Paediatric drug metabolism: From minipig to man

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Het juveniele Göttingen minivarken als translationeel model voor de studie van geneesmiddelmetabolisme bij kinderen

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Diergeneeskundige Wetenschappen aan de Universiteit Antwerpen, te verdedigen door

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**Abbreviations**

1-OH-MDZ: 1’-OH-midazolam  
4-OH-TOL: 4-OH-tolbutamide  
ABC: ATP-binding cassette  
ACT: Acetaminophen  
ADME: Absorption, distribution, metabolism, and excretion  
AhR: Aryl hydrocarbon receptor  
AICc: Adapted Akaike Information Criterions  
AL: Adult level  
ATP: adenosine triphosphate  
Bac: Baculosomes  
B.C. Before Christ  
BD: Bile duct  
BPCA: Best Pharmaceuticals for Children Act  
CAR: Constitutive androstane receptor  
CYP450: Cytochrome P450 enzymes  
DAB: 3,3’-diaminobenzidine chromogen and substrate buffer  
DX: Dextrophan  
DGA: Days of gestational age  
DHEA-S: Dehydroepiandrosterone 3-sulfate  
DIC 4-OH: Diclofenac 4-hydroxylation  
e.g.: Example given  
ELISA: Enzyme-linked immunosorbent assay  
EMA: European Medicines Agency  
EROD: Ethoxyresorufin O-deethylation  
F: Female  
FDA: Food and Drug Administration  
Frz: Frizzled receptor  
h: Hour  
HA: Hepatic artery  
HLM: Human liver microsomes  
Hnf4α: Hepatocyte nuclear factor 4alpha  
HV: Hepatic vein  
K CZ: Ketoconazole  
K M: Michaelis-Menten constant  
KPO 4 : Potassium phosphate  
L.I.: Large intestine  
LLOQ: Lower limit of quantification  
M: Male  
MEP 4-OH: S-mephenytoin 4’-hydroxylation  
MDZ 1-OH: midazolam 1’-hydroxylation  
Min: Minutes  
MLM: Minipig liver microsomes
mol/min/mg MP: Moles of metabolite formed per minute per milligram of microsomal protein
MP: Microsomal protein
MDZ: Midazolam
MROD: Methoxyresorufin O-dealkylation
NADPH: Nicotinamide adenine dinucleotide phosphate
NRS: Normal rabbit serum
NHP: Non-human primate
NSAID: Non-steroidal anti-inflammatory drug
PBPK: Physiologically based pharmacokinetic modelling
PBS: Phosphate buffered saline
PD: Pharmacodynamic
PDCO: Paediatric Committee
Pgp: P-glycoprotein
PIP: Paediatric Investigation Plan
PK: Pharmacokinetic
PND: Postnatal day
PPARα: Peroxisome proliferator-activated receptor α
PREA: Pediatric Research Equity Act
PXR: Pregnane X-receptor
RLU: Relative light unit
RT: Room temperature
sec: Seconds
S.I.: Small intestine
TBS: Tris-buffered saline
TOL 4-OH: Tolbutamide 4-hydroxylation
TST: Testosterone
TST 6-OH: Testosterone 6β-hydroxylation
UGT MES: UGT multienzyme substrate
U.S.: United States
UDPGA: Uridine-5'-diphospho-glucuronic acid
UGT: Uridine diphosphate glucuronosyltransferase
V_{max}: Maximal velocity
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Preface

In view of drug development and medicinal treatment, the vulnerable group of paediatric patients has been ignored for decades. Consequently, children have been and are often exposed to ineffective treatment and adverse drug reactions. With the implementation of 'The Paediatric Regulation' (EC No 1901-2/2006) in January 2007, extra efforts are made to facilitate the development and accessibility of medicinal products for use in the paediatric population. In this respect, animal models play a crucial role in drug development, and especially in view of paediatric drug development, the juvenile Göttingen minipig may deliver a valuable contribution. Hence, this thesis focuses on the characterization of the developing Göttingen minipig regarding key elements in the absorption, distribution, metabolism and excretion of drugs, with a focus on the small intestine and the liver.
1 Introduction

1.1 Paediatric drug development

Although the paediatric population is a very vulnerable part of society, this age group has historically been neglected when it comes to evidence-based appropriate medical care. Indeed, regulatory safety and efficacy testing of new medicinal products has been based on adult human clinical trials only for decades. Consequently, questions on the paediatric safety and efficacy of drugs with an adult authorization remained largely unanswered, as well as uncertainties on appropriate dosing (1-3). However, as will be demonstrated in this thesis, paediatric doses cannot be simply extrapolated from adult doses based on body weight, since many other important key factors are changing in a developing body too. Unfortunately, toxicity has already occurred in children after administration of drugs that were considered safe in adults. (Table 1.1) Such adverse events have urged the need for a legal context regarding safety and efficacy testing of drugs in children. (See below) Especially preterm and term neonates, but also infants and toddlers appear to be at highest risk for adverse events, due to their many dissimilarities with adult and mature individuals. The European Medicines Agency (EMA) has made a classification for the determination of the different paediatric age groups (4). (Table 1.2)

Table 1.1. Altered drug toxicity profiles in children compared with adults.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Therapeutic indication</th>
<th>Toxicity in paediatric age groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamotrigine</td>
<td>Anticonvulsant drug</td>
<td>A three-fold increased risk for hypersensitivity reactions in children compared with adults. Due to immature glucuronidation capacity (5, 6).</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Antibiotic</td>
<td>Cardiovascular collapse in neonates (grey baby syndrome). Due to immature glucuronidation capacity (6, 7).</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Anticonvulsant drug</td>
<td>Children below 6 years of age are at higher risk for the development of fatal hepatotoxicity and pancreatitis, especially in case of polytherapy with other drugs (6, 8, 9).</td>
</tr>
</tbody>
</table>
Table 1.2. Age classification of the paediatric population according to the EMA.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm newborn infants</td>
<td>&lt; 37 weeks of gestation</td>
</tr>
<tr>
<td>Term newborn infants</td>
<td>0-27 days</td>
</tr>
<tr>
<td>Infants and toddlers</td>
<td>28 days–23 months</td>
</tr>
<tr>
<td>Children</td>
<td>2-11 years</td>
</tr>
<tr>
<td>Adolescents</td>
<td>12-17 years</td>
</tr>
</tbody>
</table>

1.1.1 Historical and Legal Context

Dr Abraham Jacobi (°1830–†1919) is considered the ‘Father of American Paediatrics and Advocate for Children’s Health’. After being raised in poor conditions and being predicted not to live long due to weak health, he succeeded in graduating as a doctor of medicine at the University of Bonn and decided to stand up for children and women. In the first years of his professional career, he got involved in the difficult political climate at that time and he ended up in jail for a few years for treason and subversion against the repressive government. Afterwards, he moved to New York, where he started a practice of medicine accessible for less advantaged patients. He greatly promoted health care for children and women and dared to advocate for neglected groups like orphans, which brought him in disapproval from the health care providers at that time. However, he did not draw back and became ‘Clinical Professor of Diseases of Children at the College of Physicians and Surgeons of Columbia University’ in 1870. Afterwards, he occupied other important positions in the field of paediatric healthcare. Dr Abraham Jacobi already stated in the 19th century that children are not small adults, but that they have their own types of diseases, requiring adapted ways of treatment (10). However, it took more than one century to force pharmaceutical companies to investigate the efficacy and safety of drugs in the paediatric population. Consequently, paediatric patients have received and still receive drugs that are authorized for adults. This distorted situation puts young children at risk of ineffective treatment, toxicity and adverse drug reactions, as history has showed us (7-9, 11).
In order to facilitate the development of ‘Better Medicines for children’, the European Parliament and Council have implemented The Paediatric Regulation (EC No 1901-2/2006) in January 2007. From a practical point of view, this means that new medicinal drugs (with potential paediatric use) need to be appropriately authorized for this indication. For drugs already on the market, but still on patent, a line extension can be obtained by gathering paediatric data. Consequently, high quality research is requested in order to generate sufficient relevant information. As a reward, the pharmaceutical companies get six months of patent extension, even when the product appeared to be not appropriate for paediatric use (12).

Two tools came into life in Europe to help companies in accomplishing these mandatory studies, i.e. ‘The Paediatric Committee’ (PDCO) and the ‘Paediatric Investigation Plan’ (PIP).

A PIP is mandatory and is submitted by the pharmaceutical company. It sets out a programme for the development of a medicine in the paediatric population. The PIP aims to generate the necessary quality, safety and efficacy data through studies to support the authorisation of the medicine for use in children of all ages. The pharmaceutical company presents these data to the PDCO, or national competent authorities, as part of an application for a marketing authorisation for a new medicine, or for one covered by a patent. In some cases, a PIP may include a waiver of the studies in one or more paediatric subsets, or a deferral. Waivers can be issued if there is evidence that the medicine concerned is likely to be ineffective or unsafe in the paediatric population, or that the disease or condition targeted occurs only in adult populations, or that the medicine, or the performance of trials, does not represent a significant therapeutic benefit over existing treatments for paediatric patients (13). Preclinical studies in juvenile animals can form part of a PIP to provide a translational model for paediatric age groups in man. (See Chapter 1.2)

The PDCO is the committee at the EMA that is responsible for assessing the content of paediatric investigation plans and adopting opinions on them. This includes assessing applications for full or partial waivers and assessing applications for deferrals. This committee is composed of five members of the ‘Committee for Medicinal products for human use’, one member appointed by each European Union Member state that is not represented by the members appointed by the Committee for Medicinal products for human use, three members representing healthcare professionals and three members representing patient associations (14). This committee will evaluate the content of
submitted PIPs and will form an opinion on them in accordance with the ‘Paediatric Regulation’, and will subsequently assess the data that were generated in this PIP. The PIP forms the basis for development and authorization of a medicinal product for the paediatric population, which is submitted upon availability of adult pharmacokinetic (PK studies). This PIP has to be agreed upon and/or demanded by the PDCO and is binding for the company. Modifications in the PIP can be made during the procedure of drug development as new data come apparent. The PIP includes details on timing and the measures proposed to demonstrate quality, safety, and efficacy in the paediatric population and should cover all ages from birth to adolescence (12).

The Food and Drug Administration (FDA) in the United States (U.S.) performs the same role as the EMA and established the ‘Pediatric Rule’ (1998), ‘Best Pharmaceuticals for Children Act’ (BPCA, 2002) and the ‘Pediatric Research Equity Act’ (PREA, 2003). In the latter rule and act, respectively, the incentives and the requirements concerning paediatric drug development are defined. Both acts were amended and reauthorized in view of the ‘Food and Drug Administration Amendments Act’ in 2007. In 2012, the ‘FDA Safety and Innovations Act’ made the BPCA and PREA permanent and established the need to submit ‘Pediatric study Plans’ to the ‘Pediatric Review Committee’. The goal of the European and U.S. legislations is the same, however, their way of working differs. In Europe, all incentives and requirements fall under one legislation, with paediatric development being mandatory for all new products under development unless a waiver or deferral was granted. Additionally, the paediatric drug development is earlier discussed in the regulatory process compared with in the U.S. (in U.S. upon availability of adult PK studies). In the U.S., a Written Request (equivalent PIP) is voluntary and issued by the FDA, usually following a proposed paediatric study request from the sponsor (12). Zisowsky et al. nicely reviewed similarities and dissimilarities between the regulations in Europe and the U.S. (12).

In 2016, the ‘International Council on Harmonisation of technical requirements for pharmaceuticals for human use’ released an addendum to guideline ICHE11 to promote alignment between Europe, the U.S., Japan, Health Canada and Switzerland in view of the clinical investigation of medicinal products in the paediatric population. The final goal of this addendum is to prevent region-related differences in requirements for paediatric development and in access to medicines for children (15).
1.1.2 Why is paediatric drug development challenging?

Several challenges in the domain of paediatric drug development may explain for neglecting this vulnerable population. First, the inclusion of paediatric subjects in clinical trials has ethical limitations, but also paediatric tissues for *in vitro* studies are scarcely available, which hampers extensive research in this patient group. Additionally, this challenging kind of research might also result in a limited return on investment. However, the implementation of the Paediatric Regulation (EC No 1901-2/2006) made it mandatory for pharmaceutical companies to rise to these challenges. A final point of difficulty is the fact that a developing body is constantly changing over time, both anatomically and physiologically (e.g. lipid and water composition, distribution volumes, organ weights, blood flow, organ functions, metabolizing capacities, etc.) (2, 3, 16). These constantly varying body-dependent factors make the prediction of the absorption, distribution, metabolism and excretion (ADME) of a drug in a child of a certain age challenging. However, insight in the ADME of a drug in a developing body is crucial for paediatric drug development, especially in view of the dose selection of the first paediatric patients, but also for the generation of valid safety and efficacy data in children (12). Juvenile animal studies may be helpful in finding answers to these questions. The potential value of the Göttingen minipig as a juvenile animal model, based on the determination of determinants on the ADME of drugs, will be explored in this thesis.

1.1.3 The oral bioavailability of drugs in paediatric age groups

Oral ingestion is the most common route of drug administration (17). Subsequently, the drug will need to pass the gastrointestinal tract and the liver, prior to reaching the systemic circulation and the target organs. In both organs, important events take place that will determine the final oral bioavailability (= the degree to which a drug becomes available to the target tissue after oral administration) of the ingested compound (18). The gastrointestinal tract is the location where absorption takes place, mainly at the level of the small intestine. The rate of absorption is generally lower in neonates and very young children compared with in older children and adults. This is mainly caused by a reduced gastric emptying time, a reduced absorptive surface area of the small intestine (See Chapter 1.4.1), and/or a reduced intestinal motility. Consequently, a
delay and reduction of the peak serum concentration may be present, although the final extent of absorption appears to remain unaltered (1). Additionally, the small intestine determines the oral bioavailability of a drug by the presence of drug metabolizing enzymes and drug transporters (uptake and efflux transporters) (19-21). The portion of the compound or its metabolite that has escaped intestinal metabolism or efflux transport can reach the liver via the portal vein. Similarly as for the small intestine, drug metabolizing enzymes and drug transporters (uptake from the blood and efflux in the bile and the blood) are present to determine the oral bioavailability of the compound (18). In children, the oral bioavailability of a compound is often enhanced compared with adults, due to immaturity or absence of such drug metabolizing enzymes and drug transporters. This may lead to supra-therapeutic or even toxic plasma levels and prolonged half life when paediatric doses are extrapolated from adult doses, based on body weight or body surface area only (1-3). Therefore, it is important that developmental changes in such key determinants of the oral bioavailability of drugs are investigated.

This thesis will focus on age-dependent differences in:

- the presence and localisation of the efflux transporter P-glycoprotein in the small intestine and liver of the Göttingen minipig
- the presence, localisation and activity of drug metabolizing Cytochrome P450 enzymes and uridine diphosphate glucuronosyltransferase enzymes in the liver of the Göttingen minipig.

1.1.3.1 The drug efflux transporter P-glycoprotein

Uptake and efflux drug transporters are often referred to as Phase 0- and Phase III-transport, respectively, and together with Phase I- and Phase II- metabolizing enzymes, they are involved in the ADME of drugs (22, 23). Additionally, drug transporters can share substrate specificities with drug metabolizing enzymes, enhancing their efficiencies, but also increasing the risk for drug-drug interactions (23).

The ATP-binding cassette (ABC) superfamily represents an important group of membrane-associated proteins that facilitate transport of endogenous and exogenous compounds into and out of cells (23). The ABCB subfamily (with P-glycoprotein (Pgp) and the bile-salt export pump), the ABCC subfamily (with the multidrug resistance
proteins), and the ABCG subfamily (with the breast cancer resistance proteins) form important families and members of the ABC superfamily. They are not only involved in the absorption and the disposition of drugs, but also in significant drug-drug interactions (e.g. anti-cancer drugs) (23). In this thesis, we will focus on P-glycoprotein. P-glycoprotein (also referred to as ABCB1 or its gene MDR1) is a well-known and important efflux transporter with a protective function against cytotoxic substances, like harmful agents in a diet, but clearly also against drugs (24, 25). Pgp is often involved in the phenomenon of ‘multidrug resistance’ during cancer chemotherapy and HIV treatment, due to an induced Pgp expression at the entrance of organs of interest (25). Drugs that are substrates for Pgp are often hydrophobic and positively charged at physiological pH. However, a wide range of structurally diverse substances are a substrate for Pgp (26). Some typical Pgp substrates are digoxin, loperamide, doxorubicin, vinblastine and paclitaxel (23). Two main mechanisms have been proposed to describe the mode of action of Pgp, i.e. ‘the vacuum cleaner model’ and the ‘flippase model’. Since many Pgp substrates are lipophilic and amphipathic, they will easily accumulate in the lipid bilayer, and more specifically in the interfacial region between the lipophilic and hydrophobic parts of the lipid bilayer. To reach the drug binding pocket of Pgp, the drug needs to reach the cytoplasmic or inner leaflet of the cell membrane (27). According to the ‘vacuum cleaner model’, Pgp can interact with its substrate within the membrane and facilitate its efflux from the cytoplasmic leaflet into the extracellular space, without reaching the cytoplasm (27). (See Figure 1.1) According to the flippase model, the substrate is actively moved from the inner membrane leaflet to the outer membrane leaflet. When the outer membrane leaflet is reached, the substrates can diffuse passively back to the extracellular aqueous space (fast), or they can move back to the inner membrane leaflet by spontaneous passive flip-flop. The active outwardly directed Pgp-mediated flipping needs to work faster than the passive movement from the outer membrane to the inner membrane, in order to generate a concenctratiant gradient that is necessary for the process to work, which appears to be the case (27). (See Figure 1.1) The energy that is required to facilitate the efflux capacity of Pgp is generated by hydrolysis of adenosine triphosphate (ATP) after binding of ATP to the cytoplasmic side Pgp (28).

Pgp is present at strategic positions in the body, where it limits uptake or facilitates efflux of endogenous and exogenous compounds. Such strategic localisations comprise the luminal cell membrane of the brain capillary endothelium, the luminal membrane
of the proximal tubular epithelium of the kidney, the luminal membrane of the intestinal epithelium and the biliary canalicular membrane of the hepatocytes (23). Consequently, entry of drugs in the central nervous system and the enterocytes is limited, while excretion of its substrates is enhanced in the urine or the bile (23). In this view, drug transporters are involved in determining the absorption, distribution and excretion from the drugs. In case of pregnancy, Pgp is present in the placenta at the level of the trophoblast and is mainly upregulated in the early phase of human pregnancy and decreases towards the end of pregnancy. This can be seen as kind of protection mechanism for the foetus against potentially toxic agents in the blood of the mother at the moment that the foetus is most vulnerable (29). Indeed, during early foetal development, Pgp is absent in the developing body (30). Different organs show a different developmental pattern for Pgp expression, with detection of Pgp in liver and kidneys from 13 weeks of pregnancy onwards, while expression in the blood-brain barrier and the small intestine were not detected at that time. However, Pgp presence was detected in the brain and the small intestine of a foetus that was born prematurely at 28 weeks of gestation (30, 31). Whether this early expressed Pgp is also active in foetuses remains to be determined. However, it is known that Pgp expression and activity remains limited in neonates, resulting in increased sensitivity to neurotoxic drugs in children until 3 to 6 months of age (32). Therefore, we were interested in the developmental pattern of Pgp in the liver and the small intestine of the Göttingen minipig since they represent our organs of interest and immaturity at this level after birth, may lead to increased systemic exposure after oral administration of drugs in immature animals. Higher systemic exposure after oral administration of a drug that is a substrate for Pgp has been noted in juvenile rats and was associated with toxicity and mortality, due to immaturity of intestinal Pgp (33, 34).
1.1.3.2 Drug metabolizing enzymes

Many enzymes are involved in the biotransformation of potentially harmful endogenous and exogenous compounds. Two distinct phases are present in such processes. Phase I-biotransformation generally adds or uncovers a specific functional group that is necessary for subsequent Phase II-metabolism. Phase II-biotransformation conjugates an endogenous co-substrate with the appropriate functional group of its substrate. The final goal of these phases of biotransformation is to make the substrates more polar and hydrophilic, and hence easier to excrete via biliary or renal excretion. As such, these enzymes participate in host defence against toxic endogenous and exogenous compounds (35). In general, the drug metabolizing capacity matures with
age, with absent or very low abundance and activity at time of birth, but which increases during the first days, months and years of life (3, 16, 36-39). Consequently, in view of drug administration to immature bodies, higher systemic exposure, prolonged half-life, and potentially toxicity of drugs may occur in young children with an immature metabolizing capacity (36-38, 40). These effects should be taken into account when drugs are administered to paediatric age groups.

**Phase I-biotransformation** involves oxidation, reduction, hydration and ester hydrolysis of the compound. Examples of enzymes involved in Phase I-biotransformation (35):

- Cytochrome P450 enzymes (CYP450)
- Flavin mono-oxygenases
- Xanthine oxidases
- Peroxidases
- Amine oxidases
- Aldehyde oxidases
- Glutatione peroxidases
- Carboxylesterases
- Alcohol dehydrogenases

In this thesis, we will take a closer look at the ontogeny of the most important drug metabolizing members of the **Cytochrome P450 superfamily**. CYP450 enzymes are integrated in the membrane of the endoplasmic reticulum of many organs (e.g. liver, small intestine, kidney, lung placenta, brain) and are mainly involved in the oxidative metabolism of endogenous compounds, like steroid hormones, fatty acids and prostaglandins, but also xenobiotics including chemicals, drugs, alcohol and procarcinogens. The resulting metabolites are more hydrophilic forms that are easier to excrete from the body via the bile or the urine. Consequently, toxicity that is due to accumulation of the compound in the body is prevented. Additionally, the metabolites from the CYP450-mediated pathways often generate a better substrate for the enzymes of the Phase II-reaction, further enhancing their elimination. However, substances may
also be turned into toxic metabolites and carcinogens by CYP450 biotransformation (35, 41).

The oxidative metabolizing capacity of CYP450 enzymes is based on the transfer of electrons to its heme core, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor, NADPH-cytochrome P450 reductase and oxygen.

In short, binding of the substrate in the binding pocket of the CYP450 enzyme alters the conformation of the enzyme, and facilitates the reduction of Fe$^{3+}$ in the heme core of the enzyme into Fe$^{2+}$ by the electron donor NADPH. Subsequently, O$_2$ can bind with the ferrous state of CYP450. A new electron is provided via NADPH (or Cytochrome b5) that will further facilitate the incorporation of one oxygen atom in the substrate (= oxidized substrate), release of H$_2$O, oxidized NADPH and restoring of the oxidized ferric state of the heme core of the CYP450 enzyme. NADPH functions as an electron donor via the flavoprotein NADPH-CYP450 reductase and is generated through the cytosolic pentose pathway in vivo. However, an NADPH-regenerating system is added to the incubational mixture during in vitro studies. Upon reduction by Cytochrome b5 reductase, Cytochrome b5 can also function as a reducing agent on CYP450 (35). Figure 1.2 shows the catalytic cycle of CYP450 (42).
The nomenclature of CYP450 enzymes is based on identity in amino acid sequences. A 40% identity in amino acid sequences is considered the cut-off to place CYP450 enzymes in the same family, represented by the same first number. These families are divided into subfamilies, based on having > 59% amino acid sequence identity, which is indicated by the same letter. Being part of the same subfamily does not necessarily mean that their functionality is the same. Small differences in amino acids can result in important differences in substrate specificity and metabolizing capacity. An individual enzyme in a subfamily is indicated by a final Arabic number. In other words, this classification and nomenclature is based on sequence alignment, and not on functional similarities (43). (Table 1.3) In view of their major relevance for human drug metabolism, we will study the ontogeny of CYP1A2-, CYP2C9-, CYP2D6- and CYP3A4-orthologues in the Göttingen minipig. Together, these isoforms are responsible for the metabolism of about 70% of clinically used drugs in man (44).
Table 1.3. Overview of the most important CYP450 enzymes for the metabolism of drugs present on the human market to date.

<table>
<thead>
<tr>
<th>Superfamily</th>
<th>Family</th>
<th>Subfamily</th>
<th>Individual enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP</td>
<td>1</td>
<td>A</td>
<td>1, 2</td>
</tr>
<tr>
<td>CYP</td>
<td>2</td>
<td>B</td>
<td>6</td>
</tr>
<tr>
<td>CYP</td>
<td>2</td>
<td>C</td>
<td>8, 9, 18, 19</td>
</tr>
<tr>
<td>CYP</td>
<td>2</td>
<td>D</td>
<td>6</td>
</tr>
<tr>
<td>CYP</td>
<td>2</td>
<td>E</td>
<td>1</td>
</tr>
<tr>
<td>CYP</td>
<td>3</td>
<td>A</td>
<td>4, 5, 7, 43</td>
</tr>
</tbody>
</table>

The isoforms that are of interest in our study are marked in bold.

Phase II-biotransformation involves biosynthetic reactions, mediated via enzymes like:

- Uridine diphosphate glucuronosyltransferase (UGT)
- Sulfotransferases
- Gluthatione S-transferases
- Glucosyltransferases
- O-, N- and S-transferases
- N-acetyltransferases
- Acyltransferases

Phase II-reactions involve conjugation reactions that further increase the water solubility of their substrates, enhancing biliary or urinary excretion (35, 45).

Glucuronidation is such a conjugation reaction, exerted by uridine diphosphate glucuronosyltransferases (UGT) that adds, in presence of uridine-5'-diphosphoglucuronic acid (UDPGA) as a co-substrate, glucuronic acid to a functional group (e.g. hydroxyl, carboxyl, amino or sulfur) of its substrate (aglycone). This substrate may be a metabolite from Phase I-biotransformation (35, 45). Figure 1.3 shows the glucuronidation of three endogenous (e.g. bilirubin and estradiol) and exogenous substrates (e.g. drugs). The conjugation of glucuronic acid, containing a charged moiety, to its substrate generates a more acidic metabolite that is more water soluble at physiological pH. Hence, its transport across membranes is hampered compared with small, lipophilic and neutral molecules. The glucuronidated metabolites with a relatively low molecular weight will be easily excreted in the urine. The drugs with higher molecular weights (> 500kDa in man) will be more easily eliminated via the bile.
by diffusion or via active secretion, e.g. via the efflux transporter P-glycoprotein (18, 46).

Figure 1.3. The glucuronidation of estradiol, acetaminophen and morphine by UGT1A1, UGT1A6 and UGT2B7, respectively. Figure reproduced from Fisher et al. (2000) (47).

In Figure 1.4, the phylogenetic tree for the human UGT superfamily is presented. The UGT1 and UGT2 families only share a 41% identity in amino acid sequences. All UGT1A subfamilies share about 66% identity in amino acid sequences, while the UGT2A and UGT2B share about 59% identity in amino acid sequences (45).
1.1.3.3 Isolation of liver microsomes

Several techniques have been described to study drug metabolism in vitro, e.g. hepatocytes, liver microsomes, and cDNA-expressed CYP450 and UGT enzymes (48). Such in vitro techniques can accelerate the process of drug development since they allow a relatively fast, easy and safe screening of new compounds, and their potential as a new drug. Hepatocytes resemble the in vivo situation best, containing all enzymes, transporters, cofactors and membranes. This technique also allows for studying the inducing potential of new compounds on CYP450 expression. However, it is an expensive and time-consuming technique (48, 49). Liver microsomes are obtained after isolation of the endoplasmic reticulum from the hepatocytes by ultracentrifugation. The
disrupted endoplasmic reticulum will form small vesicles, called microsomes that contain the endoplasmic membrane-associated CYP450 enzymes, NADPH-CYP450 reductases and UGT enzymes, but they lack cytosolic drug metabolizing enzymes and cofactors (48). The cDNA-expressed enzymes or also called recombinant CYP450 or UGT microsomes only contain a specifically expressed isoform. They are mainly interesting in view of gaining information on the role of individual isoforms in the metabolism of a compound (48).

As we were interested in the ontogeny of CYP450 enzymes and UGT enzymes, which enzymes are both integrated in the membranes of the endoplasmic reticulum of the liver, we have isolated liver microsomes from the Göttingen minipigs at different ages. These liver microsomes were incubated with human probe substrates and the necessary cofactors to assess their metabolizing capacity. (See Chapters 4 and 5) As a positive control, human liver microsomes (HLM) and Cytochrome P450 Baculosomes® were included in the CYP450 activity studies, along with the minipig liver microsomes. These Cytochrome P450 Baculosomes® are microsomes prepared from insect cells infected with recombinant baculovirus containing cDNA for a specific human P450 isozyme, human NADPH-CYP450 reductase, and in some cases human Cytochrome b5. Both the HLM and the Baculosomes® are commercially available (Life Technologies, Thermo Fisher Scientific).
1.2 The non-clinical phase of paediatric drug development

Safety studies are pivotal in drug development. Both adult and juvenile animals, or their tissues, can be useful in this non-clinical phase of paediatric drug development. The EMA and the FDA have provided guidelines for non-clinical safety evaluation of paediatric products (6, 50). The guidelines stress the importance of (juvenile) animal studies in paediatric drug development.

1.2.1 The role of juvenile animal models in paediatric drug development

Between 2008 and 2016, 26% of PIPs included juvenile animal studies. About 70% of these PIPs contained juvenile animal studies that were intended for a target population of infants up to two years of age (51).

From the EMA perspective: ‘The main aim of non-clinical studies, to support the development of medicinal products to be used in paediatric patients, is to obtain information on the potentially different safety profiles from those seen in adults. Juvenile animal studies can be used to investigate findings that cannot be adequately, ethically, and safely assessed in paediatric clinical trials.’ (50)

In other words, the main aim of a juvenile study is to assess whether juvenile animals have a different sensitivity to a medicinal product compared with adult animals, and to identify effects on developing organs. To obtain these goals, a thorough consideration of species selection, and representative animal age at start (developmental stage of the target organs), study duration, choice of endpoints and routes of administration are pivotal to generate useful data. Only then is a relevant extrapolation to paediatric age groups possible (52).

Juvenile animal studies should only be performed when previous adult human studies or previous animal studies gave insufficient information to support paediatric clinical trials. However, even when certain reactions can be predicted from earlier data, a juvenile animal study may be requested to further address a specific concern, or to study reversibility or possible worsening of expected findings. The difficulty of
paediatric drug development is the fact that beside the intended main effect of the drug and its potential side effects, one also needs to take into account that there might be an effect on the ongoing developmental processes of organs or an altered vulnerability of a developing organ may be present. Additionally, the developing body may influence the pharmacokinetics and pharmacodynamics. For this reason, safety assessment of the new drug may be necessary in juvenile animals. To cut a long story short, a case-by-case analysis is necessary for each study (50).

Carleer and Karres stress in their review on juvenile animal studies and paediatric drug development that the pharmaceutical companies should provide scientifically based justifications when they omit juvenile animal studies in the initial PIP submission (53). Since both authors are members of the PDCO and the EMA, the impression is that the authorities encourage juvenile animal studies. However, some concerns have also been raised on the added value of such juvenile animal studies and on the potential request of both a rodent and a non-rodent juvenile animal model (54, 55). Baldrick reviewed in 2010 a number of juvenile studies and their outcomes. He concluded that in many cases it was not clear whether the juvenile studies really contributed to the identification of relevant new toxicity in man. Additionally, when severe adverse reactions are seen in the juvenile animal, how do you translate these findings to the paediatric population? Is toxicity in juvenile animals really a ‘new toxicity’ or is it purely related to immaturity (e.g. metabolism), what can be expected in an immature body. To overcome these uncertainties, it is important that the juvenile animal models are thoroughly characterized (56).

1.2.2 Species selection for juvenile toxicity studies

In general, one species is sufficient in juvenile studies (53). The rodent model is often preferred based on experience, historical background data and their advantages as an animal model. Additionally, they possess beneficial characteristics for juvenile studies. They have a short gestation time, are multiparous, are easy to cross-foster and do not require special or large housing facilities. Moreover, developmental systems have been investigated extensively in this species (51, 57). Hence, both the EMA and the FDA recommend the juvenile rat when applicable. Between 2008 and 2016, 76% of the juvenile animal studies were performed in the rat.
However, the only relevant species is the one that is appropriate for evaluating the toxicity endpoints relevant to the intended paediatric population (PK/PD, toxicology and feasibility) (53).

Consequently, what is the role of the non-rodent animal model in juvenile studies? First, there is the practical approach. Working with rodent pups is more challenging in view of their small size compared with dogs, minipigs and non-human primates (NHPs). However, from an ethical point of view, working with juvenile dogs and NHPs is disapproved by the society. Additionally, there is a high chance of maternal rejecting when NHP infants have been manipulated (54). The juvenile minipig may be a good alternative.

Second, rodents are far less mature at time of birth compared with man, which may result in toxicity or adverse reactions that are not relevant for the human neonatal population (54, 58). A longer gestation length results in a higher degree of maturity at birth (rodent < dog < Göttingen minipig < NHP). However, the chronological order of organ maturation may differ among organs, and among species. (Figure 1.5) For example, the neurological control of locomotion is relatively mature at birth in pigs, while it is immature in human neonates. The reverse is true for the maturity of gastrointestinal function (58). These organ-dependent developmental differences should be borne in mind when determining age groups in a preclinical setup.

Third, when the target is not expressed in rodents, or when drug metabolism significantly differs from that in man, a non-rodent animal model can be requested (50). Finally, the authorities can request both a rodent and non-rodent animal, e.g. for paediatric only indications when no adult preclinical and clinical data are available (55).

The Göttingen minipig shows some advantageous qualities among the non-rodent animal models, especially in view of juvenile studies (59). (See also Chapter 1.3) The Göttingen minipig is a continuous breeder, resulting in 2.1-2.3 litters a year. Gestation length (114 days ± 4 days) is longer than that of the dog (59-67 days), but shorter than that of the NHP (153-179 days in the Cynomolgus monkey). The offspring in both dog and minipig is large, with about 5-7 pups or piglets per litter, while the Cynomolgus monkey gives birth to only one infant. Cross-fostering and manipulating of the offspring is relatively easy in the minipig and the dog, while this is difficult in the NHP. The Göttingen minipig is also earlier sexually mature (3-5 months) compared with the dog.
(7-14 months) and the NHP (Cynomolgus monkey 36-48 months). A disadvantage of the minipig is their higher body weight compared with the two other species, especially in view of the amount of the (often expensive) compound that needs to be administered (59). Additionally, the youngest NHPs that can be purchased are about 9-12 months of age, while pregnant animals are typically not available from breeders. Consequently, when neonatal monkeys need to be included in a juvenile study, the breeding needs to occur at the research facility, which implicates logistical challenges (57).

