadrukt het belang van meer kennis over de absorptie van orale geneesmiddelen bij deze kritisch zieke kinderen. De huidige stand van kennis over de invloed van leeftijd op orale absorptie wordt beschreven in **hoofdstuk 3**. Er zijn hiaten in de kennis over het effect van groei en ontwikkeling op bijna alle processen die betrokken zijn bij de absorptie van orale geneesmiddelen (zuurgraad van de maag en de darm, snelheid van maag en darm ontlediging, galzouten, alvleesklierfunctie, omzetting en transport van geneesmiddelen in de darmwand). **Hoofdstukken 4** en **5** beschrijven specifiek het effect van leeftijd op membraan-transporteiwitten in de darmen, lever en nieren. **Hoofdstuk 4** beschrijft resultaten van *in vitro* studies, hieruit blijkt dat er verschillende ontwikkelingspatronen zijn voor verschillende transportereiwitten. Vervolgens is in **hoofdstuk 5** gekeken naar studies over geneesmiddeldispositie en farmacogenetica bij kinderen met de vraag of deze iets toevoegen aan ons inzicht in veranderingen bij individuele transporters. Over het geheel genomen blijft de rol van membraantransporters bij de farmacotherapie voor kinderen een grotendeels onontgonnen terrein. De beperkte beschikbare data wijzen er wel op dat er een effect van leeftijd is op de activiteit van transporters, maar dat dit effect per transporter verschilt. Dit suggereert dat het effect en de veiligheid van geneesmiddelen, die substraat zijn voor transporters hierdoor kan variëren bij kinderen van verschillende leeftijden.

Deel II Membraantransporters

Nieuwe data die leeftijdsafhankelijke veranderingen in de gen- en eiwitexpressie van transporters in de darmen en de lever beschrijven worden gepresenteerd in de **hoofdstukken 6**, **7** en **8**. Het betreft hier uitstroomtransporters van de ATP-binding cassette

(ABC) familie (ABCB1, ABCC2, ABCC3, ABCG2, ABCB11/BSEP), en opnametransporters van de solute carrier organic anion (SLCO) familie (OATP1B1, OATP1B3 en OATP2B1) en van de solute carrier (SLC) familie (SLC2A1/GLUT1, SLC15A1/PEPT1, SLC16A1/MCT1, SLC22A5/OCTN2).

De genexpressie van de transporters ABCB1, ABCC2, OATP1B1 en OATP1B3 in de lever bij foetussen, pasgeborenen en kinderen blijkt lager te zijn dan bij volwassenen (**hoofdstuk 6**). De genexpressie van ABCB1 en ABCC2 in de darm bij pasgeborenen is vergelijkbaar met die bij volwassenen. De genexpressie van OATP2B1 in de darm bij pasgeborenen is significant hoger dan bij volwassenen. De genexpressie van PEPT1 in de darm van pasgeborenen en jonge kinderen is iets lager dan bij adolescenten, maar dit lijkt klinisch niet relevant (**hoofdstuk 7**). Bovendien was de lokalisatie van PEPT1, ABCB1, ABCC2, en OATP2B1 eiwitten in darmcoupes van pasgeborenen, jonge kinderen en adolescenten vergelijkbaar. PEPT1, ABCB1, ABCC2 eiwitten kleurden aan in het apicale gedeelte van de borstelzoom en OATP2B1 kleurde intra-epitheliaal aan aan de basolaterale zijde van de enterocyt. **Hoofdstuk 8** beschrijft het effect van leeftijd op de eiwitexpressie in een selectie lever transporters met behulp van 'liquid chromatography tandem mass spectrometry' (LC-MS/MS). Vier patronen konden worden onderscheiden: De expressie van ABCB1, OATP1B1 en OATP2B1 is stabiel van de foetale naar de volwassen leeftijd. De expressie van ABCC2, ABCC3 en BSEP is laag op de foetale en jonge kinderleeftijd en hoog op de volwassen leeftijd, terwijl voor ABCG2, GLUT1 en OCTN2 het omgekeerde geldt. Ten slotte, de expressie van MCT1 was het hoogst bij jonge kinderen vergeleken met foetussen en volwassenen. Een belangrijke bevinding was dat de leeftijdsafhankelijke verschillen in genexpressie van ABCB1 en OATP1B1 niet in overeenstemming zijn met de verschillen in de eiwitexpressie. We mogen concluderen dat er sprake is van een transporter-specifieke rijping, en dat een discrepantie bestaat tussen de gen- en eiwitexpressie van geselecteerde transporters.

