Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
Department of Veterinary Sciences

Hepatic drug metabolism in pediatrics: investigating the neonatal and juvenile (mini)pig as a translational model

PhD thesis submitted for the degree of Doctor in Veterinary Sciences at the University of Antwerp to be defended by

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List of Abbreviations

ACN	Acetonitrile
ADME	Absorption, distribution, metabolism and excretion
BBB	Blood-brain barrier
BPCA	Best Pharmaceuticals Act for Pediatrics
$CaCl_2$	Calcium chloride
cDNA	Complement DNA
CV	Coefficient of variation
CYP	Cytochrome P450
DME(T)	Drug metabolizing enzymes (and transporters)
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
e.g.	Exempli gratia (for example)
EMA	European Medicines Agency
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
EU	European Union
FDA	Food and Drug Administration
FDAAA	FDA Amendment Act
FDAMA	FDA Modernization Act
FDASIA	FDA Safety and Innovation Act
GA	Gestational age
GD	Gestational day
GFR	Glomerular filtration rate
GIT	Gastro-intestinal tract
HD-DDA	High definition-data dependent analysis
HESI	Health and Environmental Sciences Institute
HLM	Human liver microsomes
ICH	International Conference of Harmonization
i.e.	Id est (that is)
ILSI	International Life Sciences Institute
I.V.	Intravenous
JAS	Juvenile animal study
kg	Kilogram
K_3PO_4	Potassium phosphate
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOD	Lower limit of detection

LLOQ	Lower limit of quantification
MMTS	Methyl methane-thio-sulfonate
MOA	Mode of action
MP	Microsomal protein
MPPGL	Microsomal protein per gram of liver
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
M&S	Modeling and simulation
NADPH	Nicotinamide adenine dinucleotide phosphate
NDA	New Drug Application
NEC	Necrotizing enterocolitis
NHP	Nonhuman primate
NICU	Neonatal intensive care unit
NIH	National Institute of Health
PBPK	Physiologically based pharmacokinetic modeling
PCA	Postconceptional age
PD	Pharmacodynamics
PDD	Pediatric drug development
PIP	Pediatric investigation plan
PK	Pharmacokinetics
PMA	Postmenstrual age
PNA	Postnatal age
PND	Postnatal day
P.O.	Per oral
PREA	Pediatric Research Equity Act
Q-TOF	Quadrupole- time of flight
RT-PCR	Real-time – polymerase chain reaction
SBS	Short bowel syndrome
SC	Stratum corneum
TEABC	Triethylammonium bicarbonate buffer
UDPGA	Uridine diphosphate glucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UPLC	Ultra-performance liquid chromatography
US	United States (of America)

CHAPTER 1:
General introduction

"Pediatrics does not deal with miniature men and women, with reduced doses and the same class of disease in smaller bodies but (…) has its own independent range and horizon."

Prof. dr. Abraham Jacobi
The father of American pediatrics

The human life cycle is defined by the transition of one age category into another with specific characteristics belonging to each age group. Roughly, a distinction is made between the pediatric (i.e., birth to 17 years), adult (i.e., 18 to 64 years), and older adult (65 years and older) age groups [1]. Since the adult population represents the largest subgroup and covers the largest period in the life cycle (approximately 50 years), it is not surprising that most pharmacologic research is dedicated to this age category. From an economic point of view, this age group is most interesting because of the high return on investment after a long period of drug development [2]. However, one should not forget that children and older adults also deserve proper treatment when encountering illness. In this thesis, we will focus on **pediatric drug development (PDD)** with special attention on drug metabolism in the youngest age groups and the possible use of the piglet as a translational animal model to fill the knowledge gaps still present.

1.1 Pediatric population

1.1.1 Classification

The pediatric population (i.e., birth to 17 years) comprises **2.4 billion individuals** worldwide at this moment, representing approximately **30% of the total human population** [3]. This subgroup is known to be the most vulnerable since growth and development are most pronounced during the early years of life, affecting the body's response to the administration of drugs. Within the pediatric population, a classification is made based on age (**Table 1**).

Table 1: Classification of the pediatric population. Adapted from [4].

Classification	Age
Neonates	Birth up to 1 month
Infants	1 month up to 1 year
Children	1 year to 12 years
Adolescents	13 years to 16 - 18 years

Next to the general classification, a more in-depth terminology is used for the perinatal period: the time elapsed from the first day of the last menstrual period until birth is called the **gestational age (GA)**, whereas the chronological age starting from the day of birth is called the **postnatal age (PNA)** (**Figure 1**) [5]. They should not be confused with the **postmenstrual age (PMA),** which is the sum of both GA and PNA. In the past, **postconceptional age (PCA)** was also used as a metric in the human clinic. In this case, counting starts from conception instead of the first day of the last menstrual period for PMA. Since conception can only be determined precisely during assisted reproductive procedures, this terminology is not used to avoid confusion although it is often used in a veterinary context [5].

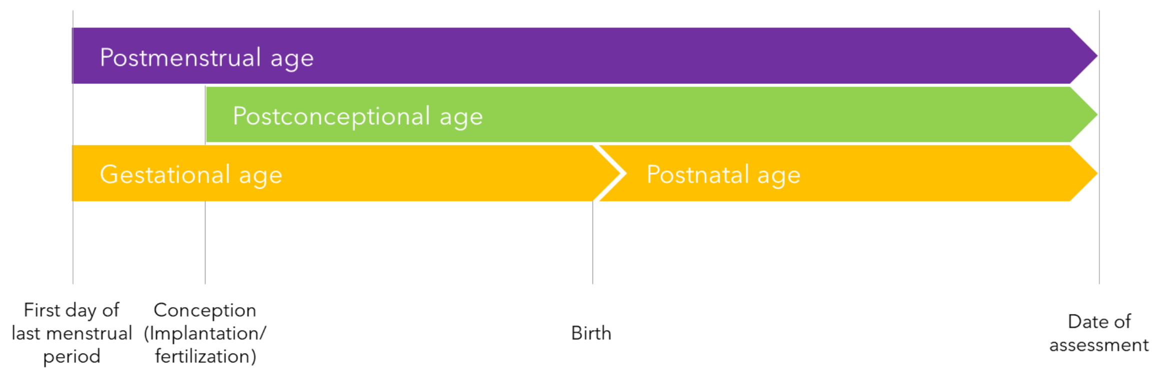

Figure 1: Perinatal age terminology. Adapted from [5].

1.1.2 Prematurity

Within the neonatal population, it is estimated that each year 15 million babies (~11% of all deliveries worldwide, ranging from 5% in northern Europe to 18% in sub-Saharan Africa) are born preterm (<37 weeks) (**Figure 2**) [6]. The rate of preterm birth fluctuates depending on the country's wealth: higher incidence rates are seen in low- to middle-income countries (e.g., India, Nigeria, Pakistan, Indonesia, and the Republic of Congo), whereas lower rates are reported in high-income regions (e.g., Canada, northern Europe, and Australia) [7]. Indications for premature birth are diverse: both maternal (e.g., pre-eclampsia, placental abruption, and placenta previa) and fetal (e.g., intrauterine growth restriction and fetal distress) factors affect its occurrence [8]. Within this preterm group, another subclassification is made based on the GA reached at birth (**Table 2**).

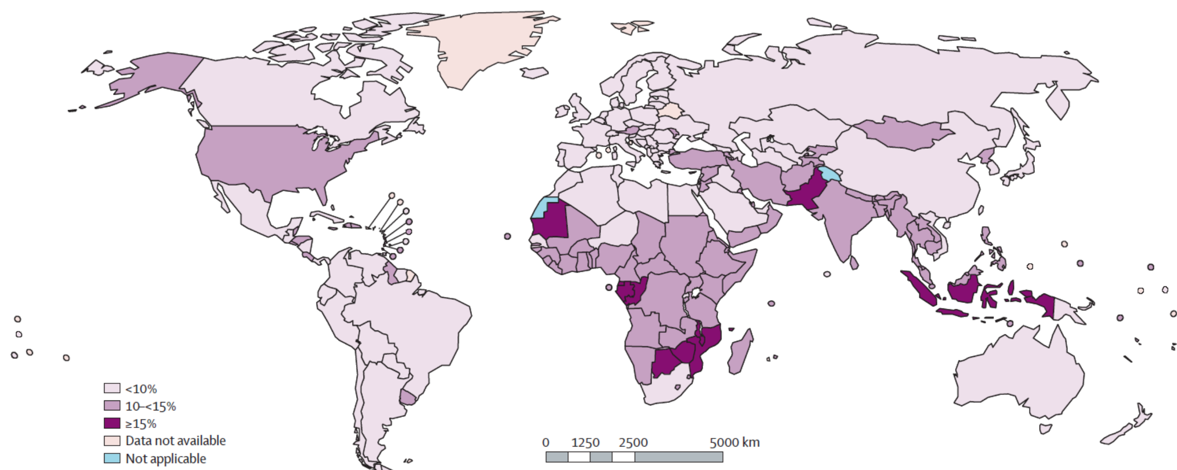

Figure 2: Estimated rate of preterm births by country for 2010. Reproduced from [6], with permission.

Preterm-born neonates are the most fragile subset within the entire pediatric population. They present with complex, challenging pathophysiological conditions (e.g., neonatal sepsis, respiratory distress, necrotizing enterocolitis (NEC), retinopathy of prematurity, and intraventricular hemorrhage) that are poorly understood and often lead to long-term comorbidities [7, 9-12]. Although the need for treatment in this group is very large, only a few drugs are authorized for use. As a result, off-label drug use is still very common, meaning that the safety and efficacy of frequently prescribed drugs (e.g., analgesics, antimicrobials, and diuretics) were not tested during drug development and were not licensed for use in this population [9, 11]. **The incidence of off-label drug use in NICUs is estimated at ~50%** [12, 13]. As a result, clinicians must determine dosing regimens based on rational scientific theories, expert medical opinions, and controlled clinical trials in other age groups [2]. As such, they need to assess whether the risk of off-label drug use is acceptable and appropriate for each patient [2]. Although decisions are made with the highest caution, the risk of adverse drug effects remains very large. These observations are a clear indication that continuous efforts should be made to improve our knowledge of developmental (patho)physiology, especially in preterm-born neonates, but also in the pediatric population in general, to advance children's health.

Table 2: Subcategories of (pre)term birth. Adapted from [4].

Classification	Gestational age
Preterm at the border of viability	22 weeks to < 24 weeks
Extremely preterm	24 to < 28 weeks
Very preterm	28 to < 32 weeks
Moderate to late preterm	32 to < 37 weeks
Early term	37 to 38 weeks
Full term	39 to 40 weeks
Later term	41 weeks

1.2 Pediatric drug development

1.2.1 Tragic historical events set the scene

Several tragedies in the 20th century have pointed out the vulnerability of the pediatric population in drug development. At the time, scientists believed that keeping children and pregnant women out of clinical studies was safer than including them [14]. However, the opposite was true, as illustrated by multiple tragic events. The administration of thalidomide to pregnant women (1959-1961), leading to gross limb malformations in their newborn babies, is probably the most well-known example [2]. Also, direct drug administration to neonates, infants, and children led to tragic toxic side effects and even death (e.g., Gray baby syndrome presenting abdominal distention, ashen-grey skin discoloration, and cardiovascular collapse in neonates due to immature glucuronidation capacity to metabolize chloramphenicol)[15]. After the thalidomide catastrophe, dr. Harry Shirkey raised the urge to consider the pediatric population in drug development: children

were becoming therapeutic orphans and as such missing out on proper medical treatment [16]. Although awareness was created in the 1960's, it lasted until the 1990's for a first step in creating a legal context to include pediatric age groups in the drug development process.

1.2.2 Legal context in the US and EU

The **US** were the first to consider the pediatric population in their legal framework for drug development. After some amendments were issued to the Federal Food and Drug Administration (FDA) in the first half of the 20th century, the **Pediatric Labeling Rule** was created in 1994 (**Figure 3**). This rule required manufacturers to explore whether existing efficacy data in adults and additional studies (pharmacokinetics, pharmacodynamics, and safety assessment) in pediatric patients could be used to extrapolate data and, as such, support additional pediatric use information in the drug's labeling [17]. Since the Pediatric Labeling Rule was not obliged for companies, only limited improvement in pediatric drug labeling was achieved [17]. Therefore, new regulations were issued in the upcoming years. In 1997, the **FDA Modernization Act (FDAMA)** was issued, providing a financial incentive of an additional 6 months of market exclusivity for companies submitting required pediatric studies [17]. This was followed by the Pediatric Labeling Rule turning into the **Pediatric Rule** in 1998 which stated that companies are required to provide safety and efficacy data in relevant pediatric age groups for the claimed indications before approval of the drug [17]. In 2002, the **Best Pharmaceuticals Act for Pediatrics (BPCA)** established a framework of incentives for companies e.g., to retrieve patent extension when pediatric studies were performed for on- and off-patent drugs [18]. Next, the **Pediatric Research Equity Act (PREA)** turned most of the Pediatric Rule into a mandatory law, including the requirements for pediatric assessment in New Drug Applications (NDA)(e.g., description of age-appropriate formulations) [17]. Both BPCA and PREA were reauthorized in 2007 by the **FDA Amendment act (FDAAA)** and were made permanent by the **FDA Safety and Innovation Act (FDASIA)** in 2012 [19, 20].

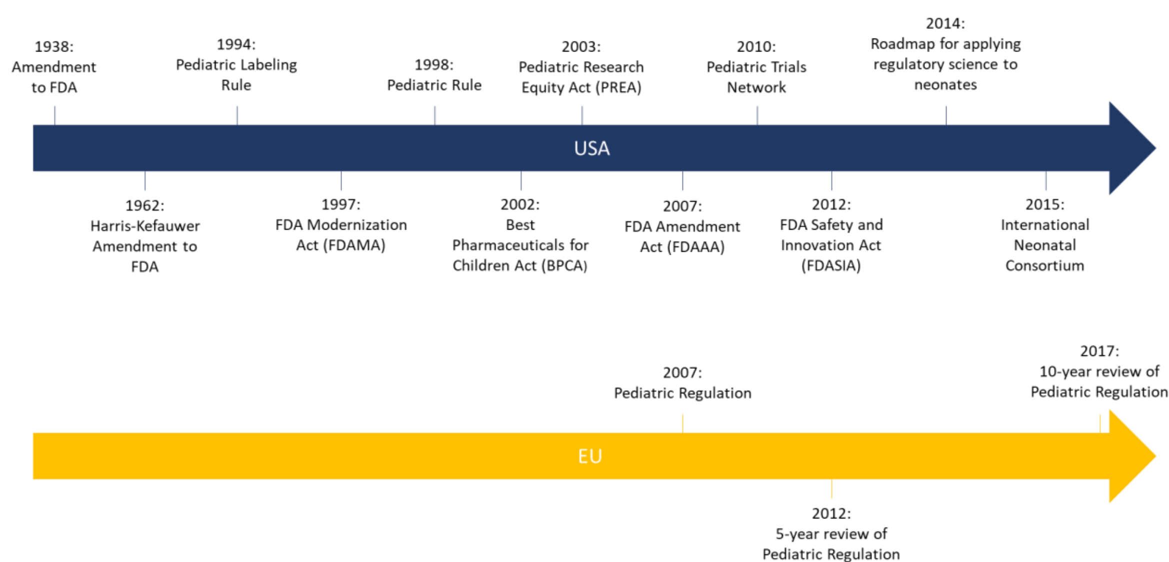

Figure 3: Milestones to promote pediatric drug development in the US and EU, including initiatives for preterm and term neonatal drug development. Adapted from [14, 19].

Only in 2007, after ten years of discussion, the **EU** enacted its first PDD law: the **Pediatric Regulation** (**Figure 3**). This regulation included filing a **pediatric investigation plan (PIP)** when applying for a new medicinal product. This document had to include the description of intended nonclinical and clinical studies (e.g., including a description of timings, formulations, etc.) to cover the entire pediatric population from birth until adolescence [21]. The PIP contains both incentives and requirements linked to PDD, in contrast to the US, where 2 legislations are enacted (i.e., BPCA covers incentives, and PREA covers requirements). After five and ten years, a review of the existing legislation was performed to assess the Pediatric Regulation's effect on PDD progress [19]. The 10-year review report showed a considerable impact on the development of pediatric medicines with increased pediatric research and increased numbers of new products with pediatric indications [22]. Nonetheless, new products often covered therapeutic areas with overlapping adult and pediatric needs, thus therapeutic advances in rare and/or unique diseases to children were still unsuccessful [22].

Both in the US and EU, waivers or deferrals can be obtained for PDD. This is the case when, e.g., the drug has no relevance to be used in (part of) the pediatric population (e.g., Alzheimer's disease drugs, eye drops for glaucoma) or when the drug availability in adults would be delayed due to pediatric trials that could not be performed in time, respectively [20].

On the international level, establishing the International Conference of Harmonization (ICH) in 1990 created a framework for harmonized guidelines for global pharmaceutical development and their regulation. Because of PDD, the **ICH E11 guideline** has provided a stepwise approach since 1999 to support and promote the timely development of medicinal products for use in the pediatric population [23].

Next to the governmental initiatives, additional actions have been taken towards improving PDD. The **Pediatric Trial Network** was established in 2010 by the National Institute of Health (NIH) in the US to promote collaborations in generating data on off-patent drugs, including optimization of trial design and increasing children's enrollment in clinical studies [24]. During the last decade, increasing attention has been given to **neonatal drug development**, as this subpopulation is not fully addressed by the existing guidance documents [4]. Therefore, the European Medicines Agency (EMA) drafted in 2007 guidelines for the investigation of medicinal products in the term and preterm neonate, which came into effect in 2010 [25]. In 2014, a workshop called "**Roadmap for Applying Regulatory Science to Neonates**" was organized by different stakeholders and discussed specific areas (e.g., innovative trial design, trials that allow for extrapolation, and description of clear clinical outcome measures) that should be tackled to improve neonatal drug development [26]. One year later, the **International Neonatal Consortium** was established and comprised a public-private partnership aiming to move towards harmonization of data collection while focusing on specific areas of therapeutic research (e.g., prevention of preterm delivery, neonatal brain, lung and gastrointestinal injury, and neonatal sepsis) [27]. In 2018, a pan-European public-private initiative, the **conect4children** project, was launched to support industry and academia in conducting pediatric clinical trials [28]. This network provides, *inter alia*, education and training

platforms, and promotes collaborations between specialist networks on national and international levels in order to improve and align pediatric clinical trial design, standards and methodologies for neonates, children and young people [28].Thus, efforts are being made to include and improve neonatal drug development. While these initiatives are still ongoing, neonatologists have to rely on handbooks [29, 30] and evidence-based research [31-34] for guidance regarding the treatment, and dose setting in particular, in their patient population.

1.2.3 Challenges encountered during pediatric drug development

Although progress has been made in PDD during the last two decades, a discrepancy between age groups remains. Adolescents are the easiest group to assess during PDD: they have matured to (almost) adult levels, making extrapolation from adult safety and efficacy data possible [35]. However, this is not the case for the youngest age groups. They undergo **extremely fast anatomical** (e.g., organ weights) and **physiological** (e.g., expression and maturation of enzymes, transporters, and neurotransmitters) **changes,** which substantially affect absorption, distribution, metabolism, and excretion (ADME) of drugs [4]. As such, a simple description of maturation based upon size or age does not provide sufficient precision to predict drug disposition and safe drug dosing regimens [4]. In preterm-born neonates, an additional dimension of **varying GA, PNA, and PMA further complicates estimations of drug disposition** [11]. The moment of birth induces large physiological changes (e.g., cortisol surge, changing cardiovascular dynamics, and enzyme maturation), which also affect ADME properties [4]. Moreover, preterm-born neonates often present with multiple comorbidities (e.g., sepsis, surgery, advanced treatment (e.g., hypothermia)) that also alter drug distribution and metabolism [11]. Thus, in-depth knowledge of anatomical and (patho)physiological particularities in (preterm-born) neonates is crucial to provide safe and effective drug therapy in this population [10, 11, 36]. Gaining such knowledge, however, is restricted by **very limited enrollment of (preterm-born) neonates and infants in clinical trials**. Both **ethical and practical challenges** hamper their participation: obtaining informed consent from parents, limited possibilities for repeated sampling due to small body size, immaturity, potential drug-drug interactions, possible need to wait for a long time to examine outcome measures, and multiple comorbidities complicate establishing safety and efficacy in the neonatal population [4, 11, 14]. Traditional control trial design is thus not feasible, especially for extremely preterm neonates [10]. As a result, alternatives should be explored. The FDA and EMA therefore support the use of juvenile animal studies (JAS) to increase our knowledge in these vulnerable age groups (**see 1.4**).

1.3 Pediatric pharmacokinetics

1.3.1 ADME properties: a general overview

Pharmacokinetics (PK) describes what the body does to an administered drug and is an important parameter during drug development. The extent to which the body can absorb, distribute, metabolize, and excrete a drug will highly impact the dose setting. Since these properties alter drastically during growth and development, a thorough understanding is needed.

Drug administration is conducted via several routes with a major distinction between the vascular (e.g., intravascular injection) and extravascular (e.g., gastrointestinal tract (oral), lungs, or skin) routes [37]. When administered intravenously no additional barriers are encountered and the drug reaches the bloodstream directly, thus skipping absorption [9]. However, intravascular administration is often linked to an increased risk of infection in neonates [9], so extravascular routes are preferred. These routes, though, are subject to varying conditions during the early weeks, months, and years of life, affecting the rate and extent of **absorption**. The thickness of the skin, for instance, is much thinner in low birthweight neonates compared to older children [38]. This may lead to a larger uptake of the compound and increases the risk of adverse effects from topical exposures [38, 39]. Altering gastric pH (cf. oral administration) is another example that causes large variations in drug stability, dissolution, and ionization, which are all important for drug absorption [37]. In addition, the drug's physicochemical properties affect the absorption rate as well [37]. Next, **distribution** to the drug's site of action is affected by altering body composition (e.g., total body water volume, percentage of fat and muscle mass), hemodynamic factors (e.g., cardiac output and systemic and regional blood flow), and protein binding (e.g., varying plasma binding protein concentrations) [9, 11, 37, 40]. The combination of these varying conditions may have a significant impact. For example, larger extracellular and total body water together with lower plasma binding proteins results in a larger volume of distribution of the free fraction of the drug, which has a significant impact on its pharmacologic effect [37]. **Drug metabolism** facilitates either clearance or activation of the compound via phase I and/or phase II reactions [9]. These processes are conducted by drug metabolizing enzymes (DME) in the liver, intestine, lungs, and kidneys [41, 42]. The resulting biotransformation aims to solubilize (and consequently eliminate) parent compounds and their metabolites by turning them into more hydrophilic entities. Within this regard, DMEs and their ontogeny play a key role. Understanding DME maturation is crucial to estimate the rate at which a drug will get metabolized. It has been shown in neonates that DMEs are immature at birth, affecting the amount of free fraction. An overall clear tendency of lowered drug metabolism in neonates is acknowledged, which should be considered when determining dosing regimens [9, 11]. Reduced drug metabolism may, for instance, result in increased drug exposure and a potential overdose when not considered during dose setting [37]. Finally, the **excretion** of the drug and its metabolites is mainly facilitated by the kidneys and thus depends on the maturation of this organ system. Since glomerular filtration rate (GFR), tubular excretion, and tubular reabsorption only reach full maturation after 3-5 months, 15 months, and 2 years of age, respectively, it is evident that renal clearance is widely variable in (preterm) neonates and infants as compared to older age groups [4, 11, 37, 40].

The above clearly shows that a range of parameters changes drastically during the early phase of life, consequently affecting drug disposition, metabolism, and clearance. A thorough understanding of each step in the process is needed to improve PDD and avoid adverse drug reactions. An overview of (patho)physiological factors potentially affecting ADME in (preterm) neonates and infants is presented in **Figure 4**. In this thesis, we will further focus on drug metabolism in the neonatal and juvenile population, which is discussed in the following paragraphs. The reader is referred to several comprehensive reviews for a complete PK overview in the neonatal population [9, 11, 36, 37, 40, 43].

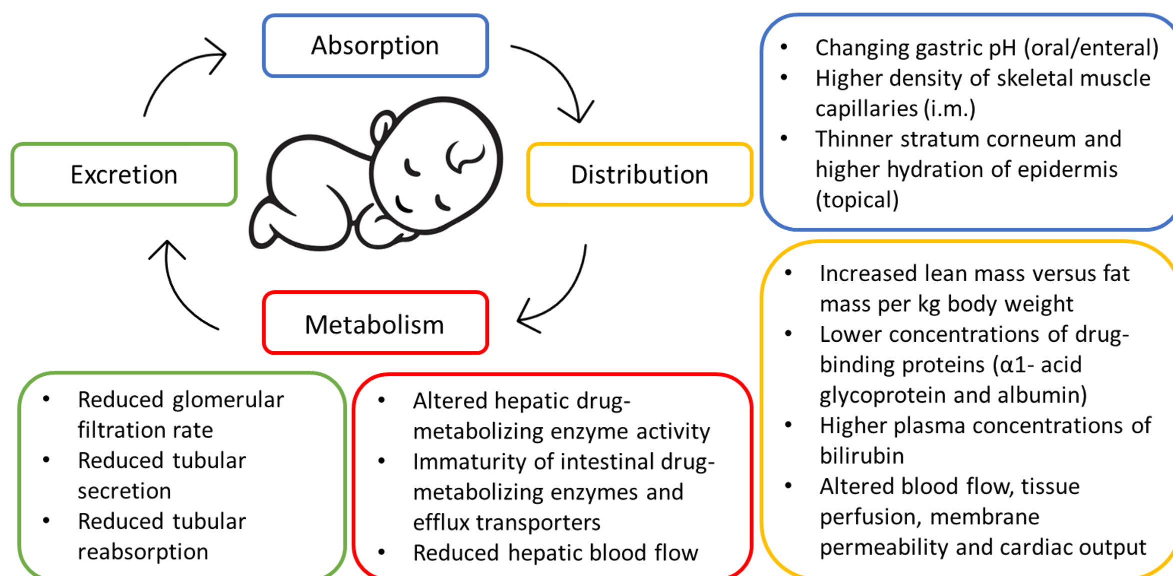

Figure 4: Prematurity- and neonatal-associated (patho)physiological conditions potentially altering ADME properties of drugs as compared to adult values. Adapted from [11].

1.3.2 Hepatic drug metabolism

The liver is the major site of action for the biotransformation of endogenous (e.g., bile acids, unsaturated fats, steroids, prostaglandins, etc.) and exogenous substrates (e.g., drugs, environmental pollutants, alcohol, procarcinogens, etc.) [44-46]. The process is typically conducted by two reaction types: phase I and phase II metabolism. **Phase I metabolism** is characterized by oxidation, reduction, and hydrolysis reactions introducing functional groups on the chemical structure of the compound. In some instances, however, these reactions will convert the substrate into its pharmacologically active form [47]. These drugs often encounter difficulties during absorption and distribution when administered in their active form, so a prodrug is used instead to overcome this issue (e.g., administration of pivampicillin hydrolyzed into ampicillin when reaching the bloodstream) [47]. Several enzyme families are responsible for phase I reactions, e.g., **cytochrome P450 (CYP)** enzymes, flavin mono-oxygenases, peroxidases, amine oxidases, aldehyde oxidases, glutathione peroxidases, carboxylesterases, and alcohol dehydrogenases [48]. **Phase II metabolism** consists of conjugation reactions (e.g., glucuronidation, acetylation, sulfation, and methylation) that add an endogenous polar compound to the parent compound or its phase I metabolite [9, 49]. As such, more hydrophilic substances are created, which facilitate drug elimination. Examples of involved DME families are, *inter alia*, **uridine 5'-diphosphoglucuronosyltransferases (UGT)**, sulfotransferases, N-acetyltransferases, and glutathione S-transferases [9]. Concerning drug metabolism, CYP and UGT enzymes are considered the most important DME families for phase I and II metabolism, respectively.

1.3.2.1 *Phase I metabolism: focus on CYP enzymes*

The heme-containing CYP family is considered the most important phase I DME superfamily as they are responsible for the metabolism of 70-80% of all marketed drugs [50]. The enzymes are integrated into the endoplasmic reticulum membrane with the catalytic domain lying on the cytosolic side [51]. A distinction between several (sub)families is made based on the amino acid sequence alignment percentage. Within the same family (indicated by an Arabic number, e.g., CYP1), 40% amino acid sequence homology is described, whereas 55% homology is present within a subfamily (indicated by a letter, e.g., CYP1A) [52]. Specific isoforms in each subfamily are further characterized by another Arabic number (e.g., CYP1A1) [52]. In the human population, 57 genes and 58 pseudogenes are documented [53]. Within this pool, 18 families are acknowledged, of which the CYP1, CYP2, and CYP3 families are considered the main drivers for drug metabolism [50]. In particular, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A7 are the most important DME in the liver [54].

A general overview of the CYP enzymes' catalytic cycle is shown in **Figure 5**. In brief, the CYP's oxidative metabolizing capacity is based on the transfer of electrons to its heme core in the presence of an electron donor (i.e., nicotinamide adenine dinucleotide phosphate (NADPH)), NADPH-cytochrome reductase and oxygen. Substrate binding in the active catalytic site of the CYP enzyme alters the conformation of the enzyme. It facilitates the reduction of Fe^{3+} in the heme core of the enzyme into Fe^{2+} by electron donor NADPH. O_2 binds subsequently with the ferrous CYP state. NADPH (or Cytochrome b5) provides a new electron that further facilitates the incorporation of an oxygen atom in the substrate (i.e., oxidized substrate), the release of H_2O, oxidized NADPH, and restoring of the oxidized ferric state of the heme core of the CYP enzyme [55]. It should be mentioned, however, that the cycle is dynamic and does not necessarily proceed step by step in a linear way [53]. As such, substrates can be bound and released along the cycle [53].

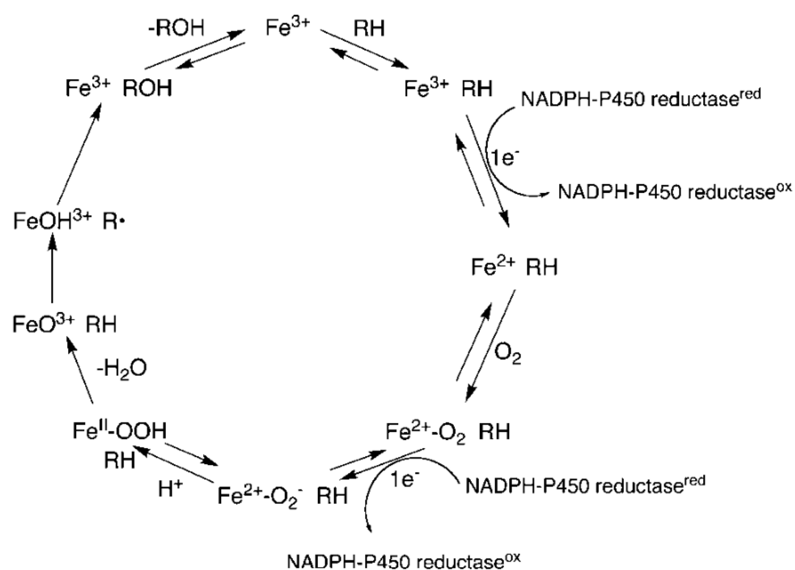

Figure 5: The cytochrome P450 catalytic cycle. RH and ROH represent the substrate and hydroxylated product (i.e., metabolite), respectively. Reproduced from [36].

