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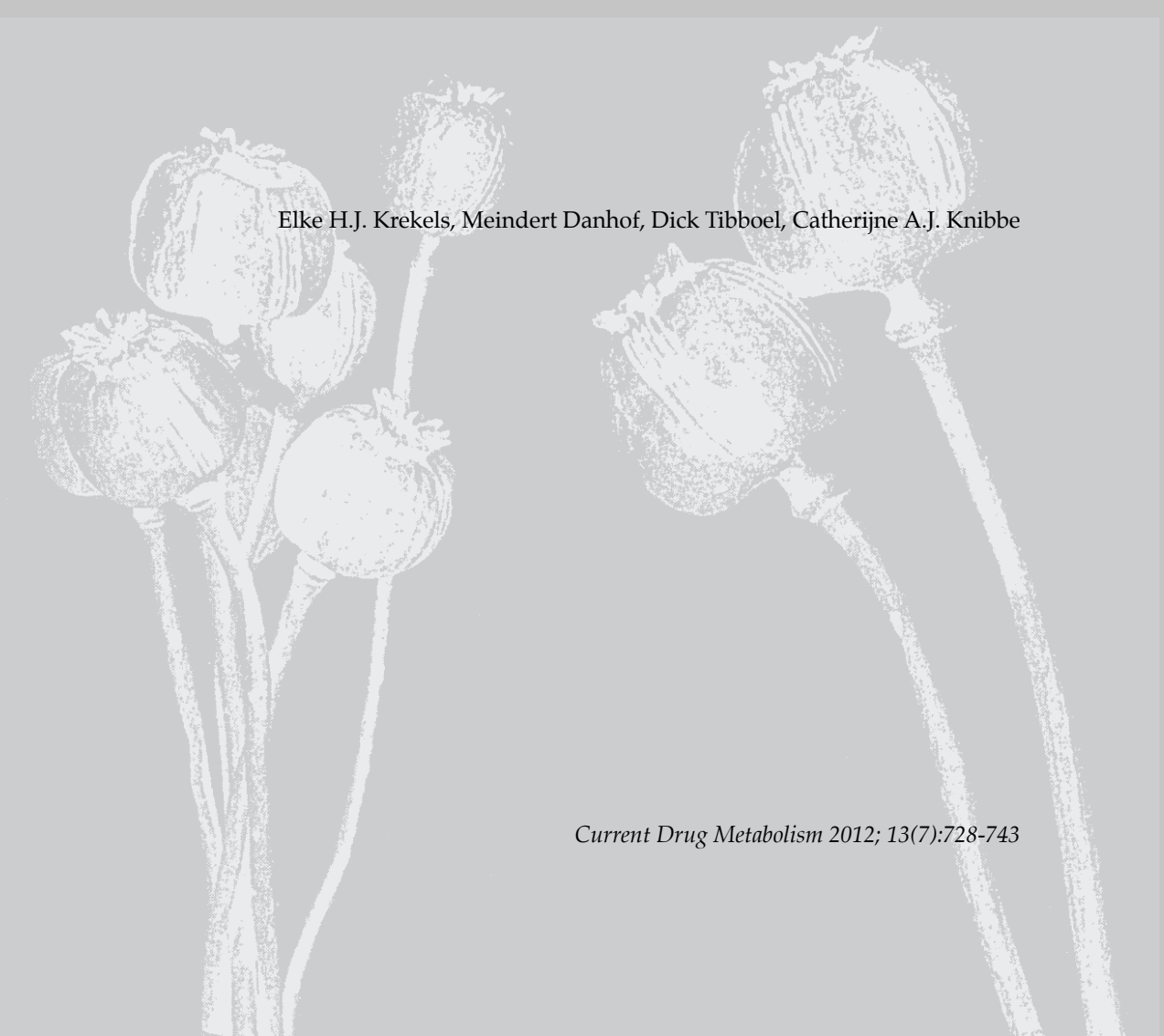
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Chapter 2

Ontogeny of Hepatic Glucuronidation; Methods and Results

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Abstract

The onset and maturation, so-called ontogeny, of hepatic glucuronidation is important for the clearance of a number of drugs in children. The current review discusses methods for studying the ontogeny of liver enzyme systems and specifically focuses on the results obtained with these methods for uridine 5'-diphosphate glucuronosyltransferases (UGTs). The number of contributing components in the biological system increases in going from mRNA transcription, to enzyme expression, *in vitro* enzyme activity, and *in vivo* glucuronidation clearance. This may result in different conclusions on UGT ontogeny when different methods are used. Various metrics to quantify glucuronidation activity, like linear or allometric scaling based on bodyweight, further disperse the conclusions on UGT ontogeny. Generally, it can be concluded that the onset of UGT expression and activity occurs after 20 weeks of gestation with a boost in expression and activity occurring in the first weeks of life. Maturation rates vary between the UGTs, but may well extend beyond the age of two years. Compared to adults, absolute doses of drugs eliminated via glucuronidation should be reduced in children. However, since the UGT isoenzymes mature differently, since substrate specificities are overlapping and since many external factors influence drug glucuronidation, it is not possible to derive general dosing recommendations for the paediatric population for these drugs. This can be improved by obtaining system specific information on each UGT isoenzyme on the basis of validated *in vivo* models that describe the ontogeny of glucuronidation and the influence of other patient characteristics like genetic polymorphisms and co-morbidities on the (intrinsic) clearance of isoenzyme specific probe drugs.

2.1 Introduction

Hepatic metabolic plasma clearance is to various extents influenced by 1) intrinsic clearance, 2) hepatic blood flow and perfusion, 3) plasma protein binding, and 4) active hepatic influx and efflux mechanisms, and intra-cellular transport processes. Intrinsic clearance is the maximum capacity to eliminate drugs in the absence of rate-limiting factors. Intrinsic clearance through hepatic biotransformation is determined by enzyme expression and enzyme activity in the liver.

Numerous enzymes responsible for the biotransformation of endogenous and exogenous compounds are present in the liver. Phase I metabolism, which entails the oxidation, reduction or hydrolysis of compounds to make them (more) polar, is carried out by various enzyme systems. Of these, the cytochrome P450 monooxygenases (CYPs) are oxidizing enzymes that have been extensively studied, since they are responsible for the majority of drug metabolism. Phase II metabolism comprises various conjugation reactions, like UDP-glucuronic acid, sulfate, glutathione, methyl and acetyl conjugation. Conjugation promotes passive renal elimination by increasing the solubility of compounds as well as active excretion of compounds through renal tubular secretion. These conjugation reactions usually result in the biological deactivation of endogenous compounds, drugs or phase I metabolites, although some examples of pharmacological active phase II metabolites are known, like for instance the morphine glucuronides ^[1]. The uridine 5'-diphosphate glucuronosyltransferases (UGTs) are the most important phase II enzymes in humans and they are the focus of the current review.

In the human genome four UGT families with glucuronidation capacity have been identified, namely the UGT1, UGT2, UGT3, and UGT8 families. The members of the UGT1 and UGT2 family are predominantly involved in detoxification of compounds and in humans the UGT1A and UGT2B subfamily have been studied most extensively in this respect. Within these two subfamilies 16 functional isoforms have been identified (see for latest nomenclature update Mackenzie *et al.* ^[2]). The UGTs are high-capacity, low-affinity enzymes that are mainly expressed in the liver, however isoforms have also found to be expressed extrahepatically ^[3-6]. Together, the UGTs glucuronidate many endogenous and exogenous compounds. Substrate specificities of the UGTs are broad and they may overlap, indicating that one isoform may glucuronidate a wide range of compounds and that one compound may be metabolized by multiple isoforms. Table I gives an overview of some of the UGT substrates and substrate specificities.

Table I. Overview of UGT substrates and substrate specificities.