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**Figure 1.5.** Maturation (as percentage of gestation) of lungs, gut, and brain, relative to term birth, weaning, and total life span, for rats, pigs and humans. The symbols for each organ indicate the time period when ontogenetic maturation is sufficient to secure general viability of preterm newborns (dark grey boxes), that is, independent breathing, digestion of non-milk food, and locomotion (in part determined by brain locomotoric control). Figure and legend are reproduced from Sangild et al. (2013) (58).
1.3 The Göttingen minipig

Pigs are versatile animals that have been largely involved in our daily life for many centuries. However, in today’s society, the only contact with pigs seems to be reduced to achievement of derivative products like a package of meat, gelatine in candies, or hairs in brushes. Therefore, some additional words about the species that was used in this thesis seem appropriate.

1.3.1 The Göttingen minipig: a general background

The first signs of domestication of European wild pigs go back to the 4th millennium B.C. However, at that time, domesticated pigs from the Near East were already introduced in Europe. Later, multiple crossbreeding between European and Asian porcine breeds occurred, resulting in a variety of external appearances (60).

The domestic pig and the wild pig are classified in the:

Order *Artiodactyla* (even-toed ungulate)

    Suborder *Suiformes*

    Family *Suidae*

    Genus *Sus*

        *Sus scrofa*

        *Sus scrofa domesticus*

Carl Linnaeus (1758) gave the name *Sus scrofa* to the wild boar. Subsequently, the domestic pig was named *Sus scrofa domesticus*, which was shortened to *Sus domesticus* by Johann Christian Polycarp Erxleben in 1777 (61).

Pigs are highly social animals. They participate in social and object play, they are able to learn spatial discriminations that depend upon working and reference memory, they can discriminate between familiar and unfamiliar humans and they are sensitive to the emotions of other pigs. In other words, pigs have more emotional and intellectual capacities than we often acknowledge (62).
The introduction of the pig as a laboratory animal led to a demand for smaller pigs that are more manageable, easier to keep, and cheaper to feed and to administer compounds to. Consequently, selective breeding created new miniature pig breeds. The growth rate of a minipig is slow. Hence, the body weight is low when these animals enter studies; generally around the time they reach sexual maturity (63). Several minipig strains have been bred (Göttingen, Yucatan, Sinclair, Hanford, etc.). The Göttingen minipig is the most commonly used strain in Western Europe. Figure 1.6 shows the origin of this breed.

![Image showing the origin of the Göttingen minipig breed](http://minipigs.dk/the-goettingen-minipig/genetic-background/)

The genetics for the entire breeding are managed in Göttingen, Germany. The entire breeding population is housed in three physical locations:

- Dalmose in Denmark (Ellegaard)
- Göttingen in Germany
- North Rose in New York, U.S.

Because of the strong exploratory and foraging behaviour of a pig in general and the impact of the pig's healthy emotional state on the productivity and reliability of experimental results, it is important to respect the minipigs welfare needs (59, 64).
1.3.2 The Göttingen minipig in biomedical research

Both rodent and non-rodent animal models are used in biomedical research. The choice of a specific animal model is a crucial step, and should be thoroughly evaluated in view of the research question. The rodent animal model (i.e. rats and mice) is predominantly used. Rodents are relatively cheap to house, to hold, and to dose. They are easy to handle and they have a short life span. Additionally, from an ethical point of view, the public opinion is less critical on their use compared with the use of non-rodent animals. Hence, rodents have extensively been characterized with many historical background data available, which further promotes their use. However, they may be unsuitable in some cases. For example, when the target is not expressed in rodents, or in case of drugs that are designed for paediatric use only, both a rodent and a non-rodent animal model may be demanded. Historically, the dog and the non-human primate are the most common non-rodent animal models (57). However, the Göttingen minipig is gaining ground. Indeed, the Göttingen minipig offers many advantages. Some examples are listed below.

- Minipigs can be bred under controlled conditions (genetically coherent, conventional or specific pathogen free, pigmented skin or non-pigmented skin) and can be housed in similar laboratory conditions as dogs (65).
- They share many anatomical and physiological similarities with man.
  - Gastrointestinal system: Both pigs and man are omnivorous and monogastric (65). Transit time and gastrointestinal pH are considered similar (66). The minipig has already proven to be a better animal model compared with the dog at the level of sensitivity towards orally administered non-steroidal anti-inflammatory drugs (NSAIDs). Dogs are prone to gastric ulceration and vomiting when NSAIDs are given, while this is not the case in pigs (67). Furthermore, dogs possess a thicker gastric mucous layer compared with man, and a lower basal gastric acid secretion in the stomach compared to humans, pigs and monkeys, which can influence the absorption of orally administered drugs (68, 69). Ulceration of the stomach as a result of stress has been found in the stomach of rodents, but not in pigs, which is advantageous
to avoid lesions that are not linked to toxicity of an administered compound (65).

- Cardiovascular system: The coronary blood supply of the heart is almost identical in pigs and humans. Blood supply to the coronary arteries is right-sided dominant and is similar to 90% of the human population without a good collateral circulation. Consequently, occlusion easily results in a total infarct. In contrast, dogs have a collateral circulation, making them a poor model (65, 66).

- Urinary system: Similar to humans, pigs have multirenulate and multipapillate kidneys with true calices (65).

- Integumentary system: Porcine skin is similar to human epidermal thickness and human dermal-epidermal thickness ratio, with a sparse hair coat. The pig forms also a good model to study wound healing and to perform reconstructive surgical treatment (65). Both local tolerance testing and the assessment of systemic toxicity after dermal application can be performed in the Göttingen minipig (66).

- The minipig is also a surgical model. Interventional catheter techniques, complex trauma procedures, (non)survival training classes, transplantations, endoscopic procedures, cardiac valve replacement and stent implantations are just some examples (65).

- Finally, the minipig has a role in the non-clinical phase of drug development, although some reluctance exists at pharmaceutical companies, mainly due to less experience compared with the Beagle dog and the NHP (66). However, the choice of an animal model should be based on the capacity of the model to generate similar pharmacokinetic, pharmacodynamic, metabolic, safety and toxicity profiles as in man (66).

In conclusion, the minipig is gaining importance in modern regulatory safety testing. They are applicable to all routes of administration, share similar anatomical and physiological characteristics with humans, and show similarities in metabolizing capacities with man, especially when considering the small intestine and liver (65, 66). (See chapter 1.4) This last characteristic will be further explored in this thesis.
Table 1.4 compiles important characteristics of the Beagle dog, the Cynomolgus monkey and the Göttingen minipig that have been discussed in Chapter 1.2.2 and Chapter 1.4.

Table 1.4. Species-related characteristics of non-rodent models.

<table>
<thead>
<tr>
<th>General remarks</th>
<th>Beagle dog</th>
<th>Cynomolgus monkey</th>
<th>Göttingen minipig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Often preferred non-rodent model</td>
<td>Most closely related to man compared with dog and minipig</td>
<td>Similar anatomy and physiology compared with man</td>
<td></td>
</tr>
<tr>
<td>Prone to vomiting</td>
<td>More difficult to handle</td>
<td>Relatively large size</td>
<td></td>
</tr>
<tr>
<td>Prone to gastric ulceration after NSAIDs</td>
<td>Special requirements for housing</td>
<td>Some reluctance due to less experience</td>
<td></td>
</tr>
<tr>
<td>Prone to cardiotoxicity after sympatomimetics and hypotensive drugs</td>
<td>Public opinion is not mild</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Public opinion is not mild</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reproductive characteristics</th>
<th>Beagle dog</th>
<th>Cynomolgus monkey</th>
<th>Göttingen minipig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant animals</td>
<td>Available from supplier</td>
<td>Typically not available, infants available &gt; 9-12 months of age</td>
<td>Available from supplier</td>
</tr>
<tr>
<td>Oestrus</td>
<td>Di-oestrus</td>
<td>Continuous breeder</td>
<td>Continuous breeder</td>
</tr>
<tr>
<td>Litters/year</td>
<td>1-2 litters</td>
<td>1 litter</td>
<td>2.2 litters</td>
</tr>
<tr>
<td>Offspring/litter</td>
<td>4-8</td>
<td>One</td>
<td>6</td>
</tr>
<tr>
<td>Gestation length</td>
<td>58-63 days</td>
<td>160-170 days</td>
<td>114 days</td>
</tr>
<tr>
<td>Manipulation offspring</td>
<td>Rather easy</td>
<td>Difficult</td>
<td>Easy, Cross-fostering</td>
</tr>
<tr>
<td>Sexual maturity</td>
<td>7-12 months of age</td>
<td>4-5 years of age</td>
<td>3-5 months of age</td>
</tr>
</tbody>
</table>
1.4 Functional morphology of the small intestine and the liver

Enzymatic degradation of food and the absorption of nutrients are the most fundamental roles of the small intestine, while the liver will further handle these absorbed nutrients. However, the function of the liver is not limited to nutrient handling, but this organ is also capable of converting potentially toxic endogenous compounds (e.g. hormones, bilirubin) and exogenous compounds (e.g. toxic substances in diet like plant toxins or aflatoxins) into less harmful and more water soluble metabolites. This metabolizing capacity appears to be present in the small intestine as well, although the liver remains the main detoxifying organ. The range of substrates that undergo metabolism has expanded from endogenous toxins and exogenous dietary toxins to a very wide variety of drugs as well (70). Therefore, we will focus on these two organs in view of their capacity to metabolize drugs, but also in view of their functional morphology that influences the absorption, distribution, metabolism and excretion of administered drugs, as discussed below.

1.4.1 The small intestine

Rodents, pigs and man are omnivores and have a relatively simple gastrointestinal tract compared with herbivores. However, they possess an important small intestinal compartment. The main function of the small intestine is digestion (degradation of fats, carbohydrates and proteins) and absorption of the degraded nutrients (70). In this respect, the small intestine also plays an important role in the absorption and the metabolism of orally ingested drugs, which will be the focus of this thesis.

The small intestine comprises three anatomical zones: the duodenum, the jejunum and the ileum, from proximal to distal, respectively. The duodenum is connected proximally with the pyloric end of the stomach, while the distal part of the ileum is connected with the caecum at the ileocaecal junction. The common bile duct and the pancreatic duct end into the duodenum (71). The wall of the digestive tract is composed of four distinct concentric layers, i.e. the tunica mucosa or innermost layer, the tela submucosa, the tunica muscularis (inner circular and an outer longitudinal smooth muscle layer), and the tunica serosa or outermost layer. The tunica mucosa consists of an epithelial layer with secretory and absorptive functions, a layer of connective tissue or lamina propria
mucosae and a thin layer of smooth muscle cells or lamina muscularis mucosae (72, 73). (Figure 1.7)

Figure 1.7. The layers of the wall of the small intestine of the pig. Part A shows a transversal section of the small intestine with several plicae circulares protruding in the intestinal lumen. Scale bar 2000 µm. Part B shows the magnification of one plica, covered with several intestinal villi and crypts. The lamina muscularis mucosae is a thin layer that lies immediately beneath the base of the villi and crypts. Scale bar 500 µm. Part C shows the magnification of one finger-like villus, lined by the enterocytes. Scale bar 200 µm. Part D shows a transversal section the different layers of the small intestinal wall. The crypts of Lieberkühn lie between the villi. The outer muscular layer takes care of the peristaltic activity of the small intestine. Scale bar 500 µm.

To enhance the absorptive surface and the contact of the enterocytes with the luminal content, the mucosa and the submucosa form circular folds or plicae circulares, perpendicular to the length of the small intestine. Additionally, the tunica mucosa shows extensions of the lamina propria and the epithelium, called villi (0.5-1.5 mm). The absorptive cells have microvilli on their surface, covered by a thick glycocalyx with several enzymes. The formation of plicae, villi and microvilli further enhances contact with orally administered compounds (71, 72). Intestinal crypts are present at the base of the villi. The villus and the crypt form a functional unit. Cell proliferation is maintained in the crypts. These new cells migrate upwards from the crypt to the top of
the villus, where they are discarded in the intestinal lumen. During their travel, they mature and acquire their secreting and metabolizing capacities. The entire epithelial cell population is renewed every four to six days (71, 72).

A network of capillary blood vessels and lymphatic vessels are present in the axis of each villus, in close proximity with the epithelium, facilitating the transport of nutrients and drugs to the liver and the systemic circulation (71). The intestinal surface area is up to 40 times smaller in neonates and children compared with adults (1, 74). The more broad-leaf shaped form of the villi in neonates and children transform into finger-shaped villi in adults. Additionally, the length and the diameter of the small intestine further increase during postnatal development (1, 71).

There is a close relationship between the degree of the maturation of the gastrointestinal tract at time of birth and the length of gestation. Rodents have a short gestation, and they are relatively immature at birth (altricial species). Consequently, they greatly depend on the dam for nutrition, thermoregulation, locomotion, etc. In contrast, the pig is more mature at time of birth (precocious species) and a major cluster of gastrointestinal changes occurs around time of birth as in humans, while these changes occur around time of weaning in rodents (75). For example, early crypt development is already present during the foetal period in man and pigs, while the formation of crypts is only observed after birth in rodents (75). A dissimilarity between pigs and man is the timing of gut closure. In pigs, this occurs after birth, allowing for transfer of macromolecules, like growth factors and immunoglobulins, during the first 24-72 h of life. In man, gut closure already occurs prenatally (75). This transfer of macromolecules proceeds in rodent until 21 days of age (75).

The structure and functionality of the intestinal tract appears to be similar to the adult already at the age of three weeks in piglets (71). However, the time of weaning, the diet and the weaning process should be taken into account as this process results in crypt depth lengthening and villus length shortening (71, 76, 77). In a non-clinical setting, minipigs are usually weaned around 28 days of age. However, this transition is rather abrupt compared with in man. In man, weaning goes more gradually, with a steady decrease in milk and a gradual increase in solid food. In man, this period occurs around 6 months up to 2-3 years of age (75).

The role of the small intestine in first line host defence and in drug metabolism has been acknowledged for many years. Indeed, the strategic location of the small intestine and
its large surface area fits well with its role in the first pass metabolism and the oral bioavailability of orally administered drugs. Both phase I- and phase II-metabolism, together with drug transporters are present in the enterocytes (21). A remarkable collaboration between intestinal CYP3A4 and the efflux transporter P-glycoprotein (Pgp) has been described. Indeed, CYP3A4 and P-glycoprotein are co-localised in the upper part of the villous enterocyte and they share many substrates, which results in complementary activities, and eventually in a lowering of oral bioavailability (20, 78). Orally ingested drugs will be absorbed by the enterocytes of the small intestine. When the compound is a substrate for both CYP3A4 and Pgp, the drug can be pumped back into the intestinal lumen, or it can be metabolized by CYP3A4. The resulting metabolite may be a better substrate for Pgp, resulting in excretion back in the intestinal lumen, or it may reach the portal vein via the capillary network in each intestinal villus. Due to this collaboration of Pgp and CYP3A4, a repeating cycle of absorption and excretion may exist along the length of the small intestine, resulting in a prolonged exposure and consequently a lowering of the oral bioavailability (20, 78). Another remarkable fact on the presence of CYP3A4 and Pgp in the small intestine is their abundance along the length of the small intestine. The abundance of CYP3A4 is highest in the proximal region of the small intestine and lowest in the distal region, while the reverse is true for P-glycoprotein. From a clinical point of view, these distribution patterns create a higher capability to metabolize drugs in the proximal region of the small intestine with a relatively low efflux capacity, while more drugs can be pumped back to the intestinal lumen at the distal part of the small intestine where the metabolizing capacity is low. Additionally, these distribution patterns may have consequences on the oral bioavailability of a drug when its absorption is mainly localised in the proximal or distal region of the small intestine and the drug is a substrate for one protein only (79).

Although the liver is considered the main player in first-pass metabolism, the small intestine has also proven to be of significant influence, mainly when doses of drugs are low (19). Cyclosporine, midazolam, nifedipine and verapamil are some of the compounds that have been described to undergo significant intestinal first-pass metabolism (19). However, much research is based on in vitro studies, which is not always easy to translate to the in vivo situation. Additionally, physiological factors like blood flow, gastric and intestinal transit times, membrane permeability and lumen pH may also influence the amount of drug that reaches the systemic circulation (19).
1.4.2 The liver

The liver is one of the most vital and largest glands in the body with roles in nutrient handling, production of plasma proteins (e.g. albumin and coagulation factors), glucose homeostasis, ammonia detoxification, synthesis and metabolism of dietary lipids, bile production and drug metabolism (46). Additionally, the foetal liver acts as a temporary location of haematopoiesis. Towards the end of gestation and in the early neonatal life, the role of intrahepatic haematopoiesis diminishes, and the haematopoietic precursor cells migrate to the bone marrow (46). Macroscopically, the liver is divided into lobes by fissures. For most mammals, the liver consists of a left lateral, a left medial, a right lateral, a right medial, a quadrate and a caudate lobe with, depending on species, the presence of a papillary and a caudate process. The right and left lobes are separated by the falciform ligament. The quadrate lobe is situated between the fissure for the *ligamentum teres* or the round ligament, which is the remnant of the *vena umbilicalis*, and the *fossa vesicae felleae* (the location for the gall bladder). The gall bladder lies between the quadrate lobe and the medial lobe of the right lobe. In general, the right lobe is the largest one, comprising the proper right lobe, the caudate lobe and the quadrate lobe. The caudate lobe lies dorsal to the *porta hepatis* and has a *sulcus venae cavae* (the groove for the caudal vena cava) and the *porta hepatis* (liver hilus) (71, 80).

The porcine liver has a left, a right and a median lobe. These lobes are separated by deep interlobular fissures. The median lobe is subdivided by the umbilical fissure, creating a left and right median lobe. In contrast with the human liver, the left liver lobe is the largest one. Additionally, a small quadrate lobe and a caudate lobe are present. The left lobe forms the largest lobe in contrast with the human liver. The caudate lobe joins the right lateral lobe on the visceral side. The gall bladder is partially embedded in the right median lobe (81). The segmental anatomy has been shown to be similar between man and pig, which makes the porcine liver a useful translational model for experimental surgery (81).

Microscopically, the adult liver shows a remarkable architectural structure, defined by a complex, but orchestrated, arrangement of (mainly) hepatocytes, cholangiocytes, endothelial cells, Kupffer cells and stellate cells. (Figure 1.8) The hepatocytes possess a polar character. The basolateral area shows increased production of membrane associated receptors and transporters for secretion of proteins associated with the sinusoidal vessels. The apical area constitutes the bile canaliculi, with membrane-
associated transporters that are related to bile secretion. Both basolateral and apical membranes have microvilli to enlarge their surface area (46). The hepatocytes are arranged in one-cell-thick plates or cords that are basolaterally lined by fenestrated endothelial cells from the sinusoidal capillaries. The hepatic cords and sinusoids radiate towards a central hepatic venule from the hepatic vein and hence frame a liver lobule. At the corner of each lobule, a triad of sections of the portal vein, the hepatic artery and the bile duct is present (46, 71, 82). (Figure 1.8) A layer of fibrous tissue encloses each individual liver lobule. This fibrous lining is clearly noticeable in the porcine species (71).

Figure 1.8. Organization of the liver. (A) One liver lobule, consisting of a central vein (CV) with hepatic cords that radiate towards the portal triads. (B) Within each lobule, a number of sinusoids is present, which are discontinuous vessels built from specialized fenestrated endothelial cells of the liver. Figure is reproduced from Gordillo et al. (2015) (82).

This well-defined organization of the liver mainly arises during postnatal maturation of the liver, represented by a thinning of hepatic plates and an increase in liver size. In a mature liver, hepatic plates are usually one-cell-thick, while at birth and during pregnancy they are about three cells thick. Mature liver characteristics are attained in man by 5 years of age (71, 83).

The liver has both a venous and an arterial blood supply. The portal vein delivers blood from the intestine, pancreas and spleen, while the hepatic artery transports the blood from the aorta. Together, they provide the liver with nutrients, hormones, endo- and xenobiotics, and oxygen (46). The adjacent position of hepatocytes and sinusoids facilitates the contact between the basolateral membrane of the hepatocytes and the blood flow. The hepatocytes produce bile that is secreted in the bile canaliculi, which are located between the apical plasma membranes of neighbouring hepatocytes and
which are lined by tight junctions. The bile flows in the opposite direction of the blood and goes into the direction of the duodenum. The bile canaliculi drain into the larger left and right hepatic bile ducts that drain into the gallbladder via the common hepatic duct and the cystic duct. When the gallbladder contracts, the bile is secreted in the duodenum via the *ductus choledochus* (71).

The liver acinus represents a functional unit of liver parenchyma, with roughly three zones along its axis (following the direction of the hepatic blood flow). Zone 1 surrounds the portal triad and it receives oxygen- and nutrients-rich blood. Zone 3 encloses the central vein. This zone receives the blood that is least oxygenated. Zone 2 is located between Zones 1 and 3. In the mature liver, there is a clear functional heterogeneity among these zones, resulting in a metabolic zonation (73, 84). (Figure 1.9) The rate of hepatic uptake of oxygen is up to 3 times higher in the periportal region compared with the perivenular region, creating a large oxygen concentration gradient in Zone 1. CYP450, UGT enzymes, and glutathione-S-transferases are mainly expressed in the perivenular region (Zone 3), creating a centrilobular expression pattern, following the difference in the presence of the endoplasmic reticulum (46, 85). In contrast, sulfotransferases, involved in sulfate conjugation, are mainly present in Zone 1 (85). Zone 2 can be seen as a kind of transition zone, with hepatocyte activities related to the most proximate zone (84).
Figure 1.9. Metabolic zonal functions. Zone 1 is located in the periportal zone, Zone 3 in the perivenous area, with Zone 2 in between. Blood flows from the portal vein (PV) (75% of blood flow) and the hepatic artery (HA) (25% of blood flow) into the direction of the hepatic vein (HV). Bile flows in the opposite direction into the bile duct (BD) (86-88).

The regulation of this metabolic zonation is not completely elucidated yet, but some important factors have been identified (87, 88). The zonation may be a response to fluctuating blood-borne factors, like gradients of oxygen, hormones (like growth hormone and thyroxin have an effect on CYP450, glucagon and insulin have an influence on carbohydrate metabolism) and transcription factors. All hepatocytes in the acinus express the gene of these metabolizing enzymes, but to a different extent depending on their exposure to this blood-borne factors. The magnitude of expression may differ depending on specific needs, representing a ‘dynamic zonal metabolism’. A compartment-like or stable zonation also exists, but is less common. The expression of gene products is limited to specific cells, independent of the metabolic state of the liver. Some examples are enzymes involved in ammonia metabolism, glutamine synthesis and carbamoylphosphate synthesis (86, 87). Together with the higher abundance of CYP450 enzymes in the perivenous region, a higher abundance of NADPH CYP450 reductase and Cytochrome b5 are present in this region compared with in the periportal hepatocytes.
Wnt/β-catenin signalling is considered a dominant player in establishing the metabolic zonation of the liver, with the presence of active β-catenin in the perivenous hepatocytes and the presence of the negative regulator adenomatous polyposis coli (APC) (tumor suppressor gene) in the periportal hepatocytes. Drug metabolism is hence largely controlled by the Wnt/β-catenin signalling pathway. In short, secreted Wnt ligands can bind to frizzled receptors (Frz). This binding leads to activation of the canonical Wnt signalling pathway. The β-catenin degradation complex will be dissociated and unphosphorylated β-catenin will accumulate and translocate into the nucleus. In collaboration with transcription factors, it can regulate transcription of the target genes (87). The influence of the Wnt/β-catenin pathway on the periportal regions consists of a down-regulation of periportal activities (87, 88). (See Figure 1.10)

It has also been shown that the Wnt/β-catenin pathway crosstalks with various nuclear receptors (Pregnane X-receptor, Constitutive androstane receptor, Aryl hydrocarbon receptor) that are involved in the regulation of CYP450 and UGT expression. In this way, the Wnt/β-catenin pathway plays an important role in the basal expression of drug metabolizing enzymes, but also a synergistic role in the induced expression that can be triggered by activation of the nuclear receptors by xenobiotics (89).

Roles for Hepatocyte Growth Factor, Hepatocyte nuclear factor 4 alfa (Hnf4α) and peroxisome proliferator-activated receptor α (PPARα) as transcription factors have also been suggested, but still need further elucidation. Roles for Hedgehog and rat sarcoma signalling, presumably cross-talking with the Wnt/β-catenin pathway, have been suggested too. However, the exact roles of these pathways also need further exploration. It seems assumable that an intensive crosstalk between different pathways results in the final hepatic zonation (85, 86, 89).
Figure 1.10. Zonation of Wnt signaling in the liver. (a) Localisation of Wnt partners adenomatous polyposis coli (APC) and unphosphorylated β-catenin. Periportal hepatocytes are enriched in APC, allowing the accumulation of active unphosphorylated β-catenin in perivenous hepatocytes. A schematic diagram of a periportal hepatocyte, within which Wnt is inactive, is shown on the left, and the consequences of Wnt activation in a perivenous hepatocyte are shown on the right. (b) Mouse models of liver-specific β-catenin inactivation (β-catenin knock-out) or activation (adenomatous polyposis coli knock-out). Figure and legend are reproduced and adapted from Monga et al., 2010 (87).
1.5 References

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2 Aims of the study

The Paediatric Regulation (EC No 1901-2/2006) has been implemented in January 2007 in order to facilitate the development and accessibility of medicinal products for use in the paediatric population. In view of safety, requests for juvenile animal studies occur, prior to the initiation of clinical trials in children, especially for the youngest age groups. The rodent is often the first choice for this type of studies as it is a well-characterized animal model, often used in (general) toxicity studies and easy to handle and house. However, rodents may not always be the most suitable juvenile animal model, e.g. when targets are not expressed in this species. As such, a juvenile non-rodent model such as the dog, minipig or non-human primate could be a better alternative. Unfortunately, these species are less characterized, especially regarding their drug metabolizing capacity. Therefore, this thesis focused mainly on the characterization of the ontogeny of drug metabolizing enzymes in the Göttingen minipig, i.e. in the liver and to a limited extent in the small intestine. Because of their value in the absorption, distribution, metabolism and excretion (ADME) of a drug, organ data and gastrointestinal pH data were analysed.

The following age groups (both sexes included) were investigated in this thesis:

<table>
<thead>
<tr>
<th>Age</th>
<th>Motivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>84-86 days of gestation</td>
<td>75% of gestation → Early preterm</td>
</tr>
<tr>
<td>108 days of gestation</td>
<td>95% of gestation → Late preterm</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>Neonatal period</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>Additional formalin-fixed liver samples and organ data from Sequani.</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>Weaning age in a preclinical setting. The gastrointestinal tract has matured significantly.</td>
</tr>
<tr>
<td>3 months</td>
<td>Male and female Göttingen minipigs reach sexual maturity (males earlier than the females)</td>
</tr>
<tr>
<td>4 months</td>
<td>Additional formalin-fixed liver samples and organ data from Sequani</td>
</tr>
<tr>
<td>5 months</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>Mature animals</td>
</tr>
</tbody>
</table>

The age groups marked in bold are the age groups that were included in each study, providing samples that were obtained by our group. The other age groups (Day 14, Day 21, 3 months, 4 months and 5 months of age) were represented by samples provided by Sequani, and were not included in the enzymatic activity assays.
The following research hypothesis was tested:

**Juvenile Göttingen minipigs show a similar ontogeny of drug metabolizing capacity as the human paediatric population (the first month of life in the Göttingen minipig ~ the first year of life in man?)**

To test this hypothesis the following research objectives were addressed:

1. Describe the localisation and developmental expression pattern of CYP3A7-, CYP3A4-, Pgp- and UGT1A-orthologues in the liver and small intestine of the Göttingen minipig. (Chapters 3 and 5)

2. Investigate the ontogeny of CYP450 and UGT activity in the liver of the Göttingen minipig, by using human probe substrates for CYP1A2, CYP2C9, CYP2D6, CYP3A4 and UGT, and their relation to developmental patterns in man. (Chapter 5)

3. Investigate whether a shift from a hepatic CYP3A7-like enzyme into a CYP3A4-like enzyme around the time of birth is present, as has been shown in man. (Chapters 3 and 4)

4. Compile and analyse gastrointestinal pH values, organ weights and body weights during development and their relation to human data. (Chapter 6)

5. Conclude on the value of the juvenile Göttingen minipig as a translational model for paediatric age groups based on *in vitro* CYP450 data. Additionally, compare the minipig with the rat and with other non-rodent animal models (Beagle dog and Cynomolgus monkey). (Chapters 4, 5 and 7)

The outcome of this research related to the objectives above will be further discussed in the General Discussion. (Chapter 7)
3 Ontogeny of CYP3A and Pgp in the liver and small intestine of the Göttingen minipig: an immunohistochemical evaluation

Adapted from:
Ontogeny of CYP3A and P-glycoprotein in the liver and the small intestine of the Gottingen minipig: an immunohistochemical evaluation.
Van Peer E, Verbueken E, Saad M, Casteleyn C, Van Ginneken C, Van Cruchten S.

3.1 Abstract

Despite the increasing use of the minipig as a non-rodent species in general and juvenile toxicity studies, knowledge on their biotransformation processes and their ontogeny is scarce. Such data are prerequisite for the correct interpretation of non-clinical studies in this species. Therefore, the aim of our investigation was to immunohistochemically document the presence of the drug transporter P-glycoprotein and the metabolizing CYP3A subfamily in the livers (n = 115) and the small intestines (n = 74) of foetal, neonatal, juvenile and adult Göttingen minipigs. Pgp was expressed in the liver in all age groups, whereas its presence in the jejunum was detected from 86 days of gestation onwards. Low expression of CYP3A was detected in the jejunums and livers from foetal and neonatal piglets. During postnatal development, the immunoreactivity for CYP3A increased in both organs. A centrilobular pattern, with a more intense staining for CYP3A of the hepatocytes surrounding the central vein, was noticed in the postnatal livers. In conclusion, the presented data suggest that the intestinal and hepatic ontogeny of P-glycoprotein and CYP3A in minipigs corresponds to that in man, in which a similar spatio-temporal expression has been reported.

3.2 Introduction

In December 2006, the European Parliament and Council issued Regulation (EC) No 1901/2006 on medicinal products for paediatric applications in order to restrict off-label drug use and consequently inadequate treatment of and adverse drug reactions in children. Furthermore, this regulation aims to ensure safe and effective medicinal
products for children (1). In this respect, knowledge on the ontogeny of biotransformation processes in children is crucial, with the liver and small intestine being the most important organs regarding the biotransformation of orally administered drugs. Consequently, data on drug metabolism in neonatal and juvenile animals are required for juvenile toxicity studies and may be requested prior to starting the clinical trials in the paediatric age groups (2). However, such data are scant for both rodent and non-rodent species. Rodents and dogs are the conventional species in non-clinical studies but rats are not always the most appropriate models to predict human drug metabolism (3, 4). The use of dogs, on the other hand, is restricted by ethical constraints and physiological characteristics. For example, the basal acid secretion in the stomach of dogs is lower than that of humans, pigs and monkeys. Furthermore, dogs possess a thicker gastric mucous layer compared to humans, which can influence the absorption of orally administered drugs (5). Pigs could be a valuable alternative non-rodent species since their anatomy and physiology of the liver and the development of the gastrointestinal system correspond to those in human beings (6, 7). Moreover, several studies in adult pigs and minipigs have demonstrated similar drug metabolizing enzymes and capacities compared to humans (8-10).

The cytochrome P450 enzyme family (CYP450) is one of the most important groups of biotransformation enzymes, with CYP3A being the largest subfamily expressed in liver and small intestine of humans (11-13). CYP3A4 and CYP3A7 are considered the adult and foetal CYP3A isoforms, respectively (14, 15). In domestic pigs and minipigs, five CYP3A isoforms have been characterized: CYP3A22, CYP3A29 v1 and v5, CYP3A39 and CYP3A46. The nucleotide sequences are 81.5-83.5% homologous to the human CYP3A4 genes, whereas the amino acid sequences for these isoforms show 75-77.8% homology. CYP3A22 and CYP3A29 v5 are considered the CYP3A4-orthologues in minipigs, with a 75% and 75.3% identity of amino acid sequences in Göttingen minipigs compared to human CYP3A4, respectively (16). Additionally, porcine CYP3A enzymes present similar biotransformation properties to the human orthologues (8, 9, 17, 18). Hermann et al. have detected a CYP3A7-like enzyme in foetal and newborn minipigs by Western blotting. However, they were not able to detect the enzyme at the mRNA level (19).

P-glycoprotein (Pgp) is an ATP-dependent drug efflux transporter, which pumps xenobiotics out of the cell. At the level of the intestine, Pgp plays an important role in determining the oral bioavailability of drugs (20, 21). In pigs, Pgp is very similar to human Pgp both genetically and functionally (22, 23).
This study aims to assess the expression and localisation of CYP3A and P-glycoprotein in the jejunum and the liver of foetal, neonatal, juvenile and adult Göttingen minipigs up to three years of age.

3.3 Materials and Methods

3.3.1 Animals and tissue samples

Livers and jejunums were obtained from healthy Göttingen minipigs. Ten pregnant sows were a kind gift from Ellegaard Göttingen Minipig A/S (Dalmose, Denmark). Sequani Limited (Ledbury, United Kingdom) kindly donated formalin fixed and paraffin embedded liver samples from Göttingen minipigs 1, 7, 14, 31 and 28 days of age and 3, 4 and 5 months of age. Janssen Research (Beerse, Belgium) kindly provided tissue samples from four adult male Göttingen minipigs. For the immunohistochemical study of the liver, the following age groups were examined: 84, 86 and 108 days of gestation (DGA 84, 86 and 108), Day 1 (within 34h after birth), Day 3, Day 7, Day 14, Day 21, Day 28, 3 Months, 4 Months, 5 Months and Adult (1.5 until 3 years of age). Immunohistochemistry of the small intestine was performed in eight age groups: 84, 86 and 108 days of gestation, Day 1 (within 24h after birth), Day 3, Day 7, Day 28 and Adult (1.5 until 3 years of age). Male and female animals were represented in each age group. (Table 3.1) Since very young age groups (foetuses and neonates) were included in this study, we decided to verify CYP3A expression by using two rabbit anti-human antibodies with porcine species reactivity: an anti-CYP3A7 and anti-CYP3A4 antibody. For detection of the CYP3A7-orthologue, samples were taken from the lowest age groups: 84 (n = 4), 86 (n = 5) and 108 (n = 8) days of gestation, Day 1 (n = 9 and n = 11 for jejunum and liver, respectively). Three pregnant sows were euthanized by electrocution and exsanguination at 84, 86 and 108 days of gestation. The foetuses were harvested and immediately placed on ice until further processing. The neonatal and juvenile piglets were naturally delivered and housed with the sow until the moment of euthanasia. The piglets were randomly allocated to a specific age group (Day 1, 3, 7, 28) in this research. They were anaesthetised by an intraperitoneal injection of natrium pentobarbital 20% (Kela NV, Hoogstraten, Belgium) (90mg/kg), followed by exsanguination. The small intestine and liver were dissected and rinsed with cold
0.01M phosphate buffered saline (pH 7.4). Samples of the liver were taken from the medial left and right liver lobes. P-glycoprotein and CYP3A4 show an increasing and decreasing expression pattern along the length of the small intestine, respectively. These expression patterns have been described in both humans and pigs (12, 24-27). Since a considerable amount of both proteins is thought to be present in the middle part of the small intestine, the jejunum was sampled in this research. Samples of both organs were taken and fixed in 4% (w/v) paraformaldehyde (2 and 24 hours (h) for jejunum and liver, respectively) in distilled water at room temperature (RT) and routinely processed to paraffin blocks. Four μm thick sections of liver and jejunum were made. The Ethical Committee of Animal Experimentation from the University of Antwerp (Belgium) approved the use of these animals in this study.