1.3.2.2 *Phase II metabolism: focus on UGT enzymes*

Since UGT-catalyzed glucuronidation is responsible for ~35% of all phase II drug metabolized reactions, UGT enzymes are considered the most important phase II DME family [56]. UGT enzymes are bound to the internal membrane of the endoplasmic reticulum and face its luminal side [42, 57]. Similar to the CYP enzymes, a distinction is made between (sub)families: each family shares at least 50% homology in their DNA sequences, whereas subfamilies share 60% homology [58]. The nomenclature is defined as follows: families are designated by an Arabic number (e.g., UGT1), subfamilies are represented by a letter (e.g., UGT1A), and specific isoforms are indicated by another Arabic number (e.g., UGT1A1) [42]. In the human population, 4 families (i.e., UGT1, UGT2, UGT3, and UGT8) and 5 subfamilies (i.e., UGT1A, UGT2A, UGT2B, UGT3A, and UGT8A) are documented, with a total of 22 proteins [49, 59]. Regarding hepatic drug metabolism, the UGT1 and UGT2 families are critical, with UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28 being key players for phase II metabolism [42, 60, 61]. UGT enzymes of the other families are mainly expressed extrahepatically and are involved in endogenous processes.

The general glucuronidation reaction scheme is represented in **Figure 6**. The working mechanism (i.e., glucuronidation) is characterized by the covalent linkage of glucuronic acid from the cofactor UDP-glucuronic acid (UDPGA) with a suitable functional group on the hydrophobic parent compound or its phase I metabolite [49, 57]. These functional groups include, *inter alia*, aromatic and aliphatic alcohols, carboxylic acids, amines, phenols, and thiols [49, 57]. Next to the glucuronide conjugate, uridine diphosphate is also produced [49].

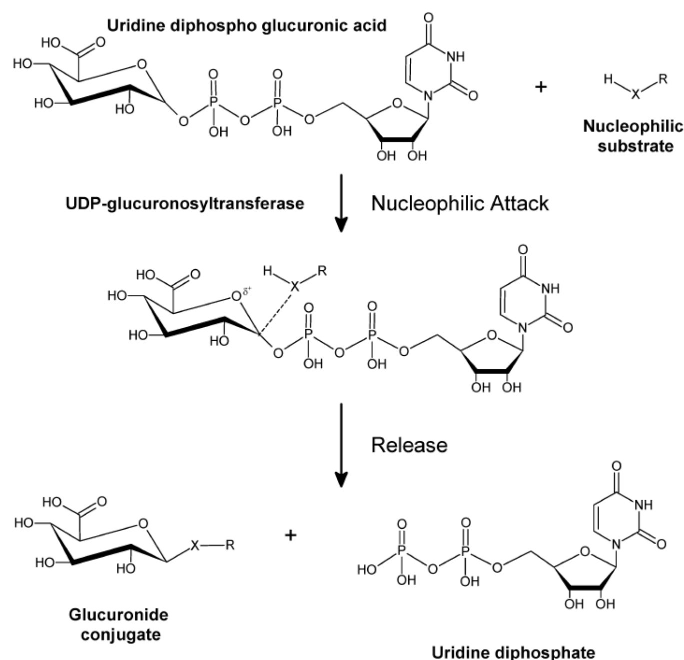

Figure 6: Glucuronidation reaction scheme. Reproduced from [41].

1.3.2.3 *mRNA, protein abundance or enzyme activity level: what to look at?*

This thesis will emphasize the different maturation profiles among the various CYP and UGT enzymes in the neonatal and juvenile populations. Large knowledge gaps still exist in our understanding of their ontogeny patterns. Although three classes of DME ontogeny patterns were described before in humans [62], the impact of age, sex (**see chapter 3**), and preterm birth (**see chapter 4**) on these patterns is still largely unknown. Additionally, one should realize that ontogeny profiles can be assessed at several levels, e.g., mRNA, protein abundance, and enzyme activity levels. Typically, protein expression is preceded by gene expression, mRNA transcription, and translation (**Figure 7**). All these processes will affect the final functionality of the resulting DME. By going backward in this causal chain, physiological insight into the different characteristics of the biological system is obtained [63]. However, by splitting these processes, some features may seem relevant at, e.g., mRNA level, but will be negligible in the overall biological system when not translated to a functional enzyme. As such, different contributing factors may be found when studying ontogeny at different levels [63]. This should thus be considered when comparing different biological levels. Studying complex biological systems *in vivo* will better represent clinical observations, but unfortunately, this is not always feasible due to practical limitations. Finally, examination of all levels will provide valuable information and will contribute to a mechanistic interpretation of the clinically observed developmental changes in DME functionalities [63]. As described in section 1.2.3, obtaining (preterm) neonatal and juvenile human samples is very difficult. Therefore, this thesis will further investigate the use of the piglet as a translational model for neonatal and juvenile CYP and UGT drug metabolism.

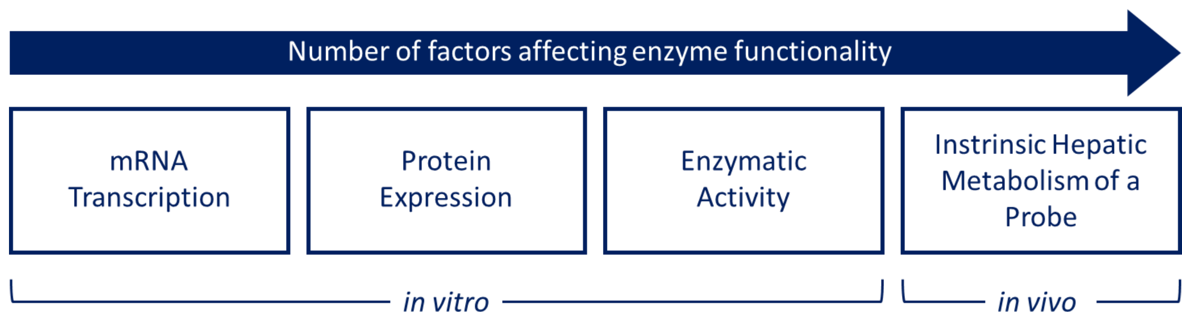

Figure 7: Schematic representation of the processes that can be studied to determine the ontogeny of enzyme systems. Adapted from [55].

1.4 The non-clinical phase of pediatric drug development

1.4.1 Role of juvenile animals models

Due to the extensive maturation during the early years of life, important structural and functional differences between children and adults, as well as between pediatric age groups exist [35, 64]. The appropriateness of adult human safety data to support pediatric studies should thus be investigated very carefully. In case of toxicity, increased sensitivity of target organs (e.g., kidney, skeleton, and central nervous system) that undergo postnatal development may be present [65]. Consequently, adult safety data may be judged insufficient to predict pediatric safety. JAS may overcome this issue and provide adequate data to define differential toxicity, safety, and effectiveness of a drug in several age ranges [66]. However, all available human safety data and standard toxicological studies must be reviewed before deciding on its use [21].

Over the years, the FDA and EMA issued guidelines for using JAS in view of PDD [35, 64]. These documents highlighted that the inclusion of JAS should be scientifically driven and considered on a case-by-case approach [21, 35, 65]. Only in defined circumstances (e.g., when findings cannot be ethically and safely assessed in pediatric clinical trials and previous animal and human safety data are insufficient) and after the application of a well-defined study design (part of the PIP) (e.g., the definition of the intended indication, age of the pediatric population, and treatment duration), JAS can be conducted [35, 64, 65, 67]. Only recently, the European guidelines were transformed into an all-including document, i.e., **"*ICH guideline S11 on nonclinical safety testing in support of development of pediatric pharmaceuticals*"** recommending international standards and promoting harmonization of the nonclinical safety assessments [68]. Considerations for additional nonclinical safety investigations and first-in pediatrics studies, study design needs, and data interpretation directions are included to provide a framework for regulators, industry, and academia [68].

The role of JAS is thus to bridge the gap between adult and pediatric populations by providing critical data to guide the pediatric clinical trial designs, drug dosing, and safety monitoring and, as such, contribute to earlier and safer access to medicines for children [21, 69, 70].

1.4.2 Species selection for juvenile safety and toxicity testing

One of the key considerations for JAS is species selection. Organ maturation of laboratory animals is rarely the same as in humans, so choosing the most adequate species will be crucial when designing JAS concerning early exposure of children to medicines [21, 70]. It is thus important that the developmental stage of the animals being studied is comparable to that in the intended pediatric population [64, 70]. Therefore, several considerations need to be taken into account, e.g., age at which the children will be treated, therapeutic use of the drug, duration of the treatment, and potential age- and species-specific differences in efficacy, PK, or toxicity [35, 64, 71].

According to the guidelines, a single animal species is usually deemed sufficient [68]. Most common is the use of the rat, but a nonrodent species (e.g., nonhuman primate (NHP), dog, minipig, or rabbit) can be appropriate when scientifically justified [65, 68]. The preference for rodents is attributable to their extensive use as laboratory animal models resulting in many favorable advantages: extensive historical background data is available as they are a widely used model, they are easy to house, transport and manage, and their short gestation time, easy cross-fostering and multiparity are also beneficial characteristics compared to nonrodent species [72]. In view of juvenile studies, however, their small size may be disadvantageous: blood sampling in young rats is limited, and sampling is terminal, thus requiring large numbers of offspring [66].

When a nonrodent species is required for the safety assessment of new pharmaceuticals, the choice should always be based on sound scientific principles [73]. In the past, there has been a tendency to use the dog as default, and if the dog was not suitable, the NHP was the next choice [73]. However, ethical and practical (e.g., small litter size, long gestation period, long preweaning periods, space requirements, and costs) concerns over the use of dogs and NHPs, have led to the search for alternatives [73, 74]. This is where the (mini)pig gained interest as a nonclinical species. First, large physiological and anatomical similarities compared to humans are observed in pigs which are extensively reviewed elsewhere [73, 75-78], though a general overview comparing human and porcine anatomy is shown in **Table 3**. Second, pigs seem a very useful species in juvenile studies as they pose practical advantages over the dog: (mini)pigs are easy to handle, they have a relatively large litter size, they reach sexual maturity earlier than dogs and NHPs, and their size allows the conduction of basic procedures such as blood sampling from an early age onwards [73, 74, 79]. It should be acknowledged, however, that less historical background data regarding drug-induced toxicities are available as compared to the dog and NHP [74]. Only a few studies have been conducted within each age range. This shortage raises concerns for the interpretation of results, potentially discouraging investigators from using this animal model [79]. Hence, the urge to further investigate the (mini)pig's potential as it presents many advantages over the dog or NHP [79].

Table 3: Summary of main similarities and differences in maturation of different organ systems in pigs and humans. Adapted from [64].

Organ System	Feature	Similarities and differences compared to man
Cardiovascular	Drainage	Different
	Main central vessels	Different relative importance
	Cardiac output	Different
	Cardiac myocyte maturation	Similar (compared to other species)
	Serum proteins (albumins and globulins)	Different in neonatal pigs and humans, but even at infant stages
Gastrointestinal	Physiology of digestion	Very similar
	Ontogeny of digestive enzymes	Similar in most cases (more than the rat)
	Neonatal gastric pH	Similar: gastric pH decreases with age and may vary due to food intake
	Gastric emptying	Maturation of gastric emptying with age has not been established in pigs. Prolonged emptying is expected in newborn pigs, as observed in humans
	Intestinal transit	Similar: intestinal myoelectrical activity is the same in human neonates and newborn pigs
	Intestinal surface	Similar: smaller than juvenile/adults, leads to similar nutrient absorption
	Microbiome	Similar: mainly consists of Firmicutes and Bacteriodetes phyla
	Liver	Similar relation to body weight in adults (about 2%)
		Slightly higher ratio in human (around 5%) than minipig (3%) neonates
Central nervous	Anatomical complexity	Similar
	Distribution of grey and white matter	Similar
	Brain growth pattern	Similar
Respiratory	Anatomy and histology	Similar
	Maturation	Faster
	Alveoli multiplication	Earlier in pigs

Renal	Nephrogenesis	Different: completes after weaning (3 weeks of age) in pig and 34-36 weeks gestational age in humans
	Glomerular filtration rate	Similar maturation: adult levels at 8 weeks (pig) and 3 to 5 months (human) of age
	Effective renal plasma flow	Within the same range in growing pigs and children
	Urinary pH	
Immune	Immune genes	High similarity (>80%)
Skeletal and neuromuscular	Development	Different: faster in pigs, in which locomotion patterns reach mature levels as early as 8 hours after birth

1.5 The pig as a nonclinical model

1.5.1 Swine in biomedical research

Over the last 30 years, pigs and minipigs are increasingly valued for their potential as translational animal models for human diseases and drug development. The major anatomical, physiological, and morphological similarities compared to humans render the pig a versatile animal model. This versatility is illustrated by their extensive use in several biomedical research domains. Depending on the research question, it should be considered that domestic pigs or minipigs are preferred (**see 1.5.2**). In the following paragraphs, "swine" is used collectively to describe both domestic pigs and minipigs.

Swine are an important animal model for **non-survival surgical training classes** for physicians [73]. *In silico* techniques cannot always adequately simulate surgical procedures, so it is common to use animal models for training purposes [73]. Since swine resemble closely to human anatomy and size, the opportunity arises to practice both general (e.g., interventional catheter techniques) and specialized (e.g., endoscopic and laparoscopic procedures) techniques [73]. In line with this, swine are a major resource for assessing **the biocompatibility and function of implanted devices** such as intravascular stents, prosthetic valves, ventricular assist devices, biliary stents, artificial bladders, ureteral stents, etc. [73]. Also, swine are valuable models to improve our understanding of disease processes in **several research areas**. For example, they have been particularly useful in studies involving the treatment of cardiovascular (e.g., atherosclerosis and myocardial infarction), digestive (e.g., liver transplantation, peritoneal dialysis, and cholecystectomy), urinary (e.g., renal hypertension, intrarenal reflux, and renal transplantation), and skin (e.g., wound healing, reconstructive techniques, burn models, and artificial skin grafts) conditions [73]. Interestingly,

long-term history of the swine in **pharmacological studies** exists, as reviewed by Ayuso et al. [72]. From the sixties onwards, swine are considered for PK assessment and toxicology studies [80-85]. Even neonatal PK studies were performed at the end of the seventies to assess the action of diuretics and their age-related effects, as such evaluating the piglet as a model for immature mammals [72, 86]. The developing awareness of the metabolic similarities between humans and swine led to a significant increase in the use of *in vivo*, *in vitro,* and *in silico* swine models to better comprehend drug metabolism and safety in the pediatric population [72, 73].

1.5.2 Does the breed matter?

Looking at the different breeds, a distinction can be made between domestic or conventional pigs and miniature pigs (i.e., mini- and micropigs). The conventional pig (e.g., Large White, Duroc, Landrace) has been domesticated for centuries, whereas minipigs are purpose-bred for research and are relatively new. The major difference between both is related to growth rate and size at sexual maturity rather than actual anatomic differences in organ structures [73]. Indeed, physiology will not be different between age-matched breeds, though organ sizes will reflect the increased size of domestic breeds compared to miniature breeds [73].

Depending on the research question and design, the animal's growth rate will be an important parameter. In the case of surgical training, the domestic pig's size will be perceived as advantageous to identify anatomical structures. In contrast, in nonclinical safety studies, their size will be a limiting factor due to the restricted availability of the test compound, which is often administered based on kg body weight^{-1}. The latter is the exact reason for the development of miniature strains: they are more manageable, easier to house in research facilities, are genetically coherent, and need less test compound when included in pharmacology, PK, and toxicologic safety evaluation studies [73]. Consequently, the interest in porcine models for drug development programs has increased rapidly since the introduction of miniature breeds [72, 73].

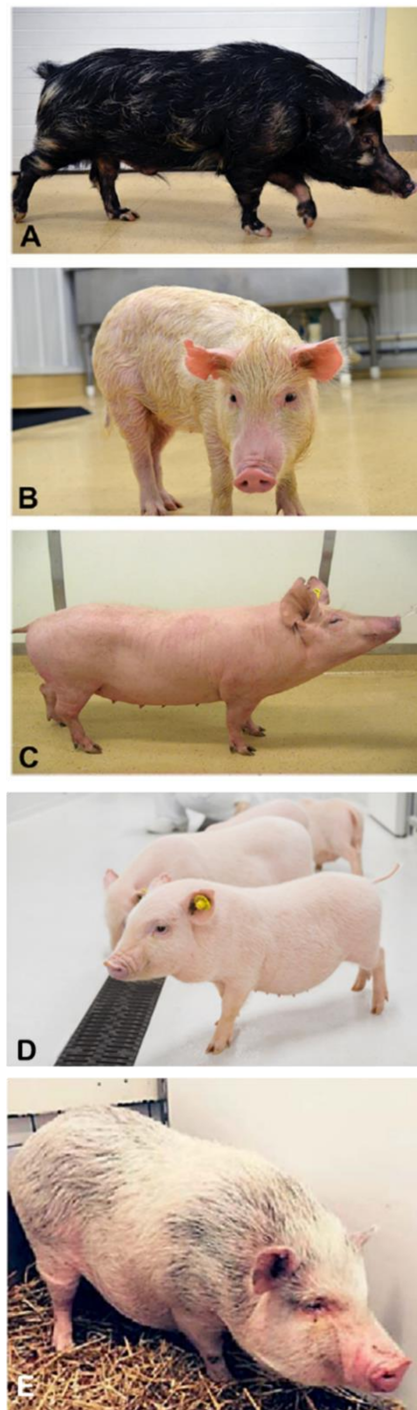

Figure 8: Photographs of the most frequently used miniature pig breeds in safety pharmacology and toxicology studies. A: Sinclair, B: Hanford, C: Yucatan, D: Göttingen E: Aachen. Adapted from [88, 89, 91].

Different mini- and micropig breeds are currently available (**Figure 8**). (Micro) Yucatan, Sinclair, and Hanford strains are mainly used in the US, whereas the Göttingen Minipig is the most popular strain in the EU [87]. However, the Aachen minipig also gained interest recently [88]. Even though all are considered miniature, large variations in basic characteristics are still present between breeds (**Table 4**). Especially regarding adult body weight, quite large ranges are observed, e.g., 30 – 45 kg for the Göttingen Minipig compared to 80 – 95 kg in the Hanford minipig [87]. Also, variations in coat and hair are prominent (**Figure 8, Table 4**). These differences should be considered when selecting the best model to meet trial requirements [89]. All breeds are currently used as translational nonclinical models in safety pharmacology and toxicology studies.

In this thesis, we will focus on (further) characterizing the Göttingen Minipig as a translational model for neonatal and juvenile hepatic drug metabolism since earlier research has shown promising results (**see 1.5.3**). However, the conventional piglet will also be included and investigated with a focus on prematurity, as these samples were available. As described above, no strong physiological differences are expected between both porcine models. Although a majority of ADME parameters shows the same developmental patterns (e.g., increasing body surface area [90, 91], lactase and sucrase ontogeny [92-95], gastric emptying time [96-98], ontogeny of phase I DMEs [99, 100] etc.), this is not the case for all (e.g., gastric pH [91, 101], maltase ontogeny [92, 93] and plasma protein concentrations [102-104]). Caution is thus warranted when aiming to compare or extrapolate between breeds.

Table 4: General characteristics of most used miniature pig breeds in safety pharmacology and toxicology studies.

Breed	Origin	Average birth weight (kg)	Average weight at sexual maturity (4 – 6 months) (kg)	Average adult weight (kg)	Coat	Research purposes	References
Göttingen	Cross breed: Minnesota Minipig x Vietnamese Potbelly pig x German Landrace	0.5 – 1	10 - 14	30 - 45	White	Safety pharmacology Systemic toxicology	[72, 73, 89, 104, 105]
Aachen	Cross breed: Vietnamese Potbelly pig x Schwäbisch Hällisch Landpig x German Landrace x Minnesota Minipig	0.4	14 - 17	45 - 50	White or dark grey and black spotted	Long-term and chronic safety studies	[88]
Sinclair	Directly derived from Hormel pigs	0.6	16 - 22	55 - 70	Black, red, white and roan	Safety pharmacology Systemic toxicology	[89, 105, 106]
Yucatan	Directly derived from 25 Yucatan pigs imported from Yucatan Peninsula in Southern Mexico	0.7	20 - 30	70 - 80	Light grey to black, hairless	Device studies Cardiovascular safety studies	[89, 105, 107, 108]
Hanford	Cross breed: Palouse x Pitman Moore x Louisiana Swamp	0.7	20 - 40	80 - 95	White	Dermal toxicology Surgical model Cardiovascular studies	[105, 109]

1.5.3 The pig as a (preterm) neonatal and juvenile animal model

The neonatal human population is extremely restricted regarding inclusion in clinical trials, and thus neonatal drug development is still considered an "orphan area" within PDD (**see 1.2.3**). Consequently, alternative models to improve our understanding of neonatal (patho)physiology are largely needed. Within this regard, the neonatal pig has gained a lot of interest during the last decades. Compared to dogs and NHP, pigs have a larger size at birth and are more like human neonates. As such, sampling at early stages is facilitated: human NICU equipment can be adapted for use in pigs, increasing their translational value [72]. Not only size matters, but also in-depth knowledge of comparative anatomy, physiology, and ADME characteristics is required to assess the feasibility of neonatal and juvenile pigs as models for PDD [72]. In the ICH S11 guidelines, many similarities at the level of organ development in pediatric age groups have been reported between humans and Göttingen Minipigs (i.e., the reference breed for the pharmaceutical industry) [68]. These similarities are also represented in **Figure 9**. In general, developmental patterns of the gastrointestinal tract (GIT), the cardiovascular, the central nervous systems, and the eye are similar between both species, while renal, immune, and reproductive development occur slightly earlier and more rapidly in humans than in pigs [72].

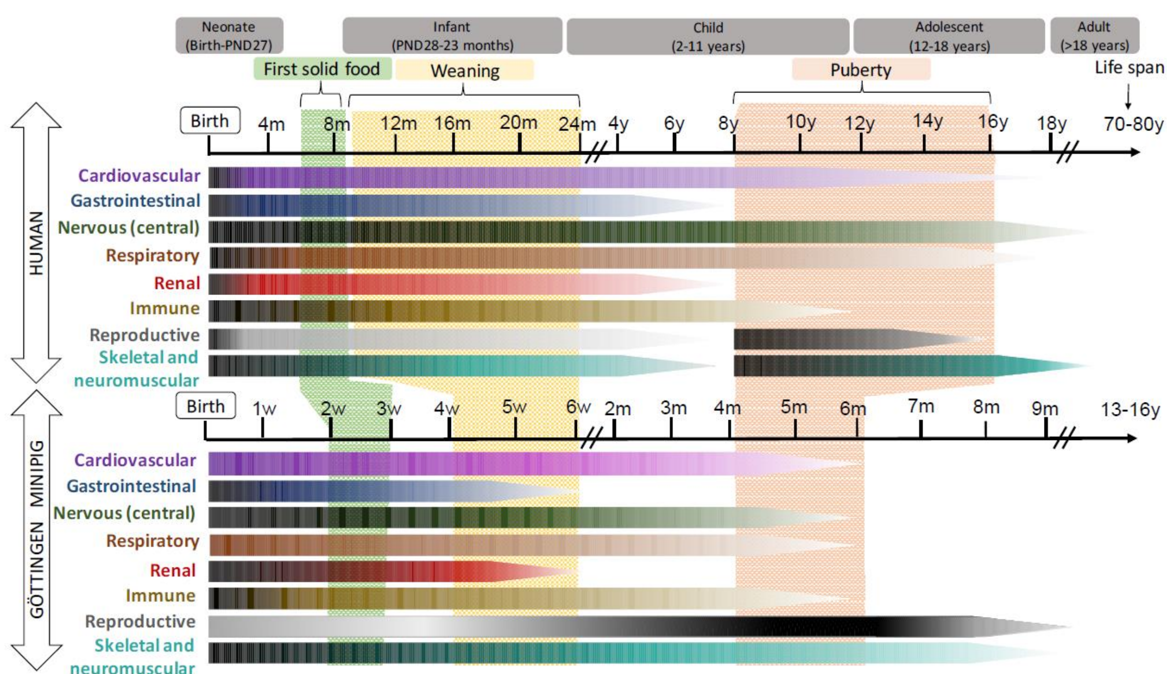

Figure 9: Schematic representation of the postnatal development of different organ systems in humans (top) and Göttingen Minipig (bottom). In the horizontal bars, the intensity of maturation processes is represented by dark (more intense) and light (less intense) tones. The time bar represents weeks (w), months (m), or years (y) of life. Reproduced from [72].

Regarding ADME, special attention goes to the function of the liver as this is the major organ for drug metabolism. In adult domestic pig [110-119] and minipig strains [118, 120-127], extensive research has been performed on phase I CYP metabolism. The observed comparability of hepatic CYP enzymes between pigs and humans supports the usefulness of the pig as a translational biotransformation model. However, the **ontogeny of CYP enzymes in the neonatal and juvenile populations is less understood**. Recently, CYP enzyme activity was assessed in domestic pigs [99], and Göttingen Minipigs [100], but data remains scarce. In view of phase II metabolism, the availability of **UGT ontogeny data is even more limited**. Few studies in conventional pig breeds and Göttingen Minipigs recently concluded that UGT enzymes are expressed from an early age with varying increases in postnatal enzyme activity [100, 128, 129]. Further characterization is thus needed to better predict drug disposition in neonatal and juvenile pigs.

In view of the preterm-born human population, the preterm pig model deserves special attention. Under the umbrella of the International Life Sciences Institute – Health and Environmental Sciences Institute (ILSI-HESI), a network on neonatal animals was created [71]. Within this collaboration, the Large White landrace pig has been used as a model of the preterm human neonate, showing that conditions in preterm human neonates are also present in preterm cesarian-section-delivered pigs at ~ 90 – 95% gestation [130]. Indeed, in this field, conventional pig breeds are the preferred choice instead of purpose-bred minipig breeds. Conventional preterm piglets are similar to human preterm neonates as they share the same pathophysiological conditions, such as respiratory failure and intestinal tract conditions. The preterm piglet is, for instance, an established translational model for NEC and short bowel syndrome (SBS) [131, 132]. Regarding ADME, preterm piglets may be a promising model. In human preterm neonates, **differences in GA, PNA, and PMA further complicate the prediction of ADME**. Little is currently known about the impact of pre- and postnatal age on DME ontogeny in this cohort resulting in an additional increased risk for adverse drug effects. Due to the similarities already shown in drug metabolism between pigs and humans, the preterm pig model may provide crucial novel information.

1.6 PBPK modeling

FDA and EMA strongly encourage the use of modeling and simulation (M&S) approaches during drug development [10, 133, 134]. These methods aim to integrate data from different domains and as such help decision making during early drug development, improve study design, reduce costs, save time and finally enhance success rates [135]. Moreover, a reduction of laboratory animal use might result on the long term.

Physiologically based pharmacokinetic (PBPK) modeling is an example of such M&S approaches. A PBPK model is based upon the integration of anatomical, physiological and biochemical data resulting from various sources such as *in silico*, *in vitro* and *in vivo* experiments (**Figure 10**) [136, 137]. A true mechanistic approach is thus provided in comparison to allometric scaling (i.e., extrapolation of drug doses solely based on body weight, not taking into account developmental differences). This mathematical concept of physiology enables **the prediction of *in vivo* metabolic**

drug clearance (and even full concentration time profiles) and as such helps **dose setting in clinical trials**. This is particularly useful for very young children (< 2 years of age) who are extremely vulnerable due to their non-linear and complex developmental changes as described earlier (**see 1.2.3**) [136]**.** Consequently, unpredictable responses to doses that seem safe and efficacious in adults, can arise in this youngest age group [36, 138]. For this reason, modeling can be very useful, but often safety data from juvenile animal (PBPK) studies are still required prior to starting clinical trials in (very) young children.

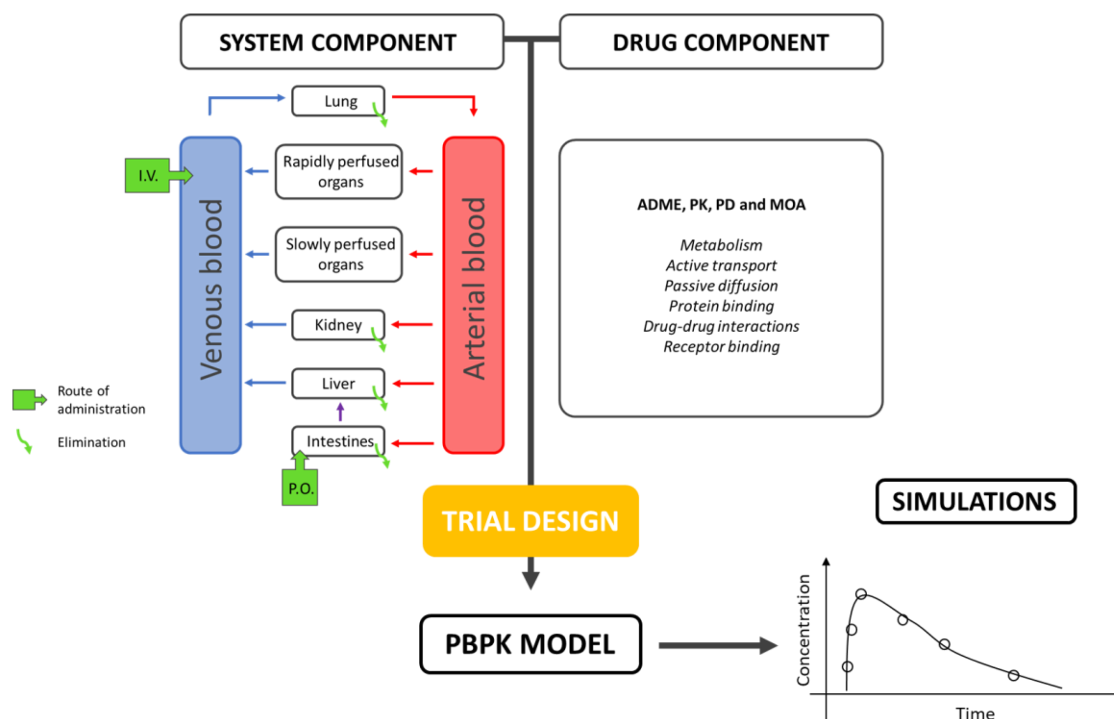

Figure 10: Principle of physiologically based pharmacokinetic (PBPK) modeling. Several parameters, i.e., the system component, drug component and trial design are considered as input to build this mathematical PBPK model. Next, simulations are performed to predict the pharmacokinetic properties of the drug of interest in the population of interest. Abbreviations: ADME, absorption – distribution – metabolism – excretion; I.V., intravenous; MOA, mode of action; PD, pharmacodynamics; PK, pharmacokinetics; P.O., per oral.