Deel III Fase II geneesmiddelen metabolisme

Het metabolisme is een van de belangrijke determinanten van de variatie in de geneesmiddeldispositie. Zogenaamde fenotyperingstudies zijn nuttig om de metabolismeroutes bij kinderen *in vivo* te ontrafelen, maar kennen praktische, ethische en wetenschappelijke bezwaren. Een alternatief is het innovatieve concept van 'microdosing' met een [^{14}C] gelabelde microdosis. Hierbij wordt een zeer lage dosering, zeer zwak radioactief gelabelde dosis van een geneesmiddel gegeven, waarna de farmacokinetiek bepaald kan worden. Dit kan doordat we met de zeer gevoelige analysemethode 'accelerated mass spectrometry', de zeer lage concentraties kunnen meten in plasma en urine. Door de zeer lage dosis kan er geen effect van het geneesmiddel zelf optreden. Deze methode om farmacokinetiek te bestuderen is hierdoor minimaal belastend voor de proefpersoon en gaat gepaard met minimaal risico. De resultaten van ons micro-

dosing onderzoek worden gepresenteerd in de **hoofdstukken 9**, **10** en **11**. In een pilot studie bleek microdosing met [^{14}C]paracetamol goed uitvoerbaar te zijn bij kinderen en veelbelovend ten aanzien van onderzoek naar de farmacokinetiek van paracetamol (**hoofdstuk 9**). In samenwerking met het Europese PAMPER consortium hebben we de ervaringen bij twee microtracer en microdosing studies geëvalueerd. We concluderen dat deze methode nuttig kan zijn voor geneesmiddelenstudies op de kinderleeftijd, mits er sprake is van een 'dosis-lineair' effect en de kosten beperkt kunnen worden (**hoofdstuk 10**). De uiteindelijke resultaten van de fenotyperingstudie waarbij de leeftijdsafhankelijke veranderingen in de glucuronidering en sulfatering van paracetamol zijn onderzocht worden beschreven in **hoofdstuk 11**. De plasma en urine metaboliet-ratio's van paracetamol-glucuronide (AAP-glu) en paracetamol-sulfaat (AAP-sul) dienden als surrogaatmarkers voor het metabolisme van paracetamol. Het betrof 50 kinderen met een mediane leeftijd van 6 maanden (spreiding 3 dagen – 6.9 jaar)] en zowel in plasma als in urine steeg de AAP-glu/AAP-sul ratio met de leeftijd viervoudig. Dit bevestigt het al bekende ontwikkelingspatroon van paracetamol sulfatering en glucuronidering. Het concept van [^{14}C]gelabeld microdosing blijkt geschikt om het fenotype van geneesmiddel metabolisme te bepalen bij kinderen en daarmee naar het effect van leeftijd te kijken.

Deel IV Algemene discussie

In **hoofdstuk 12** wordt het volgende geconcludeerd:

A) er zijn grote hiaten in de kennis over dispositieprocessen van orale geneesmiddelen, vooral op het gebied van membraantransporters;

B) de expressie van transporters in de darmen en in de lever verandert met een toenemende leeftijd volgens een transporter-specifiek patroon;

C) de glucuronidering en sulfatering van paracetamol zijn leeftijdsafhankelijk, dat wil zeggen dat er bij pasgeboren vooral sprake is van sulfatering en bij oudere kinderen vooral van glucuronidering;

D) [^{14}C]gelabeld microdosing is uitvoerbaar en veelbelovend voor fenotyperings studies bij kinderen om leeftijdsafhankelijke veranderingen in geneesmiddeldispositie te ontrafelen, het bevestigt eerder onderzoek naar het effect van leeftijd op paracetamolmetabolisme;

E) innovatieve methoden zoals microdosing en LC-MS/MS eiwitbepaling bieden uitstekende mogelijkheden om de ethische en praktische beperkingen van geneesmiddelenonderzoek bij kinderen te verminderen.