UGT isoform	substrate	UGT specific substrate	references
1A1	paracetamol, 1-naphthol, thyroxine, 4-methylumbelliferone, carvediol	bilirubin, SN-38 (irinotecan metabolite)	[7–12]
1A3	1-naphthol, 4-methylumbelliferone, thyroxine	R-lorazepam	[10,12,13]
1A4	valproic acid	trifluoperazine, lamotrigine, imipramine	[13–16]
1A5	So far no substrates have been identified for this isoenzyme.		
1A6	paracetamol, 1-naphthol, valproic acid, 4-methylumbelliferone, chloramphenicol	serotonin	[9,10,17–19]
1A7	1-naphthol, 4-methylumbelliferone		[10]
1A8	1-naphthol, 4-methylumbelliferone, valproic acid		[10,15]
1A9	paracetamol, 4-methylumbelliferone, 1-naphthol, indomethacin, valproic acid, propofol, R-oxazepam, chloramphenicol		[9,10,13,18–21]
1A10	1-naphthol, 4-methylumbelliferone, valproic acid		[10,15]
2B4	androsterone, carvediol		[11,22]
2B7	1-naphthol, 4-methylumbelliferone, valproic acid, indomethacin, testosterone, androsterone, estradiol, lorazepam, carbamazepine, R-oxazepam, epirubicin, carvediol, chloramphenicol	morphine, zidovudine	[10,11,15,19–29]
2B10	amitriptyline, imipramine, clomipramine, trimipramine		[30]
2B11	4-methylumbelliferone, 1-naphthol, 4-nitrophenol, 4-hydroxyestosterone, 4-hydroxybiphenyl, methol, estriol, 2-aminophenol, 2-hydroxyesterial.		[31]
2B15	4-methylumbelliferone, testosterone	S-oxazepam, S-lorazepam	[10,13,22,28,32]
2B17	4-methylumbelliferone, testosterone, dihydrotestosterone, androsterone		[10,22]
2B28	eugenol, 1-naphthol, testosterone, 4-methylumbelliferone		[33]

The UGT isoforms 1A2, 1A11, 1A12, 1A13, 2B24, 2B25, 2B26, 2B27, 2B29 are considered to be pseudogenes ^[2].

The ontogeny of the hepatic glucuronidation system, which in the current review refers to the onset and maturation of hepatic glucuronidation activity in the paediatric population, is considered to be an important determinant for the hepatic clearance of a number of drugs in children. Detailed description of the ontogeny of the hepatic glucuronidation system will therefore facilitate the accurate prescription of these drugs in the paediatric population. The current review discusses the age-related changes in drug glucuronidation clearance and in the underlying physiological processes of drug glucuronidation in the paediatric population. It is specified what part of the causal chain between gene expression and phenotypical hepatic clearance is studied with available techniques and various aspects that influence these techniques and the physiological processes on the causal chain of events are discussed to provide insight in factors that can cause discrepancies between findings obtained using different techniques.

2.2 Methods to Determine the Ontogeny of Enzyme Systems

The ontogeny of enzyme systems can be determined at different levels ranging from *in vitro* mRNA transcription to the intrinsic hepatic clearance of a model compound, also known as a probe, *in vivo*. Figure 1 gives an overview of the processes that are studied at the different levels. Going from left to right in the diagram, the complexity of and variability in the studied biological systems increase due to the increasing number of factors that contribute to the functionality of the enzymes, and therefore the ultimate contribution of each process to *in vivo* drug clearance decreases going from right to left.

Studying enzyme ontogeny at different levels enables a mechanistic interpretation of the clinically observed developmental changes in the functionality of drug metabolizing enzymes. By going backwards on the causal chain of events different characteristics of the biological system are studied and the number of factors that contribute to the functionality of metabolizing enzymes are reduced. By simplifying the system noise is reduced and physiological insight in the characteristics of the system is obtained. However, due to the influence of contributing factors further down the causal chain, the contribution of a particular process to the overall biological system may be negligible. These contributing factors may also result in different findings when studying ontogeny at different levels. By studying more complex systems only the net influence of all underlying physiological processes are taken into account and they might therefore represent clinical observations of the drug clearance process better. However, due to the high complexity, more data and advanced statistical tools are required to identify significant descriptors for the maturation of drug clearance capacity.

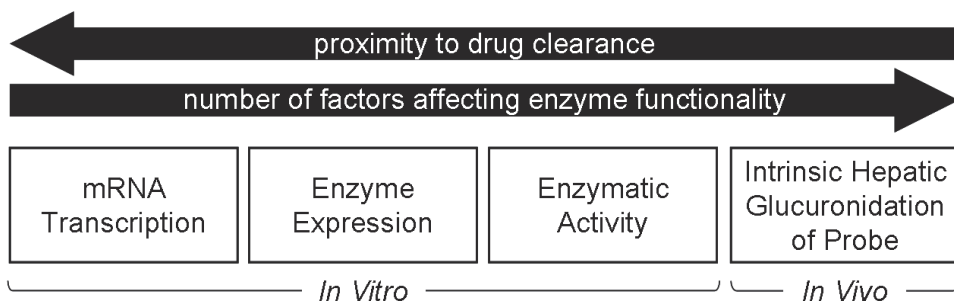


Figure 1. Schematic representation of the processes that can be studied to determine the ontogeny of enzyme systems.

2.2.1 *In Vitro* Methods

The *in vitro* methods to study enzyme ontogeny include the determination of RNA transcription, enzyme expression, and enzyme activity using human liver tissue obtained from academic or commercial liver banks. Metabolism of compounds takes place in hepatocytes, which are specialized cells that make up 70 to 80% of the liver's mass. When these cells are lysed in the laboratory, vesicles called microsomes are formed from the endoplasmic reticulum in which CYP and UGT enzymes are located. Liver homogenates, isolated hepatocytes, and microsomes can serve as experimental systems for *in vitro* studies of ontogeny of hepatic drug metabolism.

The origin of the liver tissue samples used in *in vitro* studies varies. In the past they were mostly obtained *post mortem* from liver donors that could not be matched to a recipient. Whereas currently paediatric liver tissue material is still predominantly obtained from deceased children, adult liver tissue material nowadays originates more often from tissue adjacent to removed (cancer) lesions^[34]. Since only a couple of hundred milligrams of liver tissue are necessary for these *in vitro* techniques, multiple experiments can be performed with the samples of one individual. This not only allows for replication of studies or for the testing of different conditions in the same individual, it also allows for the study of various processes like RNA transcription, enzyme expression and enzyme activity within tissue of the same individual.

Prior to using the liver tissue samples in the *in vitro* studies, they are checked for morphological and histological anomalies and often other biological and serological tests are performed on donor material to prevent the use of diseased liver samples, although this is not always possible in the paediatric population due to the limited number and size of the samples. In addition to pathophysiological changes of the liver, numerous other factors may still cause high variability in the results obtained with these samples.

These factors include 1) demographics and characteristics of the donor, like for instance age, gender, ethnicity, and genetic polymorphisms, 2) life style of the donor, such as alcohol use, smoking habits, general drug use, and dietary preferences, 3) *perimortem* or procedure related drug use and disease or trauma status in the donor prior to obtaining the tissue sample, and 4) harvesting, storage, and experimental conditions. The first two represent factors that are present in the clinical situation as well and therefore give a reflection of the variability that can be expected within a population, the second two factors mainly add noise to the data. Pooling *in vitro* material from various donors allows for the description of average trends in enzyme ontogeny, this approach will however not allow for the quantification of variability in the population nor will it allow for identification of the sources of the variability. Information on variability is as important as information on general trends and can be obtained by performing experiments in multiple samples of different individuals to obtain a measurement range rather than a single value. This can also be used to identify patient characteristics (covariates) that influence the ontogeny process.

2.2.1.1 RNA Transcription

The beginning of every physiological process is the transcription of the DNA sequence that encodes the enzymes that catalyze a process, into messenger RNA (mRNA). Studies on the onset of UGT mRNA transcription and on age-related changes in mRNA transcript levels are therefore an obvious approach to determine UGT ontogeny. The UGT1A isoforms are formed by alternative splicing of an mRNA transcript originating from a single gene, whereas the UGT2B isoforms are all encoded by independent genes^[2]. mRNA samples therefore contain distinct mRNA copies for each UGT isoform that can be uniquely identified. But it is important to keep in mind that UGT mRNA levels have been reported to be reduced under hepatic inflammation conditions^[35].

There are various techniques to detect and quantify gene transcription of which Northern blotting and quantitative real time polymerase chain reaction (qrt-PCR) are most frequently used. Northern blotting is an older technique that separates the different mRNAs in an isolated hepatic mRNA mixture by length on an agarose gel. A radio-labeled RNA probe that is complementary to the mRNA sequence of the UGT of interest will hybridize with the mRNA transcript of the UGT and the band strength of the labeled RNA on the image of the gel is then measured by autoradiography and used as a quantitative measure for specific mRNA expression.

With qrt-PCR, complementary DNA (cDNA) templates are created from an isolated hepatic RNA sample by reverse transcription. The cDNA template of the UGT of interest is subsequently amplified in successive cycles of the polymerase chain reaction in the presence of probes that give a fluorescent signal when bound to formed DNA

strands. With the amount of DNA being doubled in every cycle, the cycle in which a certain threshold in fluorescence is reached can be used to quantify the amount of mRNA in the initial sample. The DNA amplification cycles allow qrt-PCR to detect much smaller mRNA amounts than Northern blotting.