As such, **animal data** (i.e., mouse, rat, dog, and monkey) are currently also **included** in commercially available **PBPK platforms** such as Simcyp® , PK-Sim® and Gastroplus® in order to aid in selection of the most suitable species for nonclinical studies. Although **neonatal and juvenile (mini)pigs** are gaining interest for pediatric programs due to their similarities with man [74, 91, 100], this model is **not yet included** in the commercially available PBPK platforms. This is caused by a lack of historical background data. Further characterization of, *inter alia*, ADME properties is thus firstly needed in order to provide sufficient input data for the construction of porcine adult, juvenile and neonatal PBPK models. As a result, a better and more scientifically based species selection could be performed by pharmaceutical companies for drug screening and for the nonclinical phases of drug development.

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CHAPTER 2:
Thesis objectives and outline

Based on the knowledge gaps mentioned earlier in **Chapter 1**, the objectives of this thesis are:

I. To study hepatic CYP protein abundance ontogeny profiles in the developing Göttingen Minipig.
II. To investigate the correlation between hepatic CYP enzyme activity and protein abundance in the developing Göttingen Minipig.
III. To study the ontogeny of hepatic CYP3A enzyme activity in preterm and term-born domestic piglets.
IV. To study the ontogeny of hepatic UGT enzyme activity in preterm and term-born domestic piglets.
V. To investigate the effect of postconceptional and postnatal age on hepatic CYP3A and UGT enzyme activity in preterm and term-born domestic piglets.

The first part focuses on characterizing drug metabolism in the developing Göttingen Minipig. Hepatic CYP protein abundance in fetal, neonatal, juvenile, and adult Göttingen Minipigs is investigated by using LC-MS/MS in **Chapter 3**.

The second part addresses the value of the preterm-born domestic piglet as a translational animal model for human (pre)term newborns. The ontogeny of hepatic phase I (CYP3A) and phase II (UGT) enzymes and the effect of postconceptional and postnatal age on enzyme activity in preterm and term-born domestic piglets are discussed in **Chapter 4**.

A broader perspective of the results and future research opportunities are described in the General Discussion (**Chapter 5**).

Note: During the doctoral period, extensive effort was also put into the development of recombinant Göttingen Minipig CYP3A enzymes. The construction of individual isoforms allows investigation of isoform-specific characteristics (e.g., substrate-specificity) which contributes to an improved comprehension of their functionalities. As a result, comparison with human CYP isoforms is facilitated and their translational value is better understood. As this recombinant work is under IP with further valorization opportunities, it was decided to not include these data as part of the PhD thesis nor the defense.

CHAPTER 3:

Hepatic cytochrome P450 abundance and activity in the developing and adult Göttingen Minipig: pivotal data for PBPK modeling

Adapted from:
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3.1 Abstract

The Göttingen Minipig is gaining ground as nonrodent species in safety testing of drugs for pediatric indications. Due to developmental changes in pharmacokinetics (PK) and pharmacodynamics (PD), physiologically based pharmacokinetic (PBPK) models are built to better predict drug exposure in children and to aid species selection for nonclinical safety studies. These PBPK models require high quality physiological and ADME data such as protein abundance of drug metabolizing enzymes. These data are available for man and rat, but scarce for the Göttingen Minipig. The aim of this study was to assess hepatic cytochrome P450 (CYP) protein abundance in the developing Göttingen Minipig by using mass spectrometry. In addition, sex-related differences in CYP protein abundance and correlation of CYP enzyme activity with CYP protein abundance were assessed. The following age groups were included: gestational day (GD) 84 - 86 (*n* = 8), GD 108 (*n* = 6), postnatal day (PND) 1 (*n* = 8), PND 3 (*n* = 8), PND 7 (*n* = 8), PND 28 (*n* = 8) and adult (*n* = 8). Liver microsomes were extracted and protein abundance was compared to that in adult animals. Next, the CYP protein abundance was correlated to CYP enzyme activity in the same biological samples. In general, CYP protein abundance gradually increased during development. However, we observed a stable protein expression over time for CYP4A24 and CYP20A1, and for CYP51A1, a high protein expression during the fetal stages was followed by a decrease during the first month of life and an increase towards adulthood. Sex-related differences were observed for CYP4V2_2a and CYP20A1 at PND 1 with highest expression in females for both isoforms. In the adult samples, sex-related differences were detected for CYP1A1, CYP1A2, CYP2A19, CYP2E1_2, CYP3A22, CYP4V2_2a and CYP4V2_2b with higher values in female compared to male Göttingen Minipigs. The correlation analysis between CYP protein abundance and CYP enzyme activity showed that CYP3A22 protein abundance correlated clearly with the metabolism of midazolam at PND 7. These data are remarkably comparable to human data and provide a valuable step forward in the construction of a neonatal and juvenile Göttingen Minipig PBPK model.

3.2 Introduction

In recent years, the Göttingen Minipig has gained attention in view of pediatric drug development [1]. Especially for the youngest age groups (i.e. from birth up to 2 years), the Göttingen Minipig may be a better translational model than rodents due to their comparable body size and organ development but also for their similar drug metabolism [1-3]. In order to compare the juvenile Göttingen Minipig with the human pediatric population, efforts are ongoing to further characterize this animal model and build a physiologically based pharmacokinetic (PBPK) model. PBPK modeling requires three different sources of information namely (i) systemic data based upon the population of interest (e.g. organ weight, blood flow rate, amount of microsomal protein per gram of liver (MPPGL), glomerular filtration rate, ontogeny of drug metabolizing enzymes and transporters (DMET)), (ii) drug data (e.g. physicochemical parameters, drug solubility, tissue partitioning, plasma protein binding, drug-drug interactions), and (iii) trial design parameters (e.g. dose and dose regimen, route of administration, population size and demographics) [4, 5]. The integration of these various parameters results in a mathematical model that provides a bottom-up approach to predict drug exposure [5]. To validate the PBPK model, *in vivo* data are compared to the generated simulations and this leads to a feedback loop that allows for constant refinement of the model [6,

7]. This mechanistic approach has received increased attention, especially for the pediatric population, as it may achieve more accurate dose predictions compared to the traditional methods (e.g. allometric scaling) and consequently results in a better dose setting in this population [8]. Age-dependent developmental changes in physiology parameters (e.g. organ size and maturation, plasma protein binding and ontogeny of DMETs) can be specified in the pediatric PBPK model and as such considers important factors that can cause differences in exposure between adults and children of different ages [9]. Due to the nonlinear changes of these developmental changes, the allometric scaling approach (which estimates the maturation of physiological processes solely based upon body size [10]) is a rather inappropriate method to scale drug doses from the adult to the youngest pediatric population [10]. This can be outweighed by PBPK models.

With regard to the Göttingen Minipig, a preliminary PBPK model for the adult population has already been made [11, 12] and the first steps have been taken to create a model for the juvenile population [13]. Morphometric organ data and activity data of a limited group of hepatic phase I (cytochrome P450 (CYP) 3A enzymes) and phase II (UGT enzymes) DMEs are already available in juvenile groups [2, 13]. However, data on the biotransformation capacity and other ADME properties in the neonatal and juvenile Göttingen Minipig remain scarce and hamper the development of a reliable model [2]. Thus, further characterization is essential to expand our knowledge in view of the construction of a neonatal and juvenile Göttingen Minipig PBPK model.

This paper focuses on phase I drug metabolism and more specifically on CYP protein abundance in the liver of the Göttingen Minipig. CYP enzymes are one of the most important Phase I drug metabolizing enzymes, as they are responsible for the biotransformation of 70-80% of drugs in clinical use [14]. Oxidation, reduction and hydrolysis reactions of substrates will form more hydrophilic metabolites and this facilitates the biliary and renal excretion or further metabolization through phase II enzymes [15]. High homology to the human phase I drug metabolizing CYP family is described in adult minipigs (63 – 84% amino acid identity)[16-20] and the ontogeny of CYP enzyme activity in the juvenile Göttingen Minipig showed to be comparable to the corresponding age groups in human [2]. However, CYP protein abundance still has to be examined. Determination of the protein abundance levels is critical for the refinement of the neonatal and juvenile Göttingen Minipig PBPK model. In the past, mRNA levels were used as a surrogate for the protein levels of DMETs and showed to not always correlate well [7, 21, 22]. Hence, proper protein quantitation is necessary. Within this regard, the development of liquid chromatography-mass spectrometry (LC-MS) based quantitative proteomics has increased the expectations for a solid progression in the field as it is considered to be more precise and reliable than other protein quantitation techniques (e.g., Western blot, ELISA) [21]. In our research, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [23] was used to detect peptides unique to the various CYP isoforms. Based on the retrieved signal intensities of these peptides, protein abundance was determined.

The main goal of this study was to assess the ontogeny of hepatic CYP protein abundance in the Göttingen Minipig with LC-MS/MS. The CYP protein abundance was measured in liver microsomes

of both male and female minipigs, with age groups ranging from the late fetal stage to postnatal day (PND) 28. Adults were included for reference. Second, sex-related differences affecting CYP protein abundance were investigated. Third, the ontogeny profiles of CYP enzyme activity and CYP protein abundance were compared to examine the correlation between both parameters.

3.3 Material and Methods

3.3.1 Reagents

Sodium pentobarbital 20% (Kela NV, Hoogstraten, Belgium) was used for anesthesia of the animals. A 0.5M potassium phosphate (K_3PO_4) buffer was obtained from Corning Incorporated (NY, USA). Halt™ Protease Inhibitor Single-Use Cocktail (78430) and Pierce® BCA Protein Assay kit with bovine serum albumin (23225) were purchased from Thermo Fisher Scientific (MA, USA). TEABC, DTT, MMTS, $CaCl_2$ and DMSO were obtained from Sigma Aldrich (St. Louis, MO, USA). UPLC-water and ACN were purchased from Biosolve (Valkenswaard, Netherlands). Trypsin was obtained from Promega (Madison, WI, USA). Hi3 *E. coli* was purchased from Waters (Zellik, Belgium). Beta-galactosidase was obtained from Sciex (Framingham, MA, USA).

3.3.2 Animals and tissue

The protocol, use of animals and research in this study was approved by the Ethical Committee of Animal Experimentation from the University of Antwerp (Belgium) (ECD 2012-30) and adhered to the 'Principles of Laboratory Animal Care' (NIH publication Nr 85-23, revised in 1985). The animals and resulting liver microsomes that were collected and assayed by Van Peer et al. were used in this study [2]. Euthanasia of the animals, sampling of the liver and isolation of the liver microsomes were conducted and described before [2]. Ten pregnant sows were a kind gift from Ellegaard Göttingen Minipig A/S (Dalmose, Denmark). Janssen Research (Beerse, Belgium) kindly provided liver samples from four adult male Göttingen Minipigs. The following age groups were investigated: gestational day (GD) 84 - 86 (n = 8), GD 108 (n = 6), PND 1 (within 24 hours after birth) (n = 8), PND 3 (n = 8), PND 7 (n = 8), PND 28 (n = 8) and adult (n = 8). Both genders were equally represented in each age group except for PND 28 (3 males and 5 females). The fetal age groups (GD 84-86 and GD 108) refer to 75 and 95% of gestation, respectively, as normal gestation length in the minipig is 112 to 115 days. The evaluation of the fetal age groups is therefore restricted to the third trimester of fetal development. PND 28 is the age at which piglets are usually weaned in a preclinical setting. The first month of life in the Göttingen Minipig is considered to depict the first two years of life in children [24], when important changes in human CYP activity and expression occur. The ontogeny pattern of CYP protein abundance was investigated in all age groups. For the adult group, the female samples became available in a later phase. Consequently, sex-related differences for the developing age groups were determined during a first experiment using male adult samples as reference and the sex-related differences in the adult age group were determined in a second experiment when adult female samples were available. The age of adult male and female animals ranged between 18-24 months and 14-33 months, respectively. Total protein concentration of the

liver microsomes was determined by the Pierce® BCA Protein Assay Kit with bovine serum albumin as a standard.

3.3.3 CYP protein abundance: LC-MS/MS approach

A high definition – data dependent acquisition (HD-DDA) mass spectrometry (MS) set-up was used to determine protein abundance in the liver microsomes. This method consists of a full scan MS followed directly by a MS^2 analysis of the precursor ions with the highest signal intensity. These resulting MS/MS spectra were annotated in order to find unique peptides. Two different experiments were conducted. First, the CYP protein abundance maturation pattern over time was determined. Liver microsomes of all different age groups were included. Sex-related differences were investigated in all age groups except for the adult animals. In the latter group, only male individuals were included since female samples were only available in a later phase. Second, CYP protein abundance and sex-related differences were investigated in adult male and female Göttingen Minipig liver microsomes. These experiments were performed as described by Millecam et al. [23]. In brief, microsomal proteins (20 µg) of each individual pig were reduced, alkylated, and digested using trypsin prior to MS-analysis. Peptides were resuspended in 0.1% formic acid. Four hundred nanograms sample was spiked with 50 fmol beta-galactosidase and 50 fmol Hi3 *E. coli* standards before injection. The peptides were separated using a nanoscale UPLC system (nanoACQUITY UPLC, Waters, Milford, MA, United States) coupled to a Synapt G2-Si mass spectrometer (Waters). The Q-TOF Synapt G2-Si instrument was set-up for HD-DDA analysis, acquiring full scan MS and MS/MS spectra (m/z at 50–5,000) in resolution mode. Data analysis of the raw files obtained from the Synapt G2-Si was performed in Progenesis QI (Nonlinear Dynamics) version 2.3 (Waters). Peptides with charge C1 were discarded. For relative quantitation, data was normalized to all proteins. For absolute quantitation, data was normalized to Hi3 *E. coli* peptides. Peptide identification was performed with Mascot 2.5 by searching a compiled database of reviewed *Sus scrofa* entries (SwissProt), supplemented with unreviewed CYP proteins and fragments of interest, the cRAP database (laboratory proteins and dust/contact proteins1) and sequences of spiked standard proteins. For relative quantitation, the top three peptides were used and only proteins with at least one unique peptide were further considered. For absolute quantitation, proteins were quantified using the top three unique peptides against Hi3 *E. coli* peptides, and only proteins with at least one unique peptide were further considered. Protein data was exported from ProgenesisQI for further statistical analysis. All unique peptides were validated in Mascot and detection of the proteins was performed in Uniprot and NCBI. A distinction was made between the experimental evidence for the existence of the enzyme at the protein or transcript level. The difference is indicated by an underscore followed by number 1 or number 2, respectively. Evidence at the protein level means that a clear identification by mass spectrometry is available whereas evidence at the transcript level means that cDNA, RT-PCR or Northern blot data are present, but existence is not proven.

3.3.4 CYP protein abundance vs CYP enzyme activity

In this study, liver microsomes were used for which CYP enzyme activity was determined and reported before [2]. In this preceding study, liver microsomes were incubated with a cocktail of CYP substrates including phenacetin, tolbutamide, dextromethorphan and midazolam. These compounds are probe substrates for human CYP1A2, CYP2C9, CYP2D6 and CYP3A4, respectively. Thus, CYP enzyme activity maturation over time was investigated in the same biological samples used in the current work. We therefore performed a correlation study between the hepatic CYP enzyme activity and CYP protein abundance data originating from the same individuals.

3.3.5 Statistical analyses

Normality and homogeneity of variances were tested by the Shapiro-Wilk and Levene's test, respectively. A log-transformation was performed to meet the assumptions for parametric testing, if necessary. One-way ANOVA was used to examine age-related differences in CYP protein abundance. Tukey's honest significance difference *post hoc* test was used to identify differences between groups. A p-value smaller than 0.05 was considered statistically significant. The Student's t-test was used to detect sex-related differences within each age group and for each CYP isoform. If assumptions could not be met, a non-parametric Mann-Whitney U test was performed. The Bonferroni correction adjusted the threshold p-value to 0.008 for sex-related differences. A parametric Pearson correlation test was used to identify a correlation between CYP protein abundance from this study and the results from our preceding study that assessed CYP enzyme activity [2] in all age groups together and in each age group separately. A non-parametric Spearman rank correlation test was performed if assumptions for parametric testing could not be met. The Bonferroni correction adjusted the threshold p-value to 0.00012 for the multiple correlation analyses. Statistical analyses were performed in JMP® Pro 14 (SAS Institute Inc., North Carolina, USA). Graphs were made in JMP® Pro 14 (SAS Institute Inc., North Carolina, USA) and Microsoft Excel® 2016 (Microsoft Corporation, Redmond, WA, USA).

3.4 Results

3.4.1 General aspects

In the first experiment, CYP protein abundance was determined in the developing Göttingen Minipig and compared with adult values (**Figure 1**). A total of 291 proteins were identified in the liver microsomes. Twenty-one CYP enzymes were detected from which 18 had at least one unique peptide. In the second experiment, CYP protein abundance was examined in adult male and female Göttingen Minipig samples (**Figure 2**). A total of 301 proteins were identified from which 38 belonged to the CYP family. Twenty-two out of 38 CYP enzymes had at least one unique peptide. Fifteen CYP enzymes were detected in both experiments (CYP1A2, CYP2A19, CYP2C33, CYP2C33v3, CYP2C34, CYP2C36, CYP2D6, CYP2D25, CYP2E1_1, CYP3A22, CYP3A46, CYP4A21, CYP4V2_2a, CYP27A1 and CYP51A1). The additional proteins that were found in the first run were CYP3A29, CYP4A24 and CYP20A1; in the second experiment CYP1A1, CYP2B22, CYP2C32, CYP2C42, CYP2C49, CYP2E1_2 and CYP4V2_2b were retrieved.

The most abundant CYP subfamilies that were detected over the course of time in the first experiment were CYP2C, CYP2D, CYP2E and CYP3A (**Supplementary table S1**). All these subfamilies could already be detected at the late fetal stages (**Figure 1A-B**) and showed high values in the different postnatal age groups (**Figure 1C-G**). In the adult animals of the second experiment, CYP2D25 was the most prominent isoform with 28.8% in males and 28.0% in females (**Figure 2**, **Supplementary table S2**). Next to this isoform, CYP2C33 (13.0%) was the following most abundant isoform in males and CYP2A19 (32.9%) was the most abundant in females. CYP2A19 only represented 3.5% of total CYP protein abundance in males, CYP2C33 represented 6.4% of total CYP protein abundance in females.

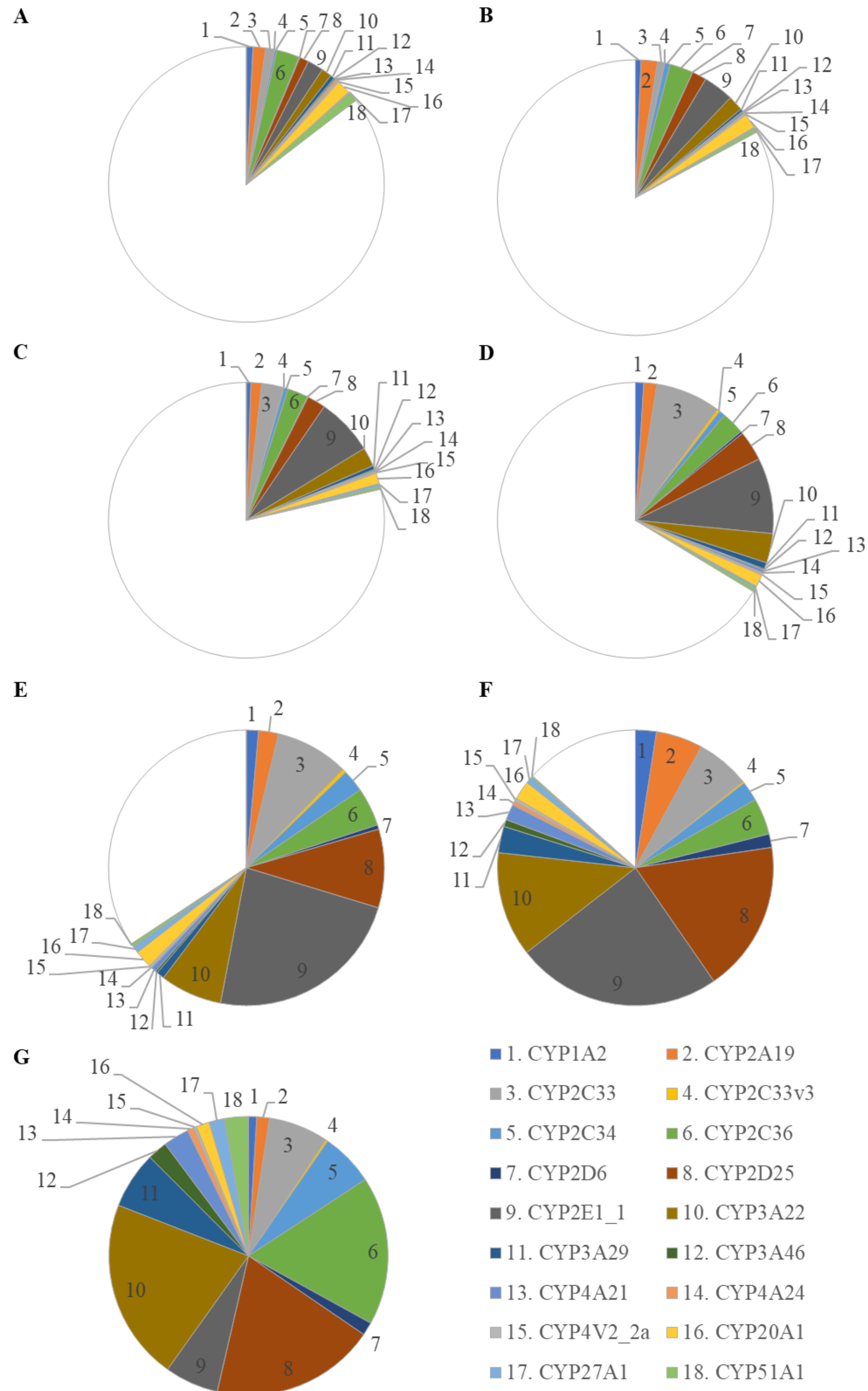

Figure 1: Ontogeny of CYP protein abundance in the developing and adult male Göttingen Minipig. The protein abundance in the male adult samples is considered as the reference and is depicted as a full pie of 100%. The different age groups are represented by GD 84 – 86 (A), GD 108 (B), PND 1 (C), PND 3 (D), PND 7 (E), PND 28 (F), and adult male (G).

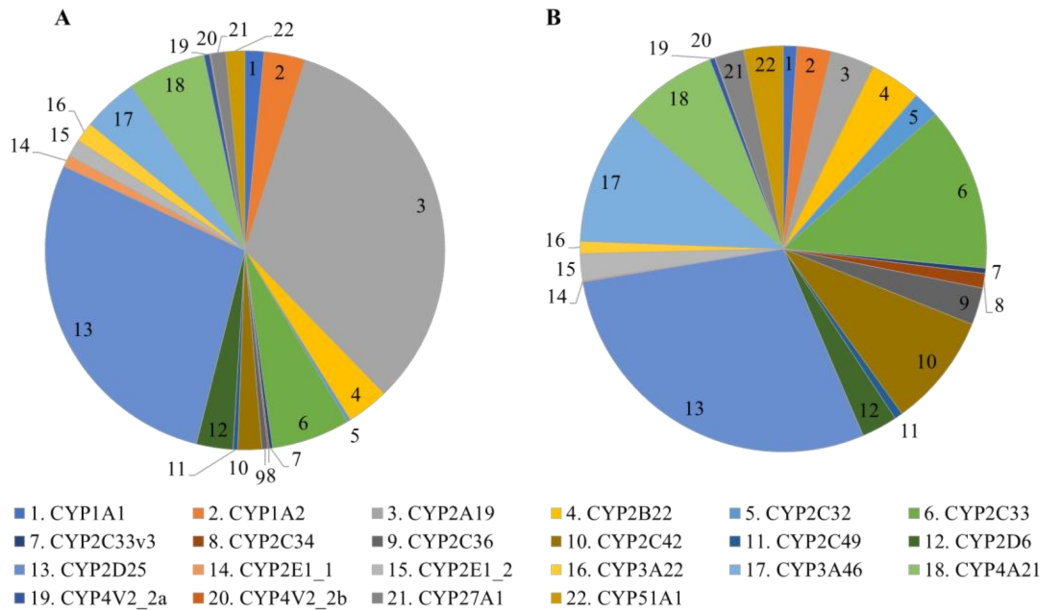

Figure 2: CYP protein abundance profile in adult female (A) and male (B) Göttingen Minipigs.

3.4.2 CYP protein abundance ontogeny profiles

The ontogeny profiles of the individual CYPs showed four different patterns (**Figure 3**). A gradual increase was observed for CYP2C33, CYP2C33v3, CYP2C34, CYP2C36, CYP3A22, CYP3A29, CYP3A46, CYP4A21, CYP4V2_2a and CYP27A1 **(Figure 3A**). The highest level of protein abundance for these isoforms was observed at the adult age. CYP1A2, CYP2A19, CYP2D6, CYP2D25 and CYP2E1_1 reached their maximum protein abundance already at PND 28 (**Figure 3B**). The relative protein abundance of CYP2D6 and CYP2D25 remained unchanged between PND 28 and adulthood, whereas the other isoforms' abundance dropped. CYP4A24 and CYP20A1 presented a stable protein abundance with no statistically significant differences between the different age groups (**Figure 3C**). CYP51A1 presented an atypical profile, with high protein abundance in the fetal stages, a drop after birth reaching high abundance again in the adult animals (**Figure 3D**).

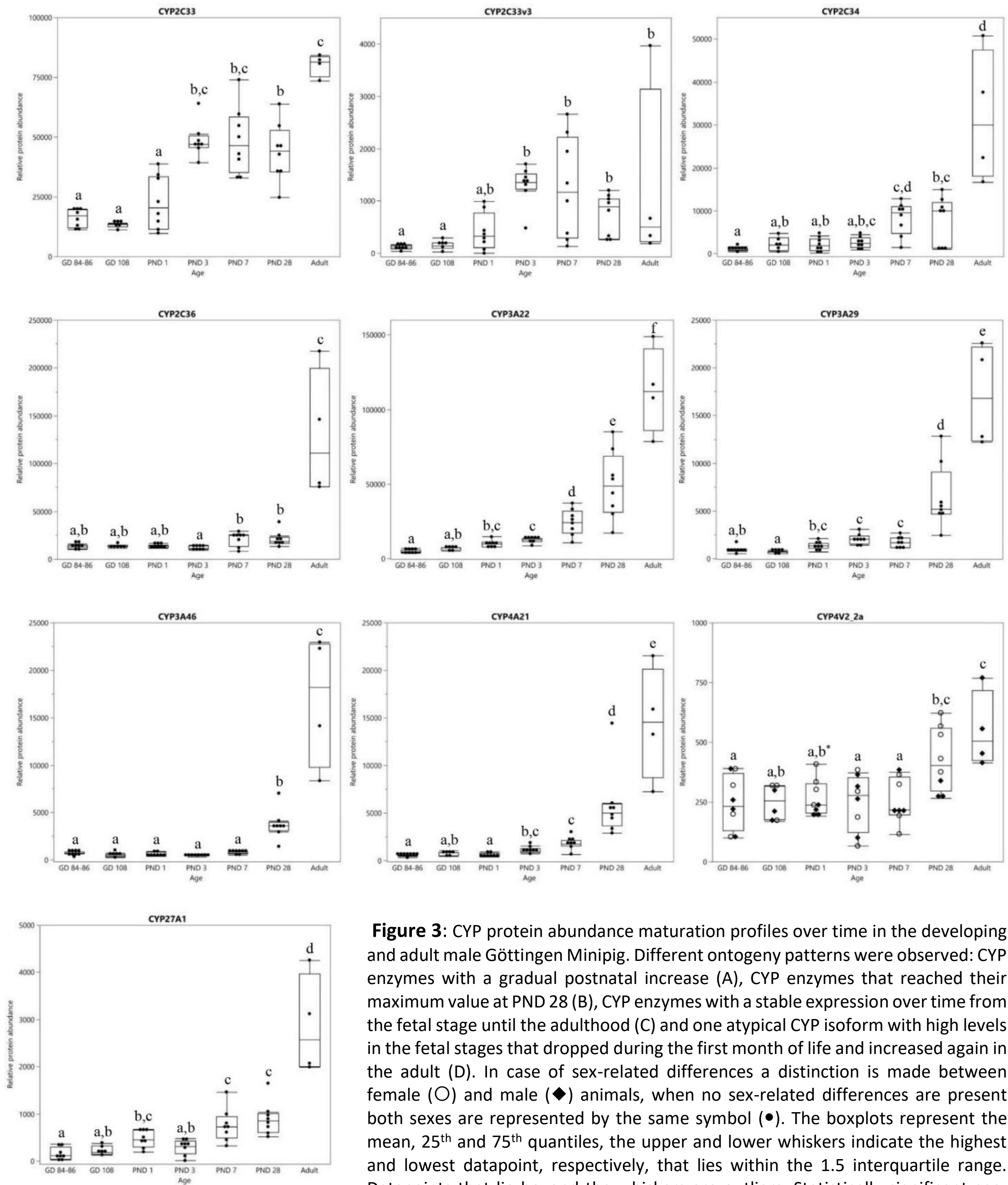

Figure 3: CYP protein abundance maturation profiles over time in the developing and adult male Göttingen Minipig. Different ontogeny patterns were observed: CYP enzymes with a gradual postnatal increase (A), CYP enzymes that reached their maximum value at PND 28 (B), CYP enzymes with a stable expression over time from the fetal stage until the adulthood (C) and one atypical CYP isoform with high levels in the fetal stages that dropped during the first month of life and increased again in the adult (D). In case of sex-related differences a distinction is made between female (○) and male (◆) animals, when no sex-related differences are present both sexes are represented by the same symbol (•). The boxplots represent the mean, 25th and 75th quantiles, the upper and lower whiskers indicate the highest and lowest datapoint, respectively, that lies within the 1.5 interquartile range. Datapoints that lie beyond the whiskers are outliers. Statistically significant age-related differences are indicated by characters ($p < 0.05$), statistically significant sex-related differences are indicated by * ($p < 0.05$).