Table 3.1. Numbers of male (M) and female (F) animals used in this study.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Liver Number of animals</th>
<th>Jejunum Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>DGA 84</td>
<td>n = 2*</td>
<td>n = 3*</td>
</tr>
<tr>
<td>DGA 86</td>
<td>n = 3*</td>
<td>n = 2*</td>
</tr>
<tr>
<td>DGA 108</td>
<td>n = 4*</td>
<td>n = 4*</td>
</tr>
<tr>
<td>Day 1</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Day 3</td>
<td>n = 6</td>
<td>n = 4</td>
</tr>
<tr>
<td>Day 7</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Day 14</td>
<td>n = 5</td>
<td>n = 4</td>
</tr>
<tr>
<td>Day 21</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Day 28</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>3 Months</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>4 Months</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>5 Months</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Adult</td>
<td>n = 4</td>
<td>n = 4</td>
</tr>
<tr>
<td>Total sum</td>
<td>n = 59</td>
<td>n = 56</td>
</tr>
</tbody>
</table>

*Both liver lobes were evaluated separately; / Age group not sampled

3.3.2 Detection of P-glycoprotein

After deparaffinization of the postnatal slides, heat induced antigen retrieval was performed by microwaving the sections at 90 Watt for 20 minutes (min) in Dako Tris buffer pH 9 (Code S2367, Dako, Glostrup, Denmark). Antigen retrieval of foetal samples was limited to 16 min to reduce background staining. Endogenous peroxidase activity
was depleted by immersion of the slides in 3% hydrogen peroxide in methanol for 10 min. The slides were subsequently incubated for 30 min at RT with 20% normal goat serum (Code X0907, Dako) to block aspecific binding sites. Then followed by incubation with the C219-antibody (1:100) (Ab C219, Covance, SIG-38710, California, U.S.) overnight at RT. On each slide, one section of tissue was not incubated with the primary antibody as a negative control. The incubation of the slides with C219 was followed by incubation with Dako Cytomation Envision+ System-HRP labelled Polymer Anti-mouse (Code K4006, Dako) for 60 min. Visualization of antibody binding was obtained by the use of 3,3'-diaminobenzidine chromogen and substrate buffer (DAB) in 10 sec (Code K3468, Dako). Counterstaining was performed with Carazzi's Haematoxylin (Klinipath, Olen, Belgium). In between subsequent steps in this procedure, except after the incubation with normal goat serum, slides were washed 3 times in 0.05 M Tris-buffered saline (TBS) for 5 min. Primary antibody and normal goat serum were diluted in TBS (0.05 M Tris-HCl, 0.9% NaCl, pH 7.4) containing 0.3% Triton X-100 (Sigma-Aldrich, Saint Louis, Missouri, U.S.) and 1% bovine serum albumin (Sigma-Aldrich). The latter solution was also used as replacement for the primary antibody as a negative control. One specific sample with well-established Pgp immunoreactivity was used as a positive control for each series of stainings.

3.3.3 Detection of the CYP3A4-orthologue

The protocol was analogous to the protocol for detection of Pgp and only divergences from the former protocol will be mentioned. Heat induced antigen retrieval was performed by microwaving the sections at 90 Watt for 18 min in Dako Citrate buffer pH 6 (Code S2369). The slides were incubated with the rabbit polyclonal anti-human anti-CYP3A4 antibody with porcine species reactivity (1:600) (ab1254, Millipore, Billerica, U.S.), overnight at 4°C. Dako Cytomation Envision+ System-HRP labelled Polymer Anti-Rabbit (Code K4002, Dako) was used after incubation with the primary antibody (30 min).

3.3.4 Detection of the CYP3A7-orthologue

The protocol was similar to the protocol used for detection of the CYP3A4-orthologue. Antigen retrieval was performed by microwaving the sections at 90 Watt in Dako Citrate buffer pH 6 (Code S2369, Dako) for 18 min. Staining was performed on foetal
samples and samples of 1-day-old animals since CYP3A7 is the foetal CYP3A isoform. The slides were incubated with the rabbit polyclonal anti-human anti-CYP3A7 antibody with porcine species reactivity (1:100) (ARP41801 T100, Aviva systems Biology Corporation, San Diego, U.S.) overnight at 4°C and with Dako Cytomation Envision+ System-HRP labelled Polymer Anti-Rabbit (Code K4002, Dako) for 60 min.

### 3.3.5 Scoring

Evaluation of the staining was performed with an Olympus BX61 microscope (Olympus Belgium, Aartselaar, Belgium). Two independent observers evaluated the staining intensity of the slides. They were blinded to the age of the piglets. For assessment of expression of Pgp, samples were scored as positive or negative in liver and jejunum. A sample was considered positive if staining was present, even though not generalized. For evaluation of the CYP3A4- and CYP3A7-orthologues in the jejunum, intensity of staining was evaluated by a score 0 to 3. Score 0 was used if no staining occurred. Score 1 represented a mild positive cytoplasmic staining of the enterocytes. The enterocytes with a moderate to limited intense staining was given score 2, whereas the samples containing enterocytes with a generalized intense staining of the cytoplasm over the entire length of the villus were given score 3.

For evaluation of the CYP3A4- and CYP3A7-orthologues in the liver, intensity of staining was evaluated with a score ranging from 0 to 5. Since differences in blood perfusion and CYP450 content have been described between right and left foetal liver lobes in several species (28-30), both were evaluated separately in the minipig foetuses. Score 0 was used in case of absence of visual staining. Score 1 and 2 represent a generalized very mild and moderate staining of the cytoplasm of all hepatocytes, respectively. In case of the presence of a few intensively stained hepatocytes among a majority of moderately stained hepatocytes, score 3 was given. Score 4 was given to samples with a zonal pattern of more intensively stained hepatocytes surrounding the central vein of each lobule. Score 5 was restricted to samples in which the intensively stained hepatocytes were reaching the fibrous tissue surrounding each liver lobule.

Specificity of immunohistochemical staining was evaluated by the use of a negative control for each sample and the absence of immunoreactivity in fibrous tissue and endothelial cells in liver and jejunum and crypt cells in the jejunum.
3.3.6 Statistical analysis

Fisher’s Exact test was used for the statistical analysis of the presence of Pgp between different age groups and between genders. Kruskal-Wallis (mean rank) test was used to statistically analyse the scoring for the CYP3A4- and CYP3A7-orthologues between different age groups, between both genders and additionally between both liver lobes in the foetuses. A $p$-value < 0.05 was considered statistically significant. All statistical analyses were performed with IBM SPSS statistics (version 20, IBM, Armonk, NY, U.S.).

3.4 Results

3.4.1 Small intestine

Pgp was localised at the brushborder from villous, but not crypt enterocytes. (Figure 3.1) Pgp could not be detected in the jejunum from foetuses at 84 days of gestation, but was present in 80% and 100% of the foetuses at 86 and 108 days of gestation, respectively. All postnatal animals showed positive staining of the jejunum, except two 1-day-old animals. Fisher’s Exact test revealed a statistically significant difference in Pgp expression between the age group 84 days of gestation and the older age groups ($p < 0.01$). Detection of CYP3A with the anti-CYP3A4 antibody revealed CYP3A expression in the cytoplasm of enterocytes lining the villus from 86 days of gestation onwards as a mild to moderate staining. From 108 days of gestation until 7 days of postnatal age, samples presented mainly score 1 or score 2. (Figures 3.2 and 3.3) The majority of the 28-day-old animals and all adult animals showed intense cytoplasmic staining corresponding to a score 3, but staining was even more intense in the adult animals. (Figure 3.4) CYP3A expression was significantly different between all age groups ($p < 0.001$) and overall CYP3A expression increased with age in the jejunum. (Figure 3.5) Detection of CYP3A with the anti-CYP3A7 antibody gave similar results to those obtained with the anti-CYP3A4 antibody in the jejunum samples from the lowest age groups. Therefore, these results are not presented. No gender-related differences were observed for expression of CYP3A or Pgp in the jejunum.
Figure 3.1. (Left) P-glycoprotein expression in the minipig jejunum. Pgp immunohistochemical staining (DAB/brown) of enterocytes from an adult male Göttingen minipig. Immunoreactivity is located at the apical membrane of the villous enterocytes. Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.

Figure 3.2. (Right) CYP3A expression in the minipig jejunum: score 1. CYP3A immunohistochemical staining (DAB/brown) of the villous enterocytes from a 1-day-old female Göttingen minipig. Mild cytoplasmic staining and a limited moderate cytoplasmic staining at the top of some villi. Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.

Figure 3.3. (Left) CYP3A expression in the minipig jejunum: Score 2. CYP3A immunohistochemical staining (DAB/brown) of intestinal villi (cross-section) in a female 28-day-old Göttingen minipig. Moderate cytoplasmic staining of the villous enterocytes. Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.

Figure 3.4. (Right) CYP3A expression in the minipig jejunum: Score 3. CYP3A immunohistochemical staining (DAB/brown) of villous enterocytes in an adult male Göttingen minipig. Intense brown staining of the entire cytoplasm of the enterocytes. Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.
3.4.2 Liver

P-glycoprotein was localised at the biliary canalicular sides of the hepatocytes (Figure 3.6) and was expressed with similar intensity in all examined liver samples. The cytoplasm of all the hepatocytes from the foetal and the 1- and 3-day-old piglets showed mild to moderate CYP3A immunoreactivity. (Figure 3.7) In the foetal samples, groups of hematopoietic stem cells were distributed among the poorly organized hepatocytes. In the neonatal animals, the hepatocytes were organized in trabeculae and the number of hematopoietic stem cells had already decreased. Small groups of more intensively stained hepatocytes, distributed among moderately stained hepatocytes, could be detected at 7 days of age. A zonal distribution with more intensively stained hepatocytes surrounding the central vein of each lobule was noticed in a few 7-day-old animals, but was clearly present at 14 days of age. The hepatocytes in the area more distal to the central vein showed a lower CYP3A expression. (Figure 3.8) The animals of 28 days of age and older showed a more pronounced zonal pattern of highly immunoreactive hepatocytes with a zone of moderately stained hepatocytes adjacent to the interlobular septa surrounding each lobule. In the adult animals, all hepatocytes were intensively stained except for a very limited number of hepatocytes close to the interlobular septa. (Figure 3.9) CYP3A was also expressed in the epithelium of the intrahepatic bile ducts.
Figure 3.6. (Left) P-glycoprotein expression in the minipig liver. Pgp immunohistochemical staining (DAB/brown) of the liver from a male 3-month-old Göttingen minipig demonstrates Pgp expression on the biliary canalicular sides of the hepatocytes. Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.

Figure 3.7. (Right) CYP3A expression in the minipig liver: Score 2. CYP3A immunohistochemical staining (DAB/brown) of the liver from a male Göttingen minipig at 86 days of gestation shows a moderate expression in the cytoplasm of all hepatocytes. Small groups of hematopoietic stem cells (indicated by black arrow) are distributed among the hepatocytes. Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.

Figure 3.8. (Left) CYP3A expression in the minipig liver: Score 4. CYP3A immunohistochemical staining (DAB/brown) of the liver from a male 7-day-old Göttingen minipig. A zonal pattern is appearing with more intensively stained hepatocytes surrounding the central vein (indicated by black arrow) of the lobule. The hepatocytes more distal to the central vein show a moderate staining. Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.

Figure 3.9. (Right) CYP3A expression in the minipig liver: Score 5. CYP3A immunohistochemical staining (DAB/brown) of liver tissue from an adult male Göttingen minipig. The hepatocytes of nearly the entire lobule are stained intensively brown. Fibrous tissue enclosing each lobule (= interlobular septa, typical for pigs) is markedly present (no CYP3A expression). Counterstaining with haematoxylin (blue nuclei). Scale bar 200 μm.
A statistically significant difference was noticed for the hepatic expression of CYP3A between the different age groups \((p < 0.001)\). In general, CYP3A expression increased with age. (Figure 3.10)

As observed for the jejunum samples, similar results were obtained with the anti-CYP3A4 and anti-CYP3A7 antibodies in the liver samples from the lowest age groups. (Data not presented.) No gender-related differences were observed for expression of CYP3A. Neither was a significant difference observed between the foetal left and right liver lobes.

![Figure 3.10. CYP3A expression in the minipig liver. The full red bars and blue checkerboard bars represent the mean scores + standard deviation for the female and male animals, respectively.](image)

### 3.5 Discussion

Recent European regulations have forced pharmaceutical companies to consider paediatric use of their newly developed compound. This has resulted in an increase of clinical trials in the paediatric age group and consequently, to a higher number of juvenile toxicity studies preceding these clinical trials (1, 2). Minipigs are increasingly being used in toxicity studies because of their anatomical and physiological similarities to human beings (31, 32).
The aim of this study was to assess the presence of CYP3A and Pgp in the jejunum and in the liver of foetal, neonatal, juvenile and adult Göttingen minipigs using immunohistochemical staining. CYP3A enzymes and Pgp are important and extensively characterized members of drug biotransformation processes. Furthermore, they share the same nuclear receptors, are co-localised and they interact in a functional manner as a significant overlap in substrates exists (33, 34). In the present immunohistochemical study, Pgp was localised at the brushborder of villous enterocytes and at the biliary canalicular sides of the hepatocytes, as described in human beings (35). Pgp was detected in all foetal and postnatal liver samples and in 0% and 80% of the intestinal samples at 84 days and 86 days of gestation, respectively. From 108 days of gestation onwards, all of the intestinal samples showed positive staining, except for two 1-day-old animals. These results are consistent with data from human literature reporting an earlier expression of Pgp in the liver than in the intestine. Van Kalken et al. found no or only weak membranous staining of Pgp in human foetal intestinal samples after 11 to 20 weeks of gestation, but they detected Pgp in bile canaliculi from 14 weeks of gestation onwards, without a clear difference in intensity of staining between the different foetal age groups (36). The foetal piglets in this research had reached about 75% and 95% of gestation (84-86 days of gestation and 108 days of gestation, respectively). Therefore, our results indicate a limited intestinal and full hepatic Pgp expression at 75% of gestation. A sow-dependent influence on the pronounced differences that were observed between the groups of 84 and 86 days of gestation cannot be excluded, since the foetuses in each age group were derived from the same sow. However, the sows were housed in the same environment and were fed with the same commercial feed.

CYP3A is considered the most abundant CYP450 enzyme in the liver and intestine of adult human beings and one of the most abundant isoforms in the porcine liver (10-13). Fakhoury et al. used a rabbit anti-human polyclonal CYP3A antibody to immunohistochemically detect CYP3A protein in human duodenum. They found CYP3A protein in all duodenal enterocytes in samples of children over 6 months of age, while they could only detect CYP3A in half of the enterocytes in samples from patients aged between 1 month and 6 months (37). In the present research, CYP3A was detected as a mild to moderate cytoplasmic staining of the villous enterocytes at 86 days of gestation, whereas it could not be detected in the jejunum at 84 days of gestation. An increasing intestinal expression of CYP3A during postnatal development was noticed. These
findings are consistent with the postnatal expression of CYP3A4 in the human intestine (38). CYP3A7 is markedly described in human foetal liver, but little is known about the presence of CYP3A7 in foetal or adult small intestine. Fakhoury et al. detected the mRNA of CYP3A7 in the duodenum of children ranging between 1 month and 17 years of age, but at much lower levels compared to the mRNAs of CYP3A4 and CYP3A5 (37). In addition, Kivistö et al. could not detect mRNA for CYP3A7 in human duodenal tissue with ages ranging from 18 to 74 years of age (39). In contrast, Burk et al. demonstrated CYP3A7 mRNA expression in adult intestinal samples, associated with the CYP3A7*1C allele in a few cases of high expression (40). A similar CYP3A expression pattern was observed with the anti-CYP3A4 and anti-CYP3A7 antibodies. This seems to contrast with the expectation of a decreasing CYP3A7 and increasing CYP3A4 expression with age, since CYP3A4 is the adult isoform and CYP3A7 is considered the foetal isoform in human beings. However, the similar expression patterns obtained by the two different antibodies may be explained by the use of polyclonal antibodies and by the fact that CYP3A4 and CYP3A7 are highly similar in gene sequence (41). Consequently, cross-reaction of the anti-CYP3A7 antibody with CYP3A4 could have occurred. CYP3A expression in foetal and neonatal livers was detected as a generalized mild to moderate staining of all hepatocytes and epithelial cells of small bile ducts. We observed an age-related zonal expression of CYP3A in the juvenile and adult livers, which has also been described in human beings (42, 43). Small groups of more intensively stained hepatocytes appeared at 7 days of age. This gradual increase in CYP3A expression resulted in a centrilobular pattern of more intensively stained hepatocytes surrounding the central vein in each lobule. This higher CYP3A expression in the hepatocytes close to the central vein might be due to an altered circulatory microenvironment, supply of nutrients, hormones and metabolites of these latter cells compared to hepatocytes in the periportal zone (28, 44). In the adult animals, the hepatocytes of the entire lobule were stained profoundly intense. Statistically significant differences in CYP3A expression were found between the age groups. Remarkably, the scores for CYP3A detected with the anti-CYP3A4 antibody were higher in animals at 84 days of gestation than those at 86 days of gestation. As mentioned above, detection of a CYP3A7-orthologue by cross-reaction is a plausible explanation. Hermann and Skaanild also reported a significantly higher CYP3A29 (porcine CYP3A4-orthologue) expression in neonatal piglets compared to foetal samples at 100 days of gestation (19). No statistically significant difference could be detected for CYP3A scores between both
foetal liver lobes. In foetal sheep, monkey and mouse livers, a higher CYP450 content was reported for the left lobe in comparison with the right lobe. A higher oxygenation due to more extensive vascularization of the foetal left liver lobe is thought to be the reason for this observation (28-30). However, this conclusion could not be drawn from our results. No significant correlation could be found between gender and CYP3A expression. This is in accordance with observations by other authors (19). However, higher CYP450 concentrations and activities in female minipigs compared to males have been reported (45).

Undoubtedly, the presented results need to be further substantiated by activity data to draw final conclusions about the role of CYP3A and Pgp in drug metabolism in the minipig. Nevertheless, these first results suggest that the ontogeny of P-glycoprotein and CYP3A is similar in Göttingen minipigs and human beings and warrant further investigation of the porcine species as a an animal model in non-clinical studies.
3.6 References


4 Is there a shift of a foetal CYP3A7-like enzyme into an adult CYP3A4-like enzyme in the liver of the Göttingen minipig?

4.1 Part A - Age-related Differences in CYP3A Abundance and Activity in the Liver of the Gottingen Minipig

Adapted from:
Age-related Differences in CYP3A Abundance and Activity in the Liver of the Gottingen Minipig.

4.1.1 Abstract

In view of paediatric drug development, regulatory authorities often request safety studies in juvenile animals. Unfortunately, knowledge on the ontogeny of the biotransformation processes in animal models remains scarce and impedes a correct interpretation of the toxicity findings, especially in non-rodents animal models. CYP3A4 is one of the most important drug metabolizing enzymes in humans and shows important similarities with CYP3A in the minipig. Therefore, the aim of this study was to assess the abundance and activity of CYP3A in liver microsomes from foetal, juvenile (Day 1, 3, 7 and 28) and adult male and female Göttingen minipigs. CYP3A abundance was studied by an indirect enzyme-linked immunosorbent assay (ELISA), whereas CYP3A activity was assessed by a biotransformation assay with Luciferin-IPA. CYP3A abundance could not be detected until Day 3. From Day 7 onwards, a gradual increase in expression was noted, leading to the highest abundance in adult animals. CYP3A activity was not detectable in foetuses and one-day-old animals. The CYP3A activity was detectable, but below the LLOQ in Day 3 animals and increased gradually with age to reach the highest level in adults. CYP3A-dependent metabolism of Luciferin-IPA in the minipig was further substantiated by its inhibition with CYP3cide, ketoconazole and the primary anti-CYP3A4 antibody from the ELISA, and its reduction by co-incubation with testosterone and midazolam. Additionally, a positive correlation was found between
CYP3A abundance and biotransformation of Luciferin-IPA (Pearson $r = 0.863; p < 0.0001$). In conclusion, both abundance and activity of CYP3A increased gradually in juvenile minipigs, but remained below the levels observed in adult animals.

### 4.1.2 Introduction

Children form a substantial and vulnerable group in the human population with regard to the use of medicines. Unfortunately, the majority of drugs on the market have not been tested nor evaluated in the paediatric age group, despite known differences in drug safety between mature and immature bodies (1, 2). The introduction of the Paediatric Regulation (EC) No 1901/2006 in December 2006 has drastically changed the efforts of pharmaceutical companies concerning paediatric drug development. This has also resulted in regulatory requests for preclinical studies in juvenile animals prior to the start of clinical trials in young patients. Unfortunately, the neonatal and young age groups remain a challenging population since important developmental changes occur in these individuals (3). Maturation of metabolizing enzymes (Phase I and II), changes in body composition (water/lipid partition), liver development and maturation of kidneys are some of the factors that impede the insight into the absorption, distribution, metabolism and excretion of drugs in children (4).

Cytochrome P450 enzymes (CYP450) are important drug metabolizing enzymes, which facilitate the excretion of drugs from the body. Unfortunately, CYP450 enzymes can also be responsible for adverse drug reactions and drug-drug interactions. Maturational differences in CYP450 activity can even increase the risk of undesired effects and under- or overdosing of drugs in the paediatric age group (2). From one year of age onwards, CYP3A4 is the most abundant member of the CYP3A subfamily in the human liver (2, 5, 6) and is responsible for the oxidative metabolism of 30-50% of human drugs on the market (7-9). CYP3A5 and CYP3A7 are also members of the human CYP3A subfamily. CYP3A7 is considered the foetal isoform, although it can be present in adult livers too. CYP3A5 shows no clear age-related expression pattern, is polymorphically expressed and has overlapping substrate specificities with CYP3A4 (6, 9-11). CYP3A7 and CYP3A5 show 88 and 83% identity of amino acids with CYP3A4, respectively (5). In view of the use of minipigs in juvenile toxicity studies, it is also important to know the developmental expression pattern of CYP3A in this species. Four CYP3A isoforms have
been identified in the porcine species, showing at least 75% identity of amino acid sequences compared to human CYP3A4, i.e. CYP3A22, CYP3A29, CYP3A39 and CYP3A46. CYP3A22 and CYP3A29 are considered CYP3A4-orthologues in minipigs, although presence of CYP3A39 and CYP3A46 in the Göttingen minipig cannot be excluded (12). The CYP3A enzymes in minipigs present even similar biotransformation properties as the human orthologues, e.g. nifedipine oxidation and testosterone 6β-hydroxylation (13-16). In addition, pigs and humans share anatomical and physiological characteristics, especially regarding many organ systems, which explain the high interest for this species as pharmacological and toxicological model (17).

Since no discrimination was made between the different minipig CYP3A isoforms in this study, we will refer to CYP3A in general when the porcine CYP3A isoforms are discussed. The aim of this study was to assess the abundance and activity of CYP3A in liver microsomes from foetal, neonatal, juvenile and adult Göttingen minipigs. CYP3A abundance was evaluated with an indirect enzyme-linked immunosorbent assay (ELISA) that was developed for quantification of human CYP3A4, whereas activity was estimated by incubation of minipig liver microsomes with Luciferin-IPA, a highly specific substrate for human CYP3A4 (18). Additionally, we evaluated the inhibitory effect of CYP3cide and ketoconazole and the primary anti-CYP3A4 antibody, and the interaction of the CYP3A substrates testosterone and midazolam on the metabolism of Luciferin-IPA.

4.1.3 Materials and Methods

4.1.3.1 Animals and tissue samples

Livers were obtained from healthy Göttingen minipigs. Ten pregnant sows were a kind gift from Ellegaard Göttingen minipig A/S (Dalmose, Denmark). Jansen Research (Beersse, Belgium) kindly provided liver samples from four adult male Göttingen minipigs.

The following age groups were investigated: 84 - 86 days of gestation (n = 8), 108 days of gestation (n = 8), Day 1 (within 24 hours after birth) (n = 8), Day 3 (n = 8), Day 7 (n = 9), Day 28 (n = 10) and Adult (n = 9). Since normal gestation length in the minipig is 112 to 115 days, the evaluation of the foetal age groups is limited to the third trimester.
of foetal development. Postnatal day 28 is considered the weaning age in the Göttingen minipig. This age range was chosen to cover the first year of life in children, as important changes in CYP3A4 expression and activity occur in this period (5, 11). Both genders were equally represented in each age group, except in groups Day 7 (Males: n = 5, Females: n = 4) and the adult age group (Males: n = 4, Females: n = 5). Liver samples from five out of ten adult sows were randomly selected in this study to obtain similar group sizes. The age of the adult males and females ranged between 18–24 months and 14–33 months, respectively. The adult sows were killed by electrical stunning, followed by exsanguination either before or after delivery, according to the desired age of their offspring. The foetuses were harvested and placed immediately on ice until further processing. The neonatal and juvenile piglets were naturally delivered and housed with the sow until euthanasia. The piglets were randomly allocated to a specific postnatal age group (Day 1 (PND1), Day 3 (PND3), Day 7 (PND7) and Day 28 (PND28)). Due to practical reasons, the piglets were not killed by electrical stunning, but they were anaesthetized by an intraperitoneal injection of sodium pentobarbital 20% (Kela NV, Hoogstraten, Belgium) (90 mg/kg), followed by exsanguination. The liver was dissected and rinsed with ice-cold 0.01 M phosphate-buffered saline (pH 7.4). Samples were taken from the lateral liver lobes and immediately snap frozen in liquid nitrogen. These samples were stored at -80°C until the isolation of liver microsomes. The Ethical Committee of Animal Experimentation from the University of Antwerp (Belgium) approved the protocol and use of the animals.

4.1.3.2 Isolation of liver microsomes

Liver tissue was thawed on ice and washed with ice-cold homogenizing buffer (0.01 M potassium phosphate (KPO₄) buffer (451201, Corning Incorporated, NY, U.S.) containing 1.15% potassium chloride). Excess of moisture was removed by blotting the tissue on paper towels. The liver tissue was minced into small pieces by means of surgical scissors and weighed. For each gram of tissue, a three-fold volume in mL of ice-cold homogenizing buffer was added. The tissue was homogenized with the Polytron® System PT 1200 E (230 V, 50 Hz) on ice for maximum 10 sec. As a final homogenization step, a motor driven Potter Elvehjem with Teflon pestle was used (1200 rpm, 5 to 10 up-and-down strokes). All homogenization steps were performed on ice. The homogenate was centrifuged at 12,000g for 20 min at 4°C. The resulting supernatant
was centrifuged at 100,000 g for 60 min at 4°C. The resulting pellet was re-suspended with homogenizing buffer and centrifuged at 100,000 g for 40 min at 4°C. The resulting microsomal pellet was re-suspended in storage buffer (0.1 M KPO₄ buffer containing 250 mM sucrose and Halt™ Protease Inhibitor Single-Use Cocktail (Thermo Fisher Scientific, MA, U.S.)) and stored at -80°C until use. Total protein concentration was determined by the Pierce® BCA Protein Assay Kit with bovine serum albumin as a standard (Thermo Fisher Scientific, MA, U.S.).

4.1.3.3 ELISA

An indirect ELISA using commercially available antibodies was used to determine CYP3A abundance in the liver microsomes from the Göttingen minipigs. De Bock et al. (2012) developed this ELISA for detection of CYP3A4 in HLM (19). The ELISA was performed according to the instructions described by De Bock et al. (2012). Microsomal samples, calibrators and validation samples were diluted to a final concentration of 10 μg of microsomal protein/mL using a carbonate-bicarbonate buffer (pH 9.4). In addition to the validation samples, CYP3A4 Baculosomes® Plus Reagent, rHuman (P2377, Life Technologies, Thermo Fisher Scientific) and HLM (HMMC-H3A4-PL040, Life Technologies, Thermo Fisher Scientific) were evaluated since they were also included in the biotransformation assays with Luciferin-IPA (see below). From each diluted sample and calibrator, 100 μL was loaded on a black 96-well MaxiSorp® microtiter plate (Nunc, Roskilde, Denmark), resulting in 1 μg of microsomal protein per well. The primary antibody, which binds to the antigen to be detected, was a CYP3A4 purified MaxPab rabbit polyclonal antibody, raised against the full-length human CYP3A4 protein (H00001576-D01P, Abnova, Taiwan). The Pierce®Goat anti-rabbit horseradish peroxidase conjugated IgG (31460, Pierce Biotechnology, Rockford, U.S.) was used as secondary antibody. Peroxidase activity was detected with the QuantaBlu™ Fluorogenic peroxidase substrate kit (Thermo Fisher Scientific), which is a fluorogenic substrate. Fluorescence was determined with the Ascent Fluoroscan (Thermo Fisher Scientific) at excitation and emission wavelengths of 320 nm and 405 nm, respectively. This ELISA was developed to quantify amounts between 2 and 300 pmol CYP3A4/mg microsomal protein (MP) in HLM with a 5-parameter logistics function with 1/x weighting factor. The ELISA was validated for sensitivity, accuracy,
precision, working range and calibration (19). The data represent the mean value of two technical replicates for each sample.

4.1.3.4 Incubation conditions for Luciferin-IPA in minipig liver microsomes

The kinetic studies were performed in non-treated Nunc™ F96 Microwell™ white Polystyrene plates (236205, Thermo Fisher Scientific). A range of seven protein concentrations of liver microsomes from an adult female Göttingen minipig was tested for linearity (0.125–8 μg / 50 μL). The kinetic profile for Luciferin-IPA (P450-Glo™ CYP3A4 Assay, V9001, Promega Corporation, Madison, U.S.) was generated from eight substrate concentration points (0.3125–40 μM). The estimated $K_m$ was 3.7 ± 0.7 μM (confidence interval: 2.3-5.1 μM). To prevent substrate inhibition in the lowest age groups and to favour biotransformation of Luciferin-IPA by the porcine 'CYP3A4-orthologue', a concentration of 1 μM Luciferin-IPA, which is below the estimated $K_m$, was chosen as final substrate concentration in the incubations. The incubation time (10 min) and microsomal protein concentration (20 μg/mL) were within the linear range for Luciferin-IPA. Figure S4.1 shows the working mechanism of the luminogenic assay with Luciferin-IPA. (See Supplementary figure at the end of Chapter 4)

In each incubation well, 1 μg of hepatic microsomal protein, 0.1 M KPO$_4$ buffer (pH 7.4), 1.3 mM NADP$^+$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride (451220 and 451200, Corning Incorporated) and 1 μM Luciferin-IPA were co-incubated. The total incubation volume was 50 μL per well. Microsomal dilutions in 0.1 M KPO$_4$ buffer were pre-incubated at room temperature (RT) for 10 min. The Luciferin-IPA and the NADPH-regenerating system in KPO$_4$ buffer were pre-incubated at RT for 10 min. The reaction was initiated by addition of the microsomal protein to the remainder of the incubation mixture. The plate was first incubated for 10 min at RT. Subsequently, 50 μL of Luciferin detection reagent with esterase (V859A and V144A, Promega Corporation) was added to each well, mixed and incubated for 20 min at RT to stabilize the luminescent signal. Luminescence was measured with a Tecan Genios (Tecan Group Ltd., Männedorf, Switzerland). The concentration of the metabolite D-Luciferin, generated by CYP3A from Luciferin-IPA, was quantified by comparing luminescence from the incubation mixtures to that from a D-Luciferin standard curve (Beetle Luciferin, Potassium Salt,
E1601, Promega Corporation). Reaction velocities were calculated in units of picomoles of D-Luciferin formed per minute per milligram of microsomal protein (pmol/min/mg MP). CYP3A4 Baculosomes® Plus Reagent, rHuman (P2377, Life Technologies, Thermo Fisher Scientific) and HLM (HMMC-H3A4-PL040, Life Technologies, Thermo Fisher Scientific) were used as a positive control. Insect cell control supersomes (456201, Corning Incorporated), lacking CYP450 enzymes, were used as a minus-P450 control. Positive and negative controls were included in each well plate and similarly treated to the minipig liver microsomes. Results from the Insect cell control supersomes were subtracted from the values obtained for the minipig liver microsomes, CYP3A4 Baculosomes® and human liver microsomes. The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were 1.18 and 3.91 nM, respectively. The data represent the mean value for each sample obtained in three separate assays, with two technical replicates within each assay.

4.1.3.5 Inhibition of biotransformation of Luciferin-IPA with CYP3cide

Ten micrograms of microsomal protein from an adult Göttingen minipig sow and from HLM (HMMC-H3A4-PL040, Life Technologies, Thermo Fisher Scientific) were pre-incubated with a range of 8 CYP3cide (20) concentrations (0–1 μM) (PZ0195, Sigma-Aldrich, St. Louis, MO, U.S.) and with 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride in 0.1 M KPO₄ buffer at 37°C for 10 min. The total incubation volume was 500 μL. Then, 10 μL of a 51 μM Luciferin-IPA solution was added to the incubation mixture resulting in a 1 μM Luciferin-IPA concentration in the final incubation mixture. After 10 min of incubation at 37°C, 50 μL of the final incubation mixture was added to 50 μL of Luciferin detection reagent with esterase in a non-treated Nunc™ F96 Microwell™ white Polystyrene plate to stabilize the signal for 20 min at RT. The continuation of the procedure was similar to the description in the previous paragraph. The reaction velocities with co-addition of CYP3cide were expressed as a percentage ratio of the control velocity with no inhibitor present.
4.1.3.6 Co-incubation of Luciferin-IPA with testosterone, midazolam or ketoconazole

Five micrograms of microsomal protein from an adult Göttingen minipig sow and from HLM (HMMC-H3A4-PL040, Life Technologies, Thermo Fisher Scientific) were co-incubated with a range of 7 testosterone concentrations (0–50 μM) (T037, Sigma-Aldrich), a range of 10 midazolam concentrations (0–50 μM) (Dormicum, Roche Holding AG, Basel, Switzerland) or a range of 6 ketoconazole concentrations (0–2 μM) (K1003, Sigma-Aldrich) together with 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 1 μM Luciferin-IPA in 0.1 M KPO₄ buffer at 37°C for 10 min. Reactions were initiated by addition of the microsomes. The total incubation volume was 250 μL. After 10 min of incubation at 37°C, 50 μL of the final incubation mixture was added to 50 μL of Luciferin detection reagent with esterase in a non-treated Nunc™ F96 Microwell™ white Polystyrene plate to stabilize the signal for 20 min at RT. The continuation of the procedure was similar to the description in the previous paragraph. The reaction velocities with co-addition of TST, MDZ or KCZ were expressed as a percentage ratio of the control velocity with no additional substrate or inhibitor present.