PCR analysis has revealed that there are no mRNA transcripts of UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, and 2B15 present in fetal liver at 20 weeks of gestation. In livers of infants with a postnatal age of 6 months, transcripts of UGT1A1, 1A3, 1A4, 1A6, 2B7, 2B10 and 2B15 were detected at levels similar to adults. At 6 months of age detectable levels of transcripts of UGT1A9 and 2B4 were present, but at lower levels than in adults. These levels further increased with age, with UGT1A9 transcript levels reaching adult values around 1.5 years and UGT2B4 transcript levels not reaching adult levels before the age of 2 years ^[36].

2.2.1.2 Enzyme Expression

mRNA transcripts are translated into amino acid chains that subsequently fold into active enzymes. However, not necessarily all mRNA transcripts are translated into enzymes, as some mRNA may be intracellularly degraded before being translated. Additionally, degradation of formed enzyme also influences the net amount of enzymes available for catalyzing cellular processes. By measuring the ontogeny of UGT enzyme expression one is therefore one step closer to the ontogeny of *in vivo* drug glucuronidation on the causal chain of events.

Techniques to detect and quantify enzyme expression, like Western blotting (also known as protein immunoblotting) and enzyme-linked immunosorbent assay (ELISA), involve the use of antibodies against the specific enzyme of interest in an isolated enzyme sample from the liver sample. Antibodies usually have very specific targets to which they bind, allowing for the detection of specific enzymes. Quantification occurs by using a label (e.g. radioactive, fluorescent) on the antibody that is detected after binding of the antibody to the enzyme. Quantification with ELISA is more precise than with Western blotting. Currently, the availability of UGT specific antibodies is scarce, which hampers the quantification of certain specific UGT isoenzymes in an enzyme sample.

Using rat antibodies that recognize a broad range of UGTs in a Western Blot analysis of human liver microsomes, it was revealed that at 18 and 27 weeks of gestation a UGT of 53 kDa is present. At term birth 3 isoforms could be observed and all isoforms that could be identified in adults with this experimental setup were found to be present at three months of age, albeit at only approximately 25% of the adult levels ^[37]. Unfortunately it is difficult to derive which UGT isoforms are described in this study as at the time

the different UGTs had not been identified and classified the way they are currently classified.

More recently, differences were found in the enzyme expression levels of UGT1A1, 1A6 and 2B7 between adults and children ranging from 7 months to 2 years of age with Western blotting. These results for enzyme expression were in agreement with finding on mRNA transcription in the same study [36]. Another study using a similar experimental setup showed an age-dependent increase in UGT2B7 enzyme expression in infants and young children. Adult expression levels of UGT2B7 enzymes in this study were only reached in the oldest paediatric age category ranging from 12 to 17 years [38].

2.2.1.3 Enzymatic Activity

After translation, enzymes can be further modified, for instance by phosphorylation and N-glycosylation, which may either enhance or inhibit the activity of an enzyme. It has for instance been suggested that UGT1A1 requires phosphorylation for the metabolism of some, but not all, of its substrates [39] and that N-glycosylation may influence the enzymatic activity of some of the enzymes of the UGT2B subfamily [40], but not on all of them [41]. Additionally, as reviewed by Ishii *et al.* UGTs can form homo- or heterodimers with each other or form complexes with other enzymes [42], and UGT activity is also influenced by the lipid composition of the membrane in which they are integrated [43]. Post-translational modifications and enzyme and lipid interactions can be studied independently, but this is not commonly done in studies of the ontogeny of enzyme systems. Rather, the net influence of gene expression, post-translational modifications, enzyme interactions and other possible contributing factors on enzymatic activity is often studied.

Contrary to the more quantitative measurements of mRNA and enzyme abundance, enzyme activity studies are of a more qualitative nature. These methods depend on the measure of either the formation of an enzyme product or the depletion of an enzyme substrate in a hepatocyte or microsome sample or liver homogenate. Individual UGTs can be investigated by using substrates specific for the UGT of interest, however due to the overlapping substrate specificities of the UGTs, few isoform selective substrates have been identified (see table I). Additionally, activity findings are neither absolute nor generalizable in that the results are specific for a particular combination of a certain enzyme and substrate and may be different for another substrate of the same enzyme. With enzyme kinetics generally being non-linear, different results are often obtained at different substrate concentrations, so it is also important to use physiologically relevant substrate concentrations in the experimental set up.

By tracking the initial formation rate of a metabolite at various substrate concentrations, the Michaelis-Menten parameters $V_{\max}$ (maximum reaction rate) and K_m (Michaelis-Menten constant) can be determined and equation 1 can subsequently

be used to determine the intrinsic clearance (Cl_{int}), which is the maximum metabolic capacity in the absence of rate-limiting factors:

$$Cl_{int} = \frac{V_{max}}{K_m} \quad (\text{Equation 1})$$

It is important to characterize the relationship between substrate concentration and enzyme reaction rate in detail, since the enzyme kinetic parameters that describe this relationship are directly correlated with the intrinsic clearance.

There is increasing evidence that some UGTs do not follow the typical non-linear behavior described by the Michaelis-Menten equation with some of their substrates ^[44–46]. This is indicative of the presence of allosteric effector sites, simultaneous binding of two substrate molecules to the enzymes active site, or cooperation with other enzymes and proteins in complexes. Technically, different methodologies should be used to accurately determine intrinsic clearance for enzymes that follow non-typical behavior, but this is not often done. Additionally, it is not clear how to correlate such non-typical *in vitro* behavior to the *in vivo* situation.

As an alternative, substrate depletion rates can be measured at a low concentrations (below K_m) as kinetics can then be assumed to be linear and measurements at a single substrate concentration suffice. The elimination rate constant (k_e) is used together with the distribution volume (V) to determine the intrinsic clearance according to equation 2:

$$Cl_{int} = k_e \cdot V \quad (\text{Equation 2})$$

The elimination rate constant is derived either from the elimination half-life of the substrate, or from the ratio between substrate concentrations at the beginning and at the end of the experiment. The distribution volume is composed of the volume of the incubation medium, the volume of the cells ($4 \cdot 10^{-9}$ mL per cell ^[47]), and the binding of the substrate to various components of the experimental system. In these studies, unspecific binding of the substrate to the experimental system or cellular uptake of the substrate without metabolism of the substrate, will increase the substrate disappearance rate at the beginning of the incubation period and will also result in over-prediction of the distribution volume. Since only one substrate concentration is used in this methodology it is very important to use relevant substrate concentrations to get physiologically meaningful results.

In the *in vitro* activity studies described above, the use of microsomes is easier and cheaper than the use of hepatocytes, however experimental protocols for microsomes are

often optimized for CYP enzymes and not for UGTs ^[48]. Numerous incubation conditions influence measured glucuronidation rates ^[49] and it has proven to be difficult to get good results for glucuronidation processes using microsomes ^[50]. Other, general advantages of using hepatocytes over microsomes for these studies are that all cofactors needed in the metabolic process are present at physiological concentrations and that the physical structure of the hepatocyte, including drug-binding cell compartments, cell membranes and transporters, is still in tact. Disadvantages of hepatocytes are that the expression and activity of many enzymes and transporters decline within hours.

The hepatocytes used in *in vitro* studies usually either originate from fresh liver samples or from cryopreserved samples. Various steps in the cryopreservation process influence the results obtained with cryopreserved hepatocytes, as reviewed by Hengstler *et al.* ^[51]. This may influence the findings on the correlation between results obtained in fresh and cryopreserved hepatocytes. Some found a good correlation for CYPs and UGTs ^[52], whereas others obtained ambiguous results for the correlation of UGT activity between cryopreserved hepatocytes and fresh hepatocytes ^[53].

In the late seventies and eighties of the 20th century, it was found that glucuronidation activity per gram of liver towards bilirubin and 2-aminophenol in fetal and neonatal liver homogenates was about 1% of the activity observed in adult liver homogenates. This study described an exponential increase in glucuronidation activity with age, to reach adult values three months postnatally. No differences were observed between term and preterm neonates ^[54]. More detailed investigation of the perinatal development of human hepatic UGT1A1 glucuronidation towards bilirubin revealed glucuronidation activity to be at 0.1% of adult activity levels between 17 and 30 weeks of gestation. Between 30 and 40 weeks of gestation activity levels increased to 1% of adult activity levels and after birth a rapid increase was observed to reach adult levels at 14 weeks of age. This postnatal increase was again found to be independent from gestational age, indicating that birth-related factors and not age-related factors are the main driving force for this increase ^[55]. In another study using microsomes the findings for bilirubin were substantiated. In term and preterm infants glucuronidation activity towards bilirubin was low and at 8 to 15 weeks activity values close to adult values were observed ^[37].