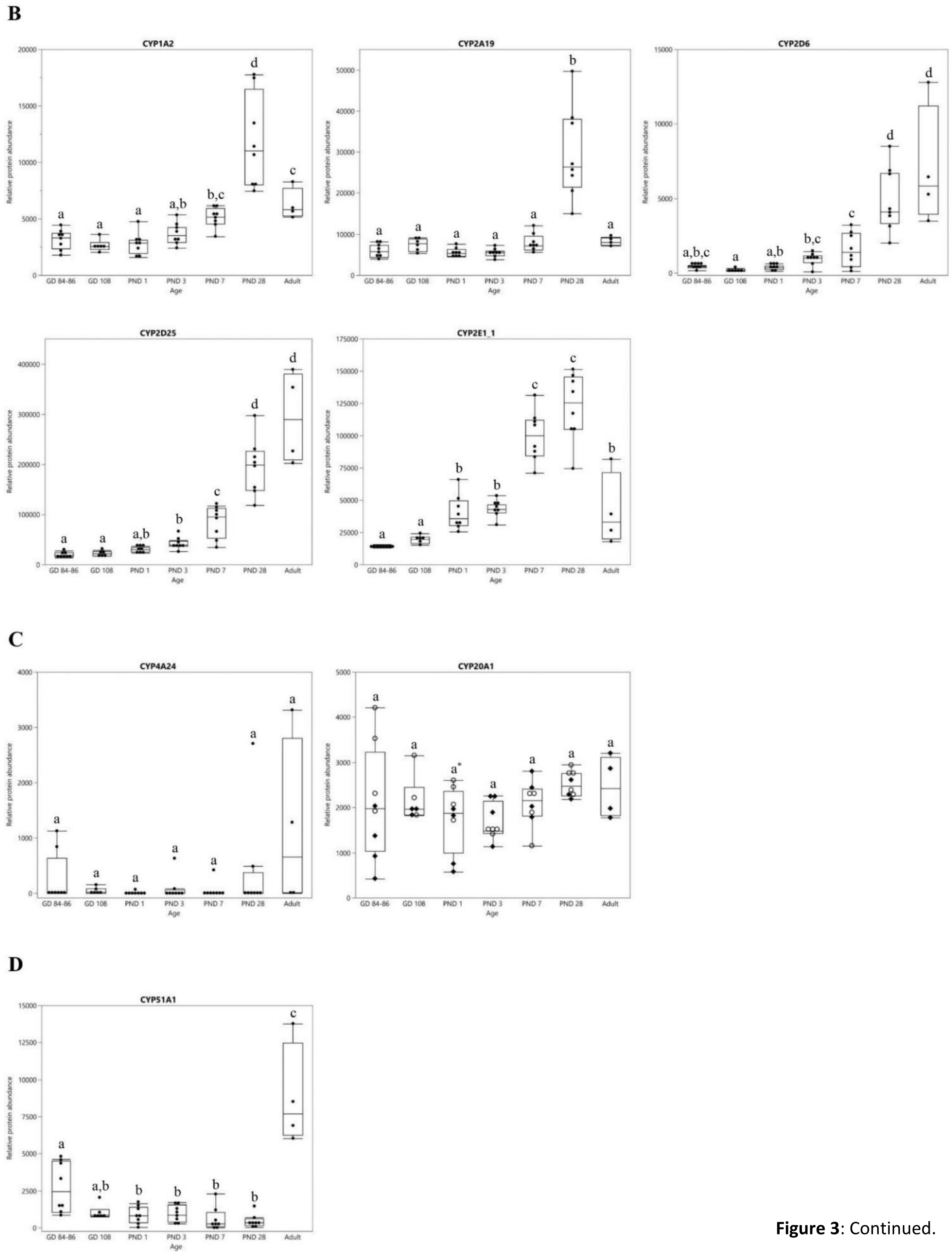

Figure 3: Continued.

3.4.3 Sex-related differences in CYP protein abundance

In the first experiment, only CYP4V2_2a (**Figure 3A**) and CYP20A1 (**Figure 3C**) showed statistically significant sex differences at PND 1. CYP protein abundance was highest in female compared to male animals for both isoforms. In the adult age groups of the second experiment, statistically significant sex-related differences were observed for CYP1A1, CYP1A2, CYP2A19, CYP2E1_2, CYP3A22, CYP4V2_2a and CYP4V2_2b with highest values observed in female animals for all isoforms (**Figure 4**).

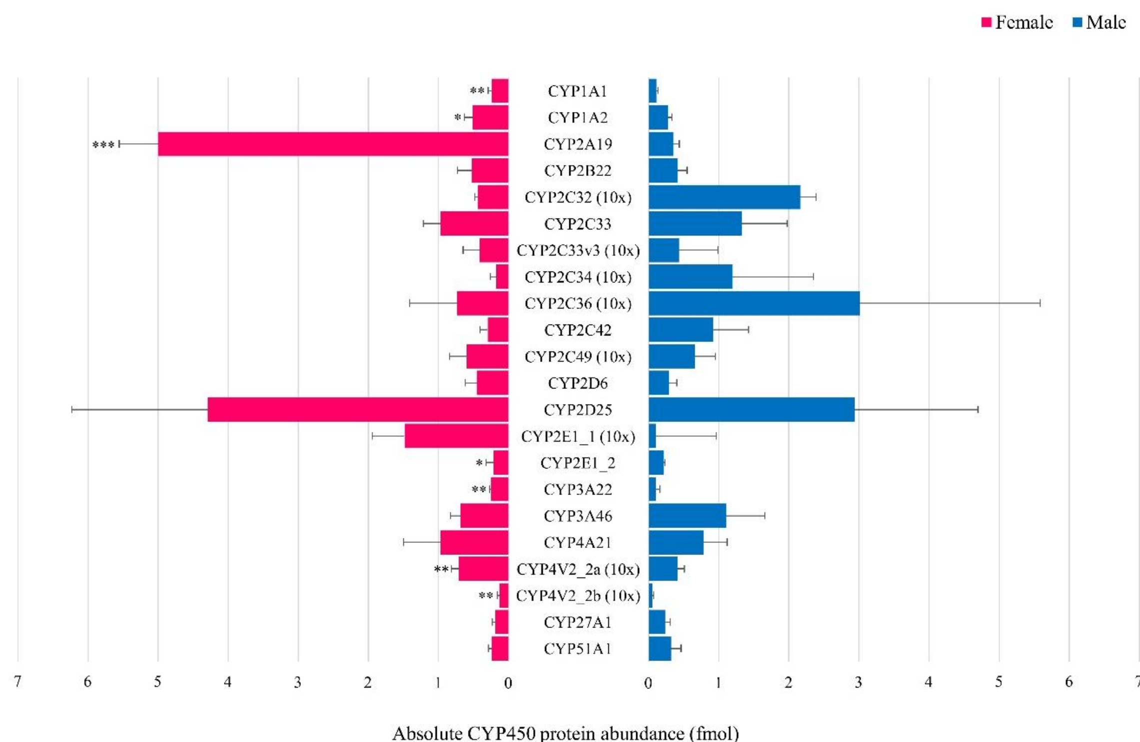

Figure 4: Comparison of the absolute CYP protein abundance in adult female (pink) and male (blue) Göttingen Minipigs. Bars represent mean ± S.D. Statistically significant sex-related differences were observed for CYP1A1, CYP1A2, CYP2A19, CYP2E1_2, CYP3A22, CYP4V2_2a and CYP4V2_2b, highest values were observed in the female Göttingen Minipig for these isoforms. For visualization purposes, the CYP2C33, CYP2C33v3, CYP2C34, CYP2C36, CYP2C49, CYP2E1_1, CYP4V2_2a and CYP4V2_2b absolute protein abundance values were magnified 10 times. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

3.4.4 CYP protein abundance vs CYP enzyme activity

A Pearson correlation analysis was performed for all age groups together per CYP. Both CYP enzyme activity (determined earlier by the metabolism of phenacetin, midazolam, tolbutamide and dextromethorphan [2]) and protein abundance showed a similar pattern of postnatal increase for most CYP enzymes, resulting in high correlations between both parameters (**Supplementary table S3**). No statistically significant correlation was observed for CYP4A24 and CYP51A1.

Second, Pearson correlation analyses were performed for each CYP per age group (**Supplementary table S4**). Only CYP3A22 showed a statistically significant correlation with midazolam metabolism at PND 7 ($p<0.00012$, $r = 0.9633$). For the youngest age group, GD 84 -86, the sample size was too small to include this group in the individual analyses per CYP, as only for 3 samples enzyme activity and protein abundance were assessed.

3.5 Discussion

This study aimed to investigate the hepatic CYP protein abundance in the developing and adult Göttingen Minipig. Since the most important changes in ADME processes occur during the first two years of life in human [25], corresponding age groups in Göttingen Minipig were investigated and compared with adult animals. This is the first study assessing changes in protein abundance along development in Göttingen Minipig, although mRNA has been used as a proxy for enzyme activity and protein abundance [17]. Since other regulatory processes downstream of transcription, such as posttranslational modifications and protein degradation [22], determine protein abundance and enzyme activity ontogeny, mRNA abundance was shown to not always be predictive [21, 22, 26]. Thus, in order to define drug disposition in the youngest age groups, it is necessary to study all processes (i.e. gene expression, protein abundance and enzyme activity) involved in the ontogeny of DMETs [26].

3.5.1 General aspects

In this research, Göttingen Minipig CYP protein abundance was determined in liver microsomes using a LC-MS/MS approach. This technique has become the new standard as immunoaffinity assays (e.g. Western Blot, ELISA etc.) have shown to be not specific enough to quantify different CYP isoforms within the same subfamily [26].

We observed some discrepancies between the two experiments regarding the number of identified CYP isoforms. Fifteen CYP enzymes were detected in both runs (CYP1A2, CYP2A19, CYP2C33, CYP2C33v3, CYP2C34, CYP2C36, CYP2D6, CYP2D25, CYP2E1_1, CYP3A22, CYP3A46, CYP4A21, CYP4V2_2a, CYP27A1 and CYP51A1) which were completed by three (CYP3A29, CYP4A24 and CYP20A1) and seven (CYP1A1, CYP2B22, CYP2C32, CYP2C42, CYP2C49, CYP2E1_2 and CYP4V2_2b) additional isoforms in the first and second experiment, respectively. This disparity can be explained by the fact that both experiments were performed separately at different times. This may cause small differences in the results. In addition, HD-DDA is confined by the limited reproducibility that derives from the stochastic precursor ion selection. Moreover, HD-DDA is not suitable for low abundant precursor ions because they may be never selected for fragmentation. So, the differences between the two experiments are more likely due to technical reasons than to real differences in protein abundance.

CYP2C, CYP2D, CYP2E and CYP3A were found to be the most abundant CYP subfamilies in the different age groups of the first experiment. These results agree with the hepatic CYP protein

abundance profile of 12-week-old conventional pigs (hybrid sow x Piétrain boars, 8 male and 8 female animals) [27]. In the latter, CYP2A was found to be the most abundant in the liver, followed by CYP2D, CYP3A, CYP2E, CYP2C and CYP1A [27]. In our second experiment, CYP2C, CYP2D and CYP3A subfamilies were the most abundant in the male adult Göttingen Minipig whereas the CYP2A, CYP2C and CYP2D subfamilies were the most abundant in adult females. This accords with previous research [23, 28], although CYP2A19 had a remarkably lower abundance (3.5%) in males in ours compared to these other studies. CYP2A19 was the most abundant CYP isoform in 2 adult male Suffolk White pigs (34%) [28] and was amongst the most abundant isoforms in 6-month-old male conventional pigs (Large white x Land race, Seghers hybrid) (12%) [23]. Based on these differences, CYP2A19 protein abundance could be breed-dependent, but data regarding its ontogeny remain scarce. Nevertheless, CYP2A19 sex-related differences with higher protein expression in female in comparison to male Göttingen Minipig, were in accordance with earlier studies [23, 29, 30]. Sex-related differences in CYP abundance will be discussed later.

3.5.2 CYP protein abundance ontogeny profiles

In general, a gradual postnatal increase in protein abundance was observed for most CYP isoforms, although with some exceptions, such as CYP4A24 and CYP20A1. These isoforms were stable over time and were already present in the late fetal stages. As CYP4A24 is not solely responsible for drug metabolism but also for physiological functions (e.g. fatty acid metabolism), this may explain this pattern [31, 32]. Moreover, this CYP4A24 maturation pattern is consistent with the human CYP4A11 ontogeny profile which shows a constant gene and protein expression over time [33]. CYP20A1, which function is still unknown, shows a fairly constant protein abundance pattern in human, similarly to what we observed in the Göttingen Minipig [33-35]. CYP51A1 demonstrated higher protein abundance in the late fetal stages than during the first month of life, which also agrees with previous findings in human [33]. CYP1A2, CYP2A19, CYP2D6, CYP2D25 and CYP2E1_1 showed their highest protein abundance at PND 28. This is in accordance with a gene expression and enzyme activity study by Heckel et al. who suggest that the ontogeny of CYP genes is completed at four weeks of age in the Göttingen Minipig [17]. Based on these findings, they suggest that 4-week-old piglets give similar pharmacological responses as adult minipigs. However, our results warrant caution since this seems not to be the case for all isoforms. CYP2C33, CYP2C34, CYP2C36, CYP3A22, CYP3A29, CYP3A46, CYP4A21, CYP4V2_2a and CYP27A1 reached their highest level only at the adult age. This illustrates again that gene expression, protein abundance and enzyme activity are not always interchangeable.

Hines et al. [36] recently classified human hepatic DMET ontogeny profiles into three different classes based upon their onset and expression pattern. Class 1 DMETs have their highest expression during the first trimester of gestation (e.g. CYP51A1), Class 2 DMETs show a constant expression from pregnancy until adulthood (e.g. CYP2B6 and CYP20A1) and Class 3 consists of DMETs with a negligible expression during gestation, and even low expression at birth that rises postnatally during maturation [33, 36]. Class 3 is the largest group within this classification comprising CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP4F11, CYP4V2 and CYP27A1 [33, 36, 37]. When comparing their results to our findings, the CYP2A, CYP2C, CYP2D, CYP3A, CYP4V,

CYP20A, CYP27A and CYP51A subfamilies belong to the same classes in both species. Thus, we can say that the hepatic CYP protein abundance ontogeny is highly comparable between Göttingen Minipig and human. However, some discrepancies regarding the CYP1A subfamily should be mentioned. In human, CYP1A1 mRNA and protein expression were detected during the first and second trimester of gestation but their expression declined to non-detectable levels towards adulthood [38-43]. CYP1A1 is thus considered to be a player in fetal xenobiotic metabolism [43]. In our study, conversely, CYP1A1 was only detected in the adult Göttingen Minipigs of the second experiment. It is debatable whether this is caused by the technical variability of both LC-MS/MS experiments, or whether it is a species-specific characteristic. In the past, CYP1A1 mRNA was detected in both fetal and adult porcine liver samples whereas CYP1A1 protein was solely retrieved in adult animals [44]. On the other hand, CYP1A2 mRNA, protein and enzyme activity were detected only after birth in human infants [41, 42, 45-47]. For this isoform, a partly comparable pattern is observed in the Göttingen Minipig: CYP1A2 protein abundance only starts to increase postnatally from PND 3 onwards, which accords to the human situation. Nevertheless, the protein is already observed in the fetal age groups which is not the case in human. Thus, using the (mini)pig as a translational model for the human CYP1A subfamily should be considered carefully.

Next to the human population, large similarities in CYP protein abundance were also observed between the Göttingen Minipig and the conventional pig. Millecam et al. investigated CYP protein abundance in conventional pigs of 2 days, 4 weeks, 8 weeks and 6-7 months of age [23]. Since the investigated age groups are not entirely the same compared to our study, caution is needed when drawing conclusions, especially for the youngest age groups. CYP1A2, CYP2C34, CYP2C36, CYP2C49, CYP2D6, CYP3A22, CYP3A29, CYP3A46, CYP4A21, CYP4V2_2a and CYP51A1 abundance increased in a similar way in the conventional pig and the Göttingen Minipig. On the other hand, CYP2E1_1 and CYP20A1 appeared to have a different ontogeny profile in the conventional pig and the Göttingen Minipig. CYP2E1_1 had a stable protein abundance profile over time in the conventional pig whereas in the Göttingen Minipig, a gradual postnatal increase was observed. This difference may be due to different environmental factors (e.g. diet, (un)controlled housing conditions etc.) and to breed-related genetic variations [48]. In humans, however, the CYP2E1 ontogeny pattern shows a gradual postnatal increase, specifically from the first month of life until 1 year of age [49, 50]. Thus, the Göttingen Minipig may be a better translational model for human CYP2E1 than the conventional pig. In addition, Göttingen Minipig CYP2E1 substrate specificity was already shown to be similar to human CYP2E1 [51]. With regard to CYP20A1, protein ontogeny remains stable from the late fetal stage until adulthood in the Göttingen Minipig whereas in the conventional pig, a significant difference is observed between the youngest age groups and the animals of 6-7 months of age [23]. Since CYP20A1 is still considered as an orphan CYP, data is limited, also in human [35]. Therefore, we cannot conclude at present which breed represents better the human situation for this enzyme.

Besides the ontogeny profiles, our study provides additional data regarding the detection of CYP proteins in fetal porcine liver samples. Earlier immunochemical assays were not able to detect CYP1A, CYP2A, CYP2E and CYP3A subfamilies in porcine fetuses [44]. The proteomic approach that

was used in this study proved to have better sensitivity than immunochemistry and provided evidence that these subfamilies are already present by the third trimester of gestation.

3.5.3 Sex-related differences in CYP protein abundance in the developing Göttingen Minipig

For the majority of the detected CYP isoforms, no sex-related differences were observed during the first month of life. This agrees with previous findings in juvenile Göttingen Minipigs and conventional pigs regarding CYP enzyme activity and protein abundance [2, 23, 27]. The fact that 28-day-old Göttingen Minipigs do not present sex-related differences and adult Göttingen Minipigs do, can be explained by the changed expression of growth and sex hormones that occurs during puberty [52]. The effect of sex hormones was for example illustrated by suppressed CYP1A, 2A and 2E1 mRNA expression in entire, i.e., non-castrated, male pigs compared to surgically and immunologically castrated male pigs from Meishan [53], Yorkshire x Landrace [54] and Large White x Landrace x Duroc [55] strains. In entire Bama miniature male pigs, a decrease of CYP2A19 and CYP2E1 mRNA expression was observed after 6 months due to sexual maturity [56]. However, suppressed CYP mRNA expression did not always result in decreased CYP protein and enzyme activity (e.g. CYP2E1) [54]. In this case, posttranslational modifications may be involved as a regulatory mechanism [54], confirming again that all parameters (i.e. gene expression, protein expression and enzyme activity) should be taken into account. Comparative data regarding CYP expression in entire and castrated male Göttingen Minipigs are not available yet, but a similar outcome may be expected.

Nevertheless, sex-related differences were present for CYP4V2_2a and CYP20A1 at PND 1, with females having higher protein abundance than males. However, these isoforms are not relevant for drug metabolism and their importance may lie elsewhere [33-35, 57].

In our preceding study, a significantly higher CYP activity was found in female Göttingen Minipigs for tolbutamide metabolism (human CYP2C9 substrate) at PND 1 and phenacetin metabolism (human CYP1A2 substrate) at PND 28 and adults [2]. These observations were not confirmed in the current study, except for the phenacetin metabolism in adults which can be linked to the higher CYP1A protein abundance in female adult Göttingen Minipigs (see next section). Regarding the other substrates (midazolam and dextromethorphan, human CYP3A4 and CYP2D6 substrates, respectively) no sex-related differences were previously determined [2]. These observations are partly confirmed in our study since CYP2D and CYP3A isoforms did not show sex-related differences during maturation. However, a discrepancy regarding sex-related differences in CYP3A activity and protein abundance is noticed for the adult age (see next section).

As described above, the sex-related differences that were observed during development (CYP4V2_2a and CYP20A1 at PND 1), were detected in CYP isoforms that are not involved in drug metabolism [33-35, 57]. Nevertheless, it may be interesting to look further into these observations as sex-related differences were observed in brain and GI metabolism before [58, 59] and were

linked to better resilience in female human and porcine neonates [60, 61]. Since no sex-related differences were observed at the transcriptional level during the first four weeks of life in Göttingen Minipig liver [17], varying posttranscriptional modifications and protein half-life between sexes, and error/noise in these kind of high throughput experiments [62] have to be considered to explain these observations. In addition, the effect of birth should also be examined as a possible factor because the only sex-related differences before puberty were observed at PND 1. Interestingly, sex-related differences regarding hormone levels have been described in neonates before [59, 63]; this supports the idea to look further into the influence of this event. Hence, it is unclear which impact these results may have, especially since these differences were observed at a single time point. In summary, further research is required to comprehend these findings but their relevance is out of the scope of drug development.

3.5.4 Sex-related differences in CYP protein abundance in the adult Göttingen Minipig

In adult Göttingen Minipig, CYP1A1, CYP1A2, CYP2A19, CYP2E1_2, CYP3A22, CYP4V2_2a and CYP4V2_2b protein abundance was significantly higher in females compared to males. This is in accordance with previous findings in the Göttingen Minipig [64-66], but also in the Yucatan minipig [67] and the conventional pig [23]. In the latter, however, sex-related differences were also observed for CYP2C36 which was not the case in our study.

In human, CYP enzyme activity studies showed higher clearance in males for CYP1A2- (Caucasian population only) [68] and CYP2E1-associated substrates [69-71], whereas CYP3A4- [72-76] and CYP2B6-associated [77] clearance were higher in females for various substrates, although conflicting data have been reported and interindividual differences may prevail [78, 79]. Conflicting results were also obtained for CYP2C9 [80-82], CYP2C19 [80, 83-85] and CYP2D6 [86-92] depending on the substrate. Moreover, CYP2C19 and CYP2D6 are highly polymorphic genes, which makes it even more challenging to draw conclusions [93, 94]. CYP gene expression studies on the other hand showed significant higher levels for females in comparison to males for amongst others CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP3A4, CYP3A5, CYP3A43, CYP27A1 and CYP51A1 [95]. Thus, in view of our results, the CYP2A and CYP3A subfamilies are comparable between human and Göttingen Minipig. As the CYP3A subfamily is clinically the most important subfamily for drug metabolism in human [96], this is an additional asset for the Göttingen Minipig as a translational model for the human population. For the other subfamilies, opposite sex-related differences between both species (CYP1A and CYP2E) or absent differences in Göttingen Minipig (CYP27A1 and CYP51A1) were observed. These differences should be considered when looking for the most appropriate animal model in nonclinical drug development.

3.5.5 CYP protein abundance vs CYP enzyme activity

Göttingen Minipig CYP enzyme activity that was previously determined and described [2] was correlated to the CYP protein abundance from this study. Since the same biological samples were used for both experiments, a one-on-one correlation between both parameters was possible. We

first performed a correlation study for all age groups together. Since most CYPs showed a postnatal increase in enzyme activity and protein abundance, high correlations were observed when comparing both parameters.

Second, correlation within each individual age group and per CYP isoform was assessed. Only CYP3A22 showed a statistically significant correlation between its protein abundance and the metabolism of midazolam at PND 7. This agrees with previous studies in minipig [2, 18, 97] and pig [23, 27, 98]. Regarding the other substrates, the results are striking since several studies have already suggested a link between CYP1A, CYP2C and CYP2D and the metabolism of phenacetin [98], tolbutamide [16, 19, 23, 27, 98, 99] and dextromethorphan [27, 100], respectively, in various minipig and pig strains. However, in the majority of those studies, no correlation analysis was conducted, but rather the ability to metabolize the substrate. Hence, no direct link between a specific CYP isoform and the substrates could be made. A word of caution is thus required when performing and interpreting such correlation and activity analyses. Moreover, these findings illustrate the importance of further characterizing the Göttingen Minipig CYP enzymes for example by the development of recombinant enzymes. This is the only way to elucidate substrate-specificity for the different CYP isoforms.

3.5.6 Future potential for PBPK modeling

The CYP protein abundance data of this study are a valuable step forward in our comprehension of CYP ontogeny in the fetal, neonatal and juvenile Göttingen Minipig. However, in order to extrapolate data from liver microsomes to the entire organ, scaling factors such as the MPPGL are required [101]. Only then the total amount of protein, enzyme activity, etc. can be calculated and implemented into the PBPK model. At this moment, MPPGL data are not yet available for the Göttingen Minipig. Once these data are obtained and the requirements are met, our CYP protein abundance data will be highly valuable for the development of a neonatal and juvenile Göttingen Minipig PBPK model.

3.6 Conclusion

This study was the first to investigate CYP protein abundance in the developing and adult Göttingen Minipig by a proteomic approach. In general, CYP protein abundance was highest in adult animals. However, several CYP proteins were already detected in the late fetal age groups and a significant postnatal increase was present during the first month of life. Sex-related differences were observed in the developing and adult Göttingen Minipig. These data are remarkably comparable to human data and provide a valuable step forward in the construction of a neonatal and juvenile Göttingen Minipig PBPK model.

3.7 Supplementary tables

Supplementary Table S1: Distribution of CYP isoforms (%) in liver microsomes of the developing Göttingen Minipig relative to adult male Göttingen Minipigs; complementary to Figure 1.

CYP	GD 84 – 86	GD 108	PND 1	PND 3	PND 7	PND 28	Adult male
1. CYP1A2	0.78	0.67	0.53	0.97	1.42	2.46	0.95
2. CYP2A19	1.47	1.91	1.24	1.52	2.32	5.45	1.46
3. CYP2C33	1.04	0.82	2.53	7.61	8.59	6.32	6.93
4. CYP2C33v3	0.03	0.04	0.11	0.38	0.47	0.22	0.28
5. CYP2C34	0.25	0.62	0.49	0.71	2.63	2.32	6.17
6. CYP2C36	2.69	2.72	2.42	2.67	4.53	4.28	17.23
7. CYP2D6	0.10	0.05	0.09	0.28	0.51	1.58	1.52
8. CYP2D25	1.08	1.70	2.15	3.53	9.22	17.74	19.07
9. CYP2E1_2	1.86	3.57	6.73	8.87	23.33	24.10	6.25
10. CYP3A22	1.20	1.79	2.24	3.44	7.26	12.29	21.06
11. CYP3A29	0.47	0.31	0.46	0.78	0.93	3.09	6.60
12. CYP3A46	0.16	0.12	0.14	0.15	0.25	0.83	2.29
13. CYP4A21	0.12	0.19	0.14	0.30	0.59	1.85	3.00
14. CYP4A24	0.22	0.04	0.00	0.09	0.09	0.49	0.75
15. CYP4V2_2a	0.19	0.20	0.19	0.21	0.25	0.37	0.34
16. CYP20A1	1.67	1.75	1.26	1.45	2.10	2.22	1.53
17. CYP27A1	0.11	0.18	0.33	0.26	0.80	0.78	1.79
18. CYP51A1	1.12	0.43	0.32	0.42	0.39	0.20	2.76
TOTAL	15	17.11	21	34	66	87	100.00

Supplementary Table S2: Distribution of CYP isoforms (%) in female and male adult Göttingen Minipig; complementary to Figure 2.

CYP	Female	Male
1. CYP1A1	1.54	1.07
2. CYP1A2	3.34	2.73
3. CYP2A19	32.87	3.49
4. CYP2B22	3.41	4.07
5. CYP2C32	0.29	2.12
6. CYP2C33	6.35	13.03
7. CYP2C33v3	0.27	0.42
8. CYP2C34	0.11	1.17
9. CYP2C36	0.48	2.95
10. CYP2C42	1.93	9.01
11. CYP2C49	0.39	0.65
12. CYP2D6	2.96	2.85
13. CYP2D25	28.00	28.82
14. CYP2E1_1	0.97	0.10
15. CYP2E1_2	1.39	2.13
16. CYP3A22	1.62	0.98
17. CYP3A46	4.47	10.86
18. CYP4A21	6.33	7.65
19. CYP4V2_2a	0.46	0.41
20. CYP4V2_2b	0.08	0.05
21. CYP27A1	1.21	2.30
22. CYP51A1	1.55	3.15
TOTAL % CYP	100.00	100.00

Supplementary Table S3: Pearson correlation analysis between CYP enzyme activity and protein abundance detected in liver microsomes of the developing and adult Göttingen Minipig. All age groups of the first experiment (GD 84-86, GD 108, PND 1, PND 3, PND 7, PND 28 and male adults) were included. CYP enzyme activity was determined before by investigating the capacity to metabolize 4 known human substrates (i.e. phenacetin, midazolam, tolbutamide and dextromethorphan) in the same liver microsomes as used in this study. The metabolite formation velocities (pmol/min/mg microsomal protein) of these substrates were used for the current correlation analyses.

	Phenacetin (human CYP1A2)		**Midazolam (human CYP3A4)**		**Tolbutamide (human CYP2C9)**		**Dextromethorphan (human CYP2D6)**	
	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
CYP1A2	0.818	<0.0001	0.7741	<0.0001	0.6994	<0.0001	0.6567	<0.0001
CYP2A19	0.6152	<0.0001	0.5515	<0.0001	0.4673	0.0012	0.476	0.0009
CYP2C33	0.7778	<0.0001	0.8254	<0.0001	0.8501	<0.0001	0.7782	<0.0001
CYP2C33v3	0.5535	<0.0001	0.555	<0.0001	0.4835	0.0009	0.6325	<0.0001
CYP2C34 (Fragment)	0.5756	<0.0001	0.6778	<0.0001	0.7107	<0.0001	0.6083	<0.0001
CYP2C36	0.453	0.0018	0.6022	<0.0001	0.6946	<0.0001	0.4723	0.0011
CYP2D6 (Fragment)	0.8117	<0.0001	0.8445	<0.0001	0.8511	<0.0001	0.7894	<0.0001
CYP2D25	0.8918	<0.0001	0.9391	<0.0001	0.9115	<0.0001	0.8635	<0.0001
CYP2E1_1	0.814	<0.0001	0.7229	<0.0001	0.6001	<0.0001	0.763	<0.0001
CYP3A	0.7841	<0.0001	0.8582	<0.0001	0.8616	<0.0001	0.7559	<0.0001
CYP3A22	0.842	<0.0001	0.9322	<0.0001	0.9147	<0.0001	0.8174	<0.0001
CYP3A46	0.6653	<0.0001	0.7538	<0.0001	0.7702	<0.0001	0.5817	<0.0001
CYP4A	0.3135	0.1664	0.37	0.0987	0.3107	0.1704	0.2432	0.2881
CYP4A21	0.7695	<0.0001	0.8724	<0.0001	0.8509	<0.0001	0.7486	<0.0001
CYP4V2_2a (Fragment)	0.3829	0.0094	0.3789	0.0103	0.4259	0.0035	0.2944	0.0496
CYP20A1	0.341	0.0219	0.3379	0.0232	0.3812	0.0098	0.3275	0.0281
CYP27A1 (Fragment)	0.625	<0.0001	0.6985	<0.0001	0.6336	<0.0001	0.6819	<0.0001
CYP51A1	-0.1734	0.2545	-0.0977	0.5232	0.0008	0.9957	-0.2204	0.1457

Supplementary Table S4.1: Pearson correlation analysis between CYP enzyme activity and protein abundance in liver microsomes originating from Göttingen Minipigs at GD 108. The analysis was performed for each CYP individually. The Bonferroni correction adjusted the threshold p-value to 0.00012. Yellow marking: assumptions for parametric testing were not met, non-parametric Spearman rank correlation analysis was performed.