Als gevolg van het wisselende affiniteit van substraten voor vaak meerdere transporters en de noodzaak een therapeutische middel te geven, zijn er tot op heden weinig goede markers om de activiteit *in vivo* te onderzoeken bij kinderen. Zodoende zijn

we momenteel in belangrijke mate afhankelijk van laboratorium studies. LC-MS/MS is een geschikte methode omdat deze verschillende transporters kan kwantificeren in een minimale hoeveelheid weefsel. Dit is van belang bij kinderen, omdat het vaak een uitdaging is om voldoende weefsel voor onderzoek te kunnen verzamelen. De volgende aspecten dienen in de toekomst onderzocht te worden: transporters in andere organen dan de darmen en lever, regulerende factoren (zoals epigenetica, transcriptiefactoren) en beïnvloedende factoren (zoals ziekte, voedingsstoffen, blootstelling aan substraten). *In vivo* studies die ons begrip over membraantransporters kunnen vergroten, zijn farmacogenetica en geneesmiddeleninteractie-studies van betrokken transportersubstraten.

De gebruikte innovatieve microdosing methode kent een minimaal veiligheidsrisico en is weinig belastend, en is daarom veelbelovend is voor geneesmiddelenstudies bij kinderen. Deze methode kan ook worden gebruikt voor andere geneesmiddelen (mits de farmacokinetiek van het middel dosis-lineair is), met name bij kwetsbare premature en kritiek zieke kinderen, maar ook in de eerste fasen van de ontwikkeling van geneesmiddelen voor kinderen waarbij een complex metabolisme wordt verwacht.

Data van *in vitro* studies naar membraantransporters en *in vivo* studies naar de fenotypering van farmacokinetiek kunnen dienen als de bron voor complexe multifactoriële modellen (op fysiologie of populatie gebaseerd) om doseringen voor kinderen te voorspellen. Het uiteindelijke doel is om te komen tot betere, op leeftijd gebaseerde geneesmiddeldoseringen met inachtneming van de maturatie van de betrokken farmacokinetische processen.

Part V

Appendices

LIST OF ABBREVIATIONS

^{14}C	Carbon 14
AAP	Paracetamol or acetominophen
AAP-glu	Paracetamol-glucuronide
AAP-sul	Paracetamol-sulfate
ABC	ATP-Binding Cassette
ALL	Acute Lymphatic Leukemia
AMS	Accelerator Mass Spectrometry
AUC	Area Under the Curve
AUC0-inf	Area Under the Curve from time zero to infinity
AUC0-t	Area Under the Curve from time zero to last observed timepoint
BCRP (*ABCG2*)	Breast Cancer Resistance Protein
BCS	Biopharmaceutics Classification System
bDNA assay	branched DNA assy
Bq	Bequerel
BSEP (*ABCB11*)	Bile Salt Export Pump
CAR	Constitutive Androstane Receptor
C_{av}	Average concentration
CES2	Carboxylesterase 2
CL/F	Oral clearance
C_{last}	Last observed concentration
Cmax	Maximum concentration
CO_2	Carbon dioxide
CT	Computer Tomography
CTIMP	Clinical Trial of Investigational Medicinal Product
CYP	Cytochrome P450
DDI	Drug-Drug Interaction
DME	Drug Metabolizing Enzyme
E-1	Elastase 1
EA	Elemental Analyzer
EMA	European Medicines Association
EMIT	Enzyme Multiplied Immunoassay Technique
FDA	US Food and Drug Administration
GA	Gestational Age
GERD	Gastroesophageal Reflux Disease
GFR	Glomerular Filtration Rate
GI	Gastrointestinal
GLUT1	Glucose transporter 1
GMP	Good Medicinal Practice