4.1.3.7 Immuno-inhibition of biotransformation of Luciferin-IPA

Immuno-inhibition of the biotransformation of Luciferin-IPA was performed by co-incubation of HLM (HMMC-H3A4-PL040, Life Technologies, Thermo Fisher Scientific) and adult Göttingen minipig liver microsomes with a range of six protein concentrations (0–2.5μg) of the primary anti-CYP3A4 antibody from the ELISA (H00001576-D01P) or of Rabbit normal serum (Code X0902, Dako). The microsomal protein (1μg per well) was pre-incubated with the primary antibody or the rabbit normal serum for 20 min at room temperature. Subsequently, reaction was started by the addition of Luciferin-IPA and NADPH-regenerating system. Incubation conditions were identical as described in section ‘Incubation conditions for Luciferin-IPA in minipig liver microsomes’. The reaction velocities with co-addition of different concentrations of primary antibody or rabbit normal serum were expressed as percentage ratios of the control velocity with no primary antibody or rabbit normal serum.
4.1.3.8 Mathematical and statistical analysis

For the ELISA-test, calibration curves were fitted and data were analysed using the Masterplex® Readerfit 2010 software (Hitachi, San Francisco, CA, U.S.). The Kruskal-Wallis (Mean Rank) test was used to detect age- and sex-related differences in CYP3A abundance for the PND28 and adult animals. A $p$-value < 0.05 was considered statistically significant. Estimation of $K_m$ and $V_{max}$ for CYP3A and Luciferin-IPA in the minipig liver microsomes was performed by a nonlinear regression analysis in GraphPad Prism Version 6.0 f (GraphPad Software, Inc., La Jolla, U.S.). Estimation of $IC_{50}$ values was performed by a non-linear regression analysis with a four-parameter logistic curve (Formula: $Y = Bottom + (Top − Bottom) / (1 + 10^{(LogIC50 − X) * HillSlope}; with the Top restricted to 100) in GraphPad Prism Version 6.0 f (GraphPad Software, Inc.). Calculation of velocities of formation of D-Luciferin was performed in Microsoft Excel® (Version 14.3.1, Microsoft Corporation, Redmond, WA, U.S.). The Kruskal-Wallis (Mean Rank) test was used to statistically analyse the results from the biotransformation assay with Luciferin-IPA and differences between age groups (PND7, PND28 and adult) and sexes. Bonferroni correction for pairwise comparisons between age groups adjusted the $p$-value to 0.025. Consequently, a $p$-value < 0.025 was considered statistically significant. Prior to the Kruskal-Wallis test, homogeneity of variances was tested with a non-parametric Levene’s Test. Statistical analyses were performed with IBM SPSS statistics (version 20; IBM, Armonk, NY, U.S.). Correlation between CYP3A abundance and formation of D-Luciferin was calculated with a Pearson correlation coefficient on the Ln-transformed data from PND 7, PND 28 and adult animals in Graphpad Prism Version 6.0 f (GraphPad Software, Inc.).
4.1.4 Results

4.1.4.1 ELISA

The results represent a semi-quantitative measurement of CYP3A in the liver of the Göttingen minipig and should be considered relative values based on the signal to human CYP3A4. Foetal, PND1 and PND3 samples were below the LLOQ of 2 pmol CYP3A/mg of MP. In samples of PND7 piglets, six out of nine samples were still below the LLOQ. The abundance of the other three samples ranged between 3.53 and 5.11 pmol CYP3A/mg of MP. At PND28, all liver samples had levels ranging between 7.19 and 35.7 pmol CYP3A/mg of microsomal MP. For the adult animals, levels ranged between 25 and 101 pmol CYP3A/mg of MP (Table 4.1), being significantly higher than in PND28 animals ($p = 0.001$). No sex-related differences were observed among PND 28 and adult animals ($p = 0.935$). (Figure 4.1) The HLM showed an abundance of 87.4 pmol CYP3A4/mg of MP. The CYP3A4 Baculosomes® showed an abundance of 140 pmol CYP3A4/mg of MP.

Table 4.1. Abundance of CYP3A in the liver of the Göttingen minipig.

<table>
<thead>
<tr>
<th>Age</th>
<th>Gender</th>
<th>Number of animals</th>
<th>CYP3A abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>Female</td>
<td>N = 4</td>
<td>Below LLOQ</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>N = 3</td>
<td>4.07 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N = 2</td>
<td>Below LLOQ</td>
</tr>
<tr>
<td>Day 28</td>
<td>Female</td>
<td>N = 5</td>
<td>19 ± 12.1</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>N = 5</td>
<td>20.8 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>N = 10</td>
<td>19.9 ± 11.1</td>
</tr>
<tr>
<td>Adult</td>
<td>Female</td>
<td>N = 5</td>
<td>74.1 ± 29.5</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>N = 4</td>
<td>66.5 ± 17.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>N = 9</td>
<td>70.7 ± 23.8</td>
</tr>
</tbody>
</table>

Results from ELISA are presented as mean ± standard deviation (± S.D.) and are expressed in “pmol/mg of microsomal protein”. These results are relative values, based on the signal to human CYP3A4. LLOQ: Lower limit of quantification.
Figure 4.1. Sex-related distribution of CYP3A abundance and formation of D-Luciferin in pmol/min/mg MP in PND 28 and adult minipig livers. Bars represent mean values ± S.D. In the adult age group, the biotransformation of Luciferin-IPA was 42% higher in females than in males ($p = 0.05$), although CYP3A abundance was at a similar level. In the younger age groups, no obvious sex-related differences in CYP3A abundance or activity were observed.

4.1.4.2 Incubations with Luciferin-IPA

A statistically significant difference in CYP450 activity was observed between the different age groups by the Kruskal-Wallis test ($p < 0.0001$). No appreciable metabolism of Luciferin-IPA was observed at 84-86 days and 108 days of gestation. (Figure 4.2) These foetal piglets had reached about 75 and 95% of gestation, respectively. Also for PND1 animals, no formation of D-Luciferin was detected. At PND3, D-Luciferin was formed at a level above the LLOD for two out of eight animals, but levels were still below the LLOQ. With one exception, all PND7 animals showed levels above the LLOD, and five animals showed levels above the LLOQ (mean ± S.D.: 31.9 ± 9.38 pmol/min/mg MP). At PND28, the rate of metabolism (93.7 ± 30.6 pmol/min/mg MP) was increased compared to the younger groups, with the exception of one animal that showed a value between the LLOD and LLOQ. The highest velocity was noted in the adult animals (264 ± 76.3 pmol/min/mg MP). No sex-related differences among age groups (PND7, PND28 and adult) were observed ($p = 0.356$). The HLM and the CYP3A4 Baculosomes® showed a velocity of 26.3 and 178 pmol/min/mg MP, respectively.
4.1.4.3 Inhibition of biotransformation of Luciferin-IPA with CYP3cide

CYP3cide inhibited biotransformation of Luciferin-IPA in both HLM and minipig liver microsomes from an adult sow. Metabolism of Luciferin-IPA in human liver microsomes was already inhibited for 90% at 0.03125 μM CYP3cide, whereas this concentration only caused 7.4% inhibition in minipig liver microsomes. However, 0.125 and 1 μM CYP3cide caused 47 and 92% inhibition in minipig liver microsomes, respectively. (Figure 4.3)
Figure 4.3. The effect of various concentrations of CYP3cide and ketoconazole on the biotransformation of Luciferin-IPA. The bullets in the upper graphs represent percentage ratios of reaction velocity (incubations in duplicate) with pre-incubation of CYP3cide, divided by the control velocity without CYP3cide. The bullets in the lower graphs represent percentage ratios reaction velocity (incubations in duplicate) with co-incubation of KCZ, divided by the control velocity without KCZ. The IC$_{50}$ values and their 95% confidence intervals are added.

4.1.4.4 Co-incubation of Luciferin-IPA with testosterone, midazolam or ketoconazole

Testosterone clearly showed a dose-related inhibition of D-Luciferin formation in the minipig liver microsomes, which was less pronounced in the HLM, whereas midazolam and ketoconazole resulted in an inhibition of the formation of D-Luciferin in both minipig liver microsomes and HLM. (Figures 4.3 and 4.4)
4.1.4.5 Immuno-inhibition of biotransformation of Luciferin-IPA

The primary anti-CYP3A4 antibody clearly inhibited the biotransformation of Luciferin-IPA in both HLM and the minipig liver microsomes in a dose-related manner. (Figure 4.5)
Figure 4.5. The effect of pre-incubation of various concentrations (in µg per well) of primary anti-CYP3A4 antibody or Normal rabbit serum (NRS) on the biotransformation of Luciferin-IPA. Red boxes represent percentage ratios of reaction velocity (incubations in duplicate) with co-incubation of Normal rabbit serum, divided by the control velocity without Rabbit normal serum. Blue dots represent percentage ratios of reaction velocity (incubations in duplicate) with co-incubation of anti-CYP3A4 antibody, divided by the control velocity without anti-CYP3A4 antibody.

4.1.4.6 Correlation between CYP3A abundance and activity

A positive correlation was found between CYP3A abundance and formation of D-Luciferin for the PND7, PND28 and adult minipigs (Pearson $r = 0.863$, $p < 0.0001$). (Figure 4.6)

Figure 4.6. Correlation between CYP3A abundance (by ELISA) and biotransformation of Luciferin-IPA. Calculation of correlation was performed on the ln-transformed data from PND7, PND28 and adult animals. A positive Pearson correlation coefficient ($r = 0.863$ ($p < 0.0001$)) was found.
4.1.5 Discussion

To gain insight into the ontogeny of CYP3A in the liver of the Göttingen minipig, we investigated the protein levels of CYP3A by means of an ELISA. This ELISA was originally developed to quantify human CYP3A4 with a primary polyclonal antibody directed against the full length of CYP3A4 (19). Since cross-reactivities of the primary antibody with CYP3A5 and CYP3A7 were not evaluated, we consider these results as abundances of CYP3A isoforms in general. No evidence exists that porcine CYP3A and human CYP3A4 bind in an identical way with the primary antibody in this ELISA. Therefore, the obtained data are semi-quantitative and show abundances relative to the abundance of CYP3A in humans. Nevertheless, the amino acid sequences of CYP3A22 and CYP3A29 in Göttingen minipigs show 75% sequence identity compared to human CYP3A4, so binding of the primary antibody with porcine CYP3A can be expected (12).

This ELISA revealed a very low or absent CYP3A expression until 7 days of age. From Day 7 onwards, a gradual increase in CYP3A abundance was noted with a significantly higher CYP3A expression in the adult animals compared to the younger animals. This postnatal increase in CYP3A expression in Göttingen minipig livers is in agreement with data from an earlier immunohistochemical study performed by our group (21) and with data on CYP3A29 mRNA expression in the liver from Bama miniature pigs, showing very low expression in neonatal animals and increasing expression with age (22). Additionally, it corresponds to human data for CYP3A4. Stevens et al. noted low CYP3A4 expression levels in HLM, with a gradually increasing expression during the first six months of life. In the age group of 5 to 15 years, they found still lower CYP3A4 levels compared to these in adults (6). This age-related onset of CYP3A4 protein is in accordance with similar observations for CYP3A4 mRNA expression (5, 23, 24). Indeed, CYP3A4 levels start to increase after birth and reach adult levels from one year of age onwards, whereas several studies suggest that the ‘foetal’ CYP3A7 isoform remains the dominant CYP3A enzyme until one year of age (2). However, Lacroix et al. could not demonstrate age-related differences in CYP3A regarding protein expression from foetal until adulthood with a polyclonal antibody directed against human CYP3A4. Apparently, this antibody was not able to discriminate between CYP3A4, CYP3A5 and CYP3A7 (5). An age-related pattern, similar to human CYP3A4 was observed in our study, which suggests that our polyclonal antibody did not cross-react with a CYP3A7-orthologue. The absence of a CYP3A7-orthologue in the Göttingen minipig might seem
unlikely as Hermann et al. reported the presence of a CYP3A7-like enzyme in foetuses at 100 days of gestation by Western Blotting, with a higher expression in foetuses (100 days of gestation) compared to Day 1. Conversely, they found a 9-fold higher CYP3A29 mRNA expression and a higher percentage of positive bands on Western Blot in the Day 1 animals compared to the foetuses, although these bands on Western Blot were still weak (25).

We assessed the CYP3A activity by incubation of minipig liver microsomes with Luciferin-IPA. In humans, Luciferin-IPA is considered a highly specific substrate for CYP3A, with a 102-fold higher enzymatic efficiency for CYP3A4 compared with CYP3A7 and a 9-fold higher enzymatic efficiency for CYP3A4 compared with CYP3A5 (18, 26). To promote binding of Luciferin-IPA to the minipig 'CYP3A4-orthologue' and to prevent substrate inhibition in the lowest age groups, the concentration of Luciferin-IPA in the incubation mixtures (1 μM) was chosen below the estimated \( K_m \) (3.659 μM). We showed that the minipig liver microsomes extensively metabolized Luciferin-IPA, supporting CYP3A activity. Additionally, pre-incubation of minipig liver microsomes with CYP3cide, which is a mechanism-based inactivator of CYP3A4, inhibited the metabolism of Luciferin-IPA although higher concentrations were needed than for HLM (20). These results suggest that the metabolic inactivation efficiency of CYP3cide is much higher in the HLM compared to the minipig liver microsomes. This is not surprising as CYP3cide already shows differences in metabolic inactivation efficiency between human CYP3A isoforms (20). CYP3A-dependent activity was further substantiated by the inhibition of D-luciferin formation by testosterone, midazolam and ketoconazole. Ketoconazole is a typical CYP3A inhibitor and is a potent inhibitor of Luciferin-IPA biotransformation in human hepatocytes (26). In pigs, ketoconazole is a potent inhibitor of CYP3A activity too (16, 27, 28). Also in our study, ketoconazole clearly inhibited the metabolism of Luciferin-IPA in both HLM and minipig liver microsomes. Testosterone and midazolam are two commonly used CYP3A4 substrates in man that are metabolized by CYP3A in pigs too (13-16, 28-30). Co-incubation of Luciferin-IPA with testosterone or midazolam resulted in a reduction of the biotransformation of Luciferin-IPA in the minipig liver microsomes. In contrast, the tested range of concentrations of testosterone did not extensively inhibit the metabolism of Luciferin-IPA in the HLM. Wang et al. studied drug-drug interactions of CYP3A substrates with CYP3A4 in HLM. In their study, testosterone was not able to inhibit nifedipine oxidation and inhibited only partially midazolam 1'-hydroxylation. Conversely, nifedipine and midazolam were able to inhibit
the 6β-hydroxylation of testosterone (31). Their study showed that the effect of interaction of two CYP3A substrates with CYP3A is substrate-dependent. Our results indicate that the effect of testosterone on Luciferin-IPA metabolism is less pronounced in the HLM than in the minipig liver microsomes, although testosterone is a well-characterized CYP3A4 substrate in humans.

The results from the biotransformation of Luciferin-IPA among different age groups reflected the results obtained by the ELISA. This age-related pattern was observed for the metabolism of midazolam in male Camborough-29 pigs as well, with very low levels at Day 1 that significantly raised at two weeks of age, and remained at a similar level until five weeks of age. From five weeks onwards, a linear increase was present till 20 weeks of age (30). These activity data are also in agreement with the age-related trend in humans. Lacroix et al. found extremely weak levels of CYP3A4 activity (6β-hydroxylation of testosterone) in the foetal liver, which increased to 30-40% of adult activities in children during the first month of life, to remain at that level until 12 months of age. Adult activities were reached after one year of age (5). Blanco et al. studied midazolam as a substrate for CYP3A4/5 in liver microsomes in age groups ranging from 6 months until 93 years of age, but could not detect age-related differences (32). In contrast, Stevens et al. published CYP3A4 activity data from children between 5 and 15 years of age that were not yet at adult levels (6). High inter-individual variation in CYP3A4 activity, as described for adults, may also be responsible for differences in the paediatric population (8, 10, 33).

Regarding sex-related differences, the mean velocity of Luciferin-IPA metabolism was 42% higher in female than in male adult minipigs, although not statistically significant \( (p = 0.05) \). Since the number of animals was limited to five and four animals per gender, a larger sample size may elucidate whether a gender difference is present or not. Skaanild and Friis (1999) found a weak but significantly higher CYP3A expression in female Göttingen minipigs than in males at 3.5 to 4 months at age (34). In man, gender differences in CYP3A4 activity have also been described \textit{in vitro} and \textit{in vivo}, with a higher CYP3A4 activity in females compared to males (35-39). Whether this difference in CYP3A activity is clinically relevant is questionable, since Chen et al. found only a minor difference in AUC in contrast to significantly higher CYP3A activity in women (38). A clear explanation for these gender-related differences has not yet been found. A differential effect of sex steroid hormones may be possible (37-39). However, data are conflicting in man. Shimada et al. found no sex-related differences in CYP3A abundance
and activity in 30 Japanese and Caucasian liver samples, neither did Snawder et al. in 40 human liver samples (7, 8).

We found a positive correlation between CYP3A abundance and biotransformation of Luciferin-IPA for the PND7, PND28 and the adult animals \( (r = 0.863; p < 0.0001) \). Skaanild and Friis also found a positive correlation between CYP3A abundance and activity in conventional pigs \( (r^2 = 0.63; p < 0.0001) \) (34). A positive correlation between CYP3A4 abundance and activity has also been described in humans for testosterone 6β–hydroxylation \( (r^2 = 0.724) \) and for bufalin 5β–hydroxylation \( (r = 0.943) \) (8, 9). Our finding of a positive correlation might not be surprising since the immune-inhibition study showed clear inhibition of Luciferin-IPA metabolism with the primary antibody of the ELISA, suggesting that the same enzyme is involved in the ELISA and activity assay.

Comparison of the results for CYP3A abundance and activity between the HLM and the adult minipig liver microsomes in this study showed a much higher activity in the adult minipig liver microsomes than in the HLM, although CYP3A abundance was at a comparable level according to the ELISA. Since the primary antibody in the ELISA was directed against the full length of the human CYP3A4, the abundance of porcine CYP3A may have been underestimated compared to the HLM, which may explain the higher activity in the adult minipig liver microsomes despite similar abundances. However, the biotransformation of Luciferin-IPA, the inhibitory potential of CYP3cide and ketoconazole and the reduction of Luciferin-IPA biotransformation by testosterone or midazolam in this study support the hypothesis that porcine CYP3A-orthologues are responsible for the metabolism of Luciferin-IPA.

To summarize, CYP3A abundance and activity in the liver of the Göttingen minipig clearly show a postnatal maturational pattern, which is in accordance with data for CYP3A4 in humans. Based on these results, we consider the juvenile Göttingen minipig to be a good model for studies involving CYP3A substrates.
4.1.6 References

4.2 Part B – Detection of CYP3A7 activity

4.2.1 Abstract

CYP3A7 is considered the main CYP3A isoform in the human foetus. It has mainly a role in physiological functions that are involved in the protection of the developing foetus. Despite the important role of human CYP3A7, little is known on its presence in foetuses from other species. Therefore, it seemed useful to investigate its presence and activity in the liver from the Göttingen minipig. Liver microsomes from foetal, Day 1, Day 3, Day 7, Day 28 and adult Göttingen minipigs were incubated with the two human CYP3A7 substrates, i.e. Luciferin-3A7 and Luciferin-BE. No significant biotransformation of these human CYP3A7 substrates was noted in foetal and neonatal minipig liver. In contrast, activity was present in 28-day-old and adult animals, and in CYP3A4 Baculosomes. These results suggest that no CYP3A7-like activity is present in the foetal and neonatal Göttingen minipig.

4.2.2 Introduction

In humans, four members are present in the CYP3A subfamily, namely CYP3A7, CYP3A4, CYP3A5 and CYP3A43. CYP3A7 is the foetal isoform, which shows a high abundance (about 36% of total CYP450 content) during the late foetal period and the first week after birth, with the first signs of transition to the main adult isoform, CYP3A4, around time of birth. The major part of this developmental switch between CYP3A7 and CYP3A4 occurs during the first year of life (1-3). CYP3A7 is highly similar to CYP3A4 and shows an 88% identity in amino acids sequence, but it displays a different substrate specificity (2). CYP3A5 shows an 83% identity of amino acids sequence with CYP3A4, has overlapping substrate specificities with CYP3A4, and shows a polymorphic expression. In general, no age-related pattern has been detected for CYP3A5. In case of a high CYP3A5 expression, this is mainly linked to the expression of the CYP3A5*1 allele (4-8). CYP3A43 is considered of little importance in the metabolism of clinically used drugs. It has a low expression in both foetal and adult liver tissues, has a 76% of identity of amino acid sequences, and displays a dissimilar substrate specificity compared with CYP3A4 (9).
CYP3A7 can be detected in adult liver too, but the abundance is generally very low and hence of little importance in postnatal life. Yet, in about 11% of adult livers, abundant CYP3A7 expression is linked to the expression of functional CYP3A7*1C or CYP3A7*1B alleles (10).

Several metabolizing capacities have been attributed to CYP3A7 (1, 3).

- The human foetal adrenals form dehydroepiandrosterone 3-sulfate (DHEA-S). CYP3A7 will hydroxylate the majority of DHEA-S at the 16α-position with the formation of 16α-DHEA-S. In addition to that, DHEA-S and other 3-S-conjugated steroids have been reported to activate the catalytic CYP3A7 activity. DHEA-S and 16α-DHEA-S will be absorbed by the maternal placenta where they play a role in oestradiol and estriol synthesis. High concentrations of DHEA-S are associated with inhibition of cell proliferation and progesterone synthesis too (6).

- CYP3A7 is also responsible for the 4-hydroxylation of the highly embryotoxic and teratogenic retinoic acids (6).

- CYP3A7 is like CYP3A4 even capable of activation of promutagens like aflatoxine B1 and aflatoxine G1 (3, 11, 12).

The goal of this research was to investigate whether liver microsomes from Göttingen minipigs are capable of the biotransformation of two human CYP3A7 substrates, namely Luciferin-BE and Luciferin-3A7.

4.2.3 Materials and methods

4.2.3.1 Animals and tissue samples

Livers were obtained from healthy Göttingen minipigs and isolation of liver microsomes was performed as described in Chapter 4.1.3.

4.2.3.2 Incubation conditions for Luciferin-BE in minipig liver microsomes

Luciferin-BE (V866C, Promega Corporation, Madison, U.S.) is a luminogenic substrate that is metabolized mainly by human CYP3A7 and CYP4F12, but also by CYP3A4 and
CYP3A5, although to a lower extent. The kinetic studies were performed in non-treated
Nunc™ F96 Microwell™ white Polystyrene plates (236205, Thermo Fisher Scientific).
According to the Technical Bulletin for the P450-Glo™ Assays (https://be.promega.com/resources/protocols/technical-bulletins/101/p450-glo-assays-protocol/), a protein concentration of 20 µg microsomal protein (MP) per well, together with 150µM of Luciferin-BE was used, to favour biotransformation of Luciferin-BE by the porcine CYP3A7-orthologue. Figure S4.1 shows the working mechanism of the luminogenic assay with Luciferin-BE. (See Supplementary figure at the end of Chapter 4)

In each incubation well, 20 µg of hepatic microsomal protein, 0.1 M KPO₄ buffer (pH 7.4), 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride (451220 and 451200, Corning Incorporated) and 150 µM Luciferin-BE were co-incubated. The total incubation volume was 50 µL per well.

Microsomal dilutions and Luciferin-BE were pre-incubated at room temperature (RT) in 0.1 M KPO₄ buffer for 10 min. The reaction was initiated by addition of the NADPH-regenerating system to the remainder of the incubation mixture. The plate was first incubated for 30 min at RT. Subsequently, 50 µL of Luciferin detection reagent (V859A, Promega Corporation) was added to each well, mixed and incubated for 20 min at RT to stabilize the luminescent signal. Luminescence was measured with a Tecan Genios (Tecan Group Ltd., Männedorf, Switzerland). The concentration of the metabolite D-Luciferin, generated from Luciferin-BE, was quantified by comparing luminescence from the incubation mixtures to that from a D-Luciferin standard curve (Beetle Luciferin, Potassium Salt, E1601, Promega Corporation). CYP3A4 Baculosomes® Plus Reagent, rHuman (P2377, Life Technologies, Thermo Fisher Scientific) was used as a positive control. Insect cell control supersomes (456201, Corning Incorporated), lacking CYP450 enzymes, were used as a minus-P450 negative control. Positive and negative controls were included in the well plate and similarly treated to the minipig liver microsomes. Results from the Insect cell control supersomes were subtracted from the values obtained for the minipig liver microsomes and CYP3A4 Baculosomes®.

The liver microsomes from five animals were used, representing 86 days of gestation (n=1), 108 days of gestation (n=1), Day 1 (n=1), Day 3 (n=1), Day 7 (n=1) and Day 28 (n=1).

The data represent the mean value for each sample of two technical replicates.
4.2.3.3 Incubation conditions for Luciferin-3A7 in minipig liver microsomes

Luciferin-3A7 (P1741, Promega Corporation) is a luminogenic substrate that is metabolized by human CYP3A7. In contrast with Luciferin-BE, the contribution of other CYP450 enzymes is very low according to the manufacturer. Figure S4.1 shows the working mechanism of the luminogenic assay with Luciferin-3A7. (See Supplementary figure S4.1 at the end of Chapter 4)

The protocol was similar to that with Luciferin-BE, except that 10 µg MP was used per well, together with 30 µM of Luciferin-3A7, and the reaction run for 30 min at 37°C. The liver microsomes from animals of 84-86 days of gestation (n=8), 108 days of gestation (n=8), Day 1 (n=8), Day 3 (n=8), Day 28 (n=2) and adult age (n=2).

4.2.3.4 Mathematical and statistical analysis

Calculation of velocities of formation of D-Luciferin was performed in Microsoft Excel® (Version 14.3.1, Microsoft Corporation, Redmond, WA, U.S.).

4.2.4 Results

4.2.4.1 Activity assays with Luciferin-BE

Activity was low, but above the Lower Limit of Quantification (LLOQ) in the foetal, Day 1 and Day 3 animals. Activity had increased about 10-fold in the Day 28 animal. Activity was highest in the CYP3A4 Baculosomes. (Figure 4.7)

4.2.4.2 Activity assays with Luciferin-3A7

Activity was absent in all 32 foetal, Day 1 and Day 3 samples. For the 28-day-old animals, activity was present in one sample and absent in the other one. The same finding was present for the two adult samples, with about 3-fold higher activity in the positive sample compared with the positive 28-day-old sample. Activity in the CYP3A4 Baculosomes was positive, but low. (Figure 4.7)
Figure 4.7. The left graph and right graph show the biotransformation of Luciferin-BE and Luciferin-3A7, respectively. Only the results for animals that showed activities above the LLOQ are presented.

4.2.5 Discussion

Based on what is known on the activity of CYP3A7 in man, one might expect to find the highest metabolizing capacities for Luciferin-BE and Luciferin-3A7 in the foetal and neonatal Göttingen minipigs. However, these age groups showed no or low metabolism of these substrates. Luciferin-BE was metabolized by all age groups, with the highest activity in the 28-day-old animal, suggesting that the metabolizing capacity matures with age. Indeed, based on the activity in the CYP3A4 Baculosomes, it seems plausible that Luciferin-BE was metabolized by similar enzymes that were involved in the metabolism of Luciferin-IPA, representing CYP3A4-like enzymes. According to the manufacturer, CYP4F12 is also involved in the metabolism of Luciferin-BE, even to a larger extent than CYP3A4 and CYP3A5. CYP4F12 shows biotransformation capacity towards inflammatory mediators and drugs like antihistaminica (13). However, the presence of a CYP4F12-like enzyme in the minipig, and its metabolizing capacity remains to be determined in order to estimate its possible contribution to our observed results. In contrast with Luciferin-BE, Luciferin-3A7 metabolism is considered performed more exclusively by CYP3A7. In general, activity was low or absent, and below the LLOQ for all the foetal, Day 1 and Day 3 animals. Only samples from one 28-
day-old and one adult sample showed clear activity, with the highest activity for the adult animal. The CYP3A4 Baculosomes showed also activity, although it was limited. In conclusion, no significant biotransformation of two human CYP3A7 substrates was noted in foetal and neonatal minipig liver. In contrast, activity was present in 28-day-old, adult animals, and in CYP3A4 Baculosomes. These results suggest that no CYP3A7-like activity is present in the foetal and neonatal Göttingen minipig, although differing substrate specificities between human CYP3A7 and a porcine CYP3A7-like isoform cannot be excluded.


## 4.2.6 References


4.3 Supplementary figure

Figure S4.1. Conversion of P450-Glo™ substrate by CYP450 enzymes. The luminogenic substrates (Luciferin-IPA, Luciferin-BE and Luciferin-3A7) are derivatives of D-luciferin, but they are not active with luciferase (A). The derivative substrate is converted by CYP450 to a luciferin product (B). The Luciferin detection reagent (LDR) contains luciferase and ATP, and is added at the end of the reaction. The LDR stops the CYP450 activity with a detergent, and initiates a luciferase reaction that generates an amount of light that is proportional to the amount of luciferin product that is formed in the first step. The chemical structures of the three luminogenic substrates are shown, with a red arrow that indicates the site of modification by the intended CYP450. The bar charts represent the selectivity of human CYP450 isoforms for the luminogenic substrates. (Figures reproduced and adapted from www.promega.com)
5  In vitro Phase I- and Phase II-Drug Metabolism in The Liver of Juvenile and Adult Göttingen Minipigs

Adapted from:

In vitro Phase I- and Phase II-Drug Metabolism in The Liver of Juvenile and Adult Göttingen Minipigs.

5.1 Abstract

In view of paediatric drug development, juvenile animal studies are gaining importance. However, data on drug metabolizing capacities of juvenile animals are scarce, especially in non-rodent species. Therefore, we aimed to characterize the in vitro biotransformation of four human CYP450 substrates and one UGT substrate in the livers of developing Göttingen minipigs. Liver microsomes from late foetal, Day 1, Day 3, Day 7, Day 28, and adult male and female Göttingen minipigs were incubated with a cocktail of CYP450 substrates, including phenacetin, tolbutamide, dextromethorphan, and midazolam. The latter are probe substrates for human CYP1A2, CYP2C9, CYP2D6, and CYP3A4, respectively. In addition, the UGT multienzyme substrate (from the UGT-Glo™ assay), which is glucuronidated by several human UGT1A and UGT2B enzymes, was also incubated with the porcine liver microsomes. For all tested substrates, drug metabolism significantly rose postnataally. At one month of age, 60.5 and 75.4% of adult activities were observed for acetaminophen and dextrophan formations, respectively, while 35.4 and 43.2% of adult activities were present for 4-OH-tolbutamide and 1’-OH-midazolam formations. Biotransformation of phenacetin was significantly higher in 28-day-old and adult females compared with males. In conclusion, maturation of metabolizing capacities occurred postnataally, as described in man.
5.2 Introduction

The ‘Paediatric Regulation’ (EC No 1901-2/2006) has been implemented in January 2007 in order to facilitate the development and accessibility of medicinal products for use in the paediatric population. In view of safety, this has resulted in requests for juvenile animal studies prior to the initiation of clinical trials in children, especially for the youngest age groups (1).

The rat and the mouse are often the first choice species for this type of studies as they are well-characterized animal models and they are easy to handle and house. However, based on findings from general toxicity studies or for pharmacological reasons, a non-rodent animal model, like the dog, non-human primate, or minipig, may be more appropriate (2). Among these non-rodent models, the Göttingen minipig shows some advantageous characteristics including physiological and anatomical similarities with humans (skin, gastrointestinal tract, cardiovascular system), earlier sexual maturity compared to dogs and monkeys and a good reproductive capacity (poloestrus with short cycle, large litter size and easy to cross-foster) (3).

Unfortunately, the ontogeny of drug metabolizing enzymes is poorly characterized in non-rodent species compared with rodents. However, further elucidation is crucial. This paper focuses on Phase I- and Phase II-metabolism in the Göttingen minipig. The cytochrome P450 superfamily (CYP450) is largely involved in Phase I-biotransformation, performing mainly oxidation, reduction or hydroxylation of substrates, during which processes an oxygen atom is inserted into its substrate to form a more hydrophilic metabolite that can be excreted more easily (4). Four human CYP450 substrates were included in this study, i.e. phenacetin for CYP1A2, tolbutamide for CYP2C9, dextromethorphan for CYP2D6 and midazolam for CYP3A4. In general, rather high percentages of sequence identity in amino acids and nucleotides between human and porcine CYP450 isoforms are present (5). (Table 5.1)

However, this does not necessarily mean that substrate specificities are similar (5). Whether all substrates in this study are metabolized by the orthologous minipig CYP450 isoenzymes remains to be determined, but e.g. phenacetin has been used before as a marker for CYP1A2 in pigs (6, 7). Furthermore, CYP1A2 seems to be a well conserved isoform among species (8) and recombinant domestic pig CYP1A2 showed good catalytic activity towards caffeine, acetanilide, methoxyresorufin and ethoxyresorufin, which are all markers for human CYP1A2 (9).
Table 5.1. CYP1A, CYP2C, CYP2D and CYP3A isoforms in (mini)pig and human beings.

<table>
<thead>
<tr>
<th>Göttingen minipig isoforms</th>
<th>Human isoforms</th>
<th>% of sequence identity of nucleotides</th>
<th>% of sequence identity of amino acids</th>
<th>Accession Nr in NCBI databank</th>
</tr>
</thead>
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<td>CYP2D21</td>
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<td>78.3%</td>
<td>D89502</td>
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<tr>
<td>CYP3A22v1</td>
<td>CYP3A4</td>
<td>81.5%</td>
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<table>
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<th>% of sequence identity of amino acids</th>
<th>Accession Nr in NCBI databank</th>
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<tr>
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<td>CYP2C9</td>
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<td>77.0%</td>
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</tr>
<tr>
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<td>NM_214422</td>
</tr>
<tr>
<td>CYP3A46</td>
<td>CYP3A4</td>
<td>83.5%</td>
<td>77.8%</td>
<td>NM_001134824</td>
</tr>
</tbody>
</table>

Percentages of sequence identity of nucleotides and amino acids between minipig and man are presented (5).