GD 108								
	Phenacetin (human CYP1A2)		Midazolam (human CYP3A4)		Tolbutamide (human CYP2C9)		Dextromethorphan (human CYP2D6)	
	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
CYP1A2	0.4389	0.3839	0.3333	0.5186	0.7008	0.1209	0.4154	0.4128
CYP2A19	0.2371	0.6511	-0.7588	0.0803	0.4024	0.4289	0.0865	0.8706
CYP2C33	-0.1593	0.7631	-0.776	0.0696	0.0248	0.9627	0.1879	0.7215
CYP2C33v3	0.6456	0.1662	-0.1644	0.7556	0.856	0.0296	0.7986	0.0568
CYP2C34 (Fragment)	0.8593	0.0283	0.5954	0.2124	0.8613	0.0275	0.4158	0.4123
CYP2C36	0.7174	0.1085	0.8224	0.0445	0.4882	0.3259	0.3795	0.4581
CYP2D6 (Fragment)	0.1993	0.705	-0.2973	0.5671	-0.0798	0.8805	0.3548	0.4901
CYP2D25	0.2223	0.6721	0.2096	0.6903	0.0745	0.8884	0.7658	0.0759
CYP2E1_2	0.5145	0.2963	-0.3035	0.5587	0.4099	0.4196	0.2853	0.5837
CYP3A22	0.2	0.704	-0.6	0.208	0.4286	0.3965	-0.0857	0.8717
CYP3A29	-0.7564	0.0818	0.1373	0.7953	-0.8955	0.0158	-0.5526	0.2555
CYP3A46	0.0446	0.9331	-0.8911	0.0171	0.0708	0.894	0.2329	0.6569
CYP4A21	-0.5475	0.2609	-0.2119	0.6869	-0.25	0.6328	-0.0488	0.9269
CYP4A24	-0.8253	0.0431	-0.5842	0.2234	-0.5404	0.2683	-0.4149	0.4134
CYP4V2_2a (Fragment)	0.4212	0.4055	0.2997	0.5639	0.3093	0.5509	0.3134	0.5453
CYP20A1	-0.0286	0.9572	-0.2571	0.6228	0.1429	0.7872	-0.1429	0.7872
CYP27A1 (Fragment)	-0.0827	0.8763	-0.3015	0.5614	0.0523	0.9216	-0.6959	0.1247
CYP51A1	-0.4857	0.3287	-0.3714	0.4685	-0.4286	0.3965	-0.0286	0.9572

Supplementary Table S4.2: Pearson correlation analysis between CYP enzyme activity and protein abundance in liver microsomes originating from Göttingen Minipigs at PND 1. The analysis was performed for each CYP individually. The Bonferroni correction adjusted the threshold p-value to 0.00012.

PND 1								
	Phenacetin (human CYP1A2)		Midazolam (human CYP3A4)		Tolbutamide (human CYP2C9)		Dextromethorphan (human CYP2D6)	
	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
CYP1A2	0.0054	0.9899	0.1158	0.7848	0.1509	0.7213	-0.0842	0.8428
CYP2A19	-0.369	0.3683	0.06	0.8877	-0.0769	0.8564	-0.1216	0.7742
CYP2C33	0.4796	0.2291	0.6201	0.101	0.7377	0.0367	0.6845	0.0611
CYP2C33v3	0.5942	0.1204	0.1301	0.7588	-0.1492	0.7244	0.6115	0.1072
CYP2C34 (Fragment)	0.504	0.2029	0.5569	0.1516	0.3836	0.3482	0.619	0.1017
CYP2C36	0.5208	0.1857	0.2027	0.6303	-0.051	0.9045	0.4908	0.2169
CYP2D6 (Fragment)	0.3352	0.417	-0.3281	0.4276	-0.0788	0.8529	0.0091	0.983
CYP2D25	0.5218	0.1847	0.3469	0.3998	0.2894	0.4869	0.1272	0.7641
CYP2E1_2	0.5909	0.123	0.9218	0.0011	0.5651	0.1444	0.699	0.0537
CYP3A22	-0.2232	0.5952	0.447	0.2669	0.264	0.5275	-0.3085	0.4573
CYP3A29	0.686	0.0603	0.3597	0.3814	0.2269	0.5889	0.807	0.0155
CYP3A46	0.1871	0.6572	-0.2291	0.5852	-0.4538	0.2587	0.0892	0.8336
CYP4A21	-0.025	0.9532	0.5194	0.1871	0.7167	0.0455	0.2013	0.6327
CYP4A24	-	-	-	-	-	-	-	-
CYP4V2_2a (Fragment)	0.2764	0.5076	0.3184	0.4421	0.7706	0.0252	0.2621	0.5306
CYP20A1	0.0042	0.9921	0.377	0.3572	0.6293	0.0946	0.1752	0.6781
CYP27A1 (Fragment)	-0.1474	0.7276	0.2492	0.5517	-0.0631	0.882	0.1031	0.8081
CYP51A1	0.0329	0.9383	0.0617	0.8847	0.1826	0.6652	0.4262	0.2924

Supplementary Table S4.3: Pearson correlation analysis between CYP enzyme activity and protein abundance in liver microsomes originating from Göttingen Minipigs at PND 3. The analysis was performed for each CYP individually. The Bonferroni correction adjusted the threshold p-value to 0.00012. Yellow marking: assumptions for parametric testing were not met, non-parametric Spearman rank correlation analysis was performed.

PND 3								
	Phenacetin (human CYP1A2)		Midazolam (human CYP3A4)		Tolbutamide (human CYP2C9)		Dextromethorphan (human CYP2D6)	
	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
CYP1A2	0.0434	0.9187	-0.2338	0.5773	-0.4363	0.2798	-0.4883	0.2195
CYP2A19	-0.0883	0.8352	-0.1716	0.6844	-0.4873	0.2206	-0.3236	0.4342
CYP2C33	0.0071	0.9867	0.0977	0.818	-0.1166	0.7834	0.0957	0.8217
CYP2C33v3	0.2143	0.6103	0.3333	0.4198	-0.1905	0.6514	-0.381	0.3518
CYP2C34 (Fragment)	0.1122	0.7914	0.068	0.873	0.5771	0.1342	0.4492	0.2642
CYP2C36	0.1211	0.7752	0.037	0.9307	0.7295	0.04	0.4915	0.2161
CYP2D6 (Fragment)	0.1826	0.6652	0.1538	0.7162	0.2522	0.5468	0.3899	0.3397
CYP2D25	0.291	0.4844	0.102	0.81	0.0182	0.966	0.1487	0.7252
CYP2E1_2	-0.2401	0.5668	0.029	0.9457	-0.579	0.1326	-0.4997	0.2073
CYP3A22	-0.0745	0.8608	0.3904	0.339	0.1895	0.6531	0.4726	0.237
CYP3A29	-0.1486	0.7255	-0.1521	0.7192	0.3461	0.401	0.3003	0.4699
CYP3A46	0.2232	0.5952	0.2851	0.4937	0.2296	0.5844	0.2713	0.5156
CYP4A21	-0.9021	0.0022	-0.6315	0.093	-0.7318	0.039	-0.5551	0.1532
CYP4A24	-	-	-	-	-	-	-	-
CYP4V2_2a (Fragment)	-0.0678	0.8732	-0.4449	0.2694	-0.1581	0.7084	-0.271	0.5162
CYP20A1	0.2239	0.594	-0.2852	0.4935	-0.0198	0.963	-0.1032	0.8078
CYP27A1 (Fragment)	-0.3418	0.4073	-0.1218	0.7738	-0.1661	0.6943	0.0195	0.9634
CYP51A1	-0.3934	0.335	-0.2499	0.5506	-0.4578	0.254	-0.4285	0.2895

Supplementary Table S4.4: Pearson correlation analysis between CYP enzyme activity and protein abundance in liver microsomes originating from Göttingen Minipigs at PND 7. The analysis was performed for each CYP individually. The Bonferroni correction adjusted the threshold p-value to 0.00012.

PND 7

	Phenacetin (human CYP1A2)		Midazolam (human CYP3A4)		Tolbutamide (human CYP2C9)		Dextromethorphan (human CYP2D6)	
	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
CYP1A2	0.645	0.0842	0.7404	0.0357	0.4977	0.2095	0.7506	0.0319
CYP2A19	0.2414	0.5647	0.6289	0.0948	0.4018	0.3238	0.5315	0.1752
CYP2C33	0.3237	0.4341	0.4675	0.2428	0.7371	0.0369	0.3444	0.4035
CYP2C33v3	0.4658	0.2447	0.5146	0.192	-0.4479	0.2658	-0.0444	0.9168
CYP2C34 (Fragment)	-0.0474	0.9112	-0.2791	0.5032	0.4367	0.2793	-0.1146	0.787
CYP2C36	-0.1513	0.7206	-0.1685	0.6899	0.5704	0.1399	0.0727	0.8642
CYP2D6 (Fragment)	0.191	0.6505	0.377	0.3573	0.6272	0.096	0.9264	0.0009
CYP2D25	0.2575	0.5381	0.6866	0.06	0.7665	0.0265	0.9349	0.0007
CYP2E1_2	0.2794	0.5027	-0.0861	0.8393	0.2674	0.522	0.0171	0.9679
CYP3A22	0.3691	0.3682	0.9633	0.0001	0.4132	0.3089	0.646	0.0835
CYP3A29	0.1736	0.681	0.8737	0.0046	0.4634	0.2475	0.5632	0.146
CYP3A46	0.1713	0.685	-0.0033	0.9938	0.4779	0.2311	0.7079	0.0486
CYP4A21	0.2306	0.5827	0.4014	0.3243	0.0424	0.9205	0.3615	0.3789
CYP4A24	0.2919	0.7081	-0.3468	0.6532	-0.6373	0.3627	-0.5363	0.4637
CYP4V2_2a (Fragment)	-0.6894	0.0586	-0.4123	0.3101	0.1397	0.7415	0.1396	0.7416
CYP20A1	0.5538	0.1544	0.4672	0.2431	0.7414	0.0353	0.7324	0.0388
CYP27A1 (Fragment)	0.8713	0.0048	0.1574	0.7098	0.1069	0.8011	0.027	0.9494
CYP51A1	-0.0193	0.9639	-0.6786	0.0643	-0.065	0.8784	-0.4985	0.2086

Supplementary Table S4.5: Pearson correlation analysis between CYP enzyme activity and protein abundance in liver microsomes originating from Göttingen Minipigs at PND 28. The analysis was performed for each CYP individually. The Bonferroni correction adjusted the threshold p-value to 0.00012.

	PND 28							
	Phenacetin (human CYP1A2)		Midazolam (human CYP3A4)		Tolbutamide (human CYP2C9)		Dextromethorphan (human CYP2D6)	
	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
CYP1A2	0.543	0.1643	-0.3066	0.4601	-0.3784	0.3553	0.3945	0.3335
CYP2A19	0.3248	0.4325	-0.1216	0.7743	-0.24	0.5669	0.1885	0.6548
CYP2C33	0.0719	0.8657	-0.0432	0.9192	-0.0469	0.9121	0.3228	0.4354
CYP2C33v3	-0.3976	0.3294	0.3682	0.3695	-0.2069	0.6213	-0.2393	0.5681
CYP2C34 (Fragment)	0.094	0.8247	0.1353	0.7494	0.1531	0.7175	0.3844	0.347
CYP2C36	-0.6337	0.0916	0.1251	0.7678	0.3849	0.3464	0.0689	0.8713
CYP2D6 (Fragment)	0.0236	0.9558	-0.0583	0.8909	0.0193	0.9638	0.4534	0.2592
CYP2D25	0.0752	0.8595	0.0481	0.91	-0.1209	0.7755	0.3474	0.3992
CYP2E1_2	0.581	0.1309	0.0285	0.9465	-0.0944	0.824	0.0403	0.9246
CYP3A22	0.0463	0.9133	0.9114	0.0016	0.4164	0.3048	-0.0504	0.9056
CYP3A29	-0.4032	0.322	0.2564	0.5399	0.324	0.4337	0.1669	0.6929
CYP3A46	-0.5439	0.1635	0.4679	0.2423	0.281	0.5002	0.1389	0.7428
CYP4A21	-0.308	0.458	0.2094	0.6187	-0.0581	0.8914	0.3864	0.3444
CYP4A24	-0.5676	0.6157	-0.068	0.9567	-0.4778	0.6828	0.4504	0.7025
CYP4V2_2a (Fragment)	-0.2012	0.6327	-0.3926	0.336	-0.4113	0.3113	-0.1125	0.7909
CYP20A1	-0.2012	0.6327	-0.3926	0.336	-0.4113	0.3113	-0.1125	0.7909
CYP27A1 (Fragment)	-0.4681	0.2421	-0.6128	0.1062	-0.5275	0.1791	-0.5305	0.1762
CYP51A1	-0.1947	0.644	0.2567	0.5393	-0.1238	0.7703	-0.1844	0.662

Supplementary Table S4.6: Pearson correlation analysis between CYP enzyme activity and protein abundance in liver microsomes originating from adult male Göttingen Minipigs. The analysis was performed for each CYP individually. The Bonferroni correction adjusted the threshold p-value to 0.00012.

	Adult male							
	Phenacetin (human CYP1A2)		Midazolam (human CYP3A4)		Tolbutamide (human CYP2C9)		Dextromethorphan (human CYP2D6)	
	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value	Pearson correlation coefficient	p-value
CYP1A2	0.8291	0.1709	0.1569	0.8431	-0.1067	0.8933	0.7025	0.2975
CYP2A19	0.9513	0.0487	0.4377	0.5623	0.4967	0.5033	0.9812	0.0188
CYP2C33	-0.7831	0.2169	-0.1096	0.8904	0.1778	0.8222	-0.6436	0.3564
CYP2C33v3	-0.3702	0.6298	0.0342	0.9658	0.4976	0.5024	-0.1633	0.8367
CYP2C34 (Fragment)	-0.8703	0.1297	-0.8883	0.1117	-0.6976	0.3024	-0.8585	0.1415
CYP2C36	-0.1703	0.8297	0.7075	0.2925	0.7137	0.2863	-0.0747	0.9253
CYP2D6 (Fragment)	0.9794	0.0206	0.3907	0.6093	0.3392	0.6608	0.9581	0.0419
CYP2D25	0.7878	0.2122	0.2417	0.7583	0.4755	0.5435	0.8718	0.1282
CYP2E1_2	0.7605	0.2395	-0.0703	0.9297	-0.2152	0.7848	0.6574	0.3426
CYP3A22	0.4236	0.5764	0.987	0.013	0.8352	0.1648	0.4608	0.5392
CYP3A29	-0.6703	0.3297	-0.2272	0.7728	-0.5463	0.4537	-0.7922	0.2078
CYP3A46	-0.5283	0.4717	0.2988	0.7012	0.5129	0.4871	-0.3815	0.6185
CYP4A21	-0.5989	0.4011	-0.0353	0.9647	0.3581	0.6419	-0.4171	0.5829
CYP4A24	-0.7825	0.2175	-0.9493	0.0507	-0.85	0.15	-0.8188	0.1812
CYP4V2_2a (Fragment)	0.148	0.852	-0.1245	0.8755	0.3787	0.6213	0.3265	0.6735
CYP20A1	0.8156	0.1844	0.2647	0.7353	0.4758	0.5242	0.8919	0.1081
CYP27A1 (Fragment)	-0.7382	0.2618	-0.5134	0.4866	-0.7658	0.2342	-0.8646	0.1354
CYP51A1	-0.0959	0.9041	0.1662	0.8338	0.6435	0.3565	0.119	0.881

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CHAPTER 4:
Ontogeny of CYP3A and UGT activity in preterm piglets: a translational model for drug metabolism in preterm newborns

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4.1 Abstract

Despite considerable progress in understanding drug metabolism in the human pediatric population, data remains scarce in preterm neonates. Improving our knowledge of the ADME properties in this vulnerable age group is of utmost importance to avoid suboptimal dosing, which may lead to adverse drug reactions. The juvenile (mini)pig is a representative model for hepatic drug metabolism in human neonates and infants, especially phase I reactions. However, the effect of prematurity on the onset of hepatic phase I and phase II enzyme activity has yet to be investigated in this animal model. Therefore, the aim of this study was to assess the ontogeny of CYP3A and UGT enzyme activity in the liver of preterm (gestational day 105 – 107) and term-born (gestational day 115 – 117) domestic piglets. In addition, the ontogeny pattern between the preterm and term group was compared to examine whether postconceptional or postnatal age affects the onset of enzyme activity. The following age groups were included: preterm postnatal day (PND) 0 (n = 10), PND 5 (n = 10), PND 11 (n = 8), PND 26 (n = 10) and term PND 0 (n = 10), PND 5 (n = 10), PND 11 (n = 8), PND 19 (n = 18) and PND 26 (n = 10). Liver microsomes were extracted, and the metabolism of CYP3A and UGT-specific substrates assessed enzyme activity. Preterm CYP3A activity was only detectable at PND 26, whereas term CYP3A activity showed a gradual postnatal increase from PND 11 onwards. UGT activity gradually increased between PND 0 and PND 26 in preterm and term-born piglets, albeit, being systematically lower in the preterm group. Thus, postconceptional age is suggested as the main driver affecting porcine CYP3A and UGT enzyme ontogeny. These data are a valuable step forward in the characterization of the preterm piglet as a translational model for hepatic drug metabolism in the preterm human neonate.

4.2 Introduction

In recent years, the understanding of drug metabolism in the human pediatric population has considerably progressed, but data on preterm neonates remain scarce [1]. Since enrollment of this vulnerable pediatric subpopulation in clinical studies is restricted by several factors (e.g., difficulty in obtaining informed parental consent, limited possibilities for repeated sampling, and general lack of pediatric trials), little is known about pharmacokinetics (PK) in preterm neonates (i.e., born before 37 weeks of gestation) [2]. Understanding the ADME (absorption, distribution, metabolism, and excretion) of drugs in this population is critical, as these patients often require pharmacological treatment to survive [2]. In addition, the level of prematurity may affect drug-metabolizing enzyme (DME) ontogeny, which is insufficiently assessed in preterm neonates [2]. Gestational age (GA) (i.e., time elapsed between the first day of the last menstrual period and birth), postnatal age (PNA) (i.e., chronological age starting from the day of birth) and postmenstrual age (PMA) (i.e., sum of GA and PNA) [3] are known to affect the degree of enzyme maturation at birth, but the influence of these parameters on DME in preterms is poorly understood [2, 4-6]. This lack of knowledge of the biotransformation processes increases the risk of suboptimal dosing, possibly resulting in adverse drug reactions [1, 2, 4, 5]. The commonly used solution of allometric scaling based upon data in older children and adults has often led to incorrect dosing regimens in preterm neonates, and

several examples of adverse events due to immature DME have been reported (e.g., Gray baby syndrome and gasping syndrome) [7, 8].

Biotransformation of xenobiotics, but also endogenous substances (e.g., bilirubin, steroid hormones, thyroid hormones, etc.), mainly occurs in the liver and is mediated by phase I and phase II reactions. Phase I enzymes aim to introduce a functional group in these compounds by oxidation, reduction, and hydrolysis reactions. The cytochrome P450 (CYP) enzyme superfamily is one of the most important groups of phase I DME, as they are responsible for the metabolism of 70-80% of all marketed drugs in clinical use [9]. Within this superfamily, the CYP3A family is the most abundant one and metabolizes a substantial fraction of prescribed drugs in children [10]. Phase II enzymes conjugate a functional group to either the parent drug or its phase I metabolites, creating more polar substances and facilitating elimination. Phase II metabolism is performed by*, inter alia*, uridine 5'-diphospho-glucuronosyltransferases (UGT), sulfotransferases, N-acetyltransferases, and glutathione S-transferases [5]. Within this group, UGT enzymes are responsible for ~35% of all phase II reactions [11]. Several studies have investigated the ontogeny pattern of CYP and UGT enzymes in the neonatal, juvenile, adolescent, and adult human populations [12-15]. However, very little data is available concerning the preterm-born neonate, since liver samples are challenging to obtain from this pediatric subpopulation. Thus, the aim of this study is to investigate their ontogeny in the preterm piglet as a surrogate to better understand the biotransformation capacity in the human preterm neonate.

The preterm pig model was characterized before and found to be representative of human preterm physiology [16]. However, a direct correlation between both species based upon GA is not possible [17], e.g., GIT maturation in the piglet is slower than in man since the development is not finished at birth and continues during the first weeks of *ex utero* life [18]. In general, it is assumed that 90% gestation in pigs represents a good model for the GIT system in preterm infants born at 75% gestation [18]. Regarding perinatal terminology, postconceptional age (PCA) (i.e., sum of GA starting from the day of conception and PNA) is used in the pig instead of PMA. Pigs have an estrous cycle [19] instead of a menstrual cycle in humans and therefore the terminology PMA is not applicable. In view of PK characteristics, multiple studies have shown considerable similarities between the juvenile population in (mini)pigs and humans [20-23]. Recent research in both the conventional pig and minipig has shown similar ontogeny profiles for CYP enzyme activity and protein abundance in comparison to human neonates and infants [20-22, 24, 25]. Despite the presence of similar ontogeny profiles, it should be taken into account that absolute enzyme activity and protein abundance levels are not necessarily the same between animal models and humans [26]. Caution is thus needed when comparing species. Concerning UGT enzymes, maturation data are limited and are primarily described in term-born neonatal and juvenile pigs [21, 27, 28]. Although fetal pig samples were included in some studies [20, 21, 25], the effect of preterm birth on the onset of phase I and II enzyme activity in piglets has not been investigated yet.

The main goal of this study was to assess the ontogeny of CYP3A and UGT enzyme activity in preterm and term-born domestic piglets. Enzyme activity was measured by the metabolism of

enzyme-specific substrates in liver microsomes of both male and female piglets, with age groups ranging from the day of birth until postnatal day (PND) 26, the weaning age. Second, the ontogeny pattern between preterm and term animals was compared to examine whether PCA (i.e., a predetermined "biological clock") or PNA (i.e., birth effect) [29] affects the onset of enzyme activity.

4.3 Materials and Methods

4.3.1 Animals and tissue

Liver samples originating from preterm and term-born piglets (Danish Landrace x Large White x Duroc) were a kind gift from the University of Copenhagen [29, 30]. All animal experiments were approved by the Danish Committee for Animal Research (license #2012-15-2934-00-193). All piglets were caesarian-section-delivered, either preterm at gestational day 105-107 (90% of gestation, 10 days before full term) or full term at gestational day 115-117 (100% of gestation). All piglets were immediately transferred to a piglet neonatal intensive care unit and reared in temperature-, moisture- and oxygen-regulated incubators. The animals were randomly allocated to a specific age group and humanely killed at the time points chosen using initial induction of anesthesia (mixture of zolazepam, tiletamine, ketamine, butorphanol, and xylazine) followed by intracardiac injection of a lethal dose of sodium pentobarbital. The following age groups were investigated: preterm PND 0 (n = 10), PND 5 (n = 10), PND 11 (n = 8), PND 26 (n = 10) and term PND 0 (n = 10), PND 5 (n = 10), PND 11 (n = 8), PND 19 (n = 18), and PND 26 (n = 10). The age groups of the term piglets cover the neonatal period up until infancy and correspond to the age range of one month to two years of age in humans [31]. The preterm group was born approximately ten days before the term group. Hence, preterm PND 11 and term PND 0 animals and preterm PND 26 and term PND 19 animals shared nearly the same PCA. Both sexes were equally represented in each age group except for preterm PND 0 (seven males and three females), term PND 5 (four males and six females), and term PND 19 (ten males and eight females). No preterm PND 19 samples were available. For the UGT activity assessment, five males and five females from the term PND 19 group were randomly selected for analysis. All term PND 19 animals were included in the CYP3A activity assessment. Isolation of the liver microsomes was performed as described by Van Peer et al. [20]. The Pierce® BCA Protein Assay Kit determined the total protein concentration of the liver microsomes, using bovine serum albumin as a standard.

4.3.2 CYP3A activity assessment

The protocol for the P450-Glo™ CYP3A4 assay (V9002; Promega Corporation, WI, USA) was executed as described earlier by our research group [20]. In brief, pig liver microsomes were incubated with Luciferin-IPA, a specific human CYP3A4 substrate. Biotransformation of this substrate by porcine CYP3A4 homologs resulted in the formation of D-Luciferin. The concentration of this metabolite was quantified based on comparing the luminescent signal of the reaction mixtures with those from a D-Luciferin (Beetle Luciferin, E1601; Promega Corporation, WI, USA) standard curve. Luminescence was measured with a Tecan Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland). Determination of the kinetic profile of Luciferin-IPA and optimization of

the substrate concentration (1 µM), incubation time (10 min), and microsomal protein (MP) concentration (20 µg/ml) within the linear range of Luciferin-IPA were described before [20]. CYP3A4 baculosomes (P2377; Thermo Fischer Scientific, MA, USA) and insect cell control supersomes (456200; Corning Incorporated, NY, USA) were used as positive and negative control, respectively. Both were included in each plate and treated equivalently to the pig liver microsomes. The study was performed in non-treated Nunc™ F96 Microwell™ white Polystyrene plates (236205; Thermo Fisher Scientific, MA, USA). Reaction velocities were calculated in units of picomoles of D-Luciferin formed per minute per milligram of microsomal protein (pmol/min/mg MP). The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were 0.97 nM and 2.3 nM, respectively. The data represent the mean value for each sample obtained in three technical replicates. The coefficient of variation (CV) for these replicates was within limits (< 10%).

4.3.3 UGT activity assessment

UGT activity was assessed by a fluorescent assay (UGT Activity Assay / Ligand Screening kit, ab273331; Abcam, Cambridge, UK) based upon the following principle: two reactions are set up in parallel for each sample. In the first reaction (i.e., plus-UDPGA reaction), liver microsomes, fluorescent UGT multienzyme substrate (glucuronidated by human UGT1A1, UGT1A3, UGT1A6, UGT1A9, and UGT2B7), and UDPGA (glucuronic acid donor) are present. The second reaction (i.e., minus-UDPGA reaction) is like the first one except for UDPGA being replaced by an equal volume of UGT assay buffer. Thus, in the first reaction, the UGT substrate becomes glucuronidated over time, depending on the UGT content. The part of the substrate that remained unmodified and the substrate that was present in the minus-UDPGA reaction will produce a fluorescent signal. The decrease in fluorescent signal, measured by the difference between both reactions, is proportional to the glucuronidation activity of the sample. A standard curve with the UGT multienzyme substrate (range 0 – 2 nmol/well) was included in each 96-well plate. Hence, the amount of unmodified substrate during the reaction could be quantified at each time point. Human liver microsomes (HLM) (H0620; Xenotech, KS, USA) were included as a positive control. Insect cell control supersomes (456200; Corning Incorporated, Corning, NY, USA), lacking UGT activity, were included as a negative control. The positive and negative controls were treated correspondingly to the pig liver microsomes.

Determination of preterm and term-born piglet UGT activity was conducted in Greiner 96 Flat Bottom Black Polystyrene Chimney plates (655900; Greiner Bio-One, Belgium). A range of six MP concentrations for the 2X sample premix preparation (6.25, 12.5, 25, 50, 100, and 200 µg/ml) and six substrate concentrations (2.5, 5, 10, 20, 40, and 80 µM) were tested for linearity. These parameters were investigated in a pool of liver microsomes originating from four term PND 26 animals (three males and one female). This pool was created by diluting the samples to the same concentration (2000 µg/ml) and the same volume (62.5 µl). Next, all diluted samples were pooled and mixed to a final volume of 250 µl. After this optimization step, the final MP concentration and substrate concentration were set to 50 µg/ml MP and 10 µM UGT multienzyme substrate, respectively. The final reaction volume (100 µl per well) consisted of 50 µl 2X sample premix (50 µg/ml MP and 0.25 µl Alamethicin), 2.5 µl 10X working solution (10 µM UGT multienzyme

substrate), 27.5 µL UGT assay buffer and 20 µL UDPGA or an equal amount of UGT assay buffer for the minus-UDPGA samples. In the first step, the 2X sample premix was prepared by adding Alamethicin to the pig liver microsomes. This mixture was kept on ice and incubated for 15 min. The 10X working solution and UGT assay buffer were added, followed by incubation at 37°C for 5 min. To start the reaction, 5X UDPGA was added, except for the minus-UDPGA samples. Immediately after, the 96-well plate was placed in a preheated Tecan Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland) at 37°C, and fluorescence was measured at Ex/Em = 415/502 nm in kinetic mode for 40 min. Reaction velocities were calculated as recommended by the manufacturer and expressed in picomoles of UGT multienzyme substrate modified per minute per milligram of microsomal protein (pmol/min/mg MP). The LLOD and LLOQ were 0.003 nmol and 0.005 nmol, respectively. The data represent the mean value for each sample obtained in two technical replicates. Prior investigation showed that duplicates for this assay led to a CV within limits (< 10%).

4.3.4 Mathematical and statistical analyses

Reaction velocities for both experiments were calculated with Microsoft Excel® 365 (Microsoft Corporation, WA, United States). Values below the LLOQ were not considered for the statistical analysis. The reaction velocity was log-transformed for both assays to meet the assumptions of normality and homoscedasticity, which were tested by the Shapiro-Wilk and Levene's tests, respectively. Statistical analyses and graphs were performed and created in JMP® Pro 16 (SAS Institute Inc., NC, United States). A *p*-value smaller than 0.05 was considered statistically significant. Both CYP and UGT data were fitted to a linear mixed model to assess the postnatal ontogeny profile. The 2-way interactions between age, group, and sex were included as fixed effects. Run-by-plates was added as a random effect to the model to correct for inter-run variability. A stepwise backward approach was used to simplify the starting model. Thus, all non-significant effects ($p > 0.05$) were removed. Tukey's honest significance *post hoc* test was used to identify differences between age groups. Term PND 19 samples were not included in this analysis since no preterm counterpart was present. An unpaired Student's t-test was performed to investigate the birth effect (PCA vs PNA). Therefore, a comparison between (1) term PND 0 and preterm PND 11 and (2) term PND 19 and preterm PND 26 was conducted, as these subgroups shared nearly the same PCA.

