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## From Baby Steps To Mature Strides: Maturation of drug metabolism and transport studied using innovative approaches

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# General introduction



The disposition of a drug is driven by various processes, such as drug metabolism, drug transport, glomerular filtration and body composition. We now know that these processes are subject to age-related changes, reflecting growth and maturation along the pediatric continuum.<sup>1-3</sup> It used to be common practice, however, to linearly adjust the dose for an adult to that of a child based on the child's bodyweight. This oversimplification of pediatric physiology commonly resulted in drug plasma concentrations either below or above adult reference concentrations. Then, a series of reports of children who experienced either severe drug toxicity or lack of effect raised awareness on this oversimplification. A classic example is the case of toxic exposure to chloramphenicol with fatal cardiovascular collapse (grey baby syndrome) in neonates as a result.<sup>4</sup> This was ascribed to underdevelopment of drug metabolism in neonates. But even recently there have been cases of serious adverse events in pediatric drug treatment partly explained by ontogeny. To illustrate this, in 2017 the US Food and Drug Administration (FDA) restricted the use of codeine and tramadol as the risk of apnea appears greater in children younger than 12 years.<sup>5,6</sup> Another example is the precipitation of ceftriaxone with calcium-containing products, which resulted in fatal cases in neonates only.<sup>7</sup>

### **Regulations on pediatric drug development**

Well, why did we have limited information on drug therapy in pediatrics when the drug development processes carried out by pharmaceutical companies are extremely regulated? Wasn't there any pediatric data when the drugs entered the market? Pediatric drug development is challenged by ethical concerns and logistical issues. In the earlier days, pharmaceutical companies were not obliged to study their compounds in children, and excluded children from experimental trials because they were considered vulnerable as developing humans. Serious adverse event such as sketched above brought realization that it is actually unethical to not conduct studies in children. For example, the drugs that could be valuable for certain disease conditions in children were made available 'off-label', but an appropriate benefit-risk analysis, including dose finding, as is mandatory for adults, was lacking. Therefore, over the years, specific regulations for pediatric drug development have been established (see Table 1 for an overview of the key landmarks). These regulations mandated pediatric research and have greatly increased expertise and activity in pediatric drug development.

### **Ontogeny of drug metabolism and membrane transport**

One of the major challenges in pediatric drug research is finding the right dose for children of different ages. We know now that most processes involved in drug disposition, including drug metabolism and membrane transport, are dependent on a child's growth and development.<sup>3</sup> Drug metabolizing enzymes are divided into phase 1 enzymes like Cytochrome P450s (CYPs) and phase 2 enzymes like UDP-glucuronosyltransferase

**Table 1** Key landmarks in pediatric medicines regulation. Adapted from *Germovsek et al.*<sup>8</sup>

Year	Regulation	Impact
1997	US FDA Modernization Act (FDAMA)	This act presented the financial incentive of an additional 6 months of market exclusivity to companies undertaking required pediatric studies
1998	US FDA Pediatric Rule	This rule permitted companies to label medicines for use in children based on extrapolation of efficacy from adult trial data, together with pediatric PKPD and safety data
2002	US Best Pharmaceutical for Children Act (BPCA)	Framework for pediatric research in both on- and off-patent drugs
2003	US Pediatric Research Equity Act (PREA)	Sponsors required to undertake clinical studies in children for new medicines and biological products
2006	EU Pediatric Regulation	Introduction of new legislation in the European Union mandating pediatric medicines research for new medicinal products
2012	US Food and Drug Administration Safety and Innovation Act (FDASIA)	BPCA and PREA became permanent in US Law

(UGTs). These drug metabolizing enzymes biotransform the parent drug into active and/or inactive metabolites. Membrane transporters are capable of moving endogenous and exogenous substrates over cell membranes in and/or out the cell.<sup>9</sup> Dependent on the characteristics, a drug may be a substrate for one or more of these drug metabolizing enzymes or transporters. As such, they are critical determinants in drug disposition.

After birth, newborns become dependent on exogenous food sources for nutrition, and the diet expands as they grow into infancy. During all changes in food exposure, the child must defend itself against potentially toxic dietary constituents, recruiting pathways not yet expressed or differentially expressed during fetal life. Hence ontogeny of drug metabolizing enzymes and transporters occurs, influencing the disposition of their endogenous and exogenous substrates over age.<sup>2,3</sup> Drug metabolizing enzymes work together with membrane transporters located in various organs to detoxify the body from exogenous compounds, like drugs and food toxins, and to maintain homeostasis of endogenous compounds. As each transporter or enzyme has its own developmental pattern, the metabolic profiles of drugs in children can significantly differ between age groups. Adjusting an adult dose based on bodyweight does not take these age-related changes into account. As such, one cannot simply perform linear size- or weight-based extrapolations from adult to pediatric doses, and dosing regimens specifically tailored to pediatrics are necessary.