GWAS	Genome-Wide Association Study
HCC	Hepatocellular carcinoma
HIV	Human Immunedeficiency Virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ICRP	International Committee on Radioactivity Protection
IHC	Immunohistochemistry
IRB	Independent Review Boards
IV	Intravenous
LC	Liquid Chromatography
LLOQ	Lower Limit Of Quantification
MATE (*SLC47A*)	Multidrug And Toxin Extrusion protein
MCT1	Monocarboxylate transporter 1
MDR1 (P-gp; *ABCB1)*	Multi-Drug Resistance 1; P-glycoprotein
miRNA	microRNA
MPA	Mycophenolic acid
mRNA	Messenger RNA
MRP *(ABCC)*	Multidrug Resistance-associated Protein
MRSA	Methicillin-Resistant *Staphylococcus Aureus*
MTX	Methotrexate
NAPQI	*N*-acetyl-p-benzoquinone imine
NOAEL	No Observed Adverse Effect Level
NTCP (*SLC10A1)*	Na^+-Taurocholate Cotransporting Polypeptide
OAT (*SLC22A*)	Organic Anion Transporter
OATP *(SLCO)*	Organic Anion Transporting Polypeptide
OCT (*SLC22A*)	Organic Cation Transporter
OCTN (*SLC22A*)	Organic cation/ergothioneine transporter
OCTT	Orocecal Transit Time
OSTα/β	Organic Solute Transporter
PAH	P-aminohippurate
PAMPER	Paediatric Accelerator Mass Spectrometry Evaluation Research study
PBPK	Physiologically-based pharmacokinetic
PD	Pharmacodynamic
PDA	Photodiode array
PEDMIC	PEDiatric MICrodosing
PEPT (*SLC15A*)	Peptide transporter
PGx	Pharmacogenetics; pharmacogenomics
PK	Pharmacokinetics
PMA	Postmenstrual age
PNA	Postnatal age
popPk	Population pharmacokinetic
PXR	Pregnane X Receptor

QC	Quality Control
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RIN	RNA integrity number
RNAse	Ribonuclease
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SLC	Solute carrier
SLCO	Solute carrier organic anion
SNP	Single-Nucleotide Polymorphism
SULT	Sulfotransferase
Sv	Sievert
$t_{1/2}$	Elimination half-life
λ_z	Terminal elimination slope
TIM	TNO gastro-Intestinal tract Model
T_{lag}	Time delay of absorption
t_{max}	Time at maximum concentration
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UK	United Kingdom; Great Britain
UPLC	Ultra-Performance Liquid Chromatography
UPLC-MS/MS	Ultra-Performance Liquid Chromatography tandem Mass Spectrometry
URAT1 (*SCL22A12*)	Urate transporter 1
US	United States of America
VSOP	Dutch collaborative patients' organization for rare and genetic diseases; Vereniging van Samenwerkende Ouder- en Patientorganisaties voor zeldzame en genetische aandoeningen
VSS/F	Apparent volume of distribution

ABOUT THE AUTHOR

Miriam Geerthe Mooij was born in Zwolle, the Netherlands, on May 30th 1986. In 2004 she received her Athenaeum degree at Agnieten College Meander in Zwolle. That year, she started her medical training at the Maastricht University in Maastricht. Miriam combined her studies with several extra-curricular activities including a board membership of the Tropical course committee of the International Federation of Medical Student Association. In her fourth year of medical school she arranged an Elective Community Medicine internship and went to Vellore (India). In her final year Miriam left for a scientific Elective internship in Paris (France) at the molecular biology department of the Université de Pierre et Marie Curie, studying the epigenetic mechanisms of the fruit fly. After returning from Paris, she spent her final internship at the pediatrics department of the Laurentius hospital in Roermond.