Experiments in microsomes on general glucuronidation activity have yielded inconsistent results. Glucuronidation of 4-methylumbelliferone, a compound glucuronidated by multiple UGT isoforms, was found to reach adult levels at 20 months of age ^[14]. Yet, in another study, the rate of glucuronidation of a series of 18 compounds including steroids, antidepressants, analgesics, opioids, flavones and coumarines, was found to not have reached adult values at 2 years of age and the difference between activity levels of 2 year-olds and adults could reach up to 40-fold ^[36]. The latter study

did however find mRNA transcription levels and enzyme expression of most UGTs to have reached adult values by the age of 6 months, underscoring the importance of other contribution factors of enzyme activity. Glucuronidation activity in microsomes towards testosterone and 1-naphthol, both compounds that are glucuronidated by a range of UGT isoforms, was found to be low at preterm and term birth and to slowly increase. At 1 year of age adult values were found not to have been reached ^[37]. Another study also found the glucuronidation capacity towards testosterone and 1-naphthol as well as towards bilirubin, androsterone, oestrone, 2-aminophenol and 4-nitrophenol to be low in the fetus and neonate ^[56].

Interestingly, in microsomes glucuronidation capacity towards serotonin, a substrate for UGT1A6, was found to be higher than adult levels in both fetal and neonatal liver ^[56]. A study in liver cells, however showed no detectable paracetamol glucuronidation in fetal liver ^[57], while paracetamol is also metabolized by UGT1A6, in addition to the isoforms 1A1 and 1A9 ^[9]. One explanation for these observations is that enzyme activity not solely depends on the enzyme, but on the combination of the enzyme and substrate together. However, based on the limited mRNA expression for all UGTs in the fetus, it is more likely that alternative hepatic elimination routes are available for serotonin in fetuses and neonates that are not or less abundantly present in adults livers.

Morphine is considered to be a specific substrate for UGT2B7 ^[23,25]. Microsomal glucuronidation activity towards morphine was found to be 6 to 10 times lower in fetal liver samples obtained between 25 to 27 weeks of gestation, than in liver samples from adults. Within this age-range a correlation with gestational age was lacking ^[58].

2.2.2 Genetic Variation in UGT Expression and Activity

Even before gene transcription and translation, the causal chain of events for drug glucuronidation starts with a genetic code on a chromosome. Mutations can occur in the promoter region of a UGT gene, potentially influencing the transcription of the gene and thereby the enzyme abundance. Additionally, mutations in genes of transcription factors that regulate UGT gene expression may also influence DNA transcription and enzyme abundance. Regulation of UGT gene expression and the influence of polymorphisms on this process have been reviewed by Mackenzie *et al.* ^[59].

Polymorphisms in the coding regions of UGT enzymes have been identified as well. Such mutations may or may not influence enzyme activity. Mutations involved in the catabolism of endogenous compounds may lead to congenital diseases. A wide range of different mutations in the UGT1A1 isoform leads for instance to various degrees of unconjugated hyperbilirubinaemia as observed in Crigler Najjar syndrome and Gilbert's syndrome ^[60], which in newborns may also lead to kernicterus. In case a mutation leads to functional changes, the k_m or V_{max} of an enzyme for a specific substrate may be altered,

or both. Therefore, other than the presence or absence of functional changes in mutant enzymes, the extent of the functional changes may also be substrate specific [8,11,28,29,61–73]. Additionally, some mutations may cause functional changes in glucuronidation activity by impacting the ability of UGT enzymes to interact with other membrane components to form complexes [74]. Since UGT1 isoforms are alternatively spliced from the same mRNA transcript, the isoforms of this subfamily share part of their genetic code. A certain polymorphism can therefore affect multiple UGT1 isoforms [75].

Genetic differences in elimination capacity prevail throughout life and studies on the impact of genetic variation on UGT enzyme expression and activity are generally performed in (tissue from) the adult population or in artificially synthesized *in vitro* enzyme systems. It is possible that (unknown) mutations in UGT genes have influenced some of the findings on UGT ontogeny, this is however not further discussed or considered in the current review.

2.2.3 Prediction of *In Vivo* Hepatic Clearance Based on *In Vitro* Enzyme Activity

Enzyme ontogeny is predominantly studied to make inferences about the maturation rate of *in vivo* drug elimination in the paediatric population. Various approaches are available to make inferences on *in vivo* hepatic clearance based on *in vitro* data obtained in hepatic material, these approaches do however not take into account extra-hepatic sources of drug glucuronidation and elimination.

It can be envisioned that *in vitro* enzyme behavior in a non-physiological medium that may lack necessary co-factors is different from the enzyme behavior *in vivo*. However when predicting *in vivo* clearance from *in vitro* data, *in vivo* and *in vitro* intrinsic clearance per unit of enzyme are assumed to be the same. Subsequently, milligram of microsomal protein per gram of liver (MPPGL) and number of hepatocytes per gram of liver (HPGL) can be used in a straightforward manner to determine the rate of metabolism per gram of liver from microsomal or hepatocyte *in vitro* clearance respectively. This in turn can be multiplied by liver weight to give an estimate of total hepatic intrinsic clearance.

For MPPGL most commonly a value of 45 mg/g is used, however a recent meta-analysis, showed geometric mean MPPGL values to be 32 mg/g in Caucasian adults with high inter-individual variability and a weak negative correlation with age [76]. No differences were found between microsomes from fresh and frozen liver samples [77]. A later analysis included data from 4 Caucasian paediatric livers (age 2, 4, 9, and 13 years) and 11 fetal livers. The geometric mean of MPPGL in fetal livers was found to be 26 mg/g. The geometric mean of MPPGL in the paediatric livers was 28 mg/g and increased to 40 mg/g at the age of 28 years, after which it slowly decreased again [77]. It should however be noted that this age-effect could only explain 10% of the observed variability observed in MPPGL.

In Caucasian adults the geometric mean of HPGL values was found to be $99 \cdot 10^6$ cells/g, with high inter-individual variability [76]. For HPGL there was also a negative correlation observed with age in adults, however no information is currently available on HPGL in paediatric livers.

As reviewed by Johnson *et al.* [78], many models exist to describe liver volume or liver weight as a function of body surface area (BSA), bodyweight, age or other covariates. Meta-analysis of these data revealed that the median liver weight in children younger than 2 years was 3.5% of the total bodyweight, whereas in adults this was only 2.2%. The authors propose the following equation to predict liver volume (LV) in the paediatric population:

$$LV = 0.722 \cdot BSA^{1.176} \qquad \text{(Equation 3)}$$

In this study a value of 1.08 kg/L for liver density was used to derive liver weight from liver volume [78]. Reference values of the International Commission on Radiological Protection (ICRP) for paediatric liver weights are 130, 330, 570, 830, and 1300 gram for respectively neonates and children of the ages of 1, 5, 10, and 15 years [79].

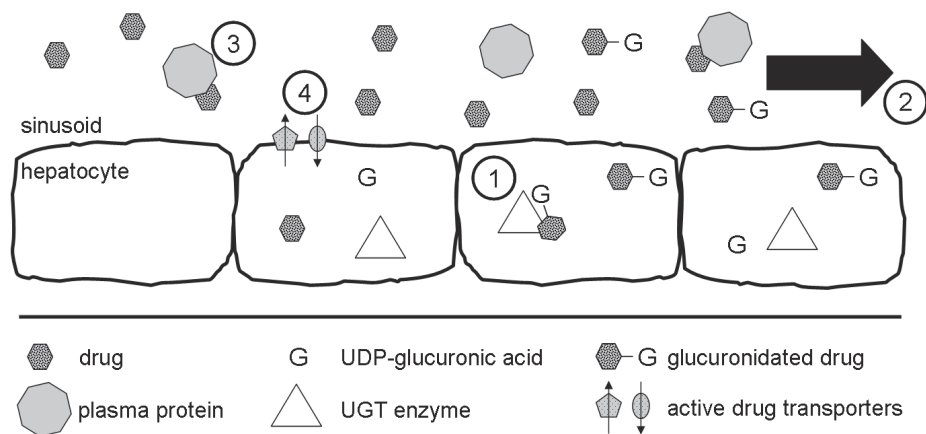


Figure 2. Schematic representation of the mechanisms involved in *in vivo* drug glucuronidation by the liver: (1) intrinsic clearance by the enzymes, (2) blood flow and perfusion, (3) plasma protein binding, and (4) active hepatic influx and efflux mechanisms.