For the CYP2C subfamily, three isoforms have been isolated in pigs, i.e. CYP2C33, CYP2C42, and CYP2C49. In Göttingen minipigs, three mRNA products were detected with primers for human CYP2C9, CYP2C19 and CYP2C8, suggesting that three CYP2C isoforms are also present in the Göttingen minipig (10). Tolbutamide has been used in several studies as a CYP2C9 substrate in pigs and minipigs (7, 11, 12) and Skaanild and Friis suggested that it is most likely biotransformed by a CYP2C9-like isoform in the porcine species, although activity was inhibited by the CYP2C19 inhibitor tranylcypromine, and not by sulfaphenazole, which is an inhibitor of human CYP2C9 (10). However, a CYP2C19-like enzyme may also metabolize this substrate as in humans, albeit at a limited level (13). For dextromethorphan, Skaanild and Friis have suggested that dextromethorphan O-demethylation is catalyzed by CYP2B instead of CYP2D in the minipig (14, 15). However, Jurima-Romet and colleagues suggested that dextromethorphan O-demethylation was catalyzed by a CYP2D6-like isoform in the domestic pig (16). In line with these findings, Sakuma et al. showed that recombinant CYP2D21, which is the CYP2D6 orthologue in the Göttingen minipig, can perform 1’-hydroxylation of bufuralol, which is another marker for human CYP2D6 (17). However,
these authors acknowledge that probably other CYP450 isoforms like CYP2B are also involved in bufuralol 1'-hydroxylation in minipigs (17). Four isoforms have been detected in the porcine CYP3A subfamily, from which CYP3A22 and CYP3A29 have been isolated in the Göttingen minipig (5, 17). The presence of CYP3A39 and CYP3A46, however, cannot be excluded. Similarly, to CYP3A dependent nifedipine oxidation and testosterone 6β-hydroxylation activities in both minipig and man, midazolam is considered a good CYP3A substrate in the porcine species as well as in man (6, 7, 11, 12, 18).

Phase II-reactions involve conjugation reactions that further increase the water solubility, enhancing the biliary or urinary excretion. Glucuronidation is such a conjugation reaction, exerted by uridine diphosphate glucuronosyltransferases (UGT) that adds glucuronic acid to a substrate, which may be a metabolite from Phase I-biotransformation (4). In the current study, UGT activity was assessed with the UGT multienzyme substrate (human UGT1A1, UGT1A8, UGT1A9, UGT1A10, UGT2B7 and UGT2B15) from the UGT-Glo™ assay (V2082, Promega, Madison, U.S.). In general, UGT enzymes are characterized by a low affinity, but a broad substrate specificity (4). Hence, human and minipig UGTs may have overlapping substrate specificities. However, different enzymatic properties of UGT among species have been described (19-21). For most human substrates, UGT activity is much higher in pigs and minipigs compared with human beings, although lower activities of specific isoforms have also been described (19-21). Additionally, the hepatic localisation of UGT1A in the developing minipig was assessed by immunohistochemical staining.

The main goal of this study was to investigate the ontogeny of Phase I- and Phase II-drug metabolizing enzymes in the Göttingen minipig using five probe substrates, representing four distinct human CYP450 activities and multiple human UGT activities. The biotransformation capacity of these enzymes was assessed in liver microsomes of both male and female minipigs, with ages ranging from the late foetal stage until Day 28 of age and in adults.
5.3 Materials and methods

5.3.1 Animals and tissue samples

Livers were obtained from healthy Göttingen minipigs. 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: 84 - 86 days of gestational age (DGA 84-86) (n = 8), 108 days of gestational age (DGA 108) (n = 8), Day 1 (within 24 hours after birth) (n = 8), Day 3 (n = 8), Day 7 (n = 9), Day 28 (n = 10) and Adult (n = 9). Since normal gestation length in the minipig is 112 to 115 days, the evaluation of the foetus age groups is limited to the third trimester of foetus development (DGA 84-86 and DGA 108 refer to 75 and 95% of gestation, respectively). Postnatal day 28 is the age at which the piglets are usually weaned in a preclinical setting. The first month of life in the minipig was chosen to cover the first year of life in children, as important changes in drug metabolizing capacity occur in this period. Both genders were equally represented in each age group, except in group Day 7 (5 Males and 4 Females) and the adult age group (4 Males and 5 Females). Liver samples from five out of ten adult sows were randomly selected in this study to obtain similar group sizes. The age of the adult males and females ranged between 18 – 24 months and 14 – 33 months, respectively. The adult sows were killed by electrical stunning, followed by exsanguination either before or after delivery, according to the desired age of their offspring. The foetuses were harvested and placed immediately on ice until further processing. The neonatal and juvenile piglets were delivered naturally and housed with the sow until euthanasia. The piglets were randomly assigned to a specific postnatal age group. Due to practical reasons, the piglets were not killed by electrical stunning, but they were anaesthetized by an intraperitoneal injection of sodium pentobarbital 20% (Kela NV, Hoogstraten, Belgium) (90 mg/kg), followed by exsanguination. Subsequently, the liver was dissected and rinsed with ice-cold 0.01 M phosphate buffered saline (PBS) (pH 7.4). Samples were taken from the lateral liver lobes (lobus hepatis sinister lateralis and lobus hepatis dexter lateralis) and were immediately snap frozen in liquid nitrogen. These samples were stored at -80°C until the isolation of liver microsomes. An interval of maximally 30 min had passed between time of death and freezng of the liver. Samples from the left medial lobes (lobus hepatis sinister medialis) were taken and fixed in 4%
paraformaldehyde (24 h) in 0.01 M PBS (pH 7.4) at room temperature (RT) and routinely processed to paraffin blocks.

5.3.2 Isolation of liver microsomes

Porcine liver tissue was thawed on ice and washed with ice-cold homogenizing buffer (0.01 M potassium phosphate (KPO₄) buffer (451201, Corning Incorporated, NY, U.S.) containing 1.15% potassium chloride). Excess of moisture was removed by blotting the tissue on paper towels. The liver tissue was minced into small pieces by means of surgical scissors and weighed. For each gram of tissue, a three-fold volume in ml of ice-cold homogenizing buffer was added. The tissue was homogenized with a Polytron® System PT 1200 E (230 V, 50 Hz) on ice for maximum 10 sec. As a final homogenization step, a motor driven Potter Elvehjem with Teflon pestle was used (1200 rpm, 5 to 10 up-and-down strokes). All homogenization steps were performed on ice. The homogenate was centrifuged at 12,000g for 20 min at 4°C. The resulting supernatant was centrifuged at 100,000g for 60 min at 4°C. The resulting pellet was re-suspended with homogenizing buffer and centrifuged at 100,000g for 40 min at 4°C. The resulting microsomal pellet was re-suspended in storage buffer (0.1 M KPO₄ buffer containing 250 mM sucrose and Halt™ Protease Inhibitor Single-Use Cocktail (78430, Thermo Fisher Scientific, MA, U.S.)) and stored at -80°C until use. Total protein concentration was determined by the Pierce® BCA Protein Assay Kit with bovine serum albumin as a standard (23225, Thermo Fisher Scientific).

5.3.3 CYP450 activity assay

Göttingen minipig liver microsomes were incubated with a cocktail stock solution containing 20 mM phenacetin, 20 mM tolbutamide, 2 mM dextromethorphan, and 2 mM midazolam in methanol (probes were kindly provided by Janssen Research, Beerse, Belgium). In the definitive incubates, stock solution was 200-fold diluted to obtain 100 µM phenacetin and tolbutamide, and 10 µM dextromethorphan and midazolam (concentration methanol was 0.5%). The probe substrate concentrations were selected to reach maximal velocities (Vₘₐₓ) and are routinely used to screen CYP450 activity in HLM at Janssen Research. Figure S5.1 shows the studied pathways of CYP450 metabolism. (See Supplementary figures at the end of Chapter 5) In order to verify that
HLM and minipig liver microsomes were functioning at $V_{\text{max}}$ in our study, HLM and adult female minipig liver microsomes were also incubated at double substrate concentrations. Phenacetin, tolbutamide, dextromethorphan and midazolam are considered probe substrates for human CYP1A2, CYP2C9, CYP2D6 and CYP3A4, respectively. Reaction velocities were calculated in units of picomoles of metabolite formed per minute per milligram of microsomal protein (pmol/min/mg MP). HLM(050B) were purchased and used as a positive control (HMMCPL050B, Life Technologies, Thermo Fisher Scientific). This preparation of HLM(050B) is composed of a pool of 33 male and 17 female human liver donors of which about the half are smokers or ex-smokers and use alcohol on a regular or occasional basis. No information on medicinal drug therapies was available. Concerning ethnicity, 41 donors were Caucasians, 4 were Hispanics and 5 were African Americans. Also recombinant human CYP1A2, CYP2C9, CYP2D6 and CYP3A4 Baculosomes® Plus Reagent (P2792, P2378, P2283, P2377, respectively, Life Technologies, Thermo Fisher Scientific) were included as a positive control (5 pmol CYP450 in 250 µl total incubate). Insect cell control supersomes (456201, Corning Incorporated, NY, U.S.), lacking CYP450 enzymes, but containing Cytochrome C reductase activity (25 nmol/min/mg protein), were used as a minus-CYP450 control. Positive and negative controls were similarly treated as the minipig liver microsomes. Results from the Insect cell control supersomes were subtracted from the values obtained for the minipig liver microsomes and the human liver microsomes. Each microsomal sample was incubated in triplicate. Additionally, correlations were made between the metabolizing velocities from this study and the results for CYP3A abundance and metabolism that were obtained in a previous study from our group in order to check substrate specificity of CYP3A for midazolam in the Göttingen minipig (22).

Three protein concentrations of liver microsomes from an adult female Göttingen minipig (50, 10 and 200 µg/ml), and three time points (10, 30 and 60 min) were tested for linearity in the presence of a mixture of 100 µM phenacetin, 100 µM tolbutamide, 10 µM dextromethorphan and 10 µM midazolam. For dextromethorphan, four additional protein concentrations (3.125 µg till 25 µg/ml) were tested with three additional time points (2, 5 and 10 min). The definitive incubation time (10 min) and microsomal protein concentration (50 µg/ml) were within the linear range for the tested substrates. Incubations of liver microsomes with the substrate mixture were performed in 1.4 ml polycarbonate tubes of a comorack-96 (MP22501, Micronic, Lelystad, The
Netherlands). In each incubation tube, 12.5 μg of hepatic microsomal protein, 0.1 M KPO₄ buffer (pH 7.4), NADPH-regenerating system (1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride (451220 and 451200, Corning Incorporated)) and a mixture of 100 μM phenacetin, 100 μM tolbutamide, 10 μM dextromethorphan and 10 μM midazolam were co-incubated. The total incubation volume was 250 μl per tube. Microsomal dilutions in 0.1 M KPO₄ buffer were pre-incubated at 37°C for 5 min. The substrate mixture and the NADPH-regenerating system in KPO₄ were also pre-incubated at 37°C for 5 min. The reaction was initiated by addition of the microsomal protein to the remainder of the incubation mixture. After ten minutes, the reactions were stopped by placing the tubes in liquid nitrogen. Frozen samples were stored at -20°C until the moment of further analysis.

Prior to analysis, all samples were thawed in the presence of DMSO supplemented with internal standard (1:1 vol:vol, 0.02 ng/μl 1-OH-midazolam-D₄). Metabolite formations (1-OH-midazolam, dextrorphan, acetaminophen, and 4-OH-tolbutamide) were quantified on an Acquity UPLC system connected to a Xevo TQ-S tandem mass spectrometer (Waters Corp., Milford, MA, U.S.) equipped with an electrospray ionization source operated in negative (OH-tolbutamide) or positive (1-OH-midazolam, dextrorphan, acetaminophen) mode. (Table 5.2) The lower limit of quantification (LLOQ) was set at 0.5 ng/ml, 0.1 ng/ml, 0.2 ng/ml and 0.1 ng/ml for acetaminophen, 4-OH-tolbutamide, dextrorphan and 1-OH-midazolam, respectively. Capillary and cone voltages were set at 3 kV and 50 V, respectively, with a de-solvation temperature of 550°C. Separation was carried out using an Acquity UPLC C18 column (1.7μm- 50 x 2.1 mm ID, Waters Corp.) at 60°C. For quantification of 1-OH-midazolam, dextrorphan, and acetaminophen, a mobile phase was established consisting of solvent A (HPLC grade water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). After a 0.5 min plateau at 95% (A) / 5% (B) a solvent gradient was started from 95% (A) / 5% (B) to 10% (A) / 90%(B) over 1.5 min at a flow rate of 0.6 ml/min to elute the compounds from the column. Finally, the system was re-equilibrated at 95% (A) / 5% (B) for an additional 0.8 min. Total run time was 2.9 min and 5 μl aliquots were injected onto the system for analysis. For quantification of 1-OH-tolbutamide a mobile phase was established consisting of solvent A (HPLC grade water containing 5% (vol:vol) methanol and 0.1% formic acid) and solvent B (methanol containing 5% (vol:vol) HPLC grade water and 0.1% formic acid). A solvent gradient was initiated from
80% (A) / 20% (B) to 40% (A) / 60% (B) over 0.8 min at a flow rate of 0.6 ml/min to elute the compounds from the column after which the system was briefly set at 100% (B) (0.1 min) before the system was re-equilibrated at 80% (A) / 20% (B) for an additional 0.8 min. Total run time was 1.8 min and 2 µl aliquots were injected into the system for analysis. All data collection, processing, and analysis was performed with Thermo Xcaliber software (Masslynx V4.1 Thermo Scientific).

Table 5.2. Overview of mass transition for CYP450 probe substrates.

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<th>Substrate</th>
<th>Metabolite</th>
<th>Retention time (min)</th>
<th>Q1-Q3 transition (g/mol)</th>
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</thead>
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<td>4-OH-Tolbutamide</td>
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<td>285.1→186</td>
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<tr>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>0.80</td>
<td>152.1→110</td>
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<tr>
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<td>Dextrorphan</td>
<td>1.05</td>
<td>258.2→157</td>
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<tr>
<td>Midazolam</td>
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<td>342.1→203</td>
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<tr>
<td></td>
<td>1-OH-Midazolam-D4(IS)</td>
<td>1.35</td>
<td>346.1→207</td>
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</tbody>
</table>

5.3.4 UGT activity assay

The protocol for the UGT-Glo™ assay (V2082, Promega Corporation, Madison, WI, U.S.) was executed following the guidelines from the manufacturer. Figure S5.2 shows a schematic presentation of the mechanism of the UGT activity assay. (See Supplementary figures at the end of Chapter 5) In brief, two parallel reactions were set up for each sample. In one reaction, the liver microsomes and the proluciferin UGT multienzyme substrate (V213B, Promega) were present together with uridine-5'-diphospho-glucuronic acid (UDPGA) (V209B, Promega) as a donor of glucuronic acid. The reaction in parallel was identical, except that UDPGA was replaced by an equal amount of ultrapure water. Hence, in the first reaction, depending on time and UGT content, a portion of the substrate becomes glucuronidated and is not able to emit light. The portion of substrate that remained unmodified, and the substrate that was present in the reaction without UDPGA, cannot become glucuronidated and will produce light. The decrease in light output, measured by comparing light output from the plus-UDPGA reaction with this from the minus-UDPGA reaction, is proportional to the glucuronidation activity of the sample. MgCl₂ (M2670, Sigma-Aldrich, St Louis, MO, U.S.)
and alamethicine (sc-200094, Santa Cruz Biotechnology, Inc., Dallas, TX, U.S.) were not provided in the kit, but were added to the assay to enhance UGT activity (23). HLM(040) and HLM(050B) were purchased and used as a positive control (HMMC-H3A4-PL040 and HMMCPL050B, Life Technologies, Thermo Fisher Scientific). Insect cell control supersomes (456201, Corning Incorporated), lacking UGT enzymes, but containing Cytochrome C reductase activity (25 nmol/min/mg protein), were used as a minus-UGT control. Positive and negative controls were similarly treated to the minipig liver microsomes. The UGT activity is expressed as the percentage of substrate that is consumed. Calculations were performed as recommended by the manufacturer. A standard curve with beetle luciferin was also present in the 96-well plate to ensure that the measured relative luminescence units (RLU) were within the quantifiable range of detection (Beetle Luciferin, Potassium Salt, E1601, Promega Corporation). The data represent the mean value for each sample obtained in two separate assays, with two technical replicates within each assay. The UGT multienzyme substrate is a substrate for human UGT1A1, UGT1A8, UGT1A9, UGT1A10, UGT2B7 and UGT2B15.

Determination of minipig UGT activity was conducted in non-treated Nunc™ F96 Microwell™ white Polystyrene plates (236205, Thermo Fisher Scientific). The UGT multienzyme substrate, provided in the kit, was used as the substrate for the UGT reaction. The UGT buffer (TES, pH 7.5) that was provided in the kit, was replaced by the more appropriate 0.1 M KPO4 buffer for the minipig UGT activity. A range of five protein concentrations (12.5, 25, 50, 100 and 200 µg/ml) from liver microsomes from an adult female Göttingen minipig, six substrate concentrations (5, 10, 20, 30, 40, and 60 µM), and three incubation time points (10, 20 and 30 min) were tested for linearity. The definitive incubation time, microsomal protein concentration (MP), and substrate concentration were set to 20 min, 50 µg/ml MP and 20 µM UGT multienzyme substrate.

The final reaction volume (40 µl per well) consisted of 0.1 M KPO4 buffer (pH 7.4), 8 mM MgCl2, 20 µM multi UGT enzyme substrate, 2 µg MP, 1 µg alamethicine and 4 mM UDPGA or an equivalent volume of ultrapure water. In a first step, 0.5 M KPO4 buffer (451201, Corning Incorporated), ultrapure water, alamethicine, UGT multienzyme substrate and microsomal protein were mixed and pre-incubated on ice. After 15 min, MgCl2 was added to the mixture and was kept at 37°C for 5 min. To start the reaction, UDPGA was added, or an equal amount of ultrapure water. The reaction was terminated after 20 min at 37°C by adding 40 µl of Luciferin detection reagent with D-cystein to each well. The 96-well plate was kept for 30 min at RT to stabilize the luminescent signal.
Luminescence was measured with a Tecan Infinite M200 pro (Tecan Group Ltd., Männedorf, Switzerland).

5.3.5 Immunohistochemical detection of UGT1A

Four micrometer thick sections were made from the left medial liver lobes of all minipigs. After deparaffinization of the slides, heat induced antigen retrieval was performed by microwaving the sections at 90 Watt for 20 min in Dako Citrate buffer pH 6 (Code S2369, Dako, Glostrup, Denmark), followed by a 20 min cool down period. Endogenous peroxidase activity was depleted by immersion of the slides in 3% hydrogen peroxide in Tris-buffered saline (TBS) for 15 min. The slides were subsequently incubated for 30 min at RT with 20% normal goat serum (Code X0907, Dako) to block aspecific binding sites. The slides were then incubated overnight with a mouse monoclonal antibody directed against human UGT1A1 (1:10) (B4:sc-271268, Santa Cruz Biotechnology, Inc.) overnight at 4°C. On each slide, one section of tissue was not incubated with the primary antibody as a negative control. Next, incubation with Dako Cytomation Envision+ System-HRP labelled Polymer Anti-mouse (Code K4006, Dako) was performed for 60 min at RT. Visualization of antibody binding was obtained by the use of 3,3'-diaminobenzidine chromogen and substrate buffer (DAB) in 60 seconds (Code K3468, Dako). Counterstaining was performed with Carazzi’s Haematoxylin (Klinipath, Olen, Belgium). In between subsequent steps in this procedure, except after the incubation with normal goat serum, slides were washed three times in 0.05 M TBS for 5 min. Normal goat serum and primary antibodies were diluted in TBS (0.05 M Tris-HCl, 0.9% NaCl, pH 7.4) containing 0.3% Triton X-100 (Sigma-Aldrich) and 1% bovine serum albumin (Sigma-Aldrich). The latter solution was also used as replacement for the primary antibody as a negative control. One specific sample with well-established UGT immunoreactivity was used as a positive control for each staining series.

Evaluation of the staining intensity was performed with an Olympus BX61 microscope (Olympus Belgium, Aartselaar, Belgium) by two independent observers. Intensity of staining was evaluated with a score ranging from 0 to 5. Score 0 was used in case of absence of visual staining. Score 1 and 2 represent a generalized very mild and moderate staining, respectively, of the cytoplasm of all hepatocytes. In case of the
presence of a few intensively stained hepatocytes among a majority of moderately stained hepatocytes, score 3 was given. Score 4 was assigned to samples with a zonal pattern of more intensively stained hepatocytes surrounding the central vein of each lobule. Score 5 was restricted to samples in which the intensely stained hepatocytes reached the interlobular fibrous tissue. Specificity of immunohistochemical staining was evaluated by the use of a negative control for each sample and the absence of immunoreactivity in fibrous tissue and endothelial cells in liver.

5.3.6 Statistical analysis

The Kruskal-Wallis test was used to detect whether a statistically significant difference was present among the investigated age groups as a whole, after performing a non-parametric Levene’s Test for homogeneity of variances. The Kruskal-Wallis test was used to examine age-related differences in biotransformation velocities for each CYP450 substrate, for the UGT substrate, and for the scores of UGT1A immunohistochemical stainings, with $p < 0.05$ as a threshold. When a statistically significant difference was present for a substrate among age groups, the Mann-Whitney test was used as a post-hoc for the pairwise comparisons between two specific age groups. The Bonferroni correction adjusted the threshold $p$-value then to 0.0025 for the CYP450 substrates and to 0.017 for the UGT substrate in accordance with the number of applied pairwise comparisons (7 and 3 age groups, for the CYP450 and the UGT substrate, respectively). Mann-Whitney test was also performed to detect sex-related differences within each age group ($p < 0.05$). Statistical analyses were performed with IBM SPSS statistics (version 22; IBM, Armonk, NY, U.S.). The Pearson correlation coefficient was calculated between the CYP450 activity data from this study and the results from a previous study that assessed CYP3A abundance and metabolism of Luciferin-IPA, as a measure of CYP3A activity, for 7-day-old, 28-day-old and adult animals (these animals showed values above the LLOQ) (22). Ln transformations of the data, analyses and graphs were made in Graphpad Prism Version 6.0 f (GraphPad Software, Inc., La Jolla, U.S.).
5.4 Results

5.4.1 CYP450 activity assay

Activities in the Insect cell negative control Supersomes approximated zero and the obtained values were subtracted from the activities in the minipig liver microsomes and HLM. All substrates were metabolized by the Göttingen minipig liver microsomes. A statistically significant age-related difference in CYP450 activity was observed by Kruskal-Wallis test, when all age groups were included (\( p < 0.05 \) for all substrates). An overview of the mean velocities ± standard deviations (in pmol/min/mg MP) for all metabolite formations per age group is presented in Table 5.3. The activities of the recombinant Baculosomes® are presented as a percentage of activity by the CYP450 isoform with the highest affinity, to provide insight into the substrate specificity. (Table 5.3) Figure 5.1 shows relative activities as a percentage of adult activities for each substrate. Acetaminophen, 4-OH-tolbutamide, dextrorphan and 1’-OH-midazolam formations remained below 2% of adult activities for the foetal age groups, representing late gestation. Although foetal relative activities were low for dextromethorphan, dextrorphan formation was already prominently present, generating a mean velocity that was comparable with the activity obtained in the batch of pooled HLM. No statistically significant differences were detected by Mann-Whitney tests when both foetal age groups were pairwise compared (\( p > 0.0025 \) for each substrate). For phenacetin, tolbutamide and dextromethorphan, relative activities of 3.63, 3.51 and 4.63% were reached at Day 1, respectively. In contrast, for midazolam, a relative activity of only 0.59% was present on the first day of life. During the first month after birth, all activities clearly rose and attained relative values of 60.5, 35.4, 75.4, and 43.2% for phenacetin, tolbutamide, dextromethorphan, and midazolam, respectively, at Day 28. For phenacetin and dextromethorphan, absolute activities were not significantly higher in adult animals compared with 28-day-old animals (\( p > 0.0025 \)), but they were significantly higher compared with 7-day-old animals and younger age groups (\( p < 0.0025 \)). The hydroxylation rates of midazolam and tolbutamide were significantly higher in adult animals compared with all younger age groups (\( p < 0.0025 \)).
Table 5.3 Metabolite formation velocities of CYP450 substrates.

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>N</th>
<th>Acetaminophen</th>
<th>4-OH-tolbutamide</th>
<th>Dextrorphan</th>
<th>1-OH-midazolam</th>
</tr>
</thead>
<tbody>
<tr>
<td>84-86 DGA</td>
<td>M</td>
<td>4</td>
<td>13.1 ± 6.07</td>
<td>2.49 ± 0.71</td>
<td>95.2 ± 88.1</td>
<td>5.76 ± 6.31</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>8.11 ± 2.45</td>
<td>2.43 ± 0.73</td>
<td>42.5 ± 73.1</td>
<td>2.01 ± 2.98</td>
</tr>
<tr>
<td>All</td>
<td>8</td>
<td></td>
<td><strong>10.6 ± 5.04</strong></td>
<td><strong>2.46 ± 0.67</strong></td>
<td><strong>68.9 ± 80.1</strong></td>
<td><strong>3.88 ± 4.99</strong></td>
</tr>
<tr>
<td>108 DGA</td>
<td>M</td>
<td>4</td>
<td>7.43 ± 2.02</td>
<td>2.16 ± 0.95</td>
<td>150 ± 83.1</td>
<td>0.69 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>7.11 ± 0.37</td>
<td>1.82 ± 0.41</td>
<td>184 ± 65.7</td>
<td>0.80 ± 0.30</td>
</tr>
<tr>
<td>All</td>
<td>8</td>
<td></td>
<td><strong>7.27 ± 1.35</strong></td>
<td><strong>1.99 ± 0.70</strong></td>
<td><strong>167 ± 71.7</strong></td>
<td><strong>0.75 ± 0.21</strong></td>
</tr>
<tr>
<td>Day 1</td>
<td>M</td>
<td>4</td>
<td>30.3 ± 32.0</td>
<td>2.90 ± 0.64</td>
<td>431 ± 329</td>
<td>2.74 ± 1.84</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>46.1 ± 5.76</td>
<td>6.54 ± 2.52</td>
<td>582 ± 169</td>
<td>5.70 ± 5.35</td>
</tr>
<tr>
<td>All</td>
<td>8</td>
<td></td>
<td><strong>38.2 ± 22.9</strong></td>
<td><strong>4.72 ± 2.59</strong></td>
<td><strong>507 ± 255</strong></td>
<td><strong>4.22 ± 4.03</strong></td>
</tr>
<tr>
<td>Day 3</td>
<td>M</td>
<td>4</td>
<td>87.6 ± 15.7</td>
<td>20.5 ± 6.66</td>
<td>3022 ± 856</td>
<td>20.8 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>67.8 ± 25.8</td>
<td>11.0 ± 5.66</td>
<td>1950 ± 905</td>
<td>17.1 ± 8.55</td>
</tr>
<tr>
<td>All</td>
<td>8</td>
<td></td>
<td><strong>77.7 ± 22.4</strong></td>
<td><strong>15.8 ± 7.62</strong></td>
<td><strong>2486 ± 997</strong></td>
<td><strong>18.9 ± 9.08</strong></td>
</tr>
<tr>
<td>Day 7</td>
<td>M</td>
<td>5</td>
<td>171 ± 47.7</td>
<td>33.5 ± 20.0</td>
<td>4865 ± 1924</td>
<td>99.4 ± 37.9</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>153 ± 90.0</td>
<td>15.7 ± 9.66</td>
<td>2577 ± 1833</td>
<td>53.3 ± 31.5</td>
</tr>
<tr>
<td>All</td>
<td>9</td>
<td></td>
<td><strong>163 ± 65.2</strong></td>
<td><strong>25.6 ± 17.9</strong></td>
<td><strong>3848 ± 2136</strong></td>
<td><strong>78.9 ± 41.0</strong></td>
</tr>
<tr>
<td>Day 28</td>
<td>M</td>
<td>5</td>
<td>325 ± 90.7</td>
<td>48.1 ± 18.5</td>
<td>6430 ± 2573</td>
<td>295 ± 113</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5</td>
<td>946 ± 152</td>
<td>47.1 ± 7.70</td>
<td>10087 ± 1993</td>
<td>318 ± 117</td>
</tr>
<tr>
<td>All</td>
<td>10</td>
<td></td>
<td><strong>636 ± 348</strong></td>
<td><strong>47.6 ± 13.4</strong></td>
<td><strong>8259 ± 2902</strong></td>
<td><strong>307 ± 109</strong></td>
</tr>
<tr>
<td>Adult</td>
<td>M</td>
<td>5</td>
<td>398 ± 196</td>
<td>173 ± 67.1</td>
<td>10390 ± 5105</td>
<td>738 ± 123</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4</td>
<td>1574 ± 318</td>
<td>104 ± 51.5</td>
<td>11408 ± 3868</td>
<td>687 ± 226</td>
</tr>
<tr>
<td>All</td>
<td>9</td>
<td></td>
<td><strong>1051 ± 670</strong></td>
<td><strong>135 ± 66.0</strong></td>
<td><strong>10956 ± 4188</strong></td>
<td><strong>710 ± 179</strong></td>
</tr>
</tbody>
</table>

HLM 1 x substrate      753 | 77.2 | 145 | 607
HLM 2 x substrate      857 | 75.3 | 156 | 682
MLM 1 x substrate      1691 | 79.5 | 6323 | 819
MLM 2 x substrate      2378 | 99.7 | 6621 | 907
CYP1A2 Bac*            100% | 3.65% | 0.04% | 0.09%
CYP2C9 Bac*            0.12% | 100% | 0.07% | 0.25%
CYP2D6 Bac*            2.63% | 13.7% | 100% | 0.11%
CYP3A4 Bac*            4.72% | 11.4% | 0.29% | 100%

Mean velocities ± standard deviations in pmol/min/mg MP are presented per age group and per sex for the minipigs (M = Males, F = Females). *Activities of the Bac(ulosomes)® are expressed as a percentage of the activity obtained in Baculosomes® with the highest activity. For the human liver microsomes (HLM) and the adult minipig liver microsomes (MLM), velocities at single and double substrate concentrations are also presented (1x and 2x substrate, respectively).
An overview of all p-values of pairwise comparisons by Mann-Whitney tests between age groups is presented in Table S5.1 in the Supplementary material. For both HLM and the adult female minipig liver microsomes, velocities were at about the same level for all substrates whether their concentrations were doubled or not, suggesting they were both functioning around \( V_{\text{max}} \) (Table 5.3).

Figure 5.1. Mean formations of metabolites from CYP450 substrates as percentages of adult activities in the developing Göttingen minipig.

By means of the Mann-Whitney test, significantly higher acetaminophen formations were observed in the 28-day-old \((p < 0.05)\) and adult \((p < 0.05)\) female animals compared with the males. For 4-OH-tolbutamide formation, a significant difference was noticeable between males and females at Day 1 \((p < 0.05)\), with higher activity in females. Furthermore, a higher dextrorphan formation was present in 28-day-old female animals compared with males, but statistical significance was weak \((p = 0.05)\). No significant sex-related differences were found for midazolam.

The strongest correlation was found between CYP3A abundance in pmol/mg MP (previous study) and 1\(^{-}\)OH-midazolam formation (Pearson \(r = 0.96\) and \(p < 0.05\)) (22). For acetaminophen, 4-OH-tolbutamide and dextrophan formations, lower Pearson
correlation coefficients were obtained ($r = 0.46, 0.79$ and $0.39$ with $p < 0.05, < 0.05$ and $> 0.05$, respectively). (Figure 5.2) Similarly, the highest correlation was found between D-luciferin formation, as a measure of CYP3A activity (previous study), and $1'\text{-OH-midazolam}$ formation (Pearson $r = 0.92$, $p < 0.05$) (22). For acetaminophen, 4-OH-tolbutamide and dextrorphan formation, lower Pearson correlation coefficients were observed ($r = 0.62, 0.82$ and $0.63$ with $p < 0.05, < 0.05$ and $< 0.05$, respectively). (Figure 5.2)

Acetaminophen formation in HLM was comparable with the mean activity in adult minipigs, but it was lower and higher compared with adult female and male minipigs, respectively. The 4-OH-tolbutamide formation was lower or similar compared with the adult minipigs. The formation of dextrorphan was extremely high in the adult minipig, showing a mean velocity in the region of 75 times the activity in the HLM. Even at 108 days of gestation, the activity in minipig liver microsomes was already at the level observed in HLM.