4.4 Results

4.4.1 Postnatal CYP3A enzyme activity

All preterm PND 0, 5, and 11 and term PND 0 and 5 values were below the LLOQ and were not included in the statistical analysis. The 2-way interactions of age*sex and group*sex were not significant ($p = 0.8925$ and $p = 0.9631$, respectively). The age*group interaction could not be assessed because only one preterm group (PND 26) was above the LLOQ. There was no significant effect of sex ($p = 0.9818$). However, a significant impact of age ($p = 0.0402$) and group ($p = 0.0021$) on CYP3A activity was detected. Accordingly, a considerably higher CYP3A enzyme activity in term-born piglets compared to preterm-born piglets was observed. On the other hand, a significantly

higher CYP3A enzyme activity at term PND 26 compared to term PND 11 was detected ($p < 0.0001$) (**Figure 1**).

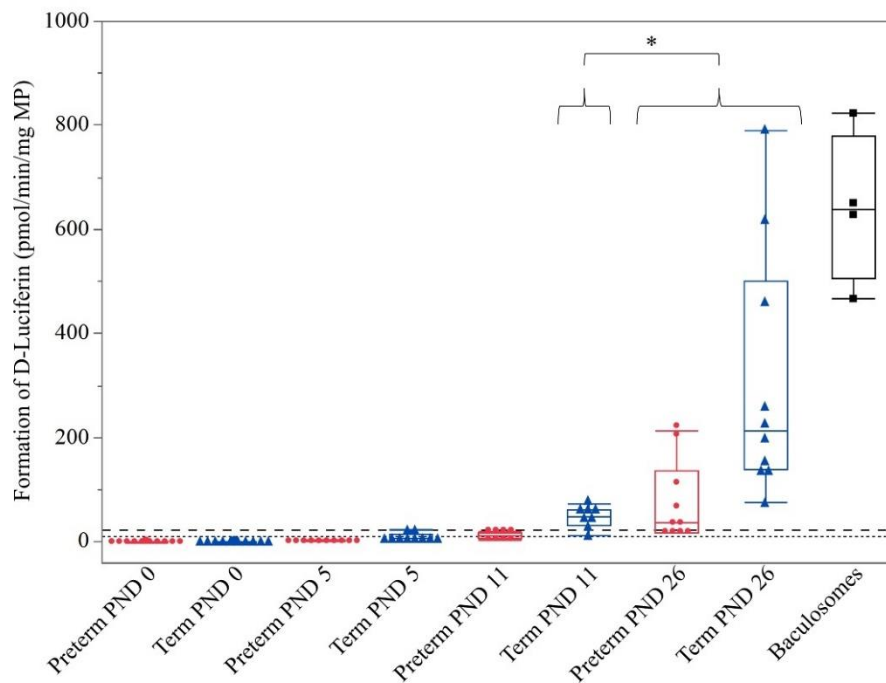

Figure 1: Hepatic CYP3A enzyme activity (D-Luciferin in pmol/min/mg MP) in preterm (red) and term-born (blue) piglets at different postnatal ages (PND). The mean value of three technical replicates for each sample is represented by a red dot (preterm group), a blue triangle (term group), or a black cube (baculosomes, positive control). A significantly higher CYP3A enzyme activity was detected at (term, preterm) PND 26 compared to (term) PND 11 (*: $p = 0.0421$). Irrespective of age, significantly higher CYP3A enzyme activity was detected in the term group compared to the preterm group ($p = 0.0021$; not shown in the graph for visual purposes). The upper (dashed) and lower (dotted) horizontal lines represent the LLOQ and LLOD, respectively. Values below the LLOQ were not considered for statistical analysis.

4.4.2 Effect of postconceptional age on CYP3A enzyme activity

Term PND 0 and preterm PND 11 were below the LLOQ. As no preterm PND 19 samples were available for comparison with CYP3A enzyme activity in term PND 11 animals, only term PND 19 and preterm PND 26 values were compared. No significant difference between both groups was detected ($p = 0.4621$) (**Figure 2**).

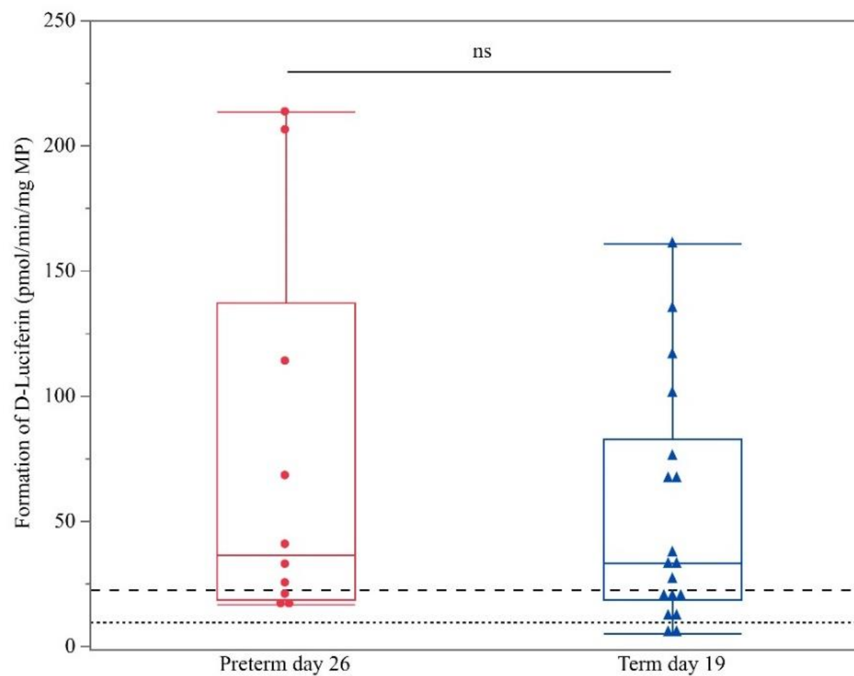

Figure 2: Hepatic CYP3A enzyme activity (D-luciferin in pmol/min/mg MP) in term PND 19 and preterm PND 26 piglets. These subgroups were compared as they shared nearly the same postconceptional age. The mean value of three technical replicates for each sample is represented by a blue triangle (term PND 19) or a red dot (preterm PND 26). The upper (dashed) and lower (dotted) horizontal lines represent the LLOQ and LLOD, respectively. Values below the LLOQ were not considered for statistical analysis. No statistically significant difference was observed (ns).

4.4.3 Postnatal UGT enzyme activity

Values for all age groups were above the LLOQ. Thus, all were included for statistical analysis. The 2-way interactions of age*group, age*sex and group*sex were not significant (p = 0.9433, p = 0.5598 and p = 0.1759, respectively). There was no significant effect of sex (p = 0.3662). However, a significant effect was detected for age (p < 0.0001). A gradual postnatal increase was observed from PND 0 until PND 26 for the formation of glucuronidated UGT multienzyme substrate (**Figure 3**). Irrespective of PNA, significantly higher glucuronidation was detected in the term group compared to the preterm group (p < 0.0001) (**Figure 3**).

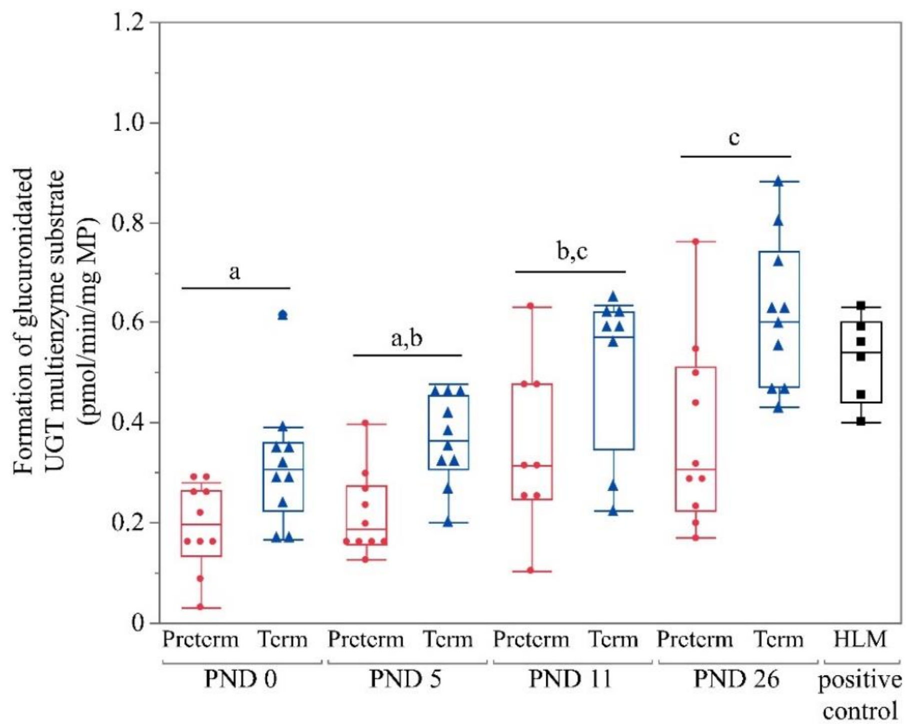

Figure 3: Hepatic UGT enzyme activity in preterm and term-born piglets (glucuronidated UGT multienzyme substrate in pmol/min/mg MP). The mean value of two technical replicates for each sample is represented by a red dot (preterm group), a blue triangle (term group), or a black cube (HLM, positive control). All values were above the LLOQ and thus included in the statistical analysis. Statistically significant age-related differences are indicated by characters ($p < 0.05$). Irrespective of age, significantly higher glucuronidation was detected in the term group compared to the preterm group ($p < 0.0001$).

4.4.4 Effect of postconceptional age on UGT enzyme activity

No significant difference was present between term PND 0 and preterm PND 11 ($p = 0.6154$) nor between term PND 19 and preterm PND 26 ($p = 0.7343$) (**Figure 4**), which shared approximately the same PCA.

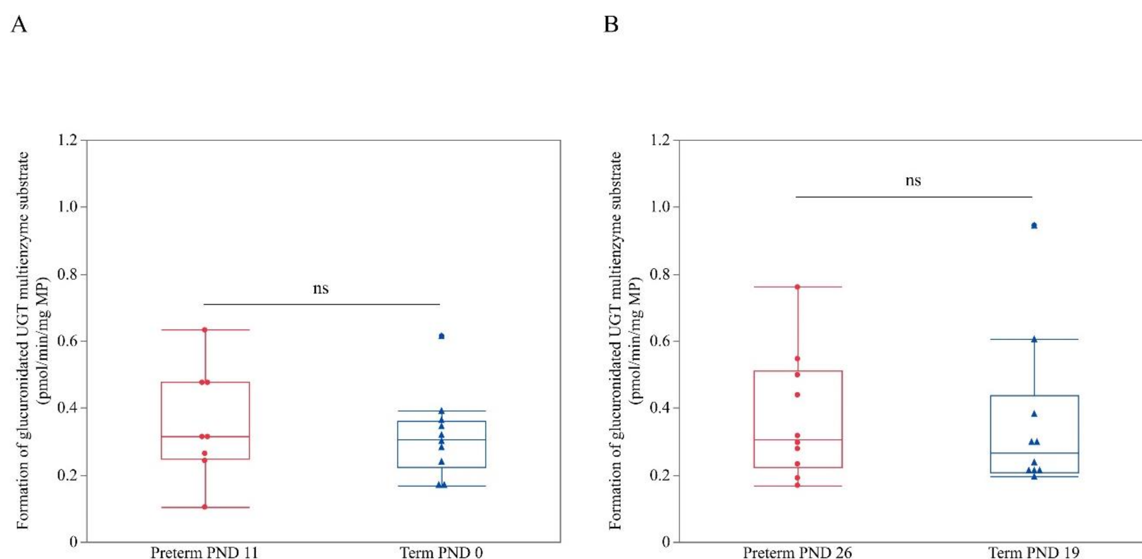

Figure 4: Hepatic UGT enzyme activity (glucuronidated UGT multienzyme substrate in pmol/min/mg MP) in preterm and term-born piglets. An unpaired Student's t-test was performed to compare UGT enzyme activity between term PND 0 and preterm PND 11 (A) and term PND 19 and preterm PND 26 (B) as these subgroups shared nearly the same postconceptional age. The mean value of two technical replicates for each sample is represented by a blue triangle (term) or a red dot (preterm). No statistically significant differences were observed (ns).

4.5 Discussion

The present study aimed to investigate CYP3A and UGT activity in the preterm piglet as a translational model for the biotransformation capacity in human (pre)term newborns. This is the first study to examine the ontogeny of hepatic phase I (CYP3A) and phase II (UGT) drug metabolism in the preterm-born piglet. Moreover, we wanted to address whether PCA or PNA drives enzyme activity.

4.5.1 Postnatal CYP3A enzyme activity

Pig CYP3A enzyme activity was determined in liver microsomes using the P450-Glo^{TM} CYP3A4 assay. This assay contained a highly specific human CYP3A4 substrate, Luciferin-IPA, which was shown to be metabolized by minipig CYP3A isoforms before [20]. Since all porcine CYP3A4 orthologs (i.e., CYP3A22, CYP3A29, CYP3A39 and CYP3A46) are present in liver microsomes, no distinction was made between isoform specific activity levels in this assay. However, earlier research detected these individual isoforms at mRNA level [32-34] and protein level [22, 25, 35, 36] with the latter showing the highest abundance of CYP3A22 in developing piglets [25]. In the preterm group, enzyme activity was only detectable at PND 26. All other age groups were below the LLOQ. In the term group, a postnatal increase in CYP3A activity was detected from PND 11 onwards. The younger age groups were below the LLOQ. These results in term-born piglets are in accordance with earlier research, in which minipig CYP3A enzyme activity was detected in term-born piglets at PND 7 with a significant increase towards PND 28 [20]. In the same study, fetal samples (gestational days 84-

86 and 108), corresponding in part to PND 0 of our preterm samples, and neonatal samples (PND 1 and 3) were included, for which values were below the LLOQ [20], as is the case in our study.

Considering the term CYP3A ontogeny profile, our results align with several human studies. CYP3A4 mRNA expression and enzyme activity are detected at very low levels in the fetus and start increasing at birth [14, 37, 38]. Later, human CYP3A4 mRNA expression, protein abundance, and enzyme activity gradually increase during postnatal life to reach 50% of adult values by 6 to 12 months of age [12, 37-39]. Although a limited number of age groups were included in this study, one can say that a similar CYP3A ontogeny pattern is observed in the pig. We focused on the pre-weaning phase, but previous research showed that CYP3A enzyme activity continues to increase gradually, after the neonatal stage, as described in humans. Millecam et al. used midazolam metabolism to measure CYP3A enzyme activity and presented a gradual postnatal increase during maturation in conventional pigs with ages ranging between PND 2 and 7 months of age [22]. This pattern was also observed in Göttingen Minipig liver microsomes incubated with midazolam. The formation of 1-OH-midazolam increased from PND 3 onwards to reach 40% of adult biotransformation capacity at PND 28 [21]. In addition, no sex-related differences in CYP3A enzyme activity ontogeny were reported in these studies [21, 22], as is the case in this work. It should be considered, however, that the animals included here did not reach sexual maturity yet and that sex-related differences indeed are observed in adult pigs [25, 40].

Next, the outcome of the term group is also in accordance with studies investigating pig CYP3A mRNA expression and protein abundance. Research has shown that neither enzyme activity nor protein abundance or mRNA expression is sufficient to predict PK characteristics as sole criterion [41, 42]. Knowing the interplay of all of them is necessary to understand the underlying mechanisms of DME development. Rasmussen et al. detected significantly higher CYP3A mRNA expression in a similar breed of adult domestic pigs (Danish Landrace x Large White x Duroc) compared to fetuses (90 days of gestation) [43]. Even in the earlier stages of life, differential gene expression was already detected between fetal (100 days of gestation) and neonatal (PND 1) Göttingen Minipigs, with the latter having higher CYP3A mRNA expression [44]. Western blot experiments showed higher protein abundance over time in pigs, but this method often lacked sensitivity. Protein expression could be measured in adult animals, but no signal was retrieved from fetal samples [43]. The introduction of proteomic approaches in recent years has overcome this practical challenge. LC-MS/MS experiments in both conventional pigs and Göttingen Minipigs showed a gradual increase in CYP3A protein abundance from the fetal stage onwards, which is in line with our findings [22, 25].

4.5.2 Effect of postconceptional age on CYP3A enzyme activity

GA, PNA and PMA are covariates used to identify age effects (i.e., predetermined "biological clock" (GA, PMA) or birth (PNA)) on, *inter alia*, drug metabolism maturation in humans. Since PMA cannot be used in pigs, PCA is used as an alternative measure in our study. The preterm and term group showed no statistically significant difference between term PND 19 and preterm PND 26, which shared approximately the same PCA. This suggests that PCA rather than birth (PNA) affects the onset of CYP3A enzyme activity in the pig. Several human studies have shown that CYP3A activity

is less mature in preterm infants compared to term newborns as measured by midazolam clearance [45-49]. However, research is not conclusive on whether GA, PNA or PMA affects the onset of CYP3A enzyme activity. For example, Allegaert et al. demonstrated PMA to be a better indicator than PNA during early life for *in vivo* CYP3A4 enzyme activity after a continuous tramadol infusion [50]. Also for other CYPs, PMA is proposed to be a better predictor than PNA since maturation occurs already before birth (e.g., CYP2D6) [51-53]. Next, Jacqz-Aigrain et al. determined a significant correlation between GA and midazolam clearance in preterm neonates (32.8 weeks GA) after a continuous midazolam infusion [49]. The same results were obtained in a population PK study of midazolam, including (pre)term neonates born between 26 and 42 weeks of gestation [54]. Moreover, the latter study postulated that PNA did not affect midazolam kinetics [54]. Other studies, however, did not detect any correlation between midazolam clearance and age, neither for GA nor PNA [37, 46, 47]. Caution is thus needed when drawing conclusions, especially when comparing species. One should acknowledge that most research assessing the relationship between CYP3A ontogeny and GA and PNA in humans is somewhat limited and was conducted twenty years ago. The current findings illustrate the importance of comprehensive PK research in preterm babies and pigs to make comparison possible.

4.5.3 Postnatal UGT enzyme activity

A UGT multienzyme substrate (specific for human UGT1A1, UGT1A3, UGT1A6, UGT1A9, and UGT2B7) was used to investigate the developmental pattern of UGT activity in preterm and term-born piglets. In contrast to the CYP experiment, UGT activity was detected for all age groups in both cohorts. In general, a gradual postnatal increase in enzyme activity during the first month of life was observed. However, a higher overall UGT activity was detected in the term group compared to the preterm group. These results are in accordance with earlier *in vitro* research in Camborough-29 pigs [28] and Gottingen Minipigs [21], which showed low UGT activity at birth, followed by an increase with age. No sex-related differences were observed, which agrees with earlier research [21].

Similar amino acid identities for the investigated UGT isoforms (~ 70 – 80%) [36, 55] and substrate-specificities (e.g., propofol, morphine, and ibuprofen) between (mini)pigs and humans have been reported [27, 56-58], but the comparison between ontogeny profiles has not been described until now. In humans, it is generally stated that hepatic UGT enzyme activity is detected from the late second or early third trimester of gestation, followed by a boost after birth reaching full capacity between 3 months (e.g., UGT1A1) and 20 years of age (e.g., UGT1A6) [59-66]. As a UGT multienzyme substrate was used in this study, a direct comparison between human and pig UGT isoforms is not possible. Though, it can be confirmed that UGT enzyme activity rises after birth during the first month of life in the pig, as is the case in human neonates.

4.5.4 Effect of postconceptional age on UGT enzyme activity

Several studies in preterm and term-born human neonates indicated that the development of UGT activity occurs irrespective of GA [59, 60, 67]. In fact, a positive correlation between UGT activity

(e.g., UGT1A3, UGT1A6, UGT1A9, and UGT2B7) and PNA was described before for several substrates (e.g., morphine, propofol, paracetamol, ibuprofen, etc.) [53, 61, 66, 68-72]. These observations contrast with what is detected in the present study: the lack of significant differences between equivalent PCA, namely (1) term PND 0 and preterm PND 11 and (2) term PND 19 and preterm PND 26, suggests that PCA and not PNA affects UGT development in the pig. It is thus tempting to assume that UGT development is regulated differently in humans and pigs. However, as we used a UGT multienzyme substrate in our study, studies with UGT-specific isoform substrates should be performed to either confirm or reject this assumption. Further characterization of UGT development in the preterm pig model is evidently needed.

4.6 Conclusion

This study was the first to investigate hepatic CYP3A and UGT enzyme activity in preterm and term-born piglets. CYP3A enzyme activity was only detected in preterm PND 26 piglets, while a gradual increase was observed in term-born piglets from PND 11 onwards. UGT enzyme activity showed a significant increase between PND 0 and PND 26 in both preterm and term-born piglets. The activity of both studied enzymes was lower in the preterm piglets, irrespective of PNA, suggesting that PCA is affecting CYP3A and UGT enzyme ontogeny in the pig. Our data are a valuable step forward in the characterization of the preterm piglet as a translational model for hepatic drug metabolism in the preterm human neonate.

4.7 References

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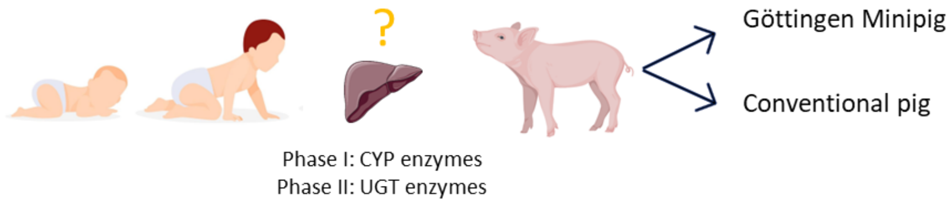
CHAPTER 5:
General discussion

ADME processes in (preterm) neonates, infants and children are substantially affected by growth and development. Thorough understanding of these characteristics is needed to comprehend how drug disposition occurs in this vulnerable population. However, several knowledge gaps still remain in the youngest age groups and this increases their risk for adverse drug effects. In particular, the ontogeny of hepatic phase I and phase II DMEs in neonates, infants and children is understudied, as well as the impact of prematurity on this ontogeny. Hence, elucidation of these developmental changes is highly needed. Clinical studies in these age groups are often considered to be not feasible due to ethical and practical restraints. Alternatives are thus explored to overcome these challenges. JAS provide valuable data in this regard, but appropriate species selection remains challenging. Traditional nonrodent species (e.g., dog and NHP) are not always the most suitable model to study hepatic drug metabolism, but the (mini)pig has been shown to be a promising alternative.

This thesis aimed to assess the potential of the (mini)pig as a translational model for biotransformation in human neonatal and juvenile populations (**Figure 1**). Therefore, hepatic phase I (CYP) and phase II (UGT) enzyme ontogeny patterns were investigated in developing Göttingen Minipigs and neonatal preterm and term-born domestic piglets. The results presented here provide another piece of the puzzle in characterizing the (mini)pig as a (preterm) neonatal and juvenile animal model and support the use of this species in PDD. In this discussion, we will further elaborate on how hepatic drug metabolism evolves beyond two years of age, to what extent biological levels such as mRNA, protein abundance and enzyme activity affect data interpretation and how prematurity affects ADME in other organ systems.

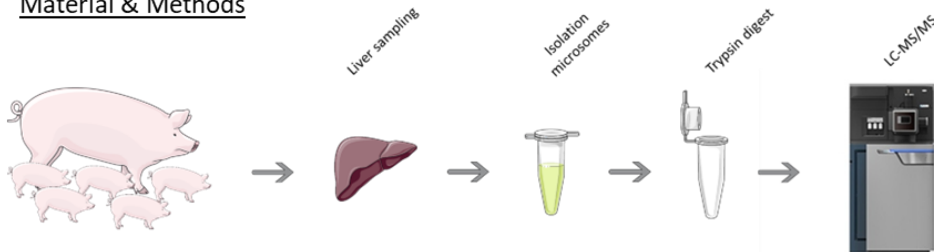
Background & research question

The pediatric population is a vulnerable group within the human population. Clinical studies are limited due to ethical and practical challenges which restrict the development of drugs in their interest. Alternatives are thus required. In this thesis, the translational value of neonatal and juvenile (mini)pigs for human pediatric hepatic drug metabolism was investigated.



Part 1: Characterization of CYP protein abundance in fetal, neonatal, juvenile and adult Göttingen Minipigs

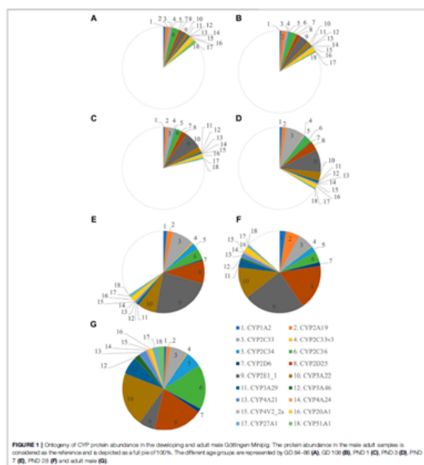
Material & Methods



Results

- Various CYP protein abundance ontogeny profiles were detected
- In general: CYP protein abundance increases postnatally with age
- Higher CYP protein abundance in adult females compared to males

≈ man



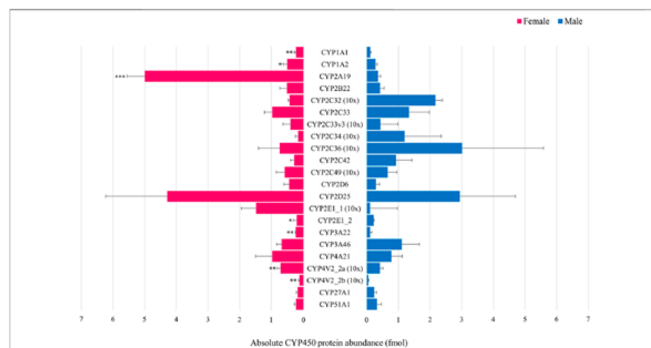
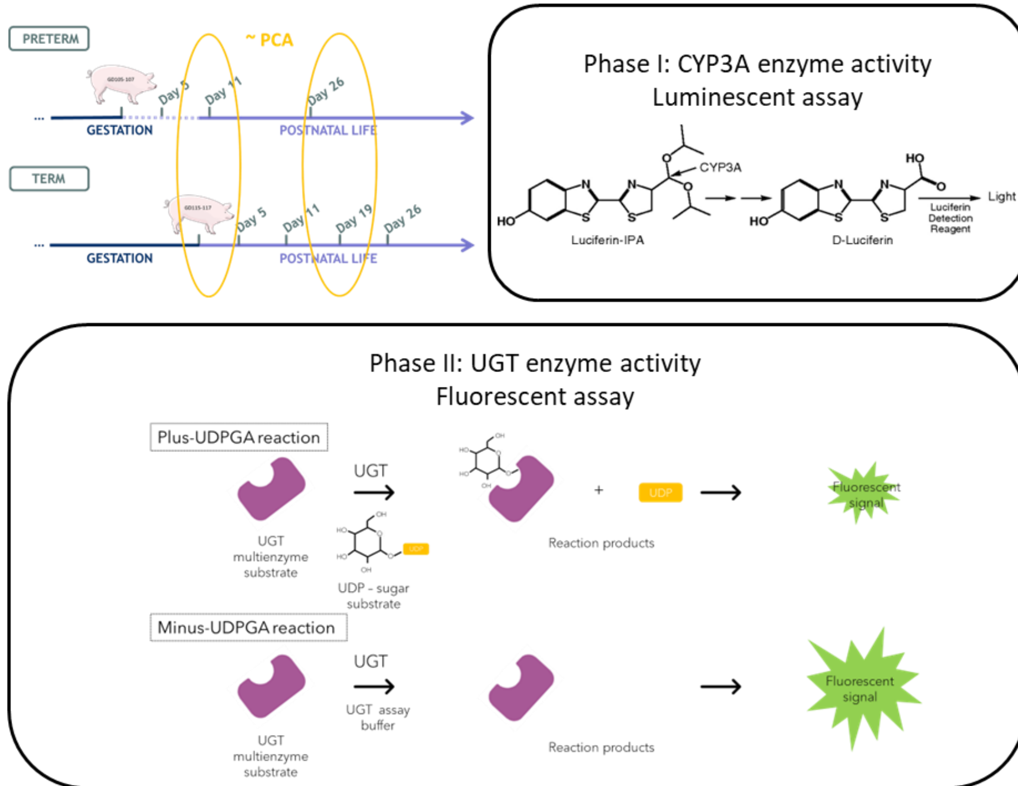


Figure 1: Summarizing figure of the research presented in this thesis.

Part 2: Characterization of CYP3A and UGT enzyme activity ontogeny in preterm and term-born conventional pigs including the effect of postconceptional and postnatal age

Material & Methods



Results

- CYP3A enzyme activity:
  - Preterm: only detected at PND 26
  - Term: gradual increase from PND 11 onwards

  } = man
- UGT enzyme activity:
  - Preterm: significant increase between PND 0 and PND 26
  - Term: significant increase between PND 0 and PND 26

  } No direct comparison to man possible
- PCA seems to affect both CYP3A and UGT enzyme activity in the pig

Table: Age effect on CYP3A and UGT enzyme activity ontogeny

	Human		Pig
CYP3A	PMA/GA	≈	PCA
UGT	PNA	≠	PCA

Figure 1: Continued.

5.1 Hepatic drug metabolism beyond the first two critical years

The age groups included in the studies presented in this thesis cover the age range of one month up to two years of age in human. Although it is recognized that major anatomical and physiological changes occur during the first two years of life, maturation does not stop after this period. Growth and development continue and each organ system will sooner or later reach its adult state. It is thus interesting to have a closer look at hepatic phase I and phase II enzyme maturation beyond two years of age.

As described before in **Chapter 3**, Hines et al. classified the human DMEs and transporters in three different classes depending on their ontogeny profiles [1, 2]. In brief, Class 1 enzymes express their highest levels in the fetus (e.g., CYP3A7), Class 2 enzymes present a rather similar level throughout gestation up to adulthood (e.g., CYP2B6, and CYP3A5) and Class 3 enzymes' expression is negligible or at a very low level in the fetus and only rises after birth (e.g., CYP2C9, CYP2D6, CYP2E1, and CYP3A4) [2]. Since no increase is expected for Class 1 and 2 enzyme expression after birth, we will focus on Class 3 enzymes. Indeed, expression levels in this group increase postnatally, but the rate to reach adult levels is significantly interindividually dependent. In general, it is stated that by one to two years of age all Class 3 isoenzyme activities reached adult values [3], but this is not the case for all.