In addition to scaling *in vitro* obtained intrinsic clearance per gram of enzyme to *in vivo* intrinsic clearance of the full liver, other contributing factors can be taken into account to derive *in vivo* hepatic clearance. Figure 2 provides a schematic representation of the

mechanisms involved in *in vivo* hepatic drug metabolism that were already mentioned in the introduction. *In vitro-in vivo* extrapolation methods that consider the different mechanisms described in figure 2 are available to make quantitative predictions of *in vivo* hepatic clearance based on *in vitro* intrinsic clearance obtained with microsomes, hepatocytes or liver homogenates.

Physiology-based pharmacokinetic software (e.g. Simcyp, PK-sim, PKQuest, GastroPlus) can integrate a wide range of *in vitro* and *in vivo* data on drug parameters and physiological parameters to aid the prediction of hepatic clearance based on historic and experimental data, first attempts to include pathological conditions like liver cirrhosis have been made as well ^[80]. In addition to the derived intrinsic hepatic clearance, physiology-based models may also use information on the age-related changes in hepatic blood flow and plasma protein binding.

Commonly used models to derive whole-blood hepatic drug clearance ($Cl_{H,B}$) are the well-stirred model (Equation 4) and the parallel-tube model (Equation 5).

$$Cl_{H,B} = \frac{(Q_{H,B} \cdot Clu_{int} \cdot f_{u,B})}{(Q_{H,B} + Clu_{int} \cdot f_{u,B})} \quad (\text{Equation 4})$$

$$Cl_{H,B} = Q_{H,B} \cdot (1 - e^{-\frac{Clu_{int} \cdot f_{u,B}}{Q_{H,B}}}) \quad (\text{Equation 5})$$

In addition to the intrinsic clearance of the full liver, which should be based on unbound drug concentrations (Clu_{int}), these models also take the unbound drug fraction in blood ($f_{u,B}$) and hepatic blood flow ($Q_{H,B}$) into consideration. Modifications of these equations are necessary to determine the more commonly used plasma drug clearance instead of whole-blood drug clearance ^[81].

Both the well-stirred model and the parallel-tube model grossly divide the liver into sinusoids, vascular channels in which arterial blood and blood from the portal vein combine, and hepatocytes, the cells that surround the sinusoids and that are responsible for the drug metabolism. The two models assume the physiological extremes in the extent of mixing of drug concentration in the sinusoids and hepatocytes. The well-stirred model assumes drug concentrations in the sinusoid and hepatocytes to be equal, whereas the parallel-tube model assumes the liver to be composed of parallel tubes along which the drug concentration decreases and the concentration driving the hepatic drug uptake to be the logarithmic mean sinusoidal concentration ^[82]. Both models assume that plasma protein binding is at steady state, that there is no diffusion delay and that no active transport systems are involved ^[81]. When the hepatic drug extraction ratio is low (<0.5) both models give similar results, but for drugs with an intermediate or high extraction

ratio predictions with the well-stirred model are generally lower than with the parallel-tube model.

In these models, commonly used adult values for hepatic blood flow are 1.35 L/min for females and 1.5 L/min for males. It has however hardly been studied whether and how hepatic blood flow changes with age in the paediatric population. Some have suggested that there are no major differences in hepatic blood flow between neonates and adults [83], this seems however unlikely, since the relative liver size decreases with age, which would imply liver blood perfusion per kilogram of liver to be lower in children compared to adults. Other possible assumptions that are often made in this case are: 1) hepatic blood flow per kilogram bodyweight is the same in children and adults, 2) hepatic blood flow per kilogram of liver is the same in children and adults, 3) hepatic blood flow is proportional to metabolic rate (parameterized by BSA), and 4) the percentage of cardiac output directed to the liver remains constant with age. Opportunely, the decreased metabolic capacity of the liver in young children decreases the drug extraction ratio, thereby making hepatic clearance less dependent on hepatic blood flow, making knowledge of paediatric hepatic blood flow less vital in the predictions of paediatric hepatic clearance. Finally, it has to be remembered that an unclosed ductus venosus can impair hepatic drug clearance in neonates, by allowing part of the blood flow to bypass the liver.

The unbound drug fraction in the paediatric population is also (potentially) influenced by various factors, namely 1) increases in the concentration of drug-binding plasma proteins from 76.7% of the adult value in neonates for human serum albumin (HSA) and 53.4% of the adult value in neonates for α 1-acid glycoprotein (AGP). The increases in the concentrations of these proteins with age have been described by various functions [84,85], 2) increases in bilirubin and free fatty acid concentrations shortly after birth, and 3) differences in the protein binding affinity of drug between children and adults [86,87]. Therefore, the value for the unbound drug fraction in blood obtained in adults cannot automatically be used in these equations for children.

With information on paediatric hepatic blood flow and plasma protein binding lacking, there is another approach that is sometimes applied to derive *in vivo* paediatric hepatic clearance based on *in vitro* data by the use of the well-stirred or parallel-tube model. In this approach *in vitro* intrinsic clearance is determined in paediatric hepatic material, this value together with adult values of the other parameters in the well-stirred or parallel-tube model are used to obtain a value for hepatic clearance. Paediatric hepatic clearance ($Cl_{H,B,paed}$) is subsequently derived from this value by the use of allometric scaling as illustrated in equation 5:

$$Cl_{H,B,paed} = Cl_{H,B} \cdot \left(\frac{W_i}{W_{std}}\right)^{0.75} \quad (\text{Equation 6})$$

In this model, the bodyweight of the individual (W_i) is divided by the bodyweight of a 'standard adult' (W_{std}) usually taken to be 70 kg. When the individual weight of the paediatric liver donor is unknown, a value can be derived from growth charts based on the age and sex of the donor. The application of allometric scaling on the organ level is however controversial [88].

So far it has proven to be difficult to characterize the influence of hepatic uptake and transporter mechanisms on drug clearance, even in adults [89]. This information is therefore rarely incorporated in physiology-based models. Pgp efflux transporters were found to be expressed in the human hepatocytes and this expression was suggested to be initiated by birth [90], however quantitative information on age-related changes in expression of hepatic uptake and transporter proteins is missing. Since hepatic transporters are already present in hepatocytes, the influence of ontogeny of transporter proteins on clearance only becomes relevant when dealing with intrinsic clearance values obtained with microsomes.

Both the well-stirred and parallel-tube model generally yield under-predictions of *in vivo* intrinsic clearance of unbound drugs up to ten-fold or even higher [49,91,92]. The assumption in both models that plasma protein binding is at steady state conditions instead of in a dynamic state has been proposed as an explanation for this under-prediction. This under-prediction is less when data obtained in hepatocytes are used compared to data obtained in microsomes, possibly because cellular uptake proteins are functionally present in hepatocytes but not in microsomes. Recently it has been found that the accuracy of the predictions of *in vivo* clearance with hepatocytes could be improved to some extent by incubating the cells with serum instead of buffer solution [93]. This system approaches the physiological situation better as drug binding to plasma proteins is already incorporated in the experimental setup, thereby eliminating the need for separate studies of the unbound drug fraction in blood. In microsomal studies the use of serum is however thought to be un-physiological, since the unbound drug fraction in microsome incubation is not an analogue for the unbound drug fraction that is available for *in vivo* metabolism. It has been suggested that the under-predictions with microsomal data could be corrected by an empirical scaling factor [46,92].

Microsomal studies on UGT1A4 activity in which trifluoperazine depletion was used as a marker for product formation to obtain Michaelis-Menten parameters, showed UGT1A4 activity to reach adult levels by 1.4 years. However, calculating hepatic clearances from the obtained *in vitro* intrinsic clearances by the use of both the well-stirred and parallel-tube model and subsequent allometric scaling, suggested *in vivo* UGT1A4 mediated hepatic glucuronidation to reach adult values only at the age of 18.9 years [14], which was however not corroborated with experimental data.

Using epirubicin as specific substrate for UGT2B7 in a study determining Michaelis-Menten parameters with human microsomes, a small age-dependent increase in UGT2B7 activity was found over the paediatric age-range, but activity in all paediatric age categories was lower than in adults. UGT2B7 enzyme expression levels in this study showed a good correlation with UGT2B7 activity levels, however adult expression levels were already reached at 12 – 17 years. By applying the allometric model directly to the *in vitro* clearances, the differences between the different paediatric age categories and between adults and the paediatric categories disappeared [38].