Since pregnancy may have an impact on CYP450 activity, individual metabolizing velocities are presented for the adult sows in Table S5.2 in the Supplementary material. Sow 1, euthanized at 84 days of gestation, showed the highest $1'\text{-OH-midazolam}$ formation and secondly highest dextrorphan formation, while Sow 5, euthanized 6 months after parturition, showed the highest acetaminophen and dextrorphan formations.
Figure 5.2. Correlations between CYP3A abundance and biotransformation of Luciferin-IPA versus biotransformation of CYP450 substrates. Calculation of correlation was performed on the ln-transformed data from PND7, PND28 and adult animals. Data on CYP3A abundance and D-Luciferin formation, as a measure of CYP3A activity were from an earlier study with the same animals (22). Correlations with CYP3A activity (left panels) and CYP3A abundance (right panels) have been made, respectively. From top to bottom, correlations for acetaminophen (ACT), 4-OH-tolbutamide (4-OH-TOL), dextrophan (DX) and 1'-OH-midazolam (1-OH-MDZ) formations have been made.
5.4.2 UGT activity

For the youngest age groups (foetal, Day 1 and Day 3), the UGT assay lacked accuracy and precision. Due to low UGT activity in these age groups, high luminescent signals for both plus-UDPGA and minus-UDPGA wells were present, resulting in similar RLU values. Moreover, values for the plus-UDPGA wells were sometimes higher in comparison with the minus-UDPGA wells. Consequently, this resulted in negative values for the percentage of used UGT substrate. Hence, these age groups were not taken into account for the statistical analysis and were omitted in Figure 5.3. UGT activities were low and similar for the foetal age groups, the 1-day-old and 3-day-old animals. At Day 7, UGT activity had increased (mean ± standard deviation: 21.3 ± 11.2% substrate consumption) compared with the younger age groups. The Kruskal-Wallis test revealed a statistically significant difference between the 7-day-old, the 28-day-old and adult animals ($p < 0.05$). At Day 28, substrate consumption (33.3 ± 7.10% substrate consumption) had increased compared with the Day 7 animals, although not statistically significant according to the Mann-Whitney test ($p > 0.017$). The highest activity was noted for the adult age group (58.6 ± 14.4% substrate consumption), which was significantly higher compared with 28-day-old animals ($p < 0.017$). No sex-related differences were found by Mann-Whitney test for Day 7, Day 28 and adult animals ($p > 0.05$ for each age group). Within the adult female age group, the highest substrate consumption was noted for the sow that was euthanized at 84 days of gestation. (Table S5.2) UGT activity was higher in the adult Göttingen minipig liver microsomes compared with HLM(050B) and HLM(040).
5.4.3 UGT1A immunohistochemistry

Kruskal-Wallis test showed a significant difference in scores among all age groups ($p < 0.05$). At 84-86 days of gestation, score 0 or 1 was given (mean score ± standard deviation: $0.9 \pm 0.5$). At 108 days of gestation, scores ranged from 1 till 3 ($2.3 \pm 0.6$). At Day 1 and at Day 3, scores 2 or 3 were assigned ($2.8 \pm 0.4$ and $2.9 \pm 0.4$, respectively). In the foetal and neonatal samples, groups of hematopoietic stem cells were distributed among the poorly organized hepatocytes. At Day 7, mainly score 3 was given, with the exception of two animals that received scores 4 and 5 ($3.4 \pm 0.8$). For 28-day-old and adult animals, scores 4 and 5 were given ($4.4 \pm 0.5$ and $4.6 \pm 0.5$, respectively). Figures 5.4, 5.5, and 5.6 represent images for scores 1, 3, and 5, respectively.
Figure 5.4. Score 1 in immunohistochemical detection of UGT1A. A mild staining among all hepatocytes is present in the liver of a foetus at 84-86 days of gestation. Scale bar 200 µm.

Figure 5.5. Score 3 in immunohistochemical detection of UGT1A. Small groups of more intensively stained hepatocytes appear close to the central vein of each lobule, though not generalized, in the liver of a 7-day-old minipig. The black arrow indicates the central vein. Scale bar 200 µm.
Figure 5.6. Score 5 in immunohistochemical detection of UGT1A. The hepatocytes of the entire liver lobules are intensely stained in the liver of an adult male minipig. The black arrow indicates the central vein. Scale bar 200 µm.

5.5 Discussion

In general, our study revealed low metabolizing capacities in foetuses, with a gradual increase during postnatal development, and maximum levels in adult animals. However, distinct developmental patterns could be distilled for the individual substrates.

Our results suggest that the maturation of phenacetin O-deethylation capacity in the minipig starts at birth, with relative activities of about 1 and 3.63% in foetuses and Day 1 animals, respectively. Mean activity in adult animals was not significantly different from the activity in 28-day-old animals that reached 60.5% of adult activity. This apparently contrasts with a rather late developmental pattern of CYP1A2 in man, as significantly lower CYP1A2 activity (caffeine N-3 demethylation) was observed in HLM from late gestational foetuses and neonates (till 4 weeks of age) compared with infants (6 weeks to 10 months of age), and with a significantly higher activity in adults compared with infants (24). These findings were further corroborated by another study in man, showing earliest appearance of CYP1A2 activity (demethylation of methoxyresorufin) during the first month of life, with achievement of 33% of the adult values at one year of age and more (25). The results from our study may indicate an earlier maturation of CYP1A2 activity in the minipig compared with man. However, differences regarding study design may also explain for this discrepancy. Different
CYP1A2 substrates were used in these human studies, which makes it delicate to make a valid comparison. Additionally, different incubation conditions, methods of detection and quantification or a variable determination of age groups may further hamper definite conclusions. Furthermore, although at a much lower level, acetaminophen formation was also performed by CYP2D6 and CYP3A4 Baculosomes® in our study. Thus, early contribution of other CYP450 isoforms in the minipig cannot be excluded. Our results were in agreement with a study in male Camborough pigs, showing very low, but detectable acetaminophen formation at Day 1, with increasing postnatal activity till 20 weeks of age (highest investigated age group) (7). A strikingly higher phenacetin O-deethylation was present in the 28-day-old and adult female minipigs compared with the males, which is in line with other studies in pigs and minipigs (26-28). An androgen based down-regulation of constitutive gene expression of hepatic CYP1A2 has been suggested in this species (27). In contrast, in man, the opposite sex difference has been reported for CYP1A2 activity in Caucasian, African American and Chinese populations, although controversy exists (29, 30). Consequently, this pronounced sex difference should be borne in mind when comparing activities in minipig liver microsomes with these in HLM. Indeed, in our study, activity in HLM was lower compared with female animals, but was higher compared with male animals. This result agrees with earlier studies that found lower CYP1A2 activities in male minipigs compared with HLM (6, 18).

The 4-hydroxylation of tolbutamide in minipig liver microsomes appeared to start at birth, with a relative activity of 3.51% at Day 1. From Day 3 onwards, activity further increased, but remained significantly lower in 28-day-old compared with adult animals, attaining only 35.4% of adult activity. Consequently, these data suggest a slow maturation of CYP2C9 activity, which agrees with results from Treluyer et al. in man (31). Indeed, these authors failed to detect CYP2C abundance and tolbutamide 4-hydroxylation in human foetal and neonatal microsomes, but found increased levels of activity from 8 days of age onwards to reach 50% of adult activity at 28 days of age, which remained similar until one year of age (31). This contrasts, however, with a later study in which human CYP2C9 abundance and activity (diclofenac 4-hydroxylation) reached about 30% of adult values towards the end of gestation. During the first five months after birth, CYP2C9 levels were significantly higher than in late gestation, but they were highly variable. About half of them achieved approximately 50% of adult levels, whereas the other part showed values that were not significantly different from
these at late gestation. In contrast, CYP2C19 abundance and activity (mephenytoin 4-hydroxylation) remained similar among foetal and early neonatal period (about 12-15% of adult values), and showed a first noticeable increase during the first 5 months of life, with highly variable expression from 5 months of age onwards. Although abundances of both isoforms increased with age, CYP2C19 was more abundant than CYP2C9 in foetal liver, while the reverse was true for postnatal liver. This early onset of CYP2C9 abundance and activity in man is in contrast to our results. However, the individual CYP2C9 and CYP2C19 abundances did not correlate well with their activities (32). Furthermore, although limited, CYP2C19 also participates in tolbutamide 4-hydroxylation, as well as other human CYP450 isoforms, as demonstrated by the human CYP2D6, CYP3A4 and CYP1A2 Baculosomes® in our study (13). Consequently, it cannot be excluded that in minipigs more than one CYP450 isoform is involved too. In male Camborough pigs, tolbutamide 4-hydroxylation was detectable, but very low at Day 1, and had significantly increased at two weeks of age, to remain at a similar level until five weeks of age, and to further increase until 20 weeks of age (7). In agreement with our results, a tendency of higher tolbutamide hydroxylation in male compared with female Göttingen minipigs has been described earlier (10). Similarly, higher CYP2C33 and CYP2C49 mRNA levels were present in 3-month- and 5-month-old male Meishan pigs compared with female animals, while this sex-related difference was not present in Landrace pigs (33). In humans, no clear sex-related differences are believed to exist for CYP2C9 and CYP2C19 (30, 32). Regarding species-related differences in our study, activity of HLM was comparable with the lowest values of the adult minipigs, which were mainly represented by female animals. In contrast, other studies report lower or similar tolbutamide hydroxylation activities in male minipigs compared with HLM (11, 18). This discrepancy may be explained by differences between batches of HLM.

Although mean dextromethorphan O-demethylation activity achieved only 1.52% of adult activity in late foetal period, it was already obviously present before birth. During postnatal development, activity further increased with age, with the achievement of 75% of adult activity at 28 days of age. Treluyer et al. observed very low or low dextromethorphan O-demethylation in human foetuses and during the first week of life, respectively, with only 25% of adult activity in 5-year-olds. Hence, birth itself was suggested as the trigger for CYP2D6 protein expression and activity (34). This contrasts with a study that detected dextromethorphan O-demethylation activity in 70% of
human liver samples from the third trimester of gestation onwards, but not earlier, and with a further significant increase after birth. However, no clear age-related differences were observed after the first week of life (35). This is in line with results for in vivo dextromethorphan O-demethylation, measured by determination of the urinary molar ratio of dextromethorphan to dextrorphan, showing no age-related differences after two weeks of age (36). However, Johnson et al. disagreed with this result and suggested a predicted progressive increase in CYP2D6 activity during the first year of life, reaching adult values at 12 months of age, when a correction for renal maturation over time was made (36, 37). Noteworthy is the highly polymorphic expression of CYP2D6 in humans, which may potentially obscure age-related trends after birth (35). As aforementioned in the introduction, controversy exists whether CYP2D or by CYP2B is involved in dextromethorphan O-demethylation in the porcine species, with most studies suggesting a major role for CYP2B (14-17). Unfortunately, the clinical relevance of CYP2B6 in man has been recognized only recently, resulting in little data on specific aspects like its ontogeny (38, 39). One study reports an increase in CYP2B6 expression with age, showing a two-fold higher CYP2B6 expression in liver samples from individuals older than 30 days of age compared with younger age groups. However, for both age groups a 25-fold variation in protein levels was present (39). Similar to our study, bufuralol hydroxylation in male Camborough piglets as a measure for CYP2D activity was far more pronounced at Day 1 compared with the metabolism of other investigated CYP450 substrates (7). Compared with HLM, dextromethorphan O-demethylation was extremely high in our study. The same trend was found for dextromethorphan O-demethylation and bufuralol hydroxylation by other researchers, suggesting higher CYP2D6-like activity in minipigs than in man (6, 7, 11, 12, 18). In addition to that, CYP2D25, the CYP2D6 orthologue in Suffolk White pigs, showed an abundance of 26% of total CYP450 content, which is considerably higher than the limited abundance of CYP2D6 in man (8). The abundance of CYP2D21 and CYP2B in the Göttingen minipig, and its potentially polymorphic character, remains to be determined.

A good correlation was found between 1'-OH-midazolam formation and both CYP3A abundance and metabolism of luciferin-IPA, obtained in an earlier study from our group, further corroborating that 1'-hydroxylation of midazolam is performed by minipig CYP3A (22). Remarkably, high Pearson r values were also present with 4-OH-tolbutamide formations (Figure 2). Some hypotheses can be made but they are not
conclusive. First, based on relative activities (Figure 1), 1’-OH-midazolam and 4-OH-tolbutamide formations follow a more similar ontogenetic pattern among all metabolite formations. Additionally, rifampicin and phenobarbital are inducers of both CYP2C and CYP3A in man as well as in pigs. This induction is regulated via the pregnane X receptor and constitutive androstane receptor (5). In contrast, CYP1A2 is not induced by these ligands, and its induction is mainly dependent on the aryl hydrocarbon receptor (5). So, it cannot be excluded that similar pathways of induction may result in more similar ontogenetic profiles. Finally, all substrates showed a postnatal increase, resulting in correlations to a certain degree for all substrates. The 1’-hydroxylation of midazolam remained below 0.6% of adult activity in the foetal and 1-day-old piglets, but increased from Day 3 onwards, to reach the highest levels in adult animals. Our activity data correspond with the postnatal ontogeny of human CYP3A4. Very low levels of CYP3A4 activity (6β-hydroxylation of testosterone) were noted in human foetal livers that increased to 30-40% and 100% of adult levels after one month and after one year of age, respectively (40). In a later study, no age-related differences were found for midazolam metabolism in HLM with age groups ranging from 0.5 to 93 years of age (41). Consequently, these latter studies suggest that CYP3A4 approximates adult levels already by one year of age. This contrasts, however, to measured CYP3A4 activities in HLM from 5- until 15-year-old children that were not yet at adult levels (42). High inter-individual variation in CYP3A4 activity, as described for adults, may also be responsible for differences in the paediatric population (43). Our results were in line with the age-related pattern that was observed for midazolam metabolism in male Camborough pigs (7). Although a trend of higher CYP3A activity in female compared with male minipigs has been described earlier (14, 22), this difference was not seen in the current study. For man, data on sex-related differences are conflicting (30). In the present study, midazolam 1-hydroxylation in HLM was at about the same level as in adult Göttingen minipigs. However, higher and lower CYP3A activities in Göttingen minipigs compared with HLM have also been reported (11, 18, 21, 22). The variability in presence of discrete sex-related differences and similarities with HLM among studies may be explained by the potential presence of four CYP3A isoforms in the porcine species, with a possible sex- and breed-related expression, and variable substrate affinities (33). UGT activities remained similar among foetal, Day 1 and Day 3 animals, with increasing activities afterwards, which was also reflected by the results of the immunohistochemical detection of UGT1A. The centrilobular pattern of UGT1A
staining, which was mainly present in the highest age groups, has also been described in adult human livers (44). Overall, human UGT1A and UGT2B proteins appear after 20 weeks of gestation and before 6 months of age, with no differences in abundances afterwards. In contrast, UGT activity appears to remain immature in children up to two years of age, with inefficient glucuronidation in neonates and young children (45). However, early activities of individual UGT isoforms with low rates may be masked by the activities of isoforms with high metabolizing capacities. Indeed, glucuronidation of morphine has already been described in human foetal liver microsomes (15-27 weeks of gestation), though reaching only 10-20% of the efficiency of adult hepatic microsomes (46). Also in the microminipig, very early maturation of glucuronidation has been reported, i.e. for 17β-estradiol (UGT1A1), with a similar activity in 1-day-old and 8-month-old animals. For other tested UGT substrates in that study, no mature activities were found at Day 1, suggesting early maturation of a specific UGT isoform (20). The use of the UGT multienzyme substrate in our study does not allow drawing conclusions about the age-related pattern of individual UGT isoforms, but provides a general view on the UGT activity in young minipigs. Regarding sex, we did not find differences in UGT activity in our study, which is in line with human data (45). The adult minipigs showed higher UGT activities compared with human beings in our study, as described by others (19-21).

An additional point that may need some discussion is the fact that pregnancy may have an impact on the presence and activity of metabolizing enzymes, as already described in women (47). In vivo studies suggest that CYP2D6 and CYP3A4 are increased during pregnancy, whereas CYP1A2 is decreased (47). Enhanced UGT1A4 activity was also observed during pregnancy, while UGT2B7 remained unchanged (47). However, it is difficult to perform studies on altered drug metabolism in this vulnerable group of population, which results in scarcity of data. The only pregnant sow, that was included in our study, showed the highest 1’-OH-midazolam formation, secondly highest dextrorphan formation and highest consumption of the UGT multienzyme substrate among all adult sows. However, the limited number of adult (pregnant) females in this study does not allow for drawing conclusions about the effect of pregnancy on metabolizing enzymes in the minipig.

In general, a higher rate of metabolism was observed in the adult minipig than in the HLM in our study. This result agrees with literature data and most probably represents a species-specific difference. For example, a two- to three-fold higher CYP450 content
has been described in the minipig compared with man (48). However, one should also take into account that human livers are more prone to loss of enzymatic activity between time of death and the moment of freezing. For laboratory animals, it is much more feasible to take measures that preserve enzymatic activities as well as possible (isolate and flush the liver immediately after moment of death and chill on ice until freezing). Additionally, a valid comparison between an animal model and ‘human beings’ as such may be hampered by the variability that exists among human liver donors that are pooled in a batch of HLM (e.g. ethnic backgrounds, feeding habits, alcohol use, drug use, smoking, gender). These differences in HLM may also cause differences in results among studies.

In conclusion, the Göttingen minipig is capable of metabolizing four typical human CYP450 substrates and one UGT substrate, with the formation of at least the same metabolite as in man. In general, the metabolic activity was highest in adult animals. However, a significant postnatal increase was already present during the first month of life, suggesting that crucial developmental changes in metabolizing capacity take place within this period. These data are useful for the interpretation of juvenile toxicity data in the Göttingen minipig but also for further strengthening of paediatric PB/PK models.
## 5.6 Supplementary tables

Table S5.1. *P*-values for pairwise comparisons between two age groups.

<table>
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<th>DGA 84-86</th>
<th>DGA 108</th>
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<th>Day 3</th>
<th>Day 7</th>
<th>Day 28</th>
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<tr>
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<tr>
<td>Day 28</td>
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Bonferroni correction adjusted the significance level for the *p*-values to 0.0025. *P*-values indicating significant differences between age groups are marked in bold.
Table S5.2. Individual velocities of CYP450 activities in sows.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Age</th>
<th>Euthanasia</th>
<th>Metabolite formation in pmol/min/mg</th>
<th>% of substrate used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MP ACT</td>
<td>4-OH- TOL</td>
</tr>
<tr>
<td>Sow 1</td>
<td>1yr 2 mo</td>
<td>84 DGA</td>
<td>1433</td>
<td>90.0</td>
</tr>
<tr>
<td>Sow 2</td>
<td>1yr 4 mo</td>
<td>Day 8 after partus</td>
<td>1690</td>
<td>79.5</td>
</tr>
<tr>
<td>Sow 3</td>
<td>2yr 9 mo</td>
<td>Day 30 after partus</td>
<td>1571</td>
<td>92.0</td>
</tr>
<tr>
<td>Sow 4</td>
<td>2yr 7 mo</td>
<td>Day 30 after partus</td>
<td>1157</td>
<td>193</td>
</tr>
<tr>
<td>Sow 5</td>
<td>2yr 5 mo</td>
<td>6 mo after partus</td>
<td>2017</td>
<td>628</td>
</tr>
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</table>

For each sow in this study, age, time point of euthanasia, individual metabolite formations of the CYP450 substrates, and percentage of consumed UGT substrate are presented. Highest and lowest activities for each substrate are marked in bold and underlined, respectively. ACT acetaminophen; 4-OH-TOL 4-OH-tolbutamide; DX dextrophan; 1-OH-MDZ 1-OH-midzaolam; UGT MES UGT multienzyme substrate
5.7 Supplementary figures

Figure S5.1. Pathways of CYP450 metabolism that were studied in Chapter 5. Phenacetin O-deethylation into acetaminophen, tolbutamide 4-hydroxylation into 4-OH-tolbutamide, dextromethorphan O-demethylation into dextrorphan, and midazolam 1’-hydroxylation into 1’-OH-midazolam, are probe reactions for detection of human CYP1A2, CYP2C19, CYP2D6 and CYP3A4, respectively. (Figures are reproduced from https://pubchem.ncbi.nlm.nih.gov/)
Figure S5.2. Conversion of the UGT multienzyme substrate by UGT enzymes. UGT enzymes attach a glucuronic acid moiety to the proluciferin substrate. During the detection step, the proluciferin is simultaneously converted to a luciferin by cyclization with D-Cysteine. Luciferase uses the luciferin analog of the initial substrate to produce light but does not produce light with the glucuronidated luciferin. Light output is inversely proportional to UGT enzymatic activity. (Figure is reproduced and adapted from www.promega.com)
## 5.8 References


6 Assessment of morphometric data and gastrointestinal pH-values in the developing Göttingen minipig

Adapted from:
Organ data from the developing Gottingen minipig: first steps towards a juvenile PBPK model.

6.1 Abstract

The Göttingen minipig is the most commonly used pig breed in preclinical drug development in Europe and has recently also been explored for physiologically based pharmacokinetic (PBPK) modelling. To develop such a model, not only physiological data from adult animals but also data from juvenile animals are required, especially when using this model for paediatric drug development. Therefore, the aim of our study was to document body and organ weights (brain, heart, lungs, liver, gastrointestinal tract, spleen and kidney), lengths of the small and large intestines and pH values of the gastrointestinal tract in Göttingen minipigs from the foetal stage until the age of 5 months. Postnatal organ and body weights were fitted to regression models to find suitable equations that could be used to estimate organ weights as a function of body weight in the neonatal and juvenile Göttingen minipig. Most organs followed a non-linear growth curve during the first 5 months of life. In general, relative organ weights were the highest during the first week of life, during which the gastric pH was more alkaline than at 28 days of age.

6.2 Introduction

Physiologically Based Pharmacokinetic Modelling (PBPK) is a mathematical modelling technique, which aims to predict absorption, distribution, metabolism and excretion (ADME) of drugs in man and animals. These models take the drug specific characteristics as well as the physiological and anatomical characteristics of the body into account, resulting in a more reliable prediction of pharmacokinetic data (1). The
different compartments in a PBPK model represent organs with inherent volumes and blood flows (2, 3). Oral dosing is often the preferred route of drug administration and therefore most PBPK models focus on gastrointestinal absorption, but models of topical and parenteral administration are also important as they represent common routes of administration (1).

With regard to orally administered drugs, several physiological and anatomical factors in the gastrointestinal tract influence drug absorption. Local pH has a significant effect on the ionisation of weakly acidic and basic compounds, which will affect their absorption (4, 5). The small intestine plays a key role in absorption due to its large absorptive surface area, determined by its length, circular folds and villi with microvilli on the enterocytes, (6, 7). Subsequently, phase I- and phase II-metabolizing enzymes and drug transporters in the enterocytes further influence absorption and exposure of orally ingested drugs (8, 9). However, first-pass metabolism largely takes place in the liver, after transportation via the portal vein. Secretion of drugs and their metabolites in the bile is largely species- and size-dependent and many compounds are secreted in the bile, resulting in a secretion back into the intestinal lumen. Here, they may be reabsorbed by intestinal cells and made available for recycling in a process known as ‘enterohepatic cycling’. When drugs enter the systemic circulation via the hepatic vein or via the lymphatic system, the target organs will be exposed to them prior to their elimination (10).

Both human data and laboratory animal data can be included in PBPK models. There are several advantages to using the Götingen minipig in toxicity studies. They share important anatomical and physiological characteristics with humans, they can be bred under controlled conditions and are easy to handle. As such, they represent a valuable alternative to dogs and nonhuman primates (11-13). Including physiological data from sexually mature minipigs has already proven to be valuable in PBPK models, but additional data are still required (3). To create and optimize a Götingen minipig PBPK model, an extensive database with anatomical and physiological data is a prerequisite. PBPK modelling is not only useful for prediction of adult PK data, but it has recently also gained much attention in paediatric drug development since the use of children in clinical studies is limited by ethical and practical concerns (2, 14, 15). The prediction of pharmacokinetics and pharmacodynamics is very challenging in children, and particularly in the neonate (16). In a developing body, the ADME of drugs is constantly changing due to the dynamic changes in absolute and relative organ weights, body
composition, blood flow, maturation of organs and the presence and activity of metabolizing enzymes and transporters (2, 5, 17). As a result of the dynamic character and complexity of the ADME processes, and given the fact that the clearance mechanisms are not fully mature in very young children, scaling paediatric doses of drugs from adult doses on the basis of body weight, height or age only may result in under- or overdosing and adverse drug reactions (15, 17). It will be helpful to integrate both anatomical and physiological data on the developing body in such PBPK models to predict more accurately the outcome of pharmacokinetics in children. Nevertheless, especially for orally ingested drugs, these predictions remain difficult in children (2).

The aim of the study was to share morphometric organ data and pH-values of the gastrointestinal tract from foetal, neonatal, juvenile, and sexually mature Göttingen minipigs. These data can be used to frame a minipig PBPK model, but may also be useful as reference data in other scientific fields. Data from 1-day-old up to 5-month-old animals were used to create equations, allowing for estimation of organ weight as a function of body weight in neonatal and juvenile animals.

6.3 Materials and Methods

6.3.1 Animals and tissue samples

Organs were obtained from healthy Göttingen minipigs at the University of Antwerp and at Sequani Limited (Ledbury, United Kingdom). Ellegaard Göttingen minipig A/S (Dalmose, Denmark) kindly donated the animals to the University of Antwerp. The following age groups were investigated at the University of Antwerp: 84-86 days of gestation (n = 18), 108 days of gestation (n = 8), Day 1 (within 24 hours after birth) (n = 10), Day 3 (n = 11), Day 7 (n = 12), Day 14 (n = 7), Day 28 (n = 14) and at Sequani Limited Day 1 (n = 10), Day 7 (n = 10), Day 14 (n = 7), Day 28 (n = 9), 3 months (n = 29), 4 months (n = 10) and 5 months (n = 10) of age. Day 28 is the age at which Göttingen minipigs are usually weaned in a pharmaceutical context. The higher ages were of interest since male and female Göttingen minipigs reach sexual maturity at 3-4 months and 4-5 months of age, respectively. In general, minipigs enter toxicological studies at 3.5-4.5 months of age when they are almost or already sexually mature (11).

Both sexes were included in each age group. Euthanasia of these animals was performed as part of other research goals (18, 19). Adult sows were euthanized by
electrocution, followed by exsanguination either before or after delivery, according to
the desired age of their offspring. The foetuses were harvested and immediately placed
on ice until further processing. The neonatal and juvenile piglets were naturally
delivered and housed with the sow until euthanasia. The piglets were randomly
allocated to a specific postnatal age group. They were anaesthetized by an
intraperitoneal injection of sodium pentobarbital 20% (Kela NV, Hoogstraten, Belgium)
(90 mg/kg), followed by exsanguination. Animals older than 28 days of age were
sedated with Rompun (xylazine hydrochloride, Baeyer, Leverkusen, Germany) and
Ketamine (ketamine hydrochloride, Ceva S.A., Libourne, France), prior to an
intravenous injection with sodium pentobarbital. Animals were not fasted prior to
euthanasia. The Ethical Committee of Animal Experimentation from the University of
Antwerp (Belgium) (Dossier 2012-30) and the Animal Health Care Committee of
Sequani Limited approved the protocol and use of the animals.

6.3.2 Morphometry and pH assessment

Body weight was assessed prior to euthanasia and exsanguination. Brain, lungs, heart,
left kidney, liver, spleen, stomach, small intestine, large intestine and caecum were
removed after exsanguination and weighed (Precision Balance BA3100P, Sartorius,
Vilvoorde, Belgium). Determination of weights, lengths and pH values of the
gastrointestinal tract was only performed at the University of Antwerp and
consequently these data are limited to the first month of life. These animals were not
fasted prior to euthanasia. The pH values of the stomach (anatomical region not
specified) and the different intestinal regions were determined, i.e. the proximal part of
the duodenum (Zone 1), the middle part of the jejunum (Zone 2), the ileum (Zone 3),
defined as the terminal part of the small intestine that is attached to the caecum via the
plica ileocaecalis (World Association of Veterinary Anatomists, 2012,
http://www.wava-amav.org/), and the middle part (Zone 2) of large intestine and
caecum. The determination of the pH was performed by bringing Dosatest® pH 1-11
indicator paper (VWR international, Fontenay/Bois Cedex, France) into contact with
the luminal side of the gastrointestinal wall. After determination of pH, all intestinal
segments were subsequently rinsed with cold 0.1 M phosphate buffered saline (pH 7.4)
before weighing.
6.3.3 Statistical analysis

The Kruskal-Wallis (Mean Rank) test was used to detect potential statistically significant differences. A $p$-value $< 0.05$ was considered significant. For organ and body weights, differences between 3-, 4- and 5-month-old animals were evaluated. Differences in pH among intestinal regions and for each separate gastrointestinal region were evaluated for the Day 1, Day 3, Day 7 and Day 28 animals. Bonferroni correction for pairwise comparisons adjusted the $p$-value for statistical significance to 0.017 for organ and body weights, and to 0.01 for the age-dependent differences in pH for each separate gastrointestinal region. Statistical analyses were performed with IBM SPSS statistics (version 20; IBM, Armonk, NY, U.S.).

6.3.4 Mathematical analysis

Calculation of descriptive values was performed with IBM SPSS statistics (version 20; IBM). For each organ, we fitted the data on organ weights (in g, on the Y-axis) as a function of body weight (in g, on the X-axis) by nonlinear curve fitting (first, second, third or fourth order polynomial) in GraphPad Prism 6.0 f (GraphPad Software, Inc., La Jolla, CA, U.S.). First, each set of organ weights was split by sex. Then, we checked whether the best-fit values of all the unshared parameters for a certain order of polynomial equation differed significantly between both datasets by F-test ($p < 0.05$). If not, one single curve was used to fit the organ data, and the data from both sexes were compiled. If the parameters differed significantly, individual equations were calculated for each sex. In case of two apparently valid models, the difference in adapted Akaike Information Criterions (AICc) was used to decide which model fitted the best. Outlier identification was performed by the ROUT-method. Additionally, the observed versus predicted values (and vice versa) for each best-fit equation were plotted in a linear regression model to check for goodness of fit, and proportional and systematic bias in the prediction of the organ weights to validate the chosen model. The same procedure was followed to fit body weights as a function of age in days.

F-test, D'Agostino-Pearson normality test, Runs test, AICc calculations and outlier detection were performed in GraphPad Prism 6.0 f (GraphPad Software, Inc.). Plotting and evaluation of linear relationships between observed and predicted data was
performed in Microsoft Excel® (Version 14.3.1, Microsoft Corporation, Redmond, WA, U.S.).

6.3.5 Preliminary validation of the model

A publicly available database, at the website of Ellegaard Göttingen minipig A/S (http://minipigs.dk/the-goettingen-minipig/background-data/), with mean body weights and mean organ weights from 2-month- and 6-month-old Göttingen minipigs, was used to perform a preliminary validation of the generated best-fit equations for brain, heart, lungs, spleen, kidney and liver as a function of body weight.

6.4 Results

6.4.1 Total body weight and absolute organ weight

The absolute weights (in g) of the brain, lungs, heart, left kidney, spleen, liver and total body weights (in g) are listed in Table 6.1. Between 3- and 4-month-old animals, statistically significant differences were present for body weights (p < 0.001) and for all organ weights (p < 0.005), except for the spleen (p = 0.024). Between 3-month- and 5-month-old animals, statistically significant differences were present for all organ and body weights (p < 0.001). No statistically significant differences were present between 4- and 5-month-old animals (p > 0.17 for all organ weights and p = 0.06 for body weights). These results indicate that body weight is still increasing between 4 and 5 months of age, but the increase is less pronounced than in younger animals. Organ growth is clearly slowing down after 4 months of age. The absolute gastrointestinal organ weights (in g) are depicted in Table 6.2.
<table>
<thead>
<tr>
<th>Age group</th>
<th>Sex</th>
<th>N</th>
<th>Brain</th>
<th>Heart</th>
<th>Lungs</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Liver</th>
<th>BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGA 84-86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>F</td>
<td>9</td>
<td>9.57 ± 1.08</td>
<td>1.54 ± 0.28</td>
<td>6.55 ± 1.65</td>
<td>0.35 ± 0.10</td>
<td>1.09 ± 0.31</td>
<td>4.09 ± 0.90</td>
<td>174 ± 37</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6-8</td>
<td>9.6 ± 1.06</td>
<td>1.54 ± 0.29</td>
<td>6.42 ± 1.75</td>
<td>0.36 ± 0.07</td>
<td>1.02 ± 0.20</td>
<td>4.71 ± 0.94</td>
<td>171 ± 32</td>
</tr>
<tr>
<td>Day 10</td>
<td>F</td>
<td>3-4</td>
<td>18.13 ± 3.33</td>
<td>3.62 ± 0.78</td>
<td>11.3 ± 2.51</td>
<td>0.59 ± 0.21</td>
<td>1.73 ± 0.41</td>
<td>11.3 ± 2.17</td>
<td>356 ± 89</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6-8</td>
<td>20.4 ± 2.18</td>
<td>2.24 ± 0.67</td>
<td>5.35 ± 1.53</td>
<td>0.62 ± 0.20</td>
<td>1.87 ± 0.54</td>
<td>10.2 ± 2.27</td>
<td>37 ± 101</td>
</tr>
<tr>
<td>Day 28</td>
<td>F</td>
<td>3</td>
<td>22.2 ± 2.00</td>
<td>6.57 ± 0.96</td>
<td>13.4 ± 2.18</td>
<td>1.97 ± 0.50</td>
<td>4.37 ± 0.67</td>
<td>25.2 ± 4.08</td>
<td>753 ± 126</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3</td>
<td>21.9 ± 2.22</td>
<td>12.6 ± 4.23</td>
<td>3.89 ± 1.41</td>
<td>3.63 ± 1.30</td>
<td>24.7 ± 9.45</td>
<td>72 ± 253</td>
<td></td>
</tr>
<tr>
<td>Day 30</td>
<td>F</td>
<td>9</td>
<td>25.3 ± 2.37</td>
<td>6.54 ± 2.23</td>
<td>13.4 ± 2.18</td>
<td>1.97 ± 0.50</td>
<td>4.37 ± 0.67</td>
<td>25.2 ± 4.08</td>
<td>753 ± 126</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10-11</td>
<td>27.4 ± 3.70</td>
<td>14.4 ± 4.24</td>
<td>3.38 ± 1.39</td>
<td>3.63 ± 1.30</td>
<td>24.7 ± 9.45</td>
<td>72 ± 253</td>
<td></td>
</tr>
<tr>
<td>Day 42</td>
<td>F</td>
<td>4</td>
<td>28.7 ± 2.13</td>
<td>10.8 ± 2.62</td>
<td>20.1 ± 5.31</td>
<td>5.93 ± 3.04</td>
<td>6.80 ± 1.49</td>
<td>45.4 ± 12.7</td>
<td>1,450 ± 480</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4</td>
<td>29.3 ± 2.29</td>
<td>7.47 ± 1.83</td>
<td>14.4 ± 4.24</td>
<td>3.38 ± 1.39</td>
<td>3.63 ± 1.30</td>
<td>24.7 ± 9.45</td>
<td>72 ± 253</td>
</tr>
<tr>
<td>Day 28</td>
<td>F</td>
<td>15</td>
<td>35.3 ± 2.90</td>
<td>19.2 ± 3.96</td>
<td>32.9 ± 6.31</td>
<td>11.8 ± 2.78</td>
<td>11.6 ± 2.74</td>
<td>71.1 ± 18.3</td>
<td>1,538 ± 290</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>14</td>
<td>35.3 ± 2.90</td>
<td>19.2 ± 3.96</td>
<td>32.9 ± 6.31</td>
<td>11.8 ± 2.78</td>
<td>11.6 ± 2.74</td>
<td>71.1 ± 18.3</td>
<td>1,538 ± 290</td>
</tr>
<tr>
<td>3 Months</td>
<td>F</td>
<td>14</td>
<td>35.3 ± 2.90</td>
<td>19.2 ± 3.96</td>
<td>32.9 ± 6.31</td>
<td>11.8 ± 2.78</td>
<td>11.6 ± 2.74</td>
<td>71.1 ± 18.3</td>
<td>1,538 ± 290</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>14</td>
<td>35.3 ± 2.90</td>
<td>19.2 ± 3.96</td>
<td>32.9 ± 6.31</td>
<td>11.8 ± 2.78</td>
<td>11.6 ± 2.74</td>
<td>71.1 ± 18.3</td>
<td>1,538 ± 290</td>
</tr>
<tr>
<td>4 Months</td>
<td>F</td>
<td>5</td>
<td>55.9 ± 4.94</td>
<td>48.7 ± 4.50</td>
<td>73.4 ± 9.46</td>
<td>55.1 ± 17.8</td>
<td>25.3 ± 2.47</td>
<td>229 ± 35.0</td>
<td>1,160 ± 1,282</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>5</td>
<td>56.2 ± 6.32</td>
<td>53.8 ± 8.78</td>
<td>73.2 ± 6.29</td>
<td>44.8 ± 8.45</td>
<td>26.9 ± 3.85</td>
<td>202 ± 27.4</td>
<td>9,400 ± 1,731</td>
</tr>
<tr>
<td>5 Months</td>
<td>F</td>
<td>4-5</td>
<td>60.7 ± 2.56</td>
<td>61.7 ± 5.49</td>
<td>78.3 ± 6.37</td>
<td>60.2 ± 11.4</td>
<td>29.2 ± 2.63</td>
<td>240 ± 21.6</td>
<td>11,800 ± 572</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4-5</td>
<td>60.7 ± 2.56</td>
<td>61.7 ± 5.49</td>
<td>78.3 ± 6.37</td>
<td>60.2 ± 11.4</td>
<td>29.2 ± 2.63</td>
<td>240 ± 21.6</td>
<td>11,800 ± 572</td>
</tr>
</tbody>
</table>