In July 2010, Miriam finished medical school (Master's degree). She then commenced a residency at the pediatrics and neonatology department of the Sint Franciscus Gasthuis hospital in Rotterdam. While obtaining clinical experience in the field, she became interested in particular in clinical pharmacology, and pursued a PhD position. Under the supervision of Dr. S.N. de Wildt and Prof. dr. D. Tibboel, she started her PhD project on the age-related changes on drug transporters and metabolism in children, at the Intensive Care and department of pediatric surgery of the Erasmus MC – Sophia children's hospital. In the second year of her PhD research Miriam was awarded with a travel grant by the Dutch Society of Clinical Pharmacology and Biopharmaceutics, which enabled her to conduct a research project at the University of Western Ontario in London (Ontario, Canada), under the guidance of Dr. R.B. Kim. Alongside her PhD Miriam was selected for the TULIPS (training upcoming leaders in pediatric science) PhD curriculum and was trained as a clinical pharmacologist.

In January 2016 Miriam started her pediatrics residency at the Leiden University Medical Centre – Willem Alexander Children's hospital in Leiden.

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9. **Mooij MG**, Nies AT, Knibbe CA, Schaeffeler E, Tibboel D, Schwab M, de Wildt SN. Development of Human Membrane Transporters: Drug Disposition and Pharmacogenetics. Clin Pharmacokinet. 2015 Sep 26.
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12. Vet NJ, Brussee JM, de Hoog M, **Mooij MG**, Verlaat CW, Jerchel IS, van Schaik RH, Koch BC, Tibboel D, Knibbe CA, de Wildt SN, SKIC. Inflammation and organ failure severely affect midazolam clearance in critically ill children. Am J Respir Crit Care Med. 2016 Jan 21.

Submitted for Publication

13. **Mooij MG**, van de Steeg E, van Rosmalen J, Windster JD, de Koning BA, Vaes WH, Tibboel D, Wortelboer HM, de Wildt SN. The effect of age on human hepatic membrane transporter protein expression.
14. **Mooij MG**, de Koning BA, Lindenbergh-Kortleve DJ, Simons-Oosterhuis I, Tibboel D, Samsom JN, de Wildt SN. Intestinal peptide transporter PEPT1 expression and tissue distribution across the pediatric age range.
15. **Mooij MG**, van Duijn E, Knibbe CA, Allegaert K, Windhorst AD, van Rosmalen J, Hendrikse NH, Tibboel D, Vaes WH, de Wildt SN. Microdosing to study ontogeny of drug metabolism: the case of [^{14}C]paracetamol.

Book chapter

16. de Koning BA, **Mooij MG**, Johnson TN, de Wildt SN. Chapter 3: Development changes in the processes governing oral drug absorption. Pediatric Formulations, Springer New York, 2014, ISBN 9781489980113, DOI 10.1007/978-1-4899-8011-3, editors Daniel Bar-Shalom, Klaus Rose.

PhD PORTFOLIO

Name PhD student:	Miriam G. Mooij
Erasmus MC Department:	Intensive Care and Department of Pediatric Surgery
PhD period:	2011-2016
Promotor:	Prof. dr. D. Tibboel
Copromotor:	Dr. S.N. de Wildt

	Year	Workload (ECTS)
General academic skills		
BROK (Basiscursus Regelgeving Klinisch Onderzoek) Erasmus MC	2011	1.0
Systematic literature search and Endnote (medical library)	2011	0.6
MolMed - Research management for PhD-students	2011	1.0
CPO mini-course	2011	0.3
MolMed - Course presenting skills	2012	1.0
Individual presentation training (Ricard Jutte)	2012, 2015	0.2
Biomedical English writing and communication	2012-2013	4.0
Erasmus MC trial IT - Introduction to Open Clinica	2013	0.1
Research skills		
MolMed - Introduction to SPSS	2011	1.0
MolMed - SNP course	2011	2.0
MolMed - Biomedical research techniques Xth edition	2011	1.5
MolMed - Genetics for dummies	2011	0.5
NIH - Principles of Clinical Pharmacology, course of webinars	2011-2012	2.0
NIH – Pediatric Clinical Pharmacology webinars – Sumner J. Yaffe Memorial	2012-2013	0.5
Radiation-course (Radiation Safety Office)	2013	0.5
CHDR - NONMEM course PKPD	2013	0.9
NIHES - Biostatistical methods 1: basic principles CCO2	2014	5.7
Symposia and workshops		
GRIP post graduate clinical pharmacology course	2013	0.3
Lareb – Introduction day and Adverse Events Day	2013, 2014	0.3
Young investigators day of Dutch Society of Pediatrics, Annual meetings *(3x)*	2012-2015	0.3
TULIPS Phd curriculum	2014-2015	1.1
Teach the Teacher course (NVKFB)	2015	0.5