First, the onset of expression varies from the start: CYP2D6, CYP2E1, UGT1A1, UGT1A4, UGT1A6 and UGT1A9 expression begin to rise directly after birth; CYP2C9, CYP2C19 and CYP3A4 expression appear during the first weeks of life; and CYP1A2 expression occurs only by one to three months of age [4]. Second, the rate at which they reach adult values, will be markedly different. Within a **few weeks**, significant increases in CYP2D6 expression occur to mature levels [5], whereas it takes **three to six months** for UGT1A1 [6, 7] and **one to two years** after birth for CYP1A2 [8], CYP2E1 [1], CYP3A4 [9] and UGT1A9 [10, 11]. Interestingly, some studies found higher values of CYP3A4 and UGT1A9 than those observed in adults during infancy [10, 12-14]. Regarding CYP2C9, CYP2C19, UGT1A4 and UGT1A6, it lasts until **post-puberty** (i.e., between 10 and 18 years of age) for adult expression levels to occur [4, 7, 11, 15, 16]. It should be mentioned, however, that large interindividual differences were observed for CYP2C9 expression during the first five months of life [15]. Some individuals showed the same expression levels as observed during late gestation whereas others presented expression levels within the range of those found in older individuals [2, 15]. These observations illustrate the **hypervariability** that is present during the perinatal period which complicates interpretation of PK studies even more [2]. When investigating adolescents, one should consider that sexual maturation is ongoing which may impact enzyme activity as well. Lower activity of CYP1A2 and CYP2E1 and higher activity of CYP3A4, CYP2A6 and CYP2B6 were indeed detected for female compared to male individuals [17]. In contrast, higher UGT2B15 expression was reported in male compared to female individuals [18].

When comparing the above data with ontogeny profiles described in (mini)pigs, marked similarities are present but comparison remains complex (**Table 1**). Both in Göttingen Minipig and conventional

pig, a slow increase of CYP2C9- and CYP3A4-like enzyme activity was observed, whereas CYP1A2- and CYP2D6-like activity increased fast during the first weeks of life [19, 20]. The slow maturation profile of CYP2C9 and CYP3A4 corresponds well with human pediatric data, but for CYP1A2 onset appears to be much earlier in (mini)pig than in human [21]. For CYP2D6 activity the pattern appears to be comparable, but enzyme activity seems much higher than in humans [22]. Similar sex-related differences in comparison to human were found as well: CYP1A2, CYP2E1 and CYP3A4 show higher values in female versus male Göttingen Minipigs and conventional pigs after puberty [23-25].

In view of phase II metabolism, data is limited regarding studies including age groups between infancy, adolescence, and adulthood. Hu et al. determined increasing UGT activity from PND 1 until week 10, followed by a decline at week 20 in male Camborough-29 pigs [26] which is in accordance with the UGT1A9 pattern described above. Another study investigated ibuprofen metabolism in 1-, 4-, and 8-week-old, and 6-7 month-old mixed breed pigs showing an increase in UGT activity from the neonatal phase onwards [20]. The latter thus also agrees with the postnatal increase observed for UGT ontogeny in humans. In addition, higher glucuronidation was observed in female compared to male 6- 7 month old animals [27]. This contrasts with observations in humans where, except for UGT2B15, no sex-related differences are detected [18].

Finally, interpretation of porcine data and comparison with human data should be done very cautiously. Research groups may use different substrates and testing conditions which may confound the results and thus species comparison [21]. Several studies emphasize, for example, that measurements at different biological levels may affect the outcome and interpretation of ontogeny profiles within the same enzyme [4, 28-30]. In the next section, we will further elaborate on this topic.

Table 1: Overview of hepatic CYP and UGT ontogeny patterns in man, conventional pig and Göttingen Minipig based upon the classification described by Hines et al. [1]. A distinction is made between the ontogeny patterns of the different biological levels (•, mRNA expression; ■, protein abundance; ◆, enzyme activity). Isoforms that are underlined showed contrasting results from different studies, isoforms indicated in red have a differing ontogeny pattern between human, conventional pig and/or Göttingen Minipig.

Ontogeny pattern	Man [2, 5-8, 29, 31-45]	Conventional pig [20, 26, 27, 46-48]	Göttingen Minipig [19, 48-50]
Class 1 **Highest expression levels are observed in the fetus and decrease after birth**	CYP1A1•,■ CYP3A7•,■,◆ CYP4A11•,■,◆ CYP4F12•,■,◆ CYP19A1•,■,◆ CYP51A1■	CYP51A1■	CYP51A1■
Class 2 **Similar expression levels throughout gestation up to adulthood**	CYP2B6■,◆ CYP2C19■,◆ CYP3A5■,◆ CYP4A11•,■ CYP4F2•,■,◆ CYP4F3•,■,◆ CYP7B1•,■,◆ CYP8B1•,■,◆ CYP20A1•,■,◆	CYP2E1_1•,■,◆	CYP4A24■ CYP20A1■
Class 3 **Negligible or very low expression in the fetus followed by an increase after birth**	CYP1A2•,■,◆ CYP2A6•,■,◆ CYP2B6■,◆ CYP2C8•,■,◆ CYP2C9•,■,◆ CYP2C19■,◆ CYP2D6•,■,◆ CYP2E1•,■,◆ CYP2J2•,■,◆ CYP3A4•,■,◆ CYP3A5■,◆ CYP4F11•,■,◆ CYP4V2•,■,◆ CYP27A1•,■,◆ UGT1A1•,■,◆ UGT1A4•,■,◆ UGT1A6•,■,◆ UGT1A9•,■,◆	CYP1A1•,■ CYP1A2■ CYP2C34■,◆ CYP2C36■,◆ CYP2C49■,◆ CYP2D6■,◆ CYP3A22•,■ CYP3A29•,■ CYP3A46•,■ CYP4A21■ CYP4V2_2a■ CYP20A1■ UGT (multienzyme)◆	CYP1A1■ CYP1A2■ CYP2A19■ CYP2C33■,◆ CYP2C34■,◆ CYP2C36■,◆ CYP2D6■,◆ CYP2D25■ CYP2E1_1■ CYP3A22■,◆ CYP3A29•,■,◆ CYP3A46■,◆ CYP4A21■ CYP4V2_2a■ CYP27A1■ UGT (multienzyme) ◆

5.2 Which biological level is the most relevant to look at?

Several biological levels (i.e., mRNA expression, protein expression, and enzyme activity) have been investigated over the years to examine CYP and UGT ontogeny. Gene expression was often assessed in this regard as a surrogate for protein expression and enzyme activity [51], but ontogeny patterns of these parameters are not necessarily interchangeable or related to each other. Various biological factors influence gene transcription (e.g., genetic polymorphisms), mRNA translation (e.g., temperature-sensitive RNA secondary structures, ribosome occupancy and ribosomal density) and protein expression (e.g., intrinsic stability, and degradation) [51-53]. However, discrepancies between these levels provide useful insights in pre- and posttranslational regulation of these enzymes.

Next, methodological constraints may also affect comparison between mRNA expression, protein abundance and enzyme activity. For example, twenty years ago Northern and Western blot were mostly used to assess mRNA and protein expression levels, respectively, whereas nowadays RT-PCR and proteomic approaches (e.g., LC-MS) are the gold standard to investigate these parameters. This methodological shift resulted in increased sensitivity, specificity and accuracy which impacted research outcomes greatly [53]. As a result, discrepancies between studies may be explained based upon the use of different techniques. Furthermore, different biological matrices (e.g., whole liver tissue, hepatocytes, or microsomes) may influence measurements as well [54].

Regarding CYP enzymes, various studies investigated correlations between the different biological levels in humans. Sumida and colleagues showed a significant correlation between **hepatic mRNA expression and enzyme activity** for CYP3A4 whereas no significant correlation was detected for CYP2E1 in adults [55]. Rodriguez-Antona extended this research by examining several other CYPs' mRNA expression and enzyme activity [56]. High correlations were observed for CYP1A1, CYP1A2, CYP3A4, CYP2D6 and CYP2B6, but no significant correlations were retrieved for CYP2A6, CYP2C9, and CYP2E1 [56]. Other studies assessed the relationship between human **hepatic CYP mRNA expression and protein abundance**. Ohtsuki et al., for instance, observed a significant correlation between mRNA expression and protein abundance for CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4, only CYP4A11 presented a poor correlation [52]. For the latter, a possible explanation would be instability during microsome preparation and storage which illustrates the influence of extrinsic factors [52]. Another study confirmed the observations for hepatic CYP3A4 with enzyme activity being well correlated with its protein abundance, but a rather poor correlation was retrieved between mRNA expression and protein abundance [57]. Finally, Drozdzik et al. examined correlations between CYP mRNA expression and protein abundance in both the liver and intestine. In the liver, significant correlations were found for CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5 whereas in the intestine CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5 were retrieved [58]. All studies suggested that the pretranslational level is the main regulatory mechanism for enzymes showing a high correlation between mRNA and either enzyme activity or protein abundance (e.g., CYP3A4). On the other hand, if no correlation was observed (e.g., CYP2E1), regulation at multiple levels (i.e., pretranscription, transcription, pretranslational,

translational and posttranslational) is proposed [55, 56, 58]. However, a discrepancy between levels is possible as illustrated by CYP2C9: a correlation was found between mRNA expression and protein abundance [52, 58], but not between mRNA expression and enzyme activity [56]. These data suggest that several regulatory mechanisms are involved. Comparing two out of three levels is thus not sufficient for a complete understanding.

For UGT, its expression is highly influenced by the presence of genetic polymorphisms and transcription factors [59, 60]. This is illustrated by strong correlations between multiple polymorphisms and mRNA expression and/or enzyme activities of UGT1A1, UGT1A3 and UGT2B17 in human liver [61]. However, the contribution of other factors is manifested by the discrepancy between several studies. On the one hand, a **lack of correlation** between hepatic mRNA expression and protein abundance and/or enzyme activity for UGT1A1, UGT1A6, UGT1A9, UGT2B7, UGT2B15 was detected [52, 62], whereas on the other hand **a significant correlation** was retrieved for hepatic UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and UGT2B15 [58, 60, 62, 63]. In this regard, the (in)ability to differentiate between protein variants may impact research outcomes. Proteomics approaches, for instance, may not discriminate functional, incompletely expressed, and nonfunctional proteins resulting from genetic variation [62]. As a result, detection or non-detection of some variants could disrupt activity-abundance correlations [62]. To our knowledge, no information is currently available concerning relationships of the different biological levels for porcine UGT ontogeny.

In conclusion, biological levels of CYP and UGT enzymes do not necessarily correlate with each other. Regarding prediction of drug metabolism and metabolic capacity, enzyme activity may be perceived as the most accurate parameter but detection is not always feasible *in vivo*, especially in preterm neonates. Therefore, determining the relationship between the different biological levels may improve our understanding of regulatory mechanisms and possibly allows translation of existing data [56]. As such, data retrieved from all biological levels should be considered complementary and will be crucial for future pharmacological research both in human and pig.

5.3 How does preterm birth affect ADME properties in other organ systems?

Preterm neonates and infants represent a patient cohort with widely different complications, affected by GA at birth, birth mode (i.e., vaginal or cesarian section), gender, prenatal insults, genetics, postnatal morbidities and treatments [64]. It is important to understand how each of these parameters influence ADME in humans in the first place and secondly whether these effects are also detected in the preterm piglet. Understanding similarities and differences between man and animal models contributes to better interpretation and extrapolation of study results as such improving drug development for the youngest age groups.

Postnatally, preterm infants are often considered to reach a physiological maturation similar to that in term infants when they reach term-corrected PCA or PMA [64], but research has shown that this is not always the case. Some organ systems will undergo “catch-up” growth to compensate for their immature status at birth whereas others will follow their “biological clock” and continue to mature independently of their PNA. Within this regard, environmental stimuli seem to play an important role. Organs that are exposed to the environment soon after birth show increased postnatal maturation in contrast to organ systems that are protected from these stimuli.

In **Chapter 4** of this thesis, the effect of PCA and PNA on hepatic CYP3A and UGT enzyme activity was investigated in preterm and term-born piglets. Based upon the obtained results, we suggest that PCA is the main driver for their maturation. The question arises, however, how ADME properties in other organ systems are affected by preterm birth in human and whether the preterm pig model shares the same drivers.

5.3.1 Absorption

5.3.1.1 Gastro-intestinal tract

GIT development is probably the most intensively investigated subject within the preterm research field. Structural and functional maturation of the GIT is needed for proper digestion and absorption of colostrum and breast milk [65] and also impacts drug disposition. However, functional processes (e.g., gastric acid production, and gastric motility) only start maturing by (roughly) 20 weeks of gestation or even later [65] which results especially for preterm neonates born <28 weeks GA in reduced GI capacities compared to term-born neonates [66-68]. In view of drug disposition, several factors in the neonatal GIT may influence ADME properties, e.g., gastric acid production, gastric emptying time, intestinal bile acid production, mucosal structure, epithelial permeability to macromolecules, absorptive surface area, digestive enzyme capacity, and the establishment of the postnatal microbiome [54]. We will discuss gastric pH and gut motility in more detail below since they are acknowledged as major contributing factors for drug absorption.

Gastric pH is considered to be crucial as it affects drug stability, dissolution and degree of ionization which all impact absorption [69]. In human neonates, **regardless of GA at birth**, gastric pH is high at birth (~ pH 7.05) and drops steadily within the following weeks (~ pH 3 – 3.5) to finally reach adult values by six months to two years of age (~ pH 1.5 – 2.5) [70-72]. However, varying pH values are reported during neonatal life and especially large differences are observed based on the feeding status of the investigated individuals. Indeed, the presence of food may change gastric pH substantially. Preterm infants, for example, receive acid buffering milk which may result in higher gastric pH values than those observed in term infants, as such affecting drug absorption [69]. Since sampling conditions (e.g., fasted or nonfasted state) often lack in reported literature, interpretation of varying pH values is difficult and no clear consensus on gastric pH ontogeny is currently available [73]. It is nevertheless important to understand how gastric pH varies in neonates compared to older age groups as it may lead to greater (e.g., acid-labile compounds, and weak bases) [74] or

reduced (e.g. weak acids) [75] absorption at the level of the stomach (i.e., due to the more alkaline environment) which consequently affects the required dose.

Research conducted in both domestic pigs and Göttingen Minipigs showed similar patterns as to what is observed in humans. A more acidic gastric pH was observed at 4 weeks PNA compared to younger age groups in Göttingen Minipigs [76]. This age-related gastric pH decrease was also described in domestic piglets when comparing different age groups around weaning [77]. Moreover, weaning appears to affect ontogeny as well since pH values in suckling piglets did not drop below 2.5 whereas pH values of 1.6-1.7 were detected at one and two weeks postweaning [77]. Finally, highly variable pH values (range 1.15 to 6) due to, *inter alia*, feeding status were described in a compilation study by Suenderhauf and Parrott in Göttingen Minipigs [78], which agrees with observations in humans as well.

Next, **gut motility** affects drug absorption by influencing peristalsis and as such the rate of exposure between the compound and the epithelial (absorptive) surface. Depending on this exposure, it may for instance take longer for a drug to reach the bloodstream and its maximum plasma level [67, 68, 75, 79]. As such, dosing regimens should be adjusted accordingly. At the level of the stomach, **gastric emptying time** is coordinated by different muscular interactions [80], but enteral food intake seems to play a role as well (e.g., formula or breast milk, caloric intake, protein type, and fat type) [54]. In general, it is stated that gastric emptying time is slower in neonates compared to adults, and preterm infants present prolonged emptying times relative to term neonates although reported data are highly variable [81-83]. The suggested reduced gastric emptying time and its effect on drug absorption is illustrated by a study investigating serum concentrations of chloramphenicol in preterm and term-born neonates in which dosing had to be increased for preterm neonates in order to obtain adequate serum levels [84]. Regarding **intestinal transit time** (measured by e.g., oral-cecal transit or small intestinal transit time), it is generally assumed that preterm infant intestinal transit time takes four times longer than adult transit time, whereas term infant transit time approximates adult values [67]. Immature proximal duodenal motor activity [80] and enteric motor system activity [85] would for instance contribute to this reduced motility and require postnatal maturation in contrast to term-born neonates. Although these observations suggest that GA is responsible for gut motility maturation, a recent meta-analysis of 49 published studies showed that **neither GA nor PNA** explained the variation in intestinal transit time in preterm neonates [86]. Interestingly, the same outcome was obtained in a meta-analysis of 66 published studies assessing gastric emptying time [87].

In order to unravel the mechanisms behind preterm GIT development, especially regarding nutrient intake and NEC, extensive research has been performed in the preterm pig model [88-93]. Moreover, Ren and colleagues recently proposed that PNA rather than advancing PCA is a key determinant of gut structure maturation (e.g., gut weight, villus height, and goblet cell density) in preterm pigs [64]. However, gut motility data in (preterm) neonatal pigs is limited. Amdi et al. showed similar gastric emptying rates between intrauterine growth-restricted (IUGR) and normal-weight domestic piglets [94]. Next, Groner et al. investigated small intestinal motility by

myoelectrical activity in neonatal piglets (PND 12 – 27), and reported similar activity patterns as seen in human neonates [95]. Detailed information, especially in the preterm piglet, concerning gut motility is thus lacking. In this sense, it is rather unique that (preterm) neonatal GI transit is better studied in humans than in a nonclinical species [54].

5.3.1.2 Skin barrier

Varying thickness of the skin in preterm neonates and their increased vulnerability for topical exposure was already mentioned in the introduction of this thesis (**Chapter 1**). Indeed, preterm-born infants have an underdeveloped epidermal barrier with few cornified layers [96, 97]. Depending on their GA, the stratum corneum (SC) will be nearly absent (i.e., 23 weeks GA) or will consist of a few layers (i.e., 26 weeks GA) [96, 97]. By 30 weeks of gestation, the barrier is still not fully competent [98]. Consequently, preterm neonates are at high risk for percutaneous toxicity. They absorb topically administered drugs to a higher extent than older children and adults resulting in increased blood levels and drug effects. Several cases illustrate this phenomenon: pentachlorophenol in laundry products [99], boric acid in talcum powder for diaper dermatitis [100], corticosteroid ointments for active atopic dermatitis [101], and hexachlorophene in antibacterial liquid soap and baby powder [102, 103] all resulted in severe adverse effects and even death due to increased absorption at the epidermal barrier [104]. It is thus clear that dosing regimens for topical application should be considered very carefully. A recent study investigating skin barrier integrity between preterm-born infants at full-term corrected age and full-term infants showed in this regard that **both GA and PNA** influence skin barrier properties [98]. As such, both should be taken into account for dose setting.

To our knowledge, no information is currently available regarding the skin barrier in preterm-born piglets. However, several studies mimicking pediatric human skin by tape-stripping adult porcine skin are found [105-107]. In these experiments, the SC is stripped off until a similar transepidermal water loss value is obtained as in the pediatric age group of interest. In addition, pig skin is known to be a good alternative for human adult skin due to their similar histological and physiological characteristics [108]. Consequently, pig skin has been used on a long-term basis as a model for, *inter alia*, wound healing [109-111], gene expression [112, 113], and skin drug bioavailability [114-116].

5.3.2 Distribution

Once drugs reach the systemic circulation, either directly via intravenous administration or indirectly after absorption through enteral (i.e., orally) or parenteral (e.g., percutaneous, intramuscular) routes, they will distribute to the various body compartments, tissues or cells [117]. The rate at which this movement happens, depends on the drug's physicochemical properties (e.g., lipophilicity, molecular weight, degree of ionization etc), as well as on population specific characteristics such as body composition, protein binding and concentrations, hemodynamics, and natural barriers (e.g., blood-brain barrier and placenta) [75, 118, 119].

Body composition changes markedly with age, as such affecting the physiological space in which the drug will distribute. Neonates and young infants have a higher proportion of total body water to body weight in comparison to older children and adults, with preterm neonates showing even higher values than term neonates [75, 120]. The ratio total body water to body weight constitutes ~90% in preterm and ~ 70 – 80% in term neonates which progressively decreases to ~60% by one to two years of age and reaches adult values by twelve years of age [121, 122]. In contrast, total body fat can be as low as 1% of total body weight in extremely preterm neonates whereas in term neonates, infants and adolescents values reach 10 – 15%, 20 – 25%, and 10 – 15%, respectively [121]. Depending on the drug's lipophilicity, large variations in distribution due to body composition may thus occur. Aminoglycosides are a well-known example to illustrate this: these drugs are highly water-soluble so the volume of distribution will be higher in preterm neonates compared to infants or children. As a result, when the same dose (mg/kg) is administered, the peak plasma concentration will be lower in preterm neonates and a loading dose may be required (but potentially with increased time intervals for consecutive administrations to avoid toxicity due to accumulation) [117, 122, 123]. The opposite is true for lipophilic drugs (e.g., diazepam, and propofol) as their distribution will be limited, thus reaching peak plasma concentrations faster [124, 125].

Protein binding also affects distribution. A lower or higher binding capacity will influence the volume of distribution and consequently the free fraction of the drug which exerts the pharmacological effect [118]. The most relevant binding proteins are albumin, α-1 glycoprotein and plasma globulins [69]. Since adult protein levels are reached during infancy (i.e., full binding capacity by one year of age), the effect of immature binding capacity is most pronounced in neonates and young infants [117]. Protein concentrations are lowest in preterm neonates and will **gradually increase with GA** [117, 126]. The reduced protein binding capacity in this group is especially of importance for drugs that are highly protein bound and have a narrow therapeutic index [127]. In fact, use of this kind of drugs (e.g., phenytoin, and ceftriaxone) is even contraindicated in the youngest age groups because they will compete with endogenous unconjugated compounds. [69, 117, 128]. Displacement of bilirubin, for instance, potentially results in kernicterus which should be avoided [117].

Differences in **hemodynamic parameters** influence distribution as well. Reduced cardiac output, organ blood flows and tissue perfusion in neonates, especially preterms, results in a slower onset time for intravenous induction compared to older children [124, 126]. Moreover, distribution to relatively under-perfused tissues of the body is delayed due to slower regional perfusion [124]. Consequently, it may take longer for the free fraction to reach its site of action.

Natural barriers such as the **blood-brain barrier** (BBB) further alter distribution. This network of complex tight junctions, extracellular matrix components and transporter mechanisms restricts diffusion between blood and brain [124, 129]. Moreover, protein binding, the drug's degree of ionization and body composition will affect penetration as well. For example, adults with an intact and functional BBB present slow crossing of drugs that are tightly protein bound and highly ionized

(e.g., morphine) [130]. In term neonates, a functional BBB is established at birth but barrier mechanisms are dynamically modulated postnatally to provide the optimal environment for the developing brain (e.g., amino acid transfer is facilitated) [131, 132]. Since the BBB is functional from 8 weeks of gestation onwards, the same rationale applies to preterm neonates [132]. Next to increased permeability for nutrients, higher concentrations of drugs with usually limited permeability may be found, together with increased efficacy at a lower drug dose [133]. Thus, in contrast to what is often believed, highly active transport systems rather than an immature BBB are responsible for the increased neonatal susceptibility for adverse drug effects [134]. In conclusion, it is important to take the continuing maturation of BBB functionalities into account when considering dose setting in (preterm) neonates. This is illustrated by several studies optimizing dosing regimens for acyclovir (i.e., treatment for neonatal herpes simplex virus), which consider PMA to avoid adverse reactions [133].

Regarding distribution parameters in the pig, Gasthuys et al. showed that body composition and protein binding are rather different between neonatal humans and pigs [135]. Pigs have a larger water to fat ratio (i.e., ~ 80%) after birth when compared to humans (i.e., ~ 70%), but reported differences disappear by infancy [135]. Additionally, humans show an increase in fat content during infancy followed by a decrease towards adulthood, whereas in pigs only an increase is observed [135]. Furthermore, albumin concentration is markedly lower in neonatal pigs (i.e., ~20% of total protein) in comparison to humans (i.e., ~50 – 60% of total protein). Differences in α-1 glycoprotein were also detected but declined by 48 hours after birth [135]. In conclusion, the resulting disparities in distribution parameters lead to differences in neonatal drug distribution between both species although these differences seem to diminish when reaching infancy.

5.3.3 Excretion: focus on renal clearance

Glomerular filtration, tubular excretion and tubular reabsorption comprise the three functions that determine renal activity. Research conducted in the seventies showed that glomerular functions were more predominant than tubular functions in infants below 32 weeks of gestation [136]. This is not surprising as tubular excretion and reabsorption are known to only reach adult levels by 15 months and 24 months of age, respectively, whereas GFR reaches mature values by 3 to 5 months of age [69, 137]. Although very little is known about tubular ontogeny processes until today [138], it is assumed that both GFR and tubular processes are lower in preterm compared to term-born neonates, as such leading to decreased clearance capacity. This assumption was confirmed by a lowered amikacin and vancomycin clearance in preterm compared to term neonates at birth, and was still observed at 4 weeks PNA [139]. Moreover, reduced amikacin clearance has been used as a valuable marker for lowered GFR in preterm neonates [140]. These observations are important since they illustrate the increased risk for high toxic drug levels in this vulnerable population. On the other hand, subtherapeutic dosing is also described in preterm infants e.g., intravenous administration of epoetin-beta led to a ~5% urinary loss because of the immature kidney [141].

Regarding the effect of preterm birth on renal clearance and GFR in particular, it was for a long time believed that GA and PMA were the most accurate descriptors [142-146]. However, recent studies

showed that **not only GA but also PNA affects GFR values**. For example, a study investigating the effect of birth on inulin clearance by a modeling approach showed that GFR increased both in function of GA and PNA [147]. In this study, preterm compared to term-born neonates of the same PMA presented higher GFR values, as such suggesting faster ontogeny [147]. Similar to the latter, fentanyl [148] and propofol [149] clearance was shown to be affected by both GA and PNA in preterm-born neonates hence suggesting that both should be considered when predicting dosing regimens.

A study comparing term neonatal renal function in humans and pigs showed a similar low GFR at birth with an increase during the first weeks of life [135]. Although GFR maturation was slower in pigs (i.e., due to slower nephrogenesis) than in man, similar values were retrieved during infancy [135]. In view of prematurity, renal maturation in the preterm pig model appears to be a blind spot. Descriptions of kidney organ weight [150] and sex-related differences [151] in preterm piglets were presented before, but no data regarding the effect of preterm birth on porcine renal clearance is available yet.

5.3.4 Conclusion

Despite extensive research during the last decades, it is still unclear to what extent prematurity is responsible for immature ADME functioning. It remains difficult to distinguish the specific effect of reduced PCA/PMA at birth from the consequences of intrinsic and extrinsic factors that present at or after birth. Moreover, widely different cofactors (e.g., GA at birth, birth mode, gender, prenatal insults, genetics, postnatal morbidities etc.) within the preterm population complicate our comprehension [64]. Several studies characterizing ADME in the (preterm) neonatal pig model have been conducted, but various knowledge gaps remain. Further exploration of the maturation of these parameters is thus needed.

5.4 General conclusion and future perspectives

The goal of this thesis was to further characterize hepatic drug metabolism in the (mini)pig and investigate the translational value of this nonclinical species regarding human PDD, with special focus on the (preterm) neonatal and juvenile population.

The following results were obtained:

i. Various CYP protein abundance ontogeny profiles were observed in the developing Göttingen Minipig, analogous to what is described in humans.
ii. CYP protein abundance was detected from the fetal stage onwards in the developing Göttingen Minipig. In general, a postnatal increase was observed for the majority of the isoforms, which is in agreement with data obtained in the human pediatric population.
iii. Higher CYP protein abundance levels were observed in adult female compared to male Göttingen Minipigs. These results partly correlate to what is described in humans.
iv. CYP3A enzyme ontogeny in the preterm pig appears to be driven by PCA which is in accordance with data obtained in human preterm neonates.
v. PCA rather than PNA is suggested to drive UGT enzyme activity in the preterm pig which contrasts with data retrieved in humans.

The generated enzyme activity and protein abundance levels contribute to a better understanding of the biotransformation capacity in the (mini)pig. Generally speaking, **large similarities with human pediatric hepatic drug metabolism were observed which encourages the use of the Göttingen Minipig and conventional pig as nonclinical species for PDD**. However, it needs to be emphasized that nonclinical species selection for JAS is not just driven by hepatic drug metabolism, as other factors are also important. Therefore, species selection has to be considered very carefully on a case-by-case basis. Choosing the best fit is of utmost importance to retrieve useful data for the pediatric population on the one hand and reduce animal use on the other hand.

Looking ahead, further elaboration of the topic remains needed. UGT ontogeny is a largely understudied field within porcine hepatic drug metabolism. The use of isoform-specific substrates will largely contribute to the comprehension of isoform-specific ontogeny profiles which will facilitate comparison with human data. Also regarding CYP enzymes, further elucidation of enzyme functionalities is needed. It is, for example, still unclear which porcine CYP3A homologue (i.e., CYP3A22, 29, 39 or 46) resembles human CYP3A4 the most. Since all isoforms are present in liver microsomes, it is difficult to distinguish their individual characteristics. Construction of **recombinant enzymes** will be of high value in this regard. This approach allows the creation of individual isoforms, as such facilitating the assessment of their individual substrate-specificities.

Next, hepatic DMEs were investigated at different biological levels (i.e., enzyme activity and protein abundance) in this thesis. Each parameter provides useful data as discussed in the previous section, but integration of all into a **PBPK model** will lift them to the next level. By combining *in vitro*, *in silico* and *in vivo* data, robust predictive models are created for dose precision in pediatric patients

and excessive animal use is avoided. Moreover, M&S approaches are strongly supported in the latest guidelines of the FDA and EMA, especially for special populations such as the pediatric population [152-154].

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Summary

The pediatric population is one of the most vulnerable age groups within the human population, especially regarding drug development. They comprise approximately 30% of the total human population, but still very little research is conducted in their interest. It was only in the late 1990's, after the occurrence of several tragic events after drug administration which lead to toxic side effects and even death (e.g., Gray baby syndrome) that the urge was raised to improve pediatric drug development. Regulatory guidelines were issued by the FDA and EMA, providing a legal framework for pharmaceutical industry. Although pediatric drug development has been encouraged and efforts are ongoing, knowledge gaps still exist. ADME (i.e., absorption, distribution, metabolism and excretion) properties are particularly understudied in the pediatric population, possibly resulting in under or overdosing. The neonatal population is especially prone to adverse drug reactions since clinical trials in this group are often perceived as not feasible or unethical. Clinicians are thus required to administer drugs off-label (i.e., without information on safety and efficacy). To overcome this issue, alternatives are explored. The use of juvenile animal studies is supported by FDA and EMA to improve our knowledge in these vulnerable age groups. In this regard, the (mini)pig has gained ground as nonclinical species. Recently, its value as a (preterm) neonatal and juvenile animal has been underlined. Newborn piglets share the same size as human neonates and can be assessed in the same way as babies in the neonatal intensive care unit. With regard to hepatic drug metabolism, large similarities have been observed between adult humans and pigs, but data in the human and porcine (preterm) neonatal and juvenile population remain scarce. In **Chapter 1**, we elaborated on the challenges that are currently encountered during pediatric drug development and the resulting search for alternatives. In **Chapter 2**, the objectives of this thesis were formulated.