### Innovation in developmental pharmacology

Better understanding of the underlying processes involved in drug disposition may aid to better predict drug disposition and create age-appropriate dosing guidelines for use in

clinical trials, thereby reducing the risks and burdens of these trials. Innovative approaches have been developed to study these developmental changes in drug metabolism and transport. First, advances in analytical methods, including liquid chromatography–mass spectrometry (LC-MS/MS) for proteomic analyses, allow to quantify the expressions of a wide variety of proteins, e.g. membrane transporters, in a small piece of organ tissue. The latter is specifically important for pediatric research where tissues are scarcely available. Second, innovative study designs using radioactive labelled microtracers allowed to study – without risk for the child – the oral bioavailability of compounds used as a marker for certain drug metabolism pathways. Feasibility of these designs to assess age-associated changes metabolism was shown for paracetamol.<sup>10,11</sup> Third, the use of modeling and simulation to support dosing recommendations in a pediatric trial or even to substitute a pediatric trial in children is supported by both the EMA and the US FDA.<sup>12,13</sup> As a result, physiologically based PK (PBPK) models, that include age-specific physiologic information, are increasingly being used, not only to aid pediatric drug development but also to improve drug therapy of existing compounds.

### **Mind the gaps and try to close them**

Although the knowledge on ontogeny of drug metabolism and transport has increased over time, important knowledge gaps remain, some of which are explained below.

#### ***Membrane transporter ontogeny in the liver and kidney***

The importance of membrane transporters in drug disposition and effect has received increasing attention in recent years.<sup>14-17</sup> In light of this, *ex vivo* transporter gene and protein expression studies using pediatric tissues allow to learn whether there are age-related changes in the expression of these membrane transporters. These studies are dependent on the availability of pediatric tissues, which is rather an exception than the rule, but these tissues may be obtained from unique biobanks.

Recently, the hepatic protein expression levels of 10 clinically relevant transporters in 25 liver samples from fetuses, neonates and young infants have been explored using LC-MS/MS.<sup>18</sup> The age-related variation in transporter protein expression appeared both transporter and organ dependent. This exploratory study was clearly informative, but the sample size was too small, however, to define transporter specific maturational patterns. While liver data is scarce, data on the ontogeny of renal membrane transporters is even scarcer. Moreover, little is known of the underlying regulatory mechanisms of ontogeny.

#### ***CYP3A ontogeny in the intestine and liver***

The drug metabolizing enzyme CYP3A is well known for its involvement in >50% of metabolized drugs, and is abundantly present in the intestine and liver. CYP3A consists

of the three main isoforms CYP3A4, -3A5 and -3A7, for which substrate specificity differs.<sup>19,20</sup> *In vitro* studies have shown that hepatic CYP3A7 abundance decreases rapidly after birth, and that hepatic and intestinal CYP3A4 abundance increases with increasing age.<sup>21-23</sup> CYP3A5 is polymorphically expressed with a stable expression from fetus to adult. This developmental pattern of CYP3A expression, established through *in vitro* studies, is supported by PK data of CYP3A substrate drugs. The benzodiazepine midazolam is a well-validated CYP3A probe with substrate specificity for CYP3A4/5 and almost no specificity for CYP3A7.<sup>24-28</sup> In preterm neonates, the intravenous midazolam clearance, reflecting hepatic CYP3A activity, was much lower (1.8 mL/kg/min) than that in infants and older children (9.1–16.7 mL/kg/min).<sup>29-32</sup> This was also seen for oral dosing, reflecting CYP3A in the intestine and liver. In preterm infants (gestational age 26-31 weeks and postnatal age 3-13 days), the oral midazolam clearance was markedly lower (0.16 L/h/kg vs 3.0 L/h/kg), and the oral bioavailability higher than those in children beyond 1 year of age (49-92% vs 21%) and in adults (49-92% vs 37%).<sup>33-35</sup> These findings suggest developmentally lower intestinal and/or hepatic CYP3A activity in preterm neonates.

Although the oral bioavailability of midazolam has been studied in children<sup>31,33-36</sup>, there is a distinct knowledge gap for term neonates to children <1 year old. This knowledge gap hampers dose predictions for oral CYP3A substrates to be prescribed to this age group.