Table 6.1. Organ and body weights in the developing Göttingen minipig. Data represent mean organ weights (in g) ± standard deviation from female and male Göttingen minipigs from late foetal until 5 months of age. Results for spleen weights may be biased by drug-induced (barbiturates, ketamine) splenomegaly or by splenic contraction due to exsanguination. Missing data: DGA: days of gestational age; BW: Body weight.
Table 6.2. Gastrointestinal weights and lengths in the developing Göttingen minipig.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Sex</th>
<th>N</th>
<th>Stomach Mean ± Standard Deviation</th>
<th>Small Int. Mean ± Standard Deviation</th>
<th>Caecum Mean ± Standard Deviation</th>
<th>Large Int. Mean ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGA 84-86</td>
<td>F</td>
<td>6-10</td>
<td>1.34 ± 0.38</td>
<td>3.77 ± 1.07</td>
<td>/</td>
<td>1.59 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6-8</td>
<td>1.36 ± 0.23</td>
<td>3.09 ± 1.11</td>
<td>/</td>
<td>1.51 ± 0.67</td>
</tr>
<tr>
<td>DGA 108</td>
<td>F</td>
<td>4</td>
<td>3.48 ± 0.71</td>
<td>10.3 ± 1.57</td>
<td>/</td>
<td>3.55 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4</td>
<td>3.40 ± 0.57</td>
<td>10.7 ± 2.84</td>
<td>/</td>
<td>4.14 ± 2.06</td>
</tr>
<tr>
<td>Day 1</td>
<td>F</td>
<td>3-4</td>
<td>4.14 ± 0.85</td>
<td>20.9 ± 4.27</td>
<td>0.53 ± 0.14</td>
<td>3.52 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3-6</td>
<td>4.04 ± 0.73</td>
<td>19.6 ± 5.37</td>
<td>0.71 ± 0.39</td>
<td>4.53 ± 0.66</td>
</tr>
<tr>
<td>Day 3</td>
<td>F</td>
<td>3-4</td>
<td>5.88 ± 1.63</td>
<td>33.8 ± 9.71</td>
<td>0.64 ± 0.19</td>
<td>5.19 ± 1.27</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3-7</td>
<td>7.61 ± 1.75</td>
<td>40.9 ± 4.84</td>
<td>0.82 ± 0.27</td>
<td>6.61 ± 0.31</td>
</tr>
<tr>
<td>Day 7</td>
<td>F</td>
<td>3-4</td>
<td>7.94 ± 2.40</td>
<td>39.2 ± 13.4</td>
<td>1.48 ± 0.47</td>
<td>9.75 ± 2.66</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6-8</td>
<td>9.13 ± 3.02</td>
<td>45.1 ± 12.3</td>
<td>1.16 ± 0.35</td>
<td>8.79 ± 2.89</td>
</tr>
<tr>
<td>Day 28</td>
<td>F</td>
<td>5</td>
<td>28.5 ± 6.48</td>
<td>106 ± 14.5</td>
<td>4.96 ± 0.86</td>
<td>28.5 ± 7.38</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>8-9</td>
<td>20.9 ± 3.73</td>
<td>93.4 ± 16.3</td>
<td>5.51 ± 2.29</td>
<td>20.4 ± 5.67</td>
</tr>
</tbody>
</table>

Data represent mean weights (in g) of the stomach, small intestine (S.I.), caecum and large intestine (L.I.) and lengths (in cm) of the small and large intestines ± standard deviation from female (F) and male (M) Göttingen minipigs as a function of age during suckling period. / missing data; DGA days of gestational age

6.4.2 Length and pH of the gastrointestinal tract

The lengths of the small and large intestines (in cm) are listed in Table 6.2. The pH values are presented in Table 6.3. Statistically significant differences in pH were detected among the three small intestinal regions at Day 3 ($p = 0.001$) and Day 7 ($p = 0.003$) with increasing pH along the small intestinal tract, but not at Day 1 ($p = 0.78$) and Day 28 ($p = 0.20$). Only in 7-day-old animals, a weakly significant difference was noted between the ileum, caecum and colon ($p = 0.044$). No significant differences were found between the caecum and colon ($p > 0.362$ for all age groups). Gastric pH differed among postnatal age groups, with a significantly different and more acidic pH at Day 28 compared to Day 1 ($p = 0.002$) and Day 7 ($p = 0.001$). The intestinal pH values remained at a more alkaline level compared to the gastric pH during the first month of life. No significant differences were found among the observed postnatal age groups for the proximal, middle or distal part of the small intestine and for the colon ($p ≥ 0.05$). For the pH in the caecum, a statistically significant difference was present among the age...
groups \((p = 0.023)\) with a significantly lower pH at Day 1 compared to Day 28 \((p = 0.005)\).

Table 6.3. Gastrointestinal pH values in the developing Göttingen minipig.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Sex</th>
<th>N</th>
<th>Stomach</th>
<th>S.I. Zone 1</th>
<th>S.I. Zone 2</th>
<th>S.I. Zone 3</th>
<th>Caecum</th>
<th>L.I. Zone 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGA 84-86</td>
<td>F</td>
<td>10</td>
<td>7.7 ± 0.5</td>
<td>7.5 ± 0.5</td>
<td>7.4 ± 0.5</td>
<td>7.8 ± 0.6</td>
<td>8.0 ± 0.5</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>8</td>
<td>7.1 ± 0.4</td>
<td>7.5 ± 0.5</td>
<td>7.1 ± 0.6</td>
<td>7.4 ± 0.5</td>
<td>7.8 ± 0.5</td>
<td>7.6 ± 0.5</td>
</tr>
<tr>
<td>DGA 84-86</td>
<td>F</td>
<td>3-4</td>
<td>5.5 ± 1.3</td>
<td>7.0 ± 0.0</td>
<td>7.4 ± 0.5</td>
<td>7.8 ± 0.6</td>
<td>7.7 ± 0.6</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1-4</td>
<td>6.5 ± 1.0</td>
<td>6.7 ± 0.6</td>
<td>7.1 ± 0.6</td>
<td>7.4 ± 0.5</td>
<td>8.0 ± 0.0</td>
<td>6.7 ± 1.2</td>
</tr>
<tr>
<td>Day 1</td>
<td>F</td>
<td>4</td>
<td>5.3 ± 0.5</td>
<td>6.3 ± 0.5</td>
<td>6.5 ± 0.6</td>
<td>6.0 ± 0.0</td>
<td>6.8 ± 0.5</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6</td>
<td>4.8 ± 0.8</td>
<td>6.5 ± 0.8</td>
<td>6.5 ± 0.8</td>
<td>7.2 ± 1.0</td>
<td>7.5 ± 0.6</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>Day 3</td>
<td>F</td>
<td>4</td>
<td>4.3 ± 0.5</td>
<td>5.8 ± 0.5</td>
<td>6.3 ± 0.5</td>
<td>7.3 ± 0.9</td>
<td>7.3 ± 0.5</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6</td>
<td>5.2 ± 1.2</td>
<td>5.8 ± 0.4</td>
<td>6.0 ± 0.0</td>
<td>6.8 ± 0.8</td>
<td>6.5 ± 0.8</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>Day 7</td>
<td>F</td>
<td>3-4</td>
<td>5.0 ± 0.0</td>
<td>5.8 ± 0.5</td>
<td>6.0 ± 0.0</td>
<td>7.3 ± 0.9</td>
<td>6.0 ± 0.0</td>
<td>6.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6-7</td>
<td>5.0 ± 0.6</td>
<td>6.1 ± 0.4</td>
<td>6.0 ± 0.6</td>
<td>7.1 ± 0.9</td>
<td>6.6 ± 0.8</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td>Day 28</td>
<td>F</td>
<td>5</td>
<td>3.8 ± 0.8</td>
<td>5.8 ± 0.5</td>
<td>5.8 ± 0.5</td>
<td>6.4 ± 0.6</td>
<td>6.4 ± 0.5</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>8</td>
<td>3.6 ± 1.2</td>
<td>6.1 ± 0.6</td>
<td>6.0 ± 0.0</td>
<td>6.1 ± 0.4</td>
<td>6.4 ± 0.5</td>
<td>6.3 ± 0.5</td>
</tr>
</tbody>
</table>

Data represent mean pH values ± standard deviation at the gastrointestinal wall from female (F) and male (M) Göttingen minipigs as a function of age during suckling period. Zones 1, 2 and 3 represent the proximal, middle and distal zone of the intestine, respectively. DGA days of gestational age

### 6.4.3 Body weight and organ weight prediction

The increase in body weight as a function of age in days was best described by a linear growth curve in male and female animals (Table 6.4 and Figure 6.1). The best-fit equations describing the body weight-dependent changes for the brain, heart, lungs, spleen, kidney, liver, stomach, small intestine and large intestine in female and male developing Göttingen minipigs are shown in Table 6.4. The body weights used to generate these equations ranged from 200 g to 13,000 g and from 300 g to 12,400 g for females and males, respectively, and covered the first 5 postnatal months for brain, heart, lungs, kidney, spleen and liver. For stomach, small intestine and large intestine,
the body weights ranged from 301 to 3,821 g and from 378 to 3,636 g for females and males, respectively, and covered the first month of life. (Table 6.4)

Table 6.4. Best-fit equations to predict organ weight as a function of body weight and body weight as a function of age in the developing Göttingen minipig.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sex</th>
<th>Equations to predict organ and body weight in g (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>F+M</td>
<td>$Y = 17.10 + 0.008051a - 5.585e^{-007}a^2 + 1.469e^{-011}$</td>
</tr>
<tr>
<td>Heart</td>
<td>F</td>
<td>$Y = 0.3466 + 0.008592a - 8.089e^{-007}a^2 + 3.730e^{-011}a^3$</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>$Y = 0.3543 + 0.008637a - 7.525e^{-007}a^2 + 4.057e^{-011}a^3$</td>
</tr>
<tr>
<td>Lungs</td>
<td>F+M</td>
<td>$Y = -1.092 + 0.02029a - 3.857e^{-006}a^2 + 3.803e^{-010}a^3 - 1.287e^{-014}a^4$</td>
</tr>
<tr>
<td>Spleen</td>
<td>F+M</td>
<td>$Y = -1.292 + 0.005224a$</td>
</tr>
<tr>
<td>Kidney</td>
<td>F</td>
<td>$Y = 0.1025 + 0.005256a - 5.127e^{-007}a^2 + 2.095e^{-011}a^3$</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>$Y = -0.4873 + 0.006445a - 1.062e^{-006}a^2 + 1.075e^{-010}a^3 - 3.774e^{-015}a^4$</td>
</tr>
<tr>
<td>Liver</td>
<td>F+M</td>
<td>$Y = -4.332 + 0.04251a - 7.151e^{-006}a^2 + 8.395e^{-010}a^3 - 3.328e^{-014}a^4$</td>
</tr>
<tr>
<td>Stomach</td>
<td>F+M</td>
<td>$Y = 0.4527 + 0.008595a$</td>
</tr>
<tr>
<td>S.I.</td>
<td>F+M</td>
<td>$Y = -1.037 + 0.05446a - 6.518e^{-006}a^2$</td>
</tr>
<tr>
<td>L.I.</td>
<td>F</td>
<td>$Y = -0.216 + 0.009642a$</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>$Y = 1.417 + 0.007046a$</td>
</tr>
<tr>
<td>Total BW</td>
<td>F+M</td>
<td>$Y = 343.9 + 77.18t$</td>
</tr>
</tbody>
</table>

*Equations cover body weights ranging from 200 to 13,000 g or first 5 months of life

*Equations cover body weights ranging from 300 to 3,800 g

*Prediction of spleen weight may be biased by drug-induced (barbiturates, ketamine) splenomegaly or by splenic contraction due to exsanguination.

*a body weight (in g); t age in days; e mathematical constant, Euler’s number; F females; M males; BW body weight

Best-fit lines, as described by the best-fit equations, and their 95% confidence interval are presented for the brain, lungs, spleen, liver, heart and kidney in Figure 6.2, and for the stomach, and small and large intestines in Figure 6.3. Residuals for spleen were not normally distributed. Among the female heart, male heart, and spleen weights, 1, 3 and 6 outliers, respectively, were detected and were excluded to create the equation.
For each best-fit equation, the observed versus predicted data (and vice versa) were plotted in a linear regression model (graphs not shown). Evaluation of these linear regressions resulted in a very good fit (mean R² ± S.D.: 0.99 ± 0.002), a very low proportional bias (mean slope ± S.D.: 1 ± 0.0003) and a very low systematic bias (Y-intercept ≤ 0.0657) for all the best-fit equations, validating these models.

Figure 6.1. Growth curve of the Göttingen minipig up to 5 months of age. Blue dots and red triangles represent body weights (in g) at different postnatal ages in male and female Göttingen minipigs, respectively. The solid line represents the best-fit line obtained by the best-fit equation with their 95% confidence intervals (dashed line).
Figure 6.2. Organ weights as a function of body weight in the developing Göttingen minipig until 5 months of age. The blue dots and red triangles represent the observed organ weights (in g) in male and female animals, respectively, for brain, lungs, spleen, liver, heart and kidney. The empty dots represent excluded outliers (by ROUT-method). The solid line represents the best-fit line obtained by the best-fit equation with their 95% confidence intervals (dashed line).
Figure 6.3. Gastrointestinal weights as a function of body weight in the developing Götingen minipig until 28 days of age. The blue dots and red triangles represent the observed organ weights (in g) in male and female animals, respectively, for stomach, small intestine and large intestine. The solid line represents the best-fit line obtained by the best-fit equation with their 95% confidence intervals (dashed line).

6.4.4 Relative organ weights

Organ weights as a percentage of body weight are plotted to visualize age-related changes in the relative size of each organ. (Figure 6.4) Relative intestinal weights and relative intestinal lengths, expressed as length in cm per 100g body weight, are shown in Figure 6.5.

Except for the spleen and the gastrointestinal tract, the highest relative organ weights were noted during the first week of life. One-day-old piglets showed a very high relative brain weight (5%), which exponentially decreased immediately after birth to level off at 0.5% of the body weight from 3 months of age onwards. The highest relative organ weights for the heart, lungs, kidney and liver were present during the first week of life. Later, a decrease with increasing age was present, which levelled off from 3 months of age onwards. In contrast, the relative spleen weight was very low in Day 1 piglets, but
had been raised 2.6-fold during the first week of life to remain at this level. The relative organ weights for the stomach, small intestine and large intestine varied between 0.85 and 0.99%, 3.43 and 5.42%, and 0.82 and 0.96%, respectively, during the first month of life, suggesting no clear age-related trend. In contrast, the relative intestinal lengths were clearly decreasing during this period, indicating a higher increase in intestinal weight compared to intestinal length.

Figure 6.4. Relative organ weights during development of the Göttingen minipig. Graphs show the age-related changes in organ weight as a percentage of body weight for the brain, heart, lungs, spleen, kidney and liver. Blue dots and error bars represent the mean value ± standard deviation for each age group. The dashed line represents the age-related trend during the first 5 months of life. BW body weight
Figure 6.5. Relative intestinal weights and lengths during the first month of life of the Göttingen minipig. Graphs on the left show the age-related changes in relative weight of small intestine and large intestine. Graphs on the right show the age-related changes in relative length of small intestine and large intestine in cm per 100 g body weight. Blue dots and error bars represent the mean value ± standard deviation for each age group. The dashed line represents the age-related trend. BW body weight.

### 6.4.5 Preliminary validation of the models

The differences in predicted and observed organ weights as a percentage of the observed organ weights (http://minipigs.dk/the-goettingen-minipig/background-data/) are presented in Table 6.5, with body weight as a dependent variable. For brain and lungs, the percentage over- or under-prediction was below 10% for both age groups. For kidney and liver, the percentage over- or under-prediction was below 17 and 13%, respectively, for both age groups. For the heart, there was an over-prediction of about 20% for both age groups. Overestimation of predicted spleen weight was clearly present for both age groups, varying between 139 and 264%.
Table 6.5. Differences in predicted (as a function of body weight) and observed organ weights as a percentage of the observed organ weight.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Sex</th>
<th>N</th>
<th>Mean Body Weight (in g)*</th>
<th>Observed Data</th>
<th>Over- or underestimation of predicted organ weights as a percentage of observed organ weights</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>23</td>
<td>4,300</td>
<td></td>
<td>Brain: -1.87, Heart: +18.6, Lungs: +1.11, Spleen: +153, Kidney: +8.86, Liver: -3.86</td>
</tr>
</tbody>
</table>

* data from http://minipigs.dk/the-goettingen-minipig/background-data/

### 6.5 Discussion

The aim of this study was to provide morphometric data of particular organs and gastrointestinal pH values in the developing Göttingen minipig. Furthermore, equations were generated to predict organ weight as a function of body weight and body weight as a function of age.

These data may be useful for integration in PBPK models, in the translation of pharmacokinetic data from the juvenile minipig to the human infant, and in pharmaceutical research using the minipig as an animal model. However, some attention should be paid to possible limitations when the presented data are used in biomedical research. The created equations were based on a limited range of not fully-grown organ and body weights. Hence, they do not reflect the entire development till adulthood, limiting the applicability of the equations to the used range of body weights. Organ weights were determined after exsanguination, while the recorded body weights included the weight of the blood. Estimation of lost and residual blood volume was not performed since collection of the presented data was part of other research goals than PBPK modelling, but would be recommended in future experiments. However, organ- and age-dependent factors are known to complicate the estimation of residual blood volumes. At necropsy in rats, Kanerva et al. detected 23 and 15% lower liver and kidney weights, respectively, in an exsanguinated group compared to a non-exsanguinated group (20). In contrast, no significant differences in absolute weights of heart, spleen...
and brain were observed between both groups (20). Linderkamp et al. reported a tendency to reduction of blood volumes per g of tissue with increasing age in 1-day-, 7-day- and 14-day-old piglets, especially for the liver, lungs and skeleton, while the blood volume in the great vessels outside the organs increased during this time period (21). Most organs did not follow a linear growth curve during the first five months of life and relative organ weights varied over time. In general, the relative organ weights were the highest during the first week of life, while the major increases in absolute organ weights were observed during the first 3 to 4 months of life. In contrast to the other organs, the spleen showed a low relative organ weight in neonates, which strongly increased during the first month of life. The absolute spleen weight followed a linear increase as a function of body weight during the first five months of life. However, the observed spleen weights, and consequently the equation to predict spleen weight from body weight, should be interpreted with caution because the spleen is a highly vascular organ and its size is largely dependent on its distension (22). Barbiturates and ketamine, used as anaesthetics in this study, can result in congestive splenomegaly, which is mainly described in dogs. This sequestration of blood is probably due to smooth muscle relaxation in the splenic capsule, following administration of these anaesthetics (23, 24). Indeed, the preliminary validation of the equations with external data from 2- and 6-month-old animals showed a large overestimation of the spleen weight. Exsanguination, on the other hand, will result in a contraction of the spleen in an attempt to compensate for the loss of blood and lack of oxygen (25). Given the aforementioned interfering factors, it is difficult to quantify the possible discrepancy between the pre-mortem and post-mortem spleen weights. Nevertheless, a linear relationship between spleen volume and body weight was also found in children aged between 1 day and 18 years (26).

Not only the juvenile Göttingen minipig, but also the human infant possesses relatively large organs. In human beings, pronounced increases in the organ weights occur in the first years of their life. The largest increase in human brain weight occurs during the first 3 years of life with the highest relative brain weight in neonates (27, 28) while the largest increase in kidney volume already takes place in the first year of life (29). In a study with 5,036 children, the median relative liver weight was 3.5% between 0 and 2 years of age, which had decreased to 2.2% for young adults of 18 years and older (30). Noda et al. reported a similar age-related trend for the liver (31). As very young individuals have relatively large organs, a body weight normalized clearance of drugs
could be expected to be fast. However, the functional immaturity of metabolizing and excreting organs, including Phase I- and Phase II- enzymes and drug transporters, and their absolutely smaller organ size compared to adults, often result in higher plasma concentrations of parent compound and potential toxicity (4, 5, 15). This statement is further supported by our recent observation of the postnatal increase in abundance and activity of CYP3A, one of the most abundant and important Phase I- enzymes, in the liver of the developing Göttingen minipig (19).

During the first 28 days of life, no clear age-related trend in the relative weight of the different intestinal segments was noted despite a clear decrease in relative intestinal length, indicating a higher increase in intestinal weight compared to intestinal length. Nevertheless, lengths of the small intestines at Day 1 and Day 28, recorded in our study were already about 27 and 60%, respectively, of the lengths that were measured in 6-month-old Göttingen minipigs by Suenderhauf and Parrott (3). This suggests an accelerated development of the small intestine during the first month of life compared to some other organ systems.

In the examined piglets (not fasted), the gastric pH was more acidic at 28 days of age compared to the younger animals. Snoeck et al. have also described this age-related decreasing trend in gastric pH in domestic piglets, when observing age groups at different time points around weaning (32). For suckling piglets (17 days of age) and at weaning (24 days of age), they found no gastric pH values below 2.5, but at 1 and 2 weeks post weaning, they detected pH values as low as 1.6-1.7 (32). Suenderhauf and Parrott compiled gastrointestinal pH values from 5 different studies (animals aged from 6 months to 2 years) and reported highly variable gastric pH values, ranging from 1.15 to 6, indicating the influence of feeding status and anatomical region (3). Also in human beings, the trend of a more alkaline gastric pH in very young children is present with adult levels of gastric acidity by 6 months till 2 years of age (6). The maturational change in gastric pH is likely a factor in the oral absorption of drugs in paediatric populations. Greater bioavailability in neonates than in older infants and children has been reported for acid-labile compounds such as penicillin G (5). Changes in the intraluminal pH in the different segments of the gastrointestinal tract affect the degree of ionisation of weak acids and weak bases, thus influencing both the amount of drug ionised available for intraluminal solubilisation and the amount unionised drug available for passive diffusion through the mucosa of the
The presence of milk in the stomach may also influence the absorption of lipid soluble drugs, and delays in gastric emptying time after feeding may explain slower oral absorption of drugs (5,6). The pH values that we determined at weaning age in different segments of the intestine, were in agreement with values reported by Snoeck et al (32). As described in human beings, the intestinal pH values in our study were also more alkaline than the gastric pH values (33). Although the gastrointestinal pH values were determined in dead animals, time lapse between euthanasia and sampling was limited to a maximum of 30 minutes. Hence, we believe that the post-mortem gastro-intestinal pH values reflect the in vivo situation.

Göttingen minipigs reach their adult body weight around two years of age (35-45 kg) and show a nearly linear increase in body weight during the first 5 months of life (3, 11, 34). During the first year of life, they gain approximately 2 kg per month, assuming that they are fed a restriction diet to prevent obesity (3). The 4-month-old female animals, included in our study, however, showed relatively high body weights compared to the observed body weights at 5 months of age and they were 2 kg heavier compared to the mean 4-month-old body female weight of 9.18 kg, reported in the Growth Data Base from Ellegaard Göttingen Minipig A/S (http://minipigs.dk/the-gottingen-minipig/background-data/). This may have misleadingly suggested that the increase in body weight is already slowing down after 4 months of age. Nevertheless, the evolution of body weight as a function of age was best described by a linear growth curve.

In conclusion, this paper provides organ and body weights, intestinal lengths and pH-values of the gastrointestinal wall in the developing Göttingen minipig, which may be of use in the construction of a PBPK model. Based on the compiled data, we have developed best-fit equations to predict brain, heart, lungs, kidney, liver, stomach, small intestine and large intestine weights as a function of body weight and body weight as a function of age.
6.6 References


7 General discussion

In this doctoral thesis, several factors that influence ADME of drugs were evaluated in the developing Göttingen minipig. This knowledge is valuable in order to determine the potential of the juvenile minipig in a preclinical context, and more specifically, as a translational model for human paediatric age groups. To evaluate this, the following questions were addressed:

1. Can we localise CYP3A7-, CYP3A4-, Pgp- and UGT1A-orthologues in the Göttingen minipig with antibodies directed against the human protein? Furthermore, are the localisation and developmental expression pattern of CYP3A7-, CYP3A4-, Pgp- and UGT1A-orthologues consistent with human data?

2. Are five human probe substrates for CYP1A2, CYP2C9, CYP2D6, CYP3A4 and UGT metabolized by the liver of the developing Göttingen minipig? If so, does this occur during the same developmental window as in man?

3. Is there a shift from a CYP3A7-like into a CYP3A4-like enzyme present in the Göttingen minipig around the time of birth, as has been shown in man?

4. How do gastrointestinal pH values, organ weights and body weights of the Göttingen minipig during development relate to human data?

5. How do the Göttingen minipig data on in vitro drug metabolism relate to other non-rodent and rodent species?
7.1 Localisation and developmental expression pattern of CYP3A7-, CYP3A4-, Pgp- and UGT1A-orthologues in the Göttingen minipig

All antibodies that were directed against human proteins also showed immunoreactivity in the small intestine and liver from the Göttingen minipig. The high percentages of identity in amino acid sequences of these porcine proteins compared with man explain for the observed cross-reactivity (1, 2). Except for CYP3A7, all detected proteins displayed similar hepatic and intestinal localisations and developmental patterns as in man (see Table 7.1), adding to the idea that minipig orthologues were detected (3-10). Indeed, CYP3A and UGT1A showed a clear postnatal expression in both the liver and the small intestine that increased with age. The appearance of a centrilobular expression pattern in the liver for CYP3A and UGT1A with age corresponds to what is described in man (6, 10-12).

The anti-CYP3A7 antibody showed weak immunoreactivity in the late foetal and neonatal liver samples, contrasting with a homogenous high immunoreactivity in man (13). Moreover, the immunoreactivities were similar to these obtained with the anti-CYP3A4 antibody, suggesting the detection of the same and most probably the ‘adult’ CYP3A4-like protein instead of a ‘foetal’ CYP3A7-like protein. Given the 88% identity in amino acid sequences of CYP3A4 and CYP3A7, reactivity of both antibodies with the same protein seems realistic. Consequently, this finding may suggest the absence of a CYP3A7-like protein in the foetal and neonatal minipig.

The investigation of Pgp was limited to its immunohistochemical localisation in this thesis. At 75% of gestation, Pgp appeared fully expressed in the liver, while this onset was later and more limited in the small intestine, as in man (5). An organ-dependent developmental expression pattern has also been reported in other species (14-16).
Table 7.1. Compilation of immunohistochemical results for Pgp and CYP3A(4) in the small intestine (S.I.) and Pgp, CYP3A(4) and UGT1A in the liver.

<table>
<thead>
<tr>
<th>Localisation of the protein</th>
<th>Human being</th>
<th>Göttingen minipig</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pgp in S.I.</strong></td>
<td>Brushborder of enterocytes villus (3, 19)</td>
<td>Brushborder of enterocytes villus</td>
</tr>
<tr>
<td><strong>Pgp in liver</strong></td>
<td>Biliary canalicular side of hepatocytes (3, 19)</td>
<td>Biliary canalicular side of hepatocytes</td>
</tr>
<tr>
<td><strong>CYP3A(4) in S.I.</strong></td>
<td>Cytoplasm of enterocytes villus (8)</td>
<td>Cytoplasm of enterocytes villus</td>
</tr>
<tr>
<td><strong>CYP3A(4) in liver</strong></td>
<td>Cytoplasm of hepatocytes (6, 10)</td>
<td>Cytoplasm of hepatocytes</td>
</tr>
<tr>
<td><strong>UGT1A in liver</strong></td>
<td>Cytoplasm of hepatocytes (11)</td>
<td>Cytoplasm of hepatocytes</td>
</tr>
</tbody>
</table>

**Developmental expression**

<table>
<thead>
<tr>
<th>Human being</th>
<th>Göttingen minipig</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pgp in S.I.</strong></td>
<td>No or weak staining at 11-20 weeks of gestation (5)</td>
</tr>
<tr>
<td><strong>Pgp in liver</strong></td>
<td>Clearly present in bile canaliculi from 14 weeks of gestation onwards</td>
</tr>
<tr>
<td><strong>CYP3A(4) in S.I.</strong></td>
<td>Present in half of paediatric samples between 1 and 6 months of age</td>
</tr>
<tr>
<td><strong>CYP3A(4) in liver</strong></td>
<td>Appearance of a centrilobular and midzonal pattern during postnatal development (12)</td>
</tr>
<tr>
<td><strong>UGT1A in liver</strong></td>
<td>Presence of diffuse staining with higher immunoreactivity in the centrilobular region in adult liver (11)</td>
</tr>
</tbody>
</table>
Little research has been done whether at these early stages Pgp is already fully active. *In vitro* research on the ontogeny of the blood-brain barrier in the guinea pig has shown low Pgp levels in capillary vessels of the brain at 59 and 77% of gestation, with a remarkable up-regulation of Pgp expression from 92% of gestation onwards, which continued to rise further postnatally. The endothelial cells of the brain were more sensitive to induction of Pgp activity by corticosteroids with increasing gestational age, which suggests that the increase of Pgp expression is reflected by an increase in Pgp activity near term, while at 59% of gestation the brain endothelial cells were not yet responsive to induction of activity (17). Additionally, in mouse brain, Pgp expression increased with gestational age, with a simultaneous increase of its efflux potential, represented by a decrease in digoxin accumulation in the foetal brain (18). Hence, it is likely that the substantial hepatic Pgp presence in late foetal stage also results in efflux capacity.

The up-regulation of Pgp in the foetal organs near term is considered an adaptive and protective function of the foetus to a decrease in maternal Pgp in the placenta. This event is orchestrated by increasing cortisol levels near term in the maternal and foetal blood, but can also be induced by synthetic glucocorticoids (17, 18). Hence, it seems likely that abundant hepatic Pgp expression in late foetal stage also results in efflux capacity.

Based upon the above immunohistochemical evaluation, the juvenile minipig seems a valuable translational model. However, these data do not allow concluding on whether the expressed proteins are also functionally active. This has been addressed, in part, in the following research question.
7.2 Metabolism of five human probe substrates for CYP1A2, CYP2C9, CYP2D6, CYP3A4 and UGT in the developing Göttingen minipig liver

In children, the first 1-2 years of life are considered a period with major differences in the ADME of administered drugs (20, 21). Consequently, it seems interesting to compare and link the first years of life in children with our data in the developing minipig. Using activity levels as a percentage of adult activities rather than absolute activities seems a good basis to compare both species and to get more insight in the translation of age groups. (Figure 7.1 and Figures 7.1a,b,c and d)

These timelines clearly show that the different substrates have different ontogenetic profiles in the Göttingen minipig. This is not surprising as human CYP450 enzymes show distinct developmental profiles too (21-24). Nevertheless, in general, Göttingen minipig liver microsomes were able to metabolize five human probe substrates, and showed the formation of the main metabolite similar as in man. For all substrates, metabolism increased with postnatal age. Phenacetin O-deethylation and dextromethorphan O-demethylation matured fast and achieved more than 60% of the adult levels in 28-day-old animals. In contrast, tolbutamide 4- and midazolam 1-hydroxylations rather slowly matured in the minipig, with levels below 45% of adult activities at 28 days of age.

During the first week of life in the Göttingen minipig, relative CYP1A2- and CYP2D6-like activity levels are reached that are comparable with these at or after the first year of life in a child (25, 26, 28). For CYP2C9- and CYP3A4-like activities, relative activities at one year of age in man relate to one month of age in the Göttingen minipig (27, 29).
Figure 7.1. Timelines for the ontogeny of metabolizing capacities as a percentage of adult activities in the liver of the Göttingen minipig (pink boxes) and man (blue boxes). For the Göttingen minipig, late foetal (starting point), Day 1, Day 3, Day 7, Day 28 and adult animals were included. For the human population, ages differed among studies. ((25, 26) for CYP1A2; (27) for CYP2C9; (28) for CYP2D6 and (29) for CYP3A4). Common relative activities are noted in the middle of the timelines, while those only linked to minipigs or man are in the upper and lower zone of the timelines, respectively.
Figure 7.1a. Graph of CYP1A2-like activity in man and minipig, at different ages. Bars represent activities as a percentage of adult activity. Pink bars represent minipig data, while blue bars represent human data 25,26).
Figure 7.1b. Graph of CYP2C9-like activity in man and minipig, at different ages. Bars represent activities as a percentage of adult activity. Pink bars represent minipig data, while blue bars represent human data (27).
Figure 7.1c. Graph of CYP2D6-like activity in man and minipig, at different ages. Bars represent activities as a percentage of adult activity. Pink bars represent minipig data, while blue bars represent human data (28,30).
Figure 7.1d. Graph of CYP3A4-like activity in man and minipig, at different ages. Bars represent activities as a percentage of adult activity. Pink bars represent minipig data, while blue bars represent human data (29).
However, translating these results from minipigs to man is not that straightforward and needs careful consideration.