2.2.4 *In Vivo* Methods

To make reliable predictions of *in vivo* clearance based on *in vitro* methods requires information on a large number of parameters, not all of which can be determined experimentally in adults, let alone in children. In addition to that, *in vitro* hepatocytes have lost normal cell attachment, polarity and have a bigger surface area than in the *in vivo* situation and the architecture of the liver also changes with age. Therefore the ontogeny of *in vivo* drug glucuronidation may be best determined *in vivo*. *In vivo* studies can be performed in both animals and humans, however animal models are not considered in the current review, as the extrapolation potential from animals to humans in ontogeny studies is unknown. Also, differences in substrate specificity between animals and humans have been reported [94].

In vivo enzyme ontogeny studies can be performed by determining drug metabolism in neonates, infants and children of various postnatal and postmenstrual ages. By looking at changes in total drug elimination from plasma, the influence of maturation in all clearance routes combined (i.e. glucuronidation, metabolism other than glucuronidation, biliary excretion and unchanged renal clearance) is determined. It is therefore important to either ensure that glucuronidation is the major route of elimination for the drug of interest or to determine the contribution of each route to the overall clearance. It should also be considered, that *in vivo* drug metabolism is not restricted to hepatic metabolism and that metabolism in organs other than the liver is also measured. The latter does not necessarily pose a problem, since the reason to study drug metabolism is generally to determine how quickly a parent drug is cleared, for instance to determine the dosing regimen, or how quickly certain metabolites are formed, which is especially important when the metabolites are pharmacologically active or toxic.

In vivo glucuronidation clearance can be derived from concentration-time profiles. With traditional methods, clearances are calculated by dividing dose by the area under the concentration-time curve (AUC). AUCs can only be derived from full concentration-time profiles, which cannot be obtained from very small children for practical and ethical reasons. Additionally, AUC calculations are sensitive to sampling times. Alternatively,

clearance can be obtained by dividing the drug infusion rate by the steady state plasma concentration, however it may take a while for the parent drug to reach steady state concentrations and even longer for drug metabolites. Additionally, determining whether steady state has been reached may prove to be difficult. Other drawbacks of traditional studies are that often only a limited number of patients are included and that usually differences in clearance capacity between groups instead of individuals are quantified, making the results on maturation processes partially dependent on the stratification of the study population.

In the past few decades advanced statistical tools have been developed to aid in the analysis of sparse and unbalanced data that are often obtained in paediatric pharmacology studies. As described by De Cock *et al.*, the population approach using non-linear mixed effects modeling is currently the preferred tool, as it analyses individuals as constituents of a population, thereby allowing for the analysis of sparse and unbalanced data and also for the identification of both within- and between-subject variability [95]. By investigating trends in the glucuronidation clearance of different individuals in the population, the best descriptor for the maturation rate can be identified. This allows for the continuous quantification of changes occurring throughout the paediatric age-range. Since incorrect conclusions can be easily drawn from sparse data, thorough model validation is imperative to ensure that the models obtained with this method are reliable.

When a drug is metabolized through multiple routes, the contribution of the glucuronidation to the total systemic clearance has to be determined. With the traditional methods the recovery of the metabolites of a drug dose in urine would be determined to establish the contribution of each route to the overall systemic drug clearance. Alternatively the drug:metabolite ratio in blood or urine would be traced in time. The latter is however not recommended as this ratio not only depends on the glucuronidation rate, but also largely depends on the fate of the metabolite. With the population approach information on the metabolites can be incorporated into the model, describing all pharmacokinetic parameters relating to both the parent drug and the metabolite, thereby allowing for the quantification of each individual elimination route, including glucuronidation.

One of the first and archetypical examples of the impact of UGT ontogeny on paediatric pharmacology was the observation in the fifties of last century that lack of UGT activity causes the grey baby syndrome in neonates that are treated with chloramphenicol [96]. Around that time it was also observed that the recovery of the acetanilide glucuronide in urine is significantly reduced in neonates and that the recovery in preterm neonates is lower than the recovery in the term neonates. The glucuronide recovery was found to increase with age, with faster maturation rates for term neonates compared to their preterm counterparts. Adult recovery levels of the glucuronide were reached around 3 months of age [97].

Studies on paracetamol ^[98–100] and salicylamide ^[99] in the seventies of the last century, showed the ratio of sulphate and glucuronide metabolites in urine to differ with age, while the total drug elimination did not increase dramatically. The sulphate fraction was found to be higher in children for both drugs, probably to compensate for the reduced glucuronidation capacity in this age-group. In 7 to 10 year old children glucuronidation fractions were found not to have reached adult values ^[99], whereas in 12 year-old children adult fractions appeared to have been reached ^[98]. In more recent studies paracetamol glucuronidation was found to increase with both postmenstrual and postnatal age ^[101–108]. These changes were quantified in different ways. Glucuronide:sulphate ratios were found to increase with gestational and postnatal age ^[101,106] and to reach adult values at 3 years of age ^[103]. The maturation half-life of total paracetamol clearance was found to be close to 3 months for term and preterm neonates ^[102,104], whereas the maturation half-life of paracetamol glucuronidation in specific was found to be 8.09 months ^[103]. It is however important to note that these half-lives do not reflect the maturation of absolute clearances, but of the clearances that are allometrically scaled to average adult bodyweights of 70 kg. Therefore, when after 4 to 6 half-lives adult clearance values are assumed to have been reached in a child, it means that clearance would be at adult values had the child been of average adult bodyweight. However, absolute clearance values in this child are still low and increasing due to the physiological increase in size (bodyweight). According to the Centers for Disease Control and Prevention (CDC) in the US, males on average reach a bodyweight of 70 kg around 18 years of age, whereas the average female does not reach this bodyweight ^[109]. Based on urine recovery studies in neonates, it has been suggested that paracetamol glucuronidation is up-regulated with multiple doses ^[106], something that has also been observed in adults ^[110]. However, our group has suggested that this observation is a possible artifact of a slower glucuronidation rate compared to the elimination rates of other routes, since this slower glucuronidation rate causes the fraction of a drugs glucuronide recovered in urine to increase at later time-intervals ^[111].

Morphine is a UGT2B7 specific substrate and the maturation of its glucuronide formation in neonates, infants and children has been widely studied. Studies on morphine and its glucuronides using traditional methodologies were not unambiguous. It is generally recognized that even at a gestational age as young as 24 weeks, neonates can glucuronidate morphine ^[112–114]. In neonates, total morphine clearance per kilogram bodyweight was reported to be positively correlated with birth weight and gestational age ^[113,115], however others could not identify an effect of gestational age on morphine pharmacokinetic parameters in neonates ^[114]. Morphine clearance per kilogram bodyweight is lower in neonates compared to infants, children and adolescents ^[112,116,117]. Adult clearance values were reported to be reached between 1 to 3 months ^[118] or between 6 months and 2.5 years ^[116]. The contribution of sulphation to morphine elimination was

found to be minor in neonates and to decrease further with age ^[119]. It is suggested that the increase in the fraction that is glucuronidated is predominantly responsible for the observed increases in morphine clearances. The fraction of morphine that is cleared through glucuronidation was found to be lower in neonates than in older patients ^[112,120] and in these neonates this fraction was found to increase with birth weight ^[113]. Others reported however that sulphation and unchanged renal excretion are still important morphine elimination routes in infants and children ^[116] and yet another group found the contribution of each elimination pathway to total morphine clearance to already be at adult ratio's in preterm neonates ^[114].

The maturation of morphine glucuronidation in the first three years of life has been quantified in different ways with the use of population modeling. In all studies absolute morphine glucuronidation clearance was found to continuously increase in this population. Two studies scaled clearances allometrically to 70 kg and subsequently described this maturation either with an exponential equation based on postnatal age ^[121] or with a sigmoidal equation based on postmenstrual age that was different for term and preterm neonates ^[122]. A third study by our group found that the changes in glucuronidation clearance was best described by a bodyweight-based exponential equation with an estimated exponent of 1.44 and that glucuronidation was decreased by about 50% in neonates younger than 10 days of age while there were no differences between term and preterm neonates except those resulting from the differences in bodyweight (Chapter 3).