	Year	Workload (ECTS)
National Conferences		
NVK annual meeting, TULIPS Late Breakers symposium, Veldhoven: *oral presentation*	2012	1.0
FIGON Dutch Medicines Days, Ede: *poster presentation*	2012	1.0
NVK annual meeting, Veldhoven: *oral presentation*	2014	1.0
Figon Dutch Medicines Day, annual meeting, Ede: *oral & poster presentation*	2014	1.0
Sophia research days, Rotterdam: *poster presentation (first prize winner)*	2014	1.0
NVK annual meeting, TULIPS Late Breakers symposium, Veldhoven: *oral presentation*	2015	1.0
International Conferences		
ASCPT annual meeting, Indianapolis, USA: *poster presentation (2x)*	2013	2.0
ESPNIC annual meeting, Rotterdam, Netherlands: *poster presentation*	2013	1.0
ESDPPP biennial meeting, Salzburg, Austria: *oral & poster presentation*	2013	2.0
IATDMCT annual meeting, Rotterdam, Netherlands: *poster presentation*	2015	1.0
ESDPPP biennial meeting, Belgrade, Servia: *oral presentation*	2015	1.0
ASCPT annual meeting, New Orleans, USA: *poster walk presentation (2x)*	2015	2.0
Teaching activities		
Supervising Master's Theses, co-supervision, medical student *(n=2)*	2012-2015	1.0
Teaching polypharmacy 3rd year medical students	2012-2015	0.2
Other		
Writing F1000 evaluations *(n=8)*	2013	1.2
Pharmacology Days, Erasmus MC –Sophia: *oral presentations (3x)*	2011-2015	0.9
Pediatric Pharmacology Research Meetings (weekly): *multiple oral presentations*	2011-2015	2.0
Clinical Pharmacology and Pharmacogenetics meetings (weekly): *multiple oral presentations*	2012-2015	1.0
Fellowship Clinical Pharmacology	2012-2015	

DANKWOORD

Dit proefschrift is tot stand gekomen dankzij de steun, mede- en samenwerking van velen, die ik graag wil bedanken. Ten eerste, de ouders en patiënten; in mijn ogen zijn jullie de echte helden, omdat in tijden van onzekerheid en ziekte jullie toch de kracht vinden om belangeloos mee te doen aan wetenschappelijk onderzoek. De ICK verpleegkundigen, altijd betrokken bij de patiënt en bereidwillig om bloed en urine af te nemen of te bellen wanneer ouders er zijn. De ICK stafleden, fellows en arts-assistenten, geïnteresseerd en behulpzaam om een patiënt te overleggen, onderzoekers op de hoogte te brengen of een keer geduldig langs ouders te lopen wanneer zij behoefte hadden aan extra informatie.

Prof. dr. Tibboel, beste Dick, vier jaar geleden kwam ik als een onervaren onderzoeker op uw geoliede onderzoeksafdeling met uiteenlopende onderzoeken en onderzoekers. Ik bewonder uw veelzijdigheid en gedrevenheid voor de wetenschap. Telkens hield u de grote lijnen in de gaten en stelde u kritische vragen wanneer ik te gedetailleerd met een 'transportertje' bezig was en met onmogelijke afkortingen gooide. Dank voor uw waardevolle begeleiding.

Dr. de Wildt, beste Saskia, de afgelopen vier jaren heb jij mij met twee dingen besmet: passie voor kinderfarmacologie en wielrennen waarvoor ik je tot op de dag van vandaag dankbaar ben. Ik heb bewondering voor jouw grote toewijding en inzet in de klinische farmacologie zowel binnen het Sophia als internationaal. Ik heb ontzettend genoten van onze discussies. Of het nu ging over radioactiviteit op de afdeling, een klinisch farmacologisch consult of wat er in de bidon moest. Terwijl we vele kilometers aflegden op de wielrenfiets waren we ook op wetenschappelijk gebied een goed team. Dank voor de afgelopen jaren!