In **Chapter 3** of this thesis, we aimed to characterize hepatic phase I drug metabolism on protein level in fetal, neonatal, juvenile and adult Göttingen Minipigs. Hepatic CYP protein abundance was investigated in liver microsomes using an LC-MS/MS approach. Various ontogeny profiles were discovered: (1) CYP enzymes with a similar protein abundance from the fetal stage up until adulthood (e.g., CYP4A24, and CYP20A1), (2) CYP enzymes with a gradual postnatal increase (e.g., CYP2C33, CYP3A22, and CYP4A21), (3) CYP enzymes that reached their maximum value at weaning (postnatal day (PND) 28) (e.g., CYP1A2, CYP2D6, and CYP2E1), and (4) one atypical CYP enzyme with high levels in the fetal stage that dropped during the first month of life and increased again in the adult (i.e., CYP51A1). Sex-related differences in the juvenile minipig were only observed for CYP4V2_2a and CYP20A1 at PND 1 with highest expression in females for both isoforms. Comparison of adult female and male Göttingen Minipigs resulted in sex-related differences for CYP1A1, CYP1A2, CYP2A19, CYP2E1_2, CYP3A22, CYP4V2_2a and CYP4V2_2b with highest expression in female minipigs. Next, CYP protein abundance was correlated with earlier observed CYP enzyme activity (investigated by metabolism of isoform-specific substrates). This analysis

retrieved a clear correlation between CYP3A22 protein abundance and the metabolism of midazolam at PND 7.

In **Chapter 4,** the translational potential of the preterm pig model for human neonatal drug metabolism was investigated, as preterm-born neonates are still considered to be "therapeutic orphans" within pediatric drug development. Since their enrollment in clinical trials is hindered by several ethical and practical challenges, alternatives for precise dosing in this population should be explored. As animal data are considered to be valuable in this respect, the aim of this study was to examine the ontogeny of CYP3A and UGT enzyme activity in the liver of preterm (90% gestation, gestational day 105 – 107) and term-born (100% gestation, gestational day 115 – 117) domestic piglets. In addition, the effect of chronological (i.e., gestational age, and postconceptional age) and postnatal age (i.e., birth effect) on the onset of enzyme activity was examined. CYP3A and UGT-specific substrates were used to assess enzyme activity ontogeny profiles. UGT activity showed a significant increase between PND 0 and PND 26 in both preterm and term-born piglets. CYP3A enzyme activity was only detected in preterm PND 26 piglets while a gradual increase was observed in term-born piglets from PND 11 onwards. In both groups, enzyme activity was lower in the preterm compared to the term group, suggesting that postconceptional age rather than postnatal age is affecting CYP3A and UGT enzyme ontogeny in the pig.

The data in Chapter 3 and 4 contribute to a better understanding of the biotransformation capacity in the (mini)pig. In general, large similarities with human pediatric hepatic drug metabolism were found which encourages the use of the pig as nonclinical species for pediatric drug development. However, it should be kept in mind that depending on the organ system, different developmental timings and species-specific characteristics are present in comparison to human neonates and infants. Moreover, the age groups included in this thesis cover the age range of one month up to two years of age in human, but maturation does not stop after this period. Inclusion of older age groups is thus needed to acquire a comprehensive understanding of the entire pediatric drug metabolism maturation process. While assessing porcine hepatic drug metabolism, we focused on protein abundance and enzyme activity as biological parameters. Each of these biological levels provides valuable data, but it should be kept in mind that one single parameter does not define the entire developmental and regulatory process. The aforementioned topics are critically addressed in **Chapter 5**, together with recommendations for future research.

Samenvatting

De pediatrische populatie is een van de meest kwetsbare groepen bij de mens, vooral als het gaat om geneesmiddelenontwikkeling. Ondanks het feit dat baby's, kinderen en adolescenten bijna 30% van de volledige wereldbevolking omvatten, wordt er nauwelijks onderzoek verricht naar geneesmiddelen in hun voordeel. Pas in de late jaren '90 werd de nood aan een verbeterde pediatrische geneesmiddelenontwikkeling erkend. Verschillende tragische gevallen waarbij een verkeerde toediening leidde tot toxische bijwerkingen en zelfs de dood (bv. Gray baby syndroom tgv een overdosis chlooramfenicol) lagen hierbij aan de grondslag. De FDA en EMA brachten vervolgens verschillende regelgevingen uit die een wettelijk kader schepten en de farmaceutische industrie aanspoorden om extra inspanningen te leveren. Desalniettemin blijft een groot gebrek aan kennis over de farmacokinetiek van geneesmiddelen bij kinderen bestaan. ADME (i.e., absorptie, distributie, metabolisme en excretie) kenmerken in het bijzonder zijn onvoldoende bestudeerd in de pediatrische populatie. Dit leidt mogelijks tot onder- of overdosering van geneesmiddelen. Vooral de neonatale populatie is hierbij vatbaar voor bijwerkingen aangezien klinisch onderzoek niet mogelijk wordt geacht in deze groep. Artsen zijn dus genoodzaakt om geneesmiddelen *off-label* voor te schrijven (i.e., zonder informatie over de veiligheid of doeltreffendheid). Om dit probleem op te lossen, worden alternatieven onderzocht. Het gebruik van juveniele diermodellen wordt hierbij aangemoedigd door de FDA en EMA om zo onze kennis in deze kwetsbare groep te vergroten. Het (mini)varken lijkt in dit opzicht een interessant alternatief. De toegevoegde waarde van (prematuur) neonatale en juveniele varkens werd recent onder de aandacht gebracht. Biggen zijn even groot als pasgeborenen en kunnen bijgevolg op dezelfde manier behandeld worden als baby's in de neonatale intensieve zorgen afdeling. Met het oog op geneesmiddelenmetabolisme in de lever, werden eerder grote overeenkomsten aangetoond tussen volwassen mensen en varkens. Data en kennis met betrekking tot de (prematuur) neonatale en juveniele populaties in zowel de mens als het varken blijven echter schaars. **Hoofdstuk 1** omschreef de uitdagingen waar de pediatrische geneesmiddelenontwikkeling momenteel mee geconfronteerd wordt en de daaruit volgende zoektocht naar alternatieven. In **Hoofdstuk 2** werden de doelen van deze thesis geformuleerd.

In Hoofdstuk 3 van deze thesis werd het fase I geneesmiddelenmetabolisme onderzocht in de lever van foetale, neonatale, juveniele en volwassen Göttingen Minipigs op eiwitniveau. CYP abundantie werd bepaald in levermicrosomen door middel van LC-MS/MS. Verschillende ontogenieprofielen werden ontdekt: (1) CYP enzymen met een gelijkaardige eiwitabundantie van de foetale fase tot en met volwassenheid (bv. CYP4A24 en CYP20A1), (2) CYP enzymen met een graduele postnatale toename (bv. CYP2C33, CYP3A22 en CYP4A21), (3) CYP enzymen die hun maximum bereikten op speenleeftijd (postnatale dag (PND) 28) (bv. CYP1A2, CYP2D6 en CYP2E1), en (4) een atypisch CYP enzym met hoge waarden tijdens de foetale fase die daalden tijdens de eerste levensmaand en nadien opnieuw stegen in de volwassen fase (i.e., CYP51A1). Geslacht-gerelateerde verschillen in de juveniele populatie werden enkel waargenomen voor CYP4V2_2a en CYP20A1 op PND 1 met de

hoogste expressie in vrouwelijke dieren voor beide isovormen. De vergelijking van volwassen vrouwelijke en mannelijke Göttingen Minipigs resulteerde in geslacht-gerelateerde verschillen voor CYP1A1, CYP1A2, CYP2A19, CYP2E1_2, CYP3A22, CYP4V2_2a en CYP4V2_2b met de hoogste expressie in vrouwelijke minivarkens. Verder werd CYP eiwitabundantie gecorreleerd aan eerder geobserveerde CYP enzymactiviteit (onderzocht met behulp van isovorm-specifieke substraten). Deze analyse toonde een duidelijk verband aan tussen CYP3A22 eiwitabundantie en de metabolisatie van midazolam op PND 7.

In **Hoofdstuk 4** werd het potentieel van het prematuur geboren varken als model voor prematuur geboren baby's onderzocht. Deze laatsten worden nog steeds als "therapeutische wezen" beschouwd binnen de pediatrische geneesmiddelenontwikkeling. Hun deelname aan klinische studies wordt door verschillende ethische en praktische bezorgdheden verhinderd, waardoor alternatieven voor nauwkeurige dosisbepaling nodig zijn. Deze studie had tot doel de ontogenie van CYP3A en UGT enzymactiviteit in de lever van prematuur (90% drachtduur, dag 105 – 107 van de dracht) en à term (100% drachtduur, dag 115 – 117 van de dracht) geboren conventionele biggen te onderzoeken. Daarnaast werd het effect van de chronologische leeftijd (i.e., leeftijd o.b.v. drachtduur) en postnatale leeftijd (i.e., leeftijd vanaf de geboorte) op de enzymactiviteit bestudeerd. CYP3A- en UGT-specifieke substraten werden gebruikt om de ontwikkeling van de enzymactiviteitprofielen te onderzoeken. De UGT-activiteit toonde een significante toename tussen PND 0 en PND 26 in zowel prematuur als à term geboren biggen. CYP3A-activiteit werd enkel gedetecteerd in prematuur geboren PND 26 biggen, terwijl een graduele toename in à term geboren biggen werd waargenomen vanaf PND 11. In beide groepen was de enzymactiviteit lager in de prematuur geboren groep. Vervolgens suggereren deze waarnemingen dat de chronologische leeftijd vanaf conceptie CYP3A- en UGT-enzymactiviteit meer beïnvloedt dan wel de postnatale leeftijd in het varken.

De data die in Hoofdstuk 3 en 4 werden verworven, draagt bij aan een beter begrip van de biotransformatiecapaciteit in het (mini)varken. Grote overeenkomsten met het geneesmiddelenmetabolisme in de humane pediatrische populatie werden waargenomen. Hierdoor wordt het gebruik van het varken als preklinisch diermodel voor pediatrische geneesmiddelenontwikkeling aangemoedigd. Verschillen in de snelheid van orgaanontwikkeling en species-specifieke kenmerken tussen de mens en het varken mogen echter niet uit het oog verloren worden tijdens deze vergelijking. Daarnaast werden in deze thesis enkel leeftijdsgroepen geïncludeerd die de leeftijdsrange van 1 maand tot en met twee jaar vertegenwoordigen in de mens. Aangezien de ontwikkeling niet stopt op tweejarige leeftijd is het insluiten van oudere pediatrische leeftijdsgroepen bijgevolg noodzakelijk. Enkel op die manier kan een volledig beeld en begrip van geneesmiddelenmetabolisme in de volledige pediatrische populatie verkregen worden. Tijdens het onderzoek naar geneesmiddelenmetabolisme in de lever van het varken werd de focus gelegd op eiwitabundantie en enzymactiviteit als biologische parameters. Onderzoek naar elk van deze biologische levels leidde tot waardevolle data, al dient men in het achterhoofd te houden dat een enkele parameter nooit de volledige ontwikkeling noch het achterliggende mechanisme zal

verklaren. De bovengenoemde onderwerpen werden kritisch besproken in **Hoofdstuk 5**, tezamen met aanbevelingen voor verder onderzoek.

Dankwoord

Zoals menig collega, vriend, vriendin of familielid zal kunnen beamen, waren de afgelopen zes jaar zeker niet altijd vanzelfsprekend. Met de nodige *ups and downs* legde ik een hele weg af die zowel op professioneel als persoonlijk vlak tot een mooie groeicurve leidde. Dat ik dit doctoraat tot een goed einde heb weten brengen, heb ik aan een ongelooflijk grote entourage te danken. Ik neem dan ook graag de tijd om iedereen in de bloemetjes te zetten die heeft bijgedragen aan dit succes.

Starten doe ik graag met mijn dank te uiten aan de leden van mijn jurypanel. Het is en blijft een wetenschappelijk project waarbij de inbreng van experts uit het vakgebied van groot belang is. **Professor Siska Croubels** en **professor Saskia de Wildt,** dank jullie wel voor de fijne, constructieve feedback op mijn werk. Ik ben ervan overtuigd dat het voor net dat tikkeltje meer gezorgd heeft om de thesis volledig op punt te stellen. **Professor Peter Bols** en **professor Ingrid De Meester**, mijn interne juryleden, ik wil jullie hartelijk danken voor de fijne opvolging gedurende de afgelopen zes jaar. Jullie hebben telkens met héél constructieve feedback bijgedragen aan de verderzetting van mijn project. Ondanks dat ik telkens met een klein hartje onze meetings voorbereidde, was het vertrouwen nadien eens zo groot om weer met volle moed verder te gaan. Bedankt daarvoor!

Uiteraard is er geen doctoraat zonder promotoren. **Steven**, dank je wel om meer dan zes jaar geleden het potentieel in mij te zien om aan dit onderzoek te beginnen. Dat het een uitdagend project was, werd al snel duidelijk. De keren dat ik met goed nieuws aan je bureau stond, zeker wat het moleculair werk betreft, waren eerder schaars. Desalniettemin denk ik dat we trots mogen zijn op het eindresultaat. Ik wil je bedanken voor de eerlijke feedback, het (snelle) naleeswerk - zeker tijdens de afgelopen maanden - en de groei die ik heb mogen doormaken als wetenschapper en als mens. Ik ben benieuwd wat de (mini)pig nog verder voor je in petto heeft. Ik wens je veel succes met het verdere onderzoek, net zoals ook op alle andere vlakken in je leven. **Chris**, dank je wel voor de kritische blik op mijn manuscripten. Je feedback zorgde telkens opnieuw voor de extra *push* om net weer een level hoger te springen. De data (en statistiek) vanuit een ander perspectief gepresenteerd krijgen door jou, heeft mij veel bijgeleerd. Ik wil je ook graag bedanken voor je luisterend oor, mildheid en vertrouwen op de momenten dat het even moeilijk ging. Een doctoraat komt er uiteindelijk zelden vanzelf. Ik wens je al het beste toe, met vooral een blakende Franse zon waarin je van tijd tot tijd even tot rust kan komen tussen alle hectiek door.

Tijdens de afgelopen periode heb ik deel mogen uitmaken van het ongelooflijk fijne team van de CoPeD groep. *Teamwork makes the dream work* en zo is het maar net. Wetenschap doe je nooit alleen en een klankbord hebben waar je op kan terugvallen is oh zo belangrijk.

Kevin. Waar moet ik beginnen 😃. *Little did we know* toen ik je het koffiemachine liet zien op je eerste werkdag. Na een week of twee werd al snel duidelijk dat we belachelijk veel gemeenschappelijke interesses hadden en zo geschiedde. Merci om me mee te nemen in de

varkenswereld, om je brede diergeneeskundige kennis te delen en om zo een betere assistent van mij te maken. Nog meer merci voor de (soms uitbundige, sorry collega's in de gang) lachsalvo's, voor de ventilatiemomentjes en om er gewoon te zijn wanneer het nodig was 😊. Ik kon me geen betere collega voorstellen om de practica samen mee te draaien. Ondertussen duim ik van aan de zijlijn (met wafels en worstjes) dat ook jij snel aan de eindmeet van je doctoraat geraakt. Go go go!

Evy, dank je wel om mij van aan de start onder je vleugels te nemen en me wegwijs te maken binnen "den anatomie". Je zorgde er telkens voor dat ik de practica op een extreem goed georganiseerde manier kon starten wat voor rust zorgde in m'n hoofd. Je steun en luisterend oor, zelfs na je vertrek uit de groep, waren en zijn superwaardevol voor mij. Ik wens je meer dan alle goeds voor de toekomst, dat verdien je zó hard! Ik ben er zeker van dat we elkaar nog genoeg tegen het lijf zullen lopen nu we binnenkort op een steenworp van elkaar zullen wonen 😉.

Chloé and **Allan**, my two little foreign buddies. I love you both so much. Chloé, we started our PhD projects more or less at the same time and it was so so valuable to me that we could share the same struggles, insecurities and doubts. Our friendship was kind of forced by having to share the same bed at the ETS in Berlin and was even lifted to the next level in Kortrijk where we had to share a bathroom WITHOUT a door or blinded windows during our enhanced LC-MS course. Who would ever believe it 😆. I really enjoyed our time together in the lab, having chitchats in between all the molecular work and sharing our little and big life problems. Dear Chloé, I truly admire who you are as a person. Little miss sunshine always looking for the bright side of life 🔆 Please never lose that vibe, it is so precious! Life hasn't been too nice lately, but you keep fighting and you will rise like a phoenix, I'm sure. Allan, the commitment and dedication that you show towards your research are truly inspiring. I have never met anyone who works so meticulously on protocols, sample preparation and data analysis. Having you by my side during my project improved my own research skills tremendously. I really enjoyed sharing the last months of our projects together: writing together, whining together, chitchatting together 😊. Our daily Teams calls kept us sane, right 😉. Finally, I wish you "veel succes" and "bon courage" for the final weeks ahead of your own defense! Yes you can!

Jente, we waren al vrienden nog voor we collega's werden, blader dus gerust verder naar de vriendensectie 😉.

Het komt in elk dankwoord terug, **Katty** en **Gunther**, en ik herhaal het graag nog eens opnieuw: dank jullie wel om de rots in de branding te zijn voor het ganse labo. Wat zouden we zonder jullie doen… Geen enkele vraag, bedenking of bestelling is te veel gevraagd. Jullie steun en betrokkenheid zijn van onschatbare waarde, niet alleen voor mij maar voor het hele team. Ik wens jullie oprecht allebei het allerbeste toe, met nog vele mooie reizen en fietstochten tussen al het werk door!

Gedurende zes jaar zijn er natuurlijk veel mensen die komen en gaan… en nog eens opnieuw terugkomen. **Sara**, dank je wel om zo'n fantastisch klankbord te zijn. Van brainstormen over (meermaals) mislukte experimenten tot planten en verbouwverhalen, van alle markten ben je thuis

😊. Je oprechte betrokkenheid en interesse in de collega's is onnavolgbaar. Wat moet je nog meer hebben? Ik stuur alle positieve *vibes* jouw kant op zodat ook jij snel verlost geraakt van die laatste loodjes!

Miriam, I am not sure whether I have to thank you for handing over the molecular work, but well… it happened anyway 😆. Thank you for listening over and over again when my experiments failed, for brainstorming on new approaches and for guiding me through statistics. Those recombinants were a hell of a ride, but all the suffering paid off (at least a bit). I wish you all the best back in Spain!

Naast de drukgevulde dagen vol experimenten, zijn er uiteraard ook minder drukke momenten met wat meer ruimte voor ontspanning. **Lieselotte**, dank je wel voor de fijne babbeltjes tussendoor en je enthousiaste aanmoedigingen in de laatste rechte lijn van mijn doctoraat. Ik moet je gelijk geven, het lucht op eens alles geschreven is. **Marjan**, dank je wel om een extra mamaatje te zijn voor iedereen binnen de groep. Je luisterend oor en goede raad verrichten wonderen 😉. **Ellen**, dank je wel om me regelmatig even uit de schrijfbubbel te komen halen om je nieuwe aanwinsten uit de zoo te showen. Ik ga nog regelmatig terugdenken aan alle zotte dieren die ik van dichtbij heb kunnen bewonderen dankzij jou! Met niet in het minst de fantastische walvis op mijn voorlaatste dag. Wauw wauw wauw… Ik ben er nog steeds van onder de indruk (met nog steeds een even grote glimlach als ik eraan terugdenk)! **Marlotte**, dank je wel om wat extra leven in de bureau te brengen tijdens het afgelopen jaar. Ik denk in het vervolg twee keer na voor ik aan iemand vraag hoe het gaat 😉. Ik wens je immens veel succes met je stalproeven, al ben ik er rotsvast van overtuigd dat alles goed komt! Tot slot bedank ik ook heel graag **Casper**, **Charlotte**, **Christel**, **Denise, Falk**, **Fien**, **Kris(tel)**, **Marina**, **Marleen** en **Steve** voor de practica samen, de fijne babbeltjes tussendoor, de gezellige lunchpauzes en de administratieve ondersteuning.

Een deel van mijn onderzoek werd uitgevoerd in samenwerking met het Laboratorium voor moleculaire biofysica, fysiologie en farmacologie. **Alain**, **Abbi**, **Evy** en **Kenny**, ik kan jullie niet genoeg bedanken voor alle hulp en ondersteuning zowel op technisch vlak als daarbuiten. Jullie aanmoedigingen en optimisme waren onuitputtelijk. Dankzij jullie voelde T5 als mijn tweede thuis. Dank jullie wel!

Naast alle collega's was ook mijn achterban van vrienden en familie ontzettend belangrijk. *It takes a village to raise a child* en zo voelde het ook voor dit doctoraat. Ik ben zo ontzettend dankbaar voor het warme, lieve en oprechte netwerk waarop ik kan terugvallen.

Lieve lieve **Anouk**, dank je wel om al meer dan tien jaar aan mijn zijde mee te wandelen. Dank je wel voor je kaartjes vol aanmoediging, trots, liefde en nog meer aanmoediging. Dat je op de meest cruciale momenten stante pede klaar stond met koffie, taart en een luisterend oor betekent alles. Zonder jou aan de zijlijn, had dit doctoraat nu niet op tafel gelegen. Ik zou hier graag nog zoveel meer schrijven en vertellen, maar laten we dat houden voor je trouwfeest. **Julie**, dank je wel voor je oh zo warme vriendschap. Ik blijf het bewonderingswaardig vinden hoe je in je eigen drukke agenda telkens de tijd kan vinden voor oprechte *quality time*. Je steun de afgelopen maanden en

jaren was zó waardevol. *I don't take it for granted*. **Emilie**, dank je wel om altijd klaar te staan met het ene idee al zotter dan het andere. Om de stress te temperen en (h)erkenning te geven bij de laatste loodjes. Het klankbord om op terug te kunnen vallen gaf rust (in de mate van het mogelijke – we moeten ook niet overdrijven natuurlijk). Lieve vriendinnen, ik koester onze vriendschap enorm, dank jullie wel.

Emmanuel en **Vincent**, samen met Emilie hebben we de volledige opleiding Biomedische Wetenschappen doorlopen en daarna waren we allemaal zot genoeg om er nog een doctoraat aan te breien ook. Emmanuel, de ambitie en flair maar ook bescheidenheid waarmee je de afgelopen jaren alles tot een goed einde bracht, zijn inspirerend en ontroerend tegelijk. Dank je wel om mij te gepasten tijde met je mopjes weer even met de voeten op de grond te zetten. Vincent, de wandelingen tijdens de middag waren een verademing. Letterlijk en figuurlijk. Dank je wel voor je luisterend oor en voor het delen van de miserie 😆. Ik ben ontzettend dankbaar voor jullie vriendschap.

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Tim, **Stijn**, **Lauren** en **Sarah**, dank je wel om al dan niet vrijwillig mee te luisteren naar mijn (soms dramatische) verhalen. Jullie oprechte interesse is hartverwarmend.

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Glenn, we leerden elkaar kennen aan de start van mijn doctoraatstraject. Je hebt me dus eigenlijk nog nooit in een volledig stressvrije versie gezien (voor zover die bestaat). Dank je wel om de afgelopen jaren mijn *inner & outer drama queen* te temperen, om rust te brengen. Om verrassend genoeg één van mijn meest kritische juryleden te zijn. Om mijn onuitstaanbaarheid toe te laten en te laten wegebben. Ik heb veel van je geduld gevraagd, maar we zijn er geraakt. Ik kijk ernaar uit om samen in rustiger vaarwater terecht te komen en binnenkort (of minder binnenkort – wie zal het zeggen) naar onze nieuwe thuishaven te varen. Dank je wel voor je steun en je liefde. *You are loved and appreciated* ❤️.

Academic Curriculum Vitae

Personalia

Last name: Buyssens
First name: Laura
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Career and Education

Academic Assistant **September 2017 – August 2023**
Laboratory of Comparative Perinatal Development
University of Antwerp
PhD thesis entitled: 'Hepatic drug metabolism in pediatrics: investigating the neonatal and juvenile (mini)pig as a translational model'
Supervisors: Prof. dr. Steven Van Cruchten & Prof. dr. Chris Van Ginneken

Master's degree in Biomedical Sciences **September 2015 – June 2017**
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
University of Antwerp
Major: Neurosciences
Minor: Research
Master thesis entitled: 'The influence of diet-induced obesity on serotonin levels and motility in the GI tract of the zebrafish'
Supervisors: Prof. dr. Guy Hubens, dr. Luc Van Nassauw & dr. Leen Uyttebroek
Graduated magna cum laude

Bachelor's degree in Biomedical Sciences **September 2012 – June 2015**
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
University of Antwerp
Bachelor thesis entitled: 'The role of sclerostin in vascular media calcification'
Supervisor: Prof. dr. Patrick D'Haese
Graduated cum laude

Honours College **September 2013 – June 2015**
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
University of Antwerp
Deep dive into science program during the bachelor's degree

Secondary school **September 2006 – June 2012**
Annuntia Instituut, Wijnegem
Majors: Sciences and Mathematics
Graduated summa cum laude

Teaching Experience

During this 6-year trajectory, part of the job consisted of teaching several practical courses and seminars:

Bachelor of Veterinary Sciences:

- ABS I & ABS II: osteology
- Organ anatomy
- Neuroanatomy
- Embryology and Teratology
- General pathology

Bachelor of Biomedical Sciences and Bachelor of Biochemistry & Biotechnology:

- Embryology and Development
- Reproduction and Embryology

Master of Biomedical Sciences:

- Laboratory Animal Sciences

Student Supervision

- Supervisor bachelor thesis Jolien Hendrix, entitled: 'The preterm piglet as a translational model for premature infants in pediatric drug development'. Academic year 2017 – 2018 (Biomedical Sciences, University of Antwerp)

- Supervisor Honours College project Morgane Van Belle, entitled: 'Constructie van recombinante CYP450 enzymen afkomstig van de Göttingen Minipig en de zebravis'. Academic year 2017 – 2018 (Veterinary Sciences, University of Antwerp)

- Supervisor bachelor thesis Luna Van Hoyweghen, entitled: ‘Improvement in the production of Göttingen Minipig recombinant CYP450 enzymes’. Academic year 2018 – 2019 (Biomedical Sciences, University of Antwerp)

- Supervisor bachelor thesis Katarina Horoba, entitled: ‘Optimalisatie van het western blot protocol bij de productie van recombinante CYP enzymen’. Academic year 2018 – 2019 (Biomedical Laboratory Technology, Karel de Grote Hogeschool)

- Supervisor bachelor thesis Charlotte van Beek, entitled: ‘De (pre)mature big als model voor de (pre)mature neonaat: focus op fase I en fase II biotransformatie in de lever’. Academic year 2019 – 2020 (Biomedical Sciences, University of Antwerp)

- Supervisor bachelor thesis Kenzie Sprangers, entitled: ‘Optimalisatie van de expressie van het recombinant Göttingen Minipig CYP3A22 enzym’. Academic year 2019 – 2020 (Biomedical Laboratory Technology, Karel de Grote Hogeschool)

Additional Scientific Trainings & Certificates

Applied Communication, University of Antwerp 2019

R workshop, University of Antwerp 2019

Basic statistics in Prism, VIB 2019

PBPK modeling for quantitative *in vitro – in vivo* extrapolation, Altertox 2018

Giving presentations in English, University of Antwerp 2018

Dose finding and trial design by simulation, Catholic University of Leuven & SGS 2018

Hands-on LC-MS training, RIC Kortrijk 2018

Introduction to (H)PLC and LC-MS, University of Antwerp 2018

Multivariate data analysis course, University of Antwerp 2018

Methods in research design, FLAMES 2018

FELASA category C, University of Antwerp 2017

Scientific Awards

Student Award for the Highest Merit 2017
Awarded by the Faculty of Pharmaceutical, Biomedical and Veterinary sciences
University of Antwerp

Conferences

11th Juvenile Toxicology Symposium 2023
Beerse, Belgium

50th Annual meeting of the European Teratology Society 2022
Antwerp, Belgium

48th Annual Meeting of the European Teratology Society 2020
Online meeting

10th Juvenile Toxicology Symposium 2019
Beerse, Belgium

47th Annual Meeting of the European Teratology Society 2019
Cologne, Germany
Short communication: 'The ontogeny of hepatic CYP450 protein abundance in the developing Göttingen Minipig'

Scientific Symposium: Celebrating 50 years with Göttingen Minipigs 2019
Wilrijk, Belgium
Oral presentation: 'The developing Göttingen Minipig and conventional pig: differences in hepatic CYP450 protein abundance and activity data'

46th Annual Meeting of the European Teratology Society 2018
Berlin, Germany
Poster presentation: 'Preterm piglets as a translational model for premature babies: focus on hepatic drug metabolism'

List of Publications

Buyssens L, Valenzuela A, Prims S, Ayuso M, Thymann T, Van Ginneken C, et al. Ontogeny of CYP3A and UGT activity in preterm piglets: a translational model for drug metabolism in preterm newborns. Frontiers in pharmacology. 2023;14:1177541.

Buyssens L, De Clerck L, Schelstraete W, Dhaenens M, Deforce D, Ayuso M, et al. Hepatic Cytochrome P450 Abundance and Activity in the Developing and Adult Göttingen Minipig: Pivotal Data for PBPK Modeling. Frontiers in pharmacology. 2021;12(535).

Valenzuela A, Ayuso M, **Buyssens L**, Bars C, Van Ginneken C, Tessier Y, et al. Platelet Activation by Antisense Oligonucleotides (ASOs) in the Göttingen Minipig, including an Evaluation of Glycoprotein VI (GPVI) and Platelet Factor 4 (PF4) Ontogeny. Pharmaceutics. 2023;15(4):1112.

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Ayuso M, **Buyssens L**, Stroe M, Valenzuela A, Allegaert K, Smits A, et al. The Neonatal and Juvenile Pig in Pediatric Drug Discovery and Development. Pharmaceutics. 2020;13(1).

Uyttebroek L, Van Remoortel S, **Buyssens L**, Popowycz N, Hubens G, Timmermans JP, et al. The Effect of Diet Induced Obesity on Serotonin in Zebrafish. J Cell Signal. 2022;3(2):115-128.

Bars C, Hoyberghs J, Valenzuela A, **Buyssens L**, Ayuso M, Van Ginneken C, et al. Developmental Toxicity and Biotransformation of Two Anti-Epileptics in Zebrafish Embryos and Early Larvae. International journal of molecular sciences. 2021;22(23):12696.