The classical study design to obtain data on oral bioavailability entails a cross-over study in which an oral and IV dose of a drug are administered alternately, with a wash-out period in between. This design is ethically and practically challenging as children are exposed twice to therapeutic drug doses with extensive blood sampling. An interesting alternative is a microtracer study with a [<sup>14</sup>C]-labelled drug. A microdose is defined as '<1/100th of the no observed adverse effect level (NOAEL) or <100 µg'.<sup>37,38</sup> The [<sup>14</sup>C]-label allows quantification of extremely low plasma concentrations by accelerator mass spectrometry (AMS) in only 10-15 µL plasma.<sup>39,40</sup> A microdose can be used in an elegant design as a microtracer in which an oral [<sup>14</sup>C]-labelled drug is administered simultaneously with therapeutic IV doses of the same unlabeled drug or *vice versa*. This allows simultaneous measurement of both the oral and IV disposition in the same subject and, with that, quantification of the oral bioavailability.<sup>10,11</sup> This approach has been shown practically and ethically feasible to study developmental changes in pharmacokinetics in children.<sup>10,11,41</sup>

Importantly, for direct extrapolation of exposure from microdose to therapeutic dose, the PK of the microdose must be linear to the PK of the therapeutic dose.<sup>42,43</sup> This may

not be the case, for example, when a therapeutic dose saturates drug metabolism pathways, plasma protein binding and/or active transporters.<sup>43</sup> Dose-linearity of the PK of a from a midazolam microdose to that of a therapeutic dose has been established in adults<sup>42,44,45</sup>, but not in children. Yet, the results in adults cannot simply be extrapolated to children due to children's developmental changes in drug metabolism, hepatic blood flow, protein binding and membrane transport.

### ***Pediatric metabolite in safety testing (MIST) study***

Due to ontogeny of processes involved in drug disposition, predicting parent and metabolite exposure of compounds with a complex metabolism is challenging in children.<sup>46</sup> In adults, a general approach to study the parent and metabolite exposures of a drug during the drug development process, is performing a mass balance and metabolite in safety testing (MIST) study to create metabolite profiles.

Just recently, advances mainly in analytical technology have enabled new approaches to MIST studies with less radioactivity exposure.<sup>47,48</sup> By using [<sup>14</sup>C]microtracers concurrently administered with a therapeutic dose, metabolites can be identified and quantified with a radioactivity exposure of even less than 0.1  $\mu$ Ci.<sup>37,38</sup> This approach not only justifies earlier radioactive exposure during drug development, but may also be used to derive metabolic profiles for vulnerable populations like children, for which higher radioactivity levels would not be ethically acceptable, even in a late stage of drug development. Yet, to the best of our knowledge, MIST microtracer studies with [<sup>14</sup>C]-labelled compounds to create complete metabolic profiles have not yet been conducted in children.

### ***Ontogeny data in literature***

The accuracy of predicting pediatric drug exposure is highly dependent on the available ontogeny profiles of drug metabolizing enzymes and transporters. While increasing pediatric data become available in literature, results are often limited in age range and fragmented in several publications. Therefore, new data are needed, in combination with better accessibility of all the available *in vitro* and *ex vivo* data. Moreover, creating high-resolution quantitative ontogeny profiles will aid to improve existing models and to specify remaining information gaps.



## AIMS AND OUTLINE OF THIS THESIS

Based on the above-mentioned knowledge gaps, the aims of this thesis are:

- To review the current literature and quantitatively describe ontogeny of hepatic membrane transporters and drug metabolizing enzymes.
- To study the ontogeny of relevant human membrane transporters gene and protein expression in pediatric hepatic and kidney tissues.
- To investigate alternative splicing as an underlying mechanism for the ontogeny of the OATP1B1 transporter
- To study the dose linearity of the pharmacokinetics of an intravenous [ $^{14}\text{C}$ ]-labeled microdose of midazolam in children.
- To study the absolute oral bioavailability and metabolism of midazolam in children by an oral [ $^{14}\text{C}$ ]-labeled microtracer study approach.
- To study the feasibility of a MIST study in children using a [ $^{14}\text{C}$ ]-labeled microtracer study approach.

### From literature to bench to clinical research

The outline of this thesis is tailored to the common approach in research; starting with literature research (Part I), going to fundamental (*ex vivo*) research on the bench (Part II), and taking it into clinical research (Part III).

First, in Part I the hepatic ontogeny of drug transporters and drug metabolizing enzymes is captured in a quantitative review in **chapter 2**. A review of the ontogeny of drug transporters in all major organs is presented in **chapter 3**.

Part II focuses on our *ex vivo* studies. **Chapter 4** and **chapter 5** address age-related changes in gene and protein expression of clinically relevant hepatic and renal transporters. To better understand observed age-related variation in transporter protein expression, in **chapter 6** alternative splicing of the OATP1B1 transporter as a mechanism for developmentally regulated expression is explored.

Part III presents the results of two clinical pediatric studies. **Chapter 7** shows the dose linearity of an intravenous [ $^{14}\text{C}$ ]midazolam microdose in children. The oral bioavailability of midazolam in children 0-6 years as determined by a [ $^{14}\text{C}$ ]midazolam microtracer study is described in **chapter 8**. **Chapter 9** presents the pilot results of the first pediatric MIST study with midazolam as an example compound.

Part IV puts the results of the studies in a broader perspective, and areas of current and future research are described in **chapter 10**. Results of the studies are summarized in **chapter 11**.

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