De leden van de kleine promotiecommissie, Prof. dr. van Gelder, Prof. dr. Allegaert, en Prof. dr. Leeder, wil ik hartelijk danken voor het beoordelen van mijn proefschrift. Prof. van Gelder, beste Teun, dank voor de leerzame klinische farmacologie en farmacogenetica bijeenkomsten. Prof. Allegaert, beste Karel, dank voor uw kritische commentaren. Prof. Leeder, dear Steve, thank you for an inspiring and productive brainstorm session in Rotterdam and for your willingness to oppose me in my PhD defense. Prof. dr. de Hoog, dr. Samsom en dr. Vaes wil ik hartelijk danken voor het plaats nemen in de grote commissie.

Alle co-auteurs wil ik bedanken voor de prettige samenwerking de afgelopen jaren. In het bijzonder onze samenwerkingspartners van TNO; dr. Wouter Vaes, dr. Esther van

Duijn, dr. Evita van de Steeg en dr. Heleen Wortelboer. Ik wil jullie danken voor de succesvolle samenwerking met laagdrempelig overleg. Het was keer op keer stimulerend om met jullie over transporters of microdosing te praten. En ik hoop van harte dat de samenwerking een lang vervolg krijgt.

Dr. Richard Kim, dr. Ute Schwarz, dr. Marianne DeGorter, dr. Mathilde Leon-Ponte, thank you for your hospitality during my stay at your department in London (Ontario) and for learning me the ins and outs of RT-PCRs. Dear Sara and Al Gallien, thank you so much for taken me into your house when I was ripped off by my landlady. Staying with you and your cats made my Canadian experience unforgettable.

Beste Ko Hagoort, je bereidheid om een manuscript snel van scherp commentaar te voorzien vanuit Rotterdam of Lombok is fantastisch. Dr. Joost van Rosmalen, bedankt voor je statistische hulp. Dr. Monique van Dijk, dank voor de klinische promovendi bijeenkomsten en met name de kerstontbijten. Annemarie Illsley, dank voor je hulp en de gezellig kletspraatjes. Chantal, Judith, Marie-Louise en Marja, bedankt voor jullie hulp. Dr. Barbara de Koning, dank voor je enthousiasme over transporters.

Joke Dunk, je steun was onmisbaar. Databases controleren, samples afdraaien of een kopje thee, je staat altijd voor elke onderzoeker klaar. Je betrokkenheid bij de patiënt, het onderzoek, en de onderzoeker is bewonderenswaardig. Hiervoor ben ik jou heel erg dankbaar. Marianne Maliepaard, dank voor je inzet in de GUTS-studie. Studenten: Mark dank voor je hulp; Jonathan, held, dank voor je enorme inzet!

Mede klinische farmacologie fellows, dank voor jullie samenwerking en farma-enthousiasme. Dr. Lidwien Hanff, altijd geïnteresseerd en enthousiast.

De club Sophia-onderzoekers is een grote familie, met onvergetelijke borrels, diners en ski-trips. Sp-1324, deze kleine ruimte liep over van gezelligheid, Nespresso en briljante research. Nienke, je hebt mij betrokken bij de DSI-studie en mij vanaf moment één ontzettend enthousiast gemaakt voor IC-onderzoek. De vele koffies en goede gesprekken over van alles en nog wat waren heerlijk. Binnenkort een dagje Blijdorp! Brabantse MC, altijd lachen en vastberaden. Bram, een gedisciplineerd en gepassioneerd anesthesist. Erik, jouw filosofische gedachten waren hilarisch en we hebben veel gelachen.