For zidovudine, which is also mainly metabolized by UGT2B7, clearance has been reported to mature rapidly with postnatal age in the first weeks of life ^[123,124]. Adult clearance values have been reported to be reached after two to eight weeks ^[123,124], whereas others report a slower increase in clearance for a subsequent two years, which is then followed by an even slower increase of absolute clearance rates during the rest of childhood and adolescence ^[125]. In preterm neonates clearance is lower ^[126,127] and maturation is slower ^[124] than in their term counterparts. In the preterms both bodyweight ^[126,127] and gestational age ^[127] have been reported to correlate with clearance rates.

The pharmacokinetics of propofol, which is mainly eliminated through glucuronidation by UGT1A9, has also been broadly studied in the paediatric and neonatal population. Studies have shown propofol metabolism in neonates to be different from adults and mainly suggest age-related changes in glucuronidation capacity for this drug in early life ^[128,129]. Kataria *et al.* have found propofol clearance per kg bodyweight to increase linearly with bodyweight in children ranging between the ages of 3 and 11 years ^[130], and Wang *et al.* described multi-directional age-related changes in total propofol clearance over the entire human age-range ^[131]. However, cautions is warranted when using propofol as an *in vivo* probe for glucuronidation, since this drug has a high

extraction ratio causing its clearance to be limited by liver blood flow rather than enzyme capacity, especially after maturation of the metabolic clearance is complete around the age of 2 years ^[132].

Additionally, some of the drugs used for the treatment of epilepsy (e.g. lamotrigine, carbamazepine, valproic acid) are also, at least partially, glucuronidated. Since these drug are often used in combination with each other and since issues with UGT-(auto) induction or inhibition are frequently encountered in this drug class ^[16], these drugs are also considered not to be very suitable as *in vivo* probes for glucuronidation.

2.2.4.1 Other Factors that Potentially Influence Pharmacokinetics *In Vivo*

When deriving paediatric drug dosages from enzyme maturation rates, it is important to consider that drug concentrations after single bolus doses or after loading doses not only depend on elimination rates but also on distribution volume. Additionally, age-related changes in the unbound drug fraction may influence the hepatic drug extraction ratio and thereby elimination rates, as well as drug effects.

In addition to maturational changes in drug glucuronidation rates and genetics, environmental factors, comedication, comorbidities and medical treatments and procedures may influence drug glucuronidation capacity. The latter three are especially important in paediatrics since ethically it is difficult to perform studies in healthy subjects. Maternal smoking for instance may influence glucuronidation capacity in the first few days of life ^[133]. A number of compounds have been identified to induce the activity of a variety of UGT isoenzymes ^[134], whereas on the other hand phenobarbital and phenytoin were found to decrease paracetamol glucuronidation in the offspring ^[135]. Hypothermia to treat hypoxic ischemic encephalopathy was found to reduce morphine clearance in neonates ^[136]. Additionally, morphine clearance was found to be different in infants undergoing cardiac surgery *versus* patients undergoing non-cardiac surgery ^[137] and the type of cardiac surgery was found to further influence morphine clearance ^[138]. Extracorporeal membrane oxygenation (ECMO) generally influences drug pharmacokinetics in numerous ways ^[139]. In neonates and infants morphine clearance was reported to be reduced in patients on ECMO ^[140–142], however others have reported no change in morphine concentration after initiation of ECMO treatment ^[143]. In neonates, maturation rates of morphine glucuronidation were found to be faster in patients on ECMO compared to non-cardiac post-operative infants, with a combination of bodyweight and postnatal age as descriptors for this process ^[140,141]. It cannot be excluded that changes in maturation rates observed during medical treatments are the result of improvements in the medical status of the patient rather than changes in maturation. Finally, clinical studies on the maturation rates of metabolic pathways are generally restricted to patients with unimpaired liver function, leaving the influence of liver failure on drug glucuronidation in the paediatric population largely unidentified.

2.3 Discussion and Conclusion

The different methodologies discussed in the current review measure different characteristics of the developing biological system. The *in vitro* methods provide mechanistic insight in different underlying processes of the clinically observed developmental changes in drug glucuronidation in the paediatric population and all methods should be regarded as complementary to each other. Table II gives a summary of the findings on maturation of the specific UGT isoenzymes with the various techniques, as discussed in the current review.

It can be seen in table II that the physiological processes in gene expression may have their own unique pattern of onset and maturation, this may be due to differences in contributing factors. Therefore different *in vitro* techniques may yield different answers to the onset and maturation of hepatic glucuronidation, even when the samples used with the various techniques are obtained from the same individual. This is nicely illustrated in the study by Strassburg *et al.*, where differences in glucuronidation activity were observed between paediatric and adult samples, while mRNA and enzyme levels in the same paediatric samples were already indistinguishable from adult values ^[36]. Also Zaya *et al.* showed the age-related increase in the enzyme expression of UGT2B7 to be more pronounced than the increase in UGT2B7 activity, and adult expression levels to be reached at 12-17 years of age, whereas activity levels remained below adult values throughout the paediatric age-range ^[38]. These findings imply that the ontogeny of UGT activity does not solely depend on the ontogeny of UGT gene expression (i.e. transcription and translation). Since UGT activity is influenced by post-translational modifications ^[39-41], maturation of UGT activity is therefore also dependent on the age-related changes in these processes. Additionally, UGT activity was found to be dependent on the formation of complexes with other UGTs, other enzymes and other proteins and on the lipid composition of the membrane it is integrated in ^[42,43], age-related changes in the expression of these components may thus further influence the ontogeny of the UGT enzyme activity. Moreover, genetic polymorphisms have also been found to influence UGT activity and alter glucuronidation capacity in adults in a substrate specific way ^[144-146].

It is also important to consider the fundamental differences between the methods that measure mRNA and enzyme quantities and the more qualitative methods that measure enzyme activity. As long as primers and antibodies are specific enough, absolute amounts of mRNA and enzyme can be quantified relatively unambiguously. The difference in the qualitative measurement of enzyme activity is that it is substrate-dependent. Findings on *in vitro* and *in vivo* enzyme activity not necessarily reflect

absolute enzyme activity, they rather describe the characteristics of a specific enzyme-substrate combination. This may limit the potential to extrapolate findings on enzyme activity from one enzyme-substrate combination to a combination of the same enzyme and another substrate. Likewise, genetic mutations may cause functional changes of an enzyme towards one substrate and not towards another.

Table II also shows that the methods that are used to derive *in vivo* clearances from *in vitro* results by incorporating various contributing components in different ways further diversify the findings on glucuronidation ontogeny. In *in vivo* studies, where external factors also influence drug glucuronidation capacity, the influence of contributing components is largest, but this setup resembles the clinical situation best. Here different scaling techniques to quantify glucuronidation capacity disperse the conclusions on glucuronidation ontogeny. In the paediatric population clearance is often expressed per kilogram or it is allometrically scaled to values of a 70 kg 'standard adult'. When clearance values expressed per kilogram or per 70 kg have reached adult values, absolute clearance values are often still low and increasing. In the study of Zaya *et al.* *in vitro* glucuronidation activity expressed per mg enzyme did not reach adult values before adulthood, whereas none of the paediatric activity levels could be discerned from adult levels when the activity levels were allometrically scaled to *in vivo* values of a 70 kg individual [38]. When expressed per 70 kg, morphine glucuronidation has reached 80% of adult levels at the age of 6 months and at 1 year adult values have been reached [121], whereas absolute glucuronidation rates have been found to be still low and exponentially increasing at least up to the age of 3 years and probably even further (Chapter 3).

Most findings suggest that absolute doses of drugs that are glucuronidated should be reduced in neonates, infants, and children and possibly also in adolescents. It is however not possible to derive more general dosing guidelines, as various UGT isoenzymes mature differently, as substrate specificities overlap and as there are many external factors that may influence drug glucuronidation. A proposed method to improve this situation is to develop validated population pharmacokinetic models for probe drugs that are metabolized by specific UGT isoenzymes. From these models information that is describing the biological system can be derived, which may include information on the maturation rate of glucuronidation in the population but also information on the influence of other factors that significantly contribute to the variability in glucuronidation capacity. It is hypothesized that this information of the biological system can be extrapolated between drugs that are metabolized by the same UGT isoenzyme [147]. Due to the fact that enzyme activity is a property of both the enzyme and its substrate, absolute clearance values can most likely not be extrapolated between drugs, however the extrapolation potential of maturational changes in clearance from one enzyme specific substrate to another specific substrate of the same enzyme is currently being investigated (Chapter 6)

[148]. The feasibility of these types of studies relies on the identification of suitable probes. Since it is largely unknown how age-related changes in the biological system affect enzyme specificities the assumption is often made that enzyme specificities are similar between artificial or adult-derived enzyme sources *in vitro* and in children *in vivo*.