Sp-2430, Dorian, Kitty, Lisette, Marlous, Manuel, Raisa en Willem. Dorian, je staat altijd voor iemand klaar, een mooie eigenschap die niemand mag overzien. Kitty, je wordt een slimme anesthesist. Marlous, je ziet altijd het positieve in de mens. Willem, blij met de nieuwste technische snufjes, ik ben benieuwd naar jouw boekje! Lisette, Manuel, Navin en Raisa dank voor de gezelligheid. Andere (oud)-onderzoekers van Prof. Tibboel of de

kinderchirurgie, dank voor jullie hulp en gezelligheid. Gerbrich, de vrolijke noot, dank voor je gezelligheid. Niina, thanks for your honesty, humour, and most of all your peptalk over a cheese fondue. All the best for you and your girls in Montreal.

Suzanne, lotgenoot in de laatste weken met blauwe vingers van het typen. Je positiviteit inspireert me, jullie grote zee-avontuur gaat bijna beginnen! Michiel, bedankt voor het nodige uitblazen en afstampen op de pedalen. En daarnaast beide bedankt voor de Mont Ventoux avonturen, evenals de andere dappere fietsers.

Lieve Alexandra, Alex, mijn promotietijd had er heel anders uitgezien zonder jou! Het was één groot avontuur: succesvol een meer overroeien in Canada, vakkundig ons eten in een (te dun) boompje hangen voor de beren; wielrennen naar Zwolle of langs 11-steden in Friesland, Jut en Jul op de spinningfiets, menig skipiste onveilig maken als een Roze Konijn en Banaan. Een blik, mailtje of foto zegt dikwijls genoeg. Dank voor je adviezen over van alles, je luisterend oor, en je steun. Je discipline, je energie en devotie zijn allen onuitputtelijk. Op naar een volgend avontuur!

Lieve Joke en Alexandra, vriendinnetjes sinds een hele lange tijd, samen in Zwolle en Maastricht. Onvergetelijke momenten hebben wij meegemaakt en jullie zijn mij ongelooflijk dierbaar. Lieve Rob, het begon allemaal met de mosselpan, en sindsdien maatjes voor het leven, dank dat je erbij bent. Timon, Franse marktjes afstruinen als de beste! Inge, dank voor het prachtige omslag. Je mag er trots op zijn! Ellen, Char en Laura, SFG-maatjes. Fleur, ik kom bij jou helemaal tot rust in het mooie Mestreech. Iris, zo trots op mijn 'kleine zusje', we blijven onafscheidelijk.

SSA, lief en leed samen delen, de heerlijke weekendjes waar geen moment stiltes vallen, en de uitgebreide diners zijn onbetaalbaar. Lieve Jolien, je vrolijkheid zal ik nooit vergeten. Verbandje voor altijd!

Lieve Kapjes en Keizers, Bertha en Bennie, dank voor jullie steun en interesse in alles. Lieve Anne-Greet, wat is het fijn om jou in de buurt te hebben. Arjan, nooit gedacht dat toen ik jouw promotie bijwoonde, ik er zelf een keer zou staan.. Gelukkig is het niet zo'n moeilijk onderwerp als de Japanse economie. Arnold, Riny, Kathelijne en Charlotte, dank voor de dierbare momenten wanneer het gaat 'sneeuwen'.

Mijn paranimfen, Sanne en Irene, wat ben ik blij dat jullie vandaag naast mij staan. Sanne, India was onvergetelijk en het begin van een hechte, dierbare vriendschap. Ik bewonder je moed en open kijk in de wereld. Irene, op de eerste dag van de geneeskunde studie heb ik je ontmoet; goudeerlijk, vrolijk en behulpzaam. St Petersburg en *All I want for Christmas* zeggen genoeg!

Lieve Hanneke, ik ben harstikke trots op jou als mijn zus. De dierenarts die stoer in de Ierse heuvels loopt. Je bent er altijd voor me. Lieve pap, ik kan je onmogelijk bedanken voor je onvoorwaardelijke steun en liefde. Je hebt mij nooit tegengehouden in mijn keuzes en je hebt altijd alles voor Hanneke en mij over. Mama en jij hebben ons geleerd altijd trouw te blijven aan onszelf.

James, *everything is fine* are the magic words, I am so happy with you in my life!