In conclusion, gaps in our knowledge on UGT ontogeny at the various *in vitro* and *in vivo* levels still exist. Enzyme expression and *in vitro* and *in vivo* enzyme activity have been studied more frequently in fetuses, neonates and infants than in older children and adolescents. The various studies that investigated UGT ontogeny at different levels generally suggest that the onset of UGT expression and hepatic glucuronidation occurs after 20 weeks of gestation, with a boost in expression and activity in the first weeks of life. The maturation rate of glucuronidation varies between the different UGT isoforms, but maturation of some UGTs may extend well beyond the age of two years.

Table II. Findings for the ontogeny of various UGT isoenzymes as determined by the various techniques discussed in this review. A. findings for the UGT1A subfamily. B. Findings for the UGT2B subfamily and C. Findings for unspecific or unspecified UGTs.

A.		
UGT isoenzyme	level of ontogeny study	findings on ontogeny
1A1	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, adult levels of transcripts have been reached at a PNA of 6 months ^[36] .
	enzyme expression	Expression levels have reached adult values between 7 months and 2 years ^[36] .
	enzymatic activity (<i>in vitro</i>)	The glucuronidation rate of bilirubin at a GA of 17 to 30 weeks is at 0.1% of adult levels ^[55] , at a GA of 30 to 40 weeks this is at 1% of adult levels ^[54,55] and after birth there is a rapid age-dependent increase that is independent of GA, to reach adult values at 8 to 15 weeks ^[37,54,55] .
	glucuronidation clearance (<i>in vitro</i>)	-
1A3	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, adult levels of transcripts have been reached at a PNA of 6 months ^[36] .
	enzyme expression	-
	enzymatic activity (<i>in vitro</i>)	-
	glucuronidation clearance (<i>in vitro</i>)	-
1A4	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, adult levels of transcripts have been reached at a PNA of 6 months ^[36] .
	enzyme expression	-
	enzymatic activity (<i>in vitro</i>)	Glucuronidation rates reach adult levels at 1.4 years, after deriving <i>in vitro</i> activity levels using the well-stirred and parallel-tube model adult levels are reached at 18.9 years ^[14] .
	glucuronidation clearance (<i>in vitro</i>)	-

1A6	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, adult levels of transcripts have been reached at a PNA of 6 months ^[36] .
	enzyme expression	Expression levels have reached adult values between 7 months and 2 years ^[36] .
	enzymatic activity (<i>in vitro</i>)	Serotonin glucuronidation is higher than adult levels in fetal and neonatal livers ^[56] , however no paracetamol glucuronidation was detectable in fetal liver ^[57] .
	glucuronidation clearance (<i>in vitro</i>)	-
1A9	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, transcripts are detectable at a PNA of 6 months, adult transcript levels of have been reached at 1.5 years ^[36] .
	enzyme expression	-
	enzymatic activity (<i>in vitro</i>)	Glucuronidation of 2-aminophenol in fetal and neonatal liver is about 1% of adult levels. Activity levels exponentially increase to reach adult values at a PNA of 3 months, with no differences in term and preterm born neonates ^[54] . Glucuronidation of 4-methylumbelliferone has reached adult values at a PNA of 20 months ^[14] . Glucuronidation of testosterone and 1-naphthol is low at birth and increases slowly. At a PNA of 1 year adult values have not been reached ^[37] . Glucuronidation rates of 18 different compounds has not reached adult values at 2 years of age, for some compounds the glucuronidation rates are up to 40-fold lower than in adults ^[36] . Acetanilide glucuronidation is reduced in neonates and this reduction is more pronounced in preterm neonates compared to their term counterparts. The age-dependent increase in glucuronidation capacity is more pronounced in term neonates compared to preterms. Adult levels are reached around 3 months ^[97] . For paracetamol and salicylamide the fraction of drugs eliminated through glucuronidation has not reached adult values at 7 to 10 years ^[99] , at 12 years adult levels have been reached ^[98] .
	glucuronidation clearance (<i>in vitro</i>)	

GA = gestational age, PNA = postnatal age

B.		
UGT isoenzyme	level of ontogeny study	findings on ontogeny
2B4	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, transcripts are detectable at a PNA age of 6 months, adult transcript levels have not been reached at 2 years ^[36] .
	enzyme expression	-
	enzymatic activity (<i>in vitro</i>)	-
	glucuronidation clearance (<i>in vitro</i>)	-
2B7	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, adult levels of transcripts have been reached at a PNA of 6 months ^[36] .
	enzyme expression	Expression levels have been found to reach adult values between 7 months and 2 years ^[36] or between 12 to 17 years ^[38] .
	enzymatic activity (<i>in vitro</i>)	Epirubicin glucuronidation capacity showed a small age-dependent increase with PNA, but in all pediatric age-ranges activity is lower than adults. After allometric scaling, <i>in vitro</i> adult clearance levels are predicted to be reached between 12 and 17 years ^[38] .
		Morphine glucuronidation can be detected at a GA of 24 to 27 weeks and is then 6 to 10 times lower than in adults ^[112-114] . No trends with age are observed in this group ^[58] .
		Morphine glucuronidation in neonates is lower than in older paediatric patients ^[112,116,117] and this fraction was found to increase with birth weight ^[113] . Others however report the ratio's in elimination routes of <i>in vitro</i> morphine clearance to already be at adult levels in preterm neonates ^[114] .
	glucuronidation clearance (<i>in vitro</i>)	Absolute morphine glucuronidation increases in the first three years of life. Clearances allometrically scaled to 70 kg mature exponentially with PNA ^[121] or sigmoidally with PMA with slower maturation in preterm neonates compared to term neonates ^[122] . Absolute morphine glucuronidation rates increase with bodyweight to the power of 1.44, with a reduction in neonates younger than 10 days and no differences between term and preterm neonates (Chapter 3).
		Zidovudine clearance rapidly increases in the first weeks of life ^[123,124] . Thereafter some report that adult clearance rates are reached at 2 to 8 weeks ^[123,124] , whereas others report a slower increase to the age of 2 years, followed by an even slower increase in absolute clearance values till adulthood ^[125] . In preterm neonates clearance was lower ^[126,127] and maturation was slower ^[124] than in term neonates.

2B10 / 2B15	mRNA transcription	No transcripts are detectable at a GA of 20 weeks, adult levels of transcripts have been reached at a PNA of 6 months [36].
	enzyme expression	-
	enzymatic activity (<i>in vitro</i>)	-
	glucuronidation clearance (<i>in vitro</i>)	-

GA = gestational age, PNA = postnatal age

C.			
UGT isoenzyme	level of ontogeny study	findings on ontogeny	
Unspecific / unspecified	mRNA transcription	-	
	enzyme expression	One unidentified UGT isoenzyme could be detected at a GA of 18 to 27 weeks, at term birth 3 isoforms could be detected and, at a PNA of 3 months all detectable adult isoforms are present at about 25% of adult level [37].	
		Glucuronidation of 2-aminophenol in fetal and neonatal liver is about 1% of adult levels. Activity levels exponentially increase to reach adult values at a PNA of 3 months, with no differences in term and preterm born neonates [54].	
	enzymatic activity (in vitro)	Glucuronidation of 4-methylumbelliferone has reached adult values at a PNA of 20 months [10].	
		Glucuronidation of testosterone and 1-naphthol is low at birth and increases slowly. At a PNA of 1 year adult values have not been reached [56].	
		Glucuronidation rates of 18 different compounds has not reached adult values at 2 years of age, for some compounds the glucuronidation rates are up to 40-fold lower than in adults [56].	
	glucuronidation clearance (in vitro)	Acetanilide glucuronidation is reduced in neonates and this reduction is more pronounced in preterm neonates compared to their term counterparts. The age-dependent increase in glucuronidation capacity is more pronounced in term neonates compared to preterms. Adult levels are reached around 3 months [97].	
		For paracetamol and salicylamide the fraction of drugs eliminated through glucuronidation has not reached adult values at 7 to 10 years [99], at 12 years adult levels have been reached [98].	
		For paracetamol the glucuronidation/ sulphation fraction increases with PNA and GA [103,100] to reach adult values at an age of 3 years [103]. When allometrically expressed per 70 kg, the maturation half-life of paracetamol glucuronidation is 8.09 months [103][144].	
GA = gestational age, PNA = postnatal age			

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