First, the number of *in vitro* and *in vivo* studies in the paediatric population is low, resulting in limited reference data. Additionally, substrates, incubation conditions and determination of age groups often differ between studies. Indeed, due to ethical and practical reasons, research in children and availability of paediatric tissues are scarce. CYP2D6 is an example that nicely demonstrates that even in man the ontogeny of CYP450 enzymes remains to be elucidated and that controversy still exists, as explained in Chapter 5. Even when the same probe reaction (dextromethorphan O-demethylation) was used in paediatric liver microsomes, one study showed very low activity in foetuses and neonates and only 25% of adult activity in 5-year-olds, while activity was already clearly detectable at late gestation in another study, with no clear age-related differences beyond the first week of life (28, 30). Furthermore, *in vivo* studies are neither conclusive for the ontogeny of CYP2D6 (31-36). These *in vivo* studies also encounter the difficulty to study the contribution of a certain CYP450 enzyme in the biotransformation of a compound during development. Indeed, not only the maturation of a certain CYP enzyme, but also other factors like functional maturation of liver and kidney, differences in body composition, changes in distribution volumes are involved in the ADME of a drug. Additionally, polymorphic gene expression results in high variability in metabolizing capacity and may mask age-related differences, both in *in vitro* and *in vivo* studies (30). In short, this example on the ontogeny of human CYP2D6 shows that even in man uncertainties remain on the exact maturation of drug metabolism, which impedes the creation of translational timelines between species. Bearing this in mind, it is challenging to translate our activity found in the minipig into paediatric data. However, our results show that the first month of life is a critical period for the ontogeny of drug metabolism in the Göttingen minipig.

A second issue is substrate specificity of CYP450 orthologues as this may differ among species. A probe substrate in one species is not necessarily a suitable marker for its CYP450 orthologue in another species (37, 38). The human CYP450 probe substrates in our study have been evaluated previously in porcine liver microsomes or in recombinant porcine CYP450 enzymes. For phenacetin, tolbutamide and midazolam, it is accepted that these compounds are metabolized by the porcine orthologues of the
intended human CYP450 enzymes (39-45). However, uncertainty remains for
dextromethorphan and probably both CYP2B and CYP2D are involved in pigs (46-48).
Ultimately, the incubation of individual recombinant Göttingen minipig CYP450
isoforms with the probe substrate of interest is the only method that generates definite
conclusions on their substrate specificities. The manufacture of these porcine
recombinant CYP450 enzymes, which are not commercially available, is currently
further explored in our research group.

A third point to consider, is that the effect of inhibitors and inducers may differ among
species even when substrate specificity is similar (38, 49).
Indeed, similarity of substrate specificity is a prerequisite for a good translational
model, but similar effects of human inducers and inhibitors are important as well to
predict drug-drug interactions in man, since many clinically used drugs act as an
inducer or inhibitor for CYP450 (37, 38). The induction of CYP450 enzymes implicates
generally the production of new proteins (50, 51). These pathways of induction involve
ligand-activated transcription factors like the constitutive androstane receptor (CAR),
the pregnane X-receptor (PXR) and the aryl hydrocarbon receptor (AhR). The binding
of a ligand, which can be structurally diverse compounds, to the ligand-binding domain
of the receptor results first in a conformational change, which allows the receptor to
interact with accessory proteins to induce transcriptional gene activation (52, 53).
CYP2B, CYP2C and CYP3A share similar inducing ligands in man and in pigs, and are
regulated via the PXR and CAR (1, 54-56). The ligand binding domains of human and
porcine CAR and PXR are highly similar, explaining for their many similarities in
inducing ligands (57, 58). In contrast, CYP1A2 is induced mainly via the AhR, although
induction via PXR and CAR has been described too (54). The human and porcine
(NP_001289955.1 in NCBI) AhR share 81% identity in amino acid sequences, but has
been studied less in the pig compared with PXR and CAR.
An overview of the potency of human CYP450 inducers can be found in Table 7.2.
Table 7.2 shows us that several human CYP450 inducers are capable to induce their
human marker activities in pigs too, although absence of induction has been reported
too. For example, phenacetin O-deethylation showed only a limited increase by a
cocktail of β-naphtoflavone, phenobarbital and dexamethasone, while CYP1A2 protein
and other CYP1A2 activities appeared clearly inducible (49, 59-61). Results are
confusing for the induction of CYP2C9-like activity. Myers et al. could not induce tolbutamide 4-, S-mephenytoin 4- and diclofenac hydroxylation with their cocktail of inducers, while Puccinelli et al. showed increase of all activities with phenobarbital (61, 63). Midazolam 1-hydroxylation was not induced by dextromethorphan, but was by rifampicin (64). CYP2D6 is considered insensitive to inducers in man. However, the cocktail of inducers increased propranolol hydroxylation and dextromethorphan O-demethylation, but not bufuralol 4-hydroxylation in pigs, while these reactions are performed by CYP2D6 in man (61). These data may further corroborate the assumption that dextromethorphan O-demethylation is not/or not entirely performed by CYP2D in the pig.

Table 7.2. The potency of human CYP450 inducers in the porcine species.

<table>
<thead>
<tr>
<th>Potency human CYP450 inducers in pig</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CYP1A2</strong></td>
<td></td>
</tr>
<tr>
<td>β-NTF</td>
<td>Increase in EROD and caffeine 3-demethylation in domestic pig (59)</td>
</tr>
<tr>
<td>OMP</td>
<td>Increase in CYP1A2 protein in domestic pig (60)</td>
</tr>
<tr>
<td>β-NTF+PB+DEX</td>
<td>Increase in EROD in Yucatan minipig (49)</td>
</tr>
<tr>
<td><strong>CYP2B</strong></td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>Increase in pentoxyresorufin-deethylation in domestic pig (62)</td>
</tr>
</tbody>
</table>
| RIF                                 | Increase in 7-ethoxy-4-(trifluoromethyl)coumarin dealkylation, 9-
|                                     | anthraldehyde oxidation and S-mephenytoin N-demethylation in domestic pig (63) |
| β-NTF+PB+DEX                        | Increase in 9-Anthraldehyde oxidation in domestic pig (55,56) |
|                                     | Increase in benzyloxyresorufin-O-debenzylation in domestic pig (55) |
|                                     | Increase in 7-ethoxy 4-(trifluoromethyl) coumarin deethylation in domestic pig (55) |
| β-NTF+PB+DEX                        | Increase in CYP2B protein+Increase in 7-methoxy-4-methyl-coumarin and 7-benzyloxyresorufin metabolism (61) |
| **CYP2C9**                          |     |
| β-NTF+PB+DEX                        | No clear increase in TOL 4-OH, DIC 4-OH, and MEP 4-OH (no activity) in domestic pigs (61) |
| PB                                  | Increase in 7-methoxy 4-(trifluoromethyl)coumarin demethylation, TOL 4-OH, paclitaxel hydroxylation and MEP 4-OH, DIC 4-OH in domestic pig (63) |
| **CYP2D6**                          |     |
| β-NTF+PB+DEX                        | Considered to be non-inducible in man (54) |
|                                     | No apparent increase in CYP2D protein |
|                                     | Increase in propranolol hydroxylation and dextromethorphan O-
|                                     | demethylation, but not in bufuralol 4-hydroxylation (all typical CYP2D6 substrates in man) (61) |
Potency human CYP450 inducers in pig

Table 7.3. The potency of human CYP450 inhibitors in the porcine species.

<table>
<thead>
<tr>
<th>Potency of human CYP450 inhibitors in pig</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CYP1A2</strong></td>
<td></td>
</tr>
<tr>
<td>Furafylline</td>
<td>No inhibition of phenacetin O-deethylation in Bama minipig liver microsomes</td>
</tr>
<tr>
<td>α-naphthoflavone</td>
<td>Inhibition of ethoxyresorufin O-deethylation</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>Inhibition of ethoxyresorufin O-deethylation</td>
</tr>
<tr>
<td><strong>CYP2B</strong></td>
<td></td>
</tr>
<tr>
<td>Orphenadrine</td>
<td>Inhibition of dextromethorphan O-demethylation (indication for CYP2B activity) in Göttingen minipigs</td>
</tr>
<tr>
<td><strong>CYP2C9</strong></td>
<td></td>
</tr>
<tr>
<td>Quercetin (inhibitor of human CYP2C8)</td>
<td>Inhibition of tolbutamide 4'-hydroxylation and S-mephénytoin 4'-demethylation in domestic pigs</td>
</tr>
<tr>
<td>Sulphaphenazole</td>
<td>No inhibition of diclofenac 4'-hydroxylation in domestic pigs</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>No inhibition of tolbutamide 4'-hydroxylation and S-mephénytoin 4'-demethylation and hydroxylation and diclofenac hydroxylation in domestic pigs</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Potency of human CYP450 inhibitors in pig</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Quinidine</td>
<td><strong>No inhibition</strong> of dextromethorphan O-demethylation in Bama minipig liver microsomes</td>
</tr>
<tr>
<td>Quinine and quinidine</td>
<td>Inhibition of dextromethorphan O-demethylation in domestic pig</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CYP3A4/5</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>Inhibition of nifedipine oxidation and testosterone 6β-hydroxylation in Bama minipig liver microsomes</td>
<td>(39)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of verapamil metabolism in domestic pig</td>
<td>(70)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of Luciferin-IPA metabolism in Göttingen minipig liver microsomes</td>
<td>(67)</td>
</tr>
<tr>
<td>CYP3cide</td>
<td>Inhibition of nifedipine oxidation and testosterone 6β-hydroxylation and midazolam 1-hydroxylation in Bama minipig liver microsomes</td>
<td>(45)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of Luciferin-IPA metabolism in Göttingen minipig liver microsomes</td>
<td>(67)</td>
</tr>
<tr>
<td>Triacetyloleandomycin</td>
<td>Inhibition of nifedipine oxidation in Brno White Göttingen minipig microsomes</td>
<td>(40)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of nifedipine oxidation and testosterone 6β-hydroxylation in domestic pig liver microsomes</td>
<td>(70)</td>
</tr>
</tbody>
</table>

These tables show that several, but not all human CYP450 inducers and inhibitors are potent in the porcine species as well as in other animal models (37, 38). Noteworthy is the fact that sometimes controversy exists between studies using the pig, as can be noted for the inducing potential of dexamethasone on CYP3A activity and the inhibitory capacity of quinidine for dextromethorphan O-demethylation. Of course, the presence of pig breed-dependent differences cannot be excluded.

Finally, more than one CYP450 subfamily can be involved in the biotransformation of a substrate, with the formation of the same or (an)other metabolite(s). By varying the incubation conditions, one can promote the intended pathway as the affinity for the substrate may shift (72). This was shown in our study by using human recombinant CYP450 Baculosomes, mainly for phenacetin and tolbutamide. However, the intended CYP450 isoforms showed clearly the highest affinity for their probe substrate. Additionally, the use of an appropriate substrate concentration combined with linear incubation conditions for concentration of microsomal protein and time, promotes the intended pathway (54, 72). In our experiment, substrate concentrations were the same as performed at Janssen Research for evaluation of the intended CYP450 enzymes in human liver microsomes, and linearity of protein concentration and incubation time
was determined. In man, these four probe substrates have proved to be valid for the evaluation of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 (54, 72). However, it remains difficult to predict to which extent other CYP450 enzymes were involved in the metabolism of our four substrates in the minipig liver microsomes, since this also depends on their relative abundances and substrate affinities. Figure 7.2 shows the relative CYP450 abundances in the pig and in man, based on a study in two Suffolk White pigs and on a large meta-analysis of 50 studies in man, respectively (73, 74).

Based on these charts, it appears that the relative abundances of CYP1A, CYP2C and CYP3A in pigs are lower than in man. In contrast, CYP2A and CYP2D show much higher abundances in Suffolk White pigs than in man. Consequently, it cannot be excluded that the contribution of additional CYP450 enzymes (like CYP2A and CYP2D) is more pronounced in pigs than in man. However, it remains unclear whether effort was made to detect other CYP450 isoforms in the pig (e.g. CYP2B22, CYP2C42, CYP3A22 and CYP3A46) or that these isoforms were not present/detected in the Suffolk White pig. If these isoforms are present but not detected, and hence not integrated in the chart, relative abundances will change. The relative CYP450 abundances in the Göttingen minipig remain to be determined. Hence, it seems premature to make assumptions for the Göttingen minipig, based on the relative CYP450 abundances in the Suffolk White pig.
The total abundance of CYP450 has been studied in the Göttingen minipig (870-1030 pmol/mg MP) and appears to be 2-3-fold higher compared with domestic pig (220-550 pmol/mg MP) (61, 65, 75). In man, levels of 250-430 pmol CYP450/mg MP have been described (76, 77). The higher CYP450 content per mg MP in Göttingen minipigs may explain in part for the higher metabolizing capacities that we observed in the minipig compared with HLM.
7.3 Detection of a CYP3A7-like enzyme in the Göttingen minipig liver

CYP3A7 is the most abundant CYP3A isoform in human foetal liver, while CYP3A4 becomes the most abundant isoform in adult liver. At time of birth, the amount of CYP3A7 decreases and the amount of CYP3A4 gradually increases (29). Although CYP3A7 and CYP3A4 share a high identity of amino acid sequences (88%), they have their own distinct substrate specificities. The latter is important to discriminate both isoforms. Indeed, due to their high similarity in amino acid sequences, cross-reactivity of anti-CYP3A4 or anti-CYP3A7 antibodies may hamper research by detecting both isoforms simultaneously, as was shown earlier in man (29). In spite of this, we detected a similar developmental expression pattern when using the anti-CYP3A7 and anti-CYP3A4 antibodies, which seemed only similar to that of human CYP3A4 (78). By ELISA, we detected a pattern that corresponded with the ontogeny of CYP3A4 (67). Absence of a minipig CYP3A7-orthologue or a lack of reactivity with the human antibody might explain for this.

In a next attempt to answer our question on the presence of a shift from a CYP3A7-like enzyme into a CYP3A4-like enzyme, we evaluated the metabolizing capacity of the Göttingen minipig liver for three human luminogenic substrates, i.e. Luciferin-BE and Luciferin-CYP3A7 for CYP3A7 and Luciferin-IPA for CYP3A4. For Luciferin-IPA, quantifiable levels of activity were detected from Day 7 onwards and showed a good correlation with the results from the ELISA for CYP3A4. Luciferin-IPA metabolism was inhibited with the CYP3A4 inhibitors ketoconazole and CYP3cide, and was reduced by co-incubation with two other CYP3A4 substrates, i.e. midazolam and testosterone. Additionally, the antibody that was used in the ELISA was able to inhibit the metabolism of Luciferin-IPA in the minipig liver microsomes, further corroborating our assumption that Luciferin-IPA was a valid substrate to assess CYP3A activity in the minipig. In contrast, Luciferin-BE and Luciferin-3A7 were not metabolized by the foetal or neonatal minipig liver microsomes. Higher biotransformation rates were present in 28-day-old and adult animals. Latter activities were probably related with CYP3A4-like activity. Again, this second study suggested the absence of a CYP3A7-like orthologue or a different substrate specificity compared with man (67).

In literature, only one study has reported the detection of a CYP3A7-like enzyme in the porcine species, more specifically by Western Blot in the liver of foetal and neonatal
Göttingen minipigs. The authors used an antibody that was raised against human CYP3A7 and they detected a protein that revealed higher abundances in foetal livers than in neonatal livers. However, they failed to detect the enzyme at the mRNA level (79). The detection of a CYP3A7-orthologue has neither been described in rats, although the ontogeny of CYP3A has been studied most extensively in this species. Moreover, one of these studies states that a foetal-neonatal dominant CYP3A subtype like CYP3A7 is not present in rat. However, they provided no references for this statement and only used postnatal animals themselves (80). Nevertheless, this remark makes sense, especially in view of a study on CYP3A phylogonomics in primates (81). This study suggests that the presence or absence of different CYP3A isoforms and their substrate specificities probably represent complex adaptive responses during evolutionary development. CYP3A7 seems to have evolved with the origin of the Hominidae, comprising human beings, chimpanzees, orang-oetans and bonobos. Additionally, exon 6 of CYP3A7 is thought to be switched with that of CYP3A4, and consequently CYP3A7 became the foetal dominant CYP3A gene in hominoids. During the further course of evolution, CYP3A7 underwent further functional changes resulting in even dissimilar substrate specificities between CYP3A7 in man and chimpanzee. Moreover, CYP3A43 is present in the chimpanzee, rhesus monkey and man, but disappeared in the marmoset while CYP3A67 arose from a duplication of CYP3A7 and can be found in the chimpanzee and the orang-oetang, but got lost in humans (81). So, these data and the lack of metabolism of Luciferin-BE and Luciferin-3A7 in foetal and neonatal minipig liver microsomes suggest the absence of a CYP3A7-like enzyme in the Göttingen minipig.
7.4 Value of the morphometric data and pH values during development

The first years of life in a child and the first months of life in the Göttingen minipig are characterized by important changes in the anatomy and physiology of the body, all with their impact on the ADME of administered drugs (20, 22, 82). Figure 7.3 shows a compilation of some developmental windows in the minipig and man that are relevant in this context.

![Developmental windows diagram]

Figure 7.3. Compilation of developmental windows based on morphometric data and gastric pH values from the Göttingen minipig and man. The pink bars represent the timelines in the Göttingen minipig, while the blue bars represent the timelines in man.

Very high relative organ weights are present in the first week of life in both the minipig and man. A nice example that demonstrates their relevance is the very high relative brain weight and consequently cerebral blood flow in the neonate. This has implications on the vulnerability of this organ when potentially neurotoxic drugs are administered to a neonate, especially in combination with the rather immature blood-brain barrier and low amount of blood proteins at that moment (22, 82). Consequently, larger fractions of administered drugs will reach the central nervous system compared with more mature bodies, with a higher risk for toxicity (22, 82). The highest increase in absolute organ weights in the Göttingen minipig is present during the first four months.
in life. This means that the increase in absolute organ weights will be of lower impact by the time that they generally enter toxicity studies (reaching of sexual maturity) than for younger age groups. Also relative organ weights remain rather stable after that time point.

At the end of the first month of life, gastric pH had decreased and levels were similar to that of adult minipigs, although the piglets were not weaned yet. However, they were already able to eat hay, which was found in their stomachs. The presence of this decrease in gastric pH during the suckling period and the ability to administer compounds orally from young ages onwards, make the minipig an interesting model in view of compounds that need to be orally administered to breastfed or formula-fed babies.

The development of the small intestine of the Göttingen minipig also deserves some extra attention. As can be seen from Figure 7.4, the relative weight of the small intestine is already increasing prior to birth, indicating that the absolute weight of the small intestine increases faster than the body weight, while this is not the case for the intestinal length. This enhanced prenatal development was not seen in the other investigated organs. These findings suggest that the functional morphology of the small intestine already prepares for the adaptations necessary for postnatal life and ingestion of food. This finding might be not so surprising since the small intestine of the porcine species is quite mature at time of birth, although to a lower extent as in man. Based on the functionality of intestinal digestive enzymes, the three-week-old pig resembles the three-month-old baby best. In contrast, rodents have a far less mature intestinal system and only show major developmental changes at time of weaning (83, 84).

So, based upon the above data most pronounced changes in organ weights and pH values occur during the first month of life and the development of the small intestine shows an earlier pattern. These physiological data are valuable for the further optimization of a Göttingen minipig PBPK model, especially in view of the importance of PBPK models in paediatric drug development (85-87). This said, we only evaluated the first five months of life on the Göttingen minipig and, as such, the entire growth process until adulthood was not covered by the present study.
Figure 7.4. Relative weight and length of the small intestine (S.I.) of the Göttingen minipig during late foetal period and the first month of life. DGA: days of gestational age; BW: body weight
7.5 Drug metabolism in rodent and other non-rodent species

The ontogeny of hepatic CYP450 has been extensively characterized in rats, but is only scarcely documented in Beagle dogs and non-human primates.

The available data in rats, i.e. the ontogeny of CYP1A2, CYP2C, CYP2D and CYP3A, are summarized in Tables 7.4, 7.5, 7.6 and 7.7, respectively. CYP1A2 protein and activity, and probably also mRNA, is absent or very low in the late foetal stage and at time of birth, but starts to increase during the first week of life. Around time of weaning and shortly thereafter, much higher CYP1A2 activity levels than in adult rats are present. These levels drop to adult levels when reaching sexual maturity. (Table 7.4)

For CYP2C, several isoforms have been described in the rat. In general, CYP2C mRNA is absent in the late foetal stage and at birth, but with a postnatal increase to reach adult levels around or after time of puberty. CYP2C11 and CYP2C12 show pronounced sex-related differences after the first month of life, with CYP2C11 being the dominant isoforms in males and CYP2C12 in females. Activity remains low during the first month of life, to become clearly present at 8 weeks of age. (Table 7.5)

The CYP2D subfamily in rats consists of at least four isoforms. Depending on its isoforms, postnatal increases at the mRNA level are present or not. Bufuralol 1'-hydroxylation, which is considered to be representative for CYP2D activity, increased postnatally with an earlier peak in females compared with males, although no sex-related differences were described at the protein level. (Table 7.6)

Similar to the CYP2C and CYP2D subfamilies, several CYP3A isoforms are present in the rat. In general, expression and activity of CYP3A isoforms increase with postnatal age, but sex-related differences largely influence their presence after the first or second month of life, with the disappearance of certain CYP3A mRNAs and CYP3A-related activities in female rats when they reach sexual maturity or adulthood. (Table 7.7) None of the studies mention the presence of a CYP3A7-like enzyme in the foetal rat.
Table 7.4. Ontogeny of CYP1A2 in the rat.

<table>
<thead>
<tr>
<th>CYP450 subfamily or isoform</th>
<th>Prenatally: late foetal stage</th>
<th>Birth/Day 1</th>
<th>Postnatally</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| CYP1A2                      | mRNA                          | Day 4: low, increase with age afterwards  
|                             |                               | Day 16 = Week 8 and 12 
|                             |                               | Day 30 > Week 8 and 12  | (80) |

<table>
<thead>
<tr>
<th>Protein</th>
<th>Low and homogenous immunoreactivity in late foetal livers</th>
<th>Low and homogenous immunoreactivity in neonatal livers</th>
<th>From Day 10-15: centrilobular pattern</th>
<th>(88)</th>
</tr>
</thead>
</table>
|         | No CYP1A2 protein by SDS-Page                           | No CYP1A2 protein by SDS-Page                          | Day 3: 25% of AL  
|         |                                                          |                                                       | Day 42 (early puberty): 45% of AL  
|         |                                                          |                                                       | Day 42-63: AL  | (89) |

| Activity | No MROD | No MROD | Day 3: low MROD  
|          |         |         | Day 5: 15% of AL  
|          |         |         | Day 21: 150% AL  
|          |         |         | Day 28: 170% AL  
|          |         |         | Day 42: dropped to AL  
|          |         |         | EROD increased gradually with age  
|          |         |         | Peak levels at Day 21 (F) and Day 26 (M) : > AL  
|          |         |         | Decreased afterwards to AL  
|          |         |         | Day 4: very low EROD  
|          |         |         | Day 16: low EROD  
|          |         |         | Day 30: higher EROD  
|          |         |         | Week 8 and Week 12: similar activity levels, but higher than at Day 30  | (90) |

MROD: methoxyresorufin O-dealkylation; EROD: 7-ethoxyresorufin deethylation; F: female; M: male; AL: adult level
### Table 7.5. Ontogeny of CYP2C in the rat

<table>
<thead>
<tr>
<th>CYP450 subfamily or isoform</th>
<th>Prenatally: late foetal stage</th>
<th>Birth/Day 1</th>
<th>Postnatally</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| CYP2C6 mRNA                  | Not present                   | Increasing expression with age  
  Week 3-Week 6: maximal levels  
  Day 4: low  
  Day 30 = week 8 and week 12 | (88) |
| CYP2C7 mRNA                  | Not present                   | Up till two weeks of age: very low  
  Between 4 and 12 weeks of age: increase with age  
  Week 12: F = 2X M | (88) |
| CYP2C11 mRNA                 | Not present                   | Low until puberty  
  Between 4 and 6 weeks of age: increase in M up to AL, not F  
  Day 4: low in M and F  
  Day 30: low in M and increased in F  
  Between Week 8 and 12: much higher levels than before in M, but low levels in F | (88) |
| CYP2C12 mRNA                 | Not present                   | Low until puberty  
  Between 2 and 8 weeks of age: increase in F up to AL, not M | (88) |
| CYP2C23 mRNA                 | Present at 50% of AL at Day 1 | Expression stabilizes at Day 7, not affected by sexual maturity | (88) |
| CYP2C6 Protein               | Homogeneous expression among all hepatocytes in 6-week-old rats | (88) |
| CYP2C Activity               | Testosterone 2α-hydroxylation activity, as a marker for CYP2C activity was very low during the first month of life in M and F, with much higher activity at week 8 and 12 | (80) |

F: female; M: male; AL: adult level
Table 7.6. Ontogeny of CYP2D in the rat.

<table>
<thead>
<tr>
<th>CYP450 subfamily or isoform</th>
<th>Prenatally: late foetal stage</th>
<th>Birth/Day 1</th>
<th>Postnatally</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2D1 mRNA</td>
<td>Not present</td>
<td>Elevated levels</td>
<td>Week 1-40: Levels unaffected by postnatal age</td>
<td>(91) (88) (92)</td>
</tr>
<tr>
<td>CYP2D2 mRNA</td>
<td></td>
<td></td>
<td>Week 1-40: Levels unaffected by postnatal age</td>
<td>(92)</td>
</tr>
<tr>
<td>CYP2D3 mRNA</td>
<td></td>
<td>Week 1-7: Increase with age in M</td>
<td>Week 1-14: Increase with age in F</td>
<td>(92)</td>
</tr>
<tr>
<td>CYP2D4 mRNA</td>
<td>Not present</td>
<td>Elevated levels</td>
<td>Week 1 = Week 3 Afterwards increase with maximal levels at 14 weeks of age No sex-related differences</td>
<td>(91) (88) (92)</td>
</tr>
<tr>
<td>CYP2D Protein</td>
<td></td>
<td>Postnatal increase in bufuralol 1'-hydroxylation Week 14: maximal in M Week 7: maximal in F (caused by increase in CYP2D3?) At one week of age, only CYP2D2 is involved with a high affinity for bufuralol; at 7 weeks of age, at least one other CYP2D isoform is involved, with a lower affinity component (probably CYP2D3)</td>
<td>(92)</td>
<td></td>
</tr>
</tbody>
</table>

F: female; M: male; AL: adult level
Table 7.7. Ontogeny of CYP3A in the rat.

<table>
<thead>
<tr>
<th>CYP450 subfamily or isoform</th>
<th>Prenatally: late foetal stage</th>
<th>Birth/Day 1</th>
<th>Postnatally</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A1 mRNA</td>
<td>Detectable in a 16 days old foetus rat liver</td>
<td>Week 1-8: decrease in both F and M</td>
<td>(80)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gradual increase</td>
<td>Day 14: maximal level in F, with similar level on Day 21 and 28</td>
<td>(94)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 56 and Day 84: decrease with age</td>
<td></td>
</tr>
<tr>
<td>CYP3A2 mRNA</td>
<td>CYP3A2 mRNA detectable in a 16 day-old-fetus</td>
<td>Day 7-18: 3- to 4- fold of AL in M</td>
<td>(90)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 42: AL in M Postnatal increase in F</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 42: 2% of male level in F CYP3A2 disappears in adult female rat?</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 4-3 months: high levels in M that are at a similar level First month of life: F = M Week 8 and Week 12: not present in F</td>
<td>(80)</td>
<td></td>
</tr>
<tr>
<td>CYP3A9 mRNA</td>
<td>Day 4: low Week 1 -Week 8: increase with age in M and F Week 12: decreased expression</td>
<td>(80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A18 mRNA</td>
<td>Day 4: low Week 1-Week 4: increase with age in M and F Week 8 and Week 12: Absent in F</td>
<td>(80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 8: highest level in M Week 12: decreased in M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A23 mRNA</td>
<td>Week 1-Week 8: increase with age in M and F Week 12: decreased</td>
<td>(80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP450 subfamily or isoform</td>
<td>Prenatally: late foetal stage</td>
<td>Birth/Day 1</td>
<td>Postnatally</td>
<td>Ref.</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>CYP3A2 protein</td>
<td></td>
<td>Day 3-Day 21: diffuse staining of all hepatocytes. Day 28: appearance of a centrilobular pattern Day 56 and Day 84: disappearance of centrilobular pattern Increase with age during first two months of life Day 150 and 240: decreased</td>
<td>(94)</td>
<td></td>
</tr>
<tr>
<td>CYP3A activity</td>
<td></td>
<td>Day 1-Day 20: increase in the 6β-hydroxylation of testosterone (linked with CYP3A2 and not CYP3A1) in M and F. After Day 20: decrease in F After Day 20: further increase in M up to one year of age (CYP2C may also be involved in 6β-hydroxylation of testosterone) Day 1-Day 18: increase with age in N-demethylation of N-ethylmorphine in F Day 1-Day 26: increase with age in N-demethylation of N-ethylmorphine in M Day 4-30: increase in testosterone 6β-hydroxylation in F and M Adult F: activity absent</td>
<td>(95) (96)</td>
<td></td>
</tr>
</tbody>
</table>

F: female; M: male; AL: adult level

Based on the abovementioned observations, it appears that the rat has mainly a postnatal development of CYP450 protein expression and activity, as in man. However, important differences with man occur during ontogeny. The major increase in CYP1A2 activity in rats around weaning has not been observed in man (24-26). In contrast, CYP1A2 is described to have a ‘delayed’ developmental pattern compared with other CYP450 enzymes, with only about 35% of adult CYP1A2 activity levels in children between 1 until 9 years of age (26). Neither did we find higher CYP1A2 activity in our
Göttingen minipigs at 28 days of age compared with adults, although these piglets were not yet weaned then. Nevertheless, a gradual increase in CYP1A2 activity has been reported in Camborough pigs during the first 20 weeks of life, which further suggests the absence of peak activities of CYP1A2 around time of weaning in the porcine species (44).

No sex-dependent expression or activity of CYP2C isoforms has been reported for man, although this is clearly present in the rat by time of puberty (97, 98). In the porcine species, a tendency of higher CYP2C expression and activity in male animals has been described and was also present in our study (Chapter 6) (42). However, the sex-dependent expression of CYP2C isoforms in the pig appears to be breed-dependent (99). Furthermore, CYP2C activity develops later in rat and minipig (around puberty) compared with CYP1A2 (around weaning), and it appears to be vice versa in man (25-27).

In contrast to man and minipig, CYP2D is represented by several isoforms in the rat, with isoform-dependent expression and activity patterns over time, which makes it difficult to compare its development between species (1). Furthermore, the polymorphic expression pattern of CYP2D6 in man makes it difficult to extract a clear ontogenetic pattern for CYP2D6 (30).

For CYP3A, sex-dependent differences are very pronounced in the rat during postnatal development, with, in general, a clear decrease of CYP3A expression and activity in female animals over time. This is in contrast to human and porcine data on higher CYP3A activity in females compared with males, although still some controversy in man exists (46, 67, 97, 100-103). CYP3A activity starts during the first month after birth in rat with CYP3A2 mRNA levels already detectable at late gestation and at time of birth. Hence, the ontogeny of CYP3A seems to occur earlier than CYP1A2 and CYP2C in the rat. This is in agreement with detection of 5% of adult activity already at late gestation and 10-15% of adult activity during the first week of life in man (29).

In conclusion, although the rat shows a postnatal development of CYP450, important differences with man and (mini)pig are present. Clear sex-dependent differences appear during postnatal development for CYP2C and CYP3A, which are not present in man and (mini)pig. Additionally, the peak around weaning in CYP1A2 activity seems to be specific for the rat. In view of these dissimilarities with man, the value and trustworthiness of the rat as animal model for human CYP450 ontogeny may be questionable.
Data on the ontogeny of CYP450 in the Beagle dog are very scarce, which makes its relevance for paediatric drug metabolism difficult to interpret. Tanaka et al. have incubated liver microsomes from male Beagle dogs at different ages (1 week, 3 weeks, 7 weeks, 15 weeks, 30 weeks and 150 weeks (adult)) with a range of compounds. Caffeine N-demethylation, erythromycin N-demethylation and phenytoin hydroxylation were used to assess CYP1A2-like, CYP3A4/5-like and CYP2C9-like activity, respectively. Caffeine N-demethylation showed a postnatal increase with a doubling of activity at week 15 compared with week 1. Thereafter, activity remained similar (104). However, Mise et al demonstrated in 2008 that caffeine in dogs is not metabolized by CYP1A2 (105). To our knowledge, the metabolism of caffeine has not been attributed to a specific CYP450 enzyme in the dog yet. The erythromycin N-demethylation showed no clear age-related differences, while the phenytoin hydroxylation demonstrated a clear postnatal increase during the first 15 weeks of life, with about 10-fold higher activity at 15 weeks compared with at one week of age. Afterwards, activity decreased to the levels observed in the liver microsomes from the first week after birth for the 150-week-old dogs (104). According to our knowledge, these latter two reactions have not conclusively been contributed to the intended CYP450 isoforms in the dog yet. So, although these three reactions appear to represent different ontogenetic profiles in the Beagle dog, it remains unclear which CYP450 enzymes were involved. Nevertheless, it appears that maximal CYP450 activities were present at 15 weeks of age, which is about six to seven weeks after weaning age.

The Cynomolgus monkey (Macaca fascicularis) is a commonly used non-human primate model in general toxicity studies, but data on drug metabolism in juvenile animals are very limited. One study has investigated the developmental gene expression of CYP450 enzymes in this species (foetuses from second and third trimester of gestation, one-month-old, 6-month-old, 12-month-old, 18-month-old and 2 to 3-year-old animals) (106). The mfCYP1A1 mRNA levels were very low during late gestation, with a clear increase postnatally to reach the highest levels at 12 months of age. Afterwards, the expression slightly decreased. According to the authors, mfCYP1A1 is the orthologue for CYP1A2 in man. The gene expression levels of mfCYP2C9, mfCYP2D17 and mfCYP3A4 greatly increased from foetal to postnatal ages. Despite the evolutionary close relation of the Cynomolgus monkey with man, the mfCYP3A7 followed a dissimilar expression
pattern than CYP3A7 in man. Expression was very low in foetal livers and increased postnatally (106). This finding further corroborates the assumptions made by Qiu et al. that the foetal dominant CYP3A7 isoform is restricted to man, and consequently also explains for the absence of detection in the foetal Göttingen minipig in our group (81).

In conclusion, the rat appears to show some significant differences with man in their CYP450 ontogeny, which should be taken into account when interpreting the results from juvenile toxicity studies in the species. Based on the results from our studies, the Göttingen minipig may be a more advantageous animal model. However, further investigation is needed. Due to the lack of studies in dogs and NHPs, it is too premature to decide which non-rodent animal model resembles man most.
7.6 Conclusion

Based on the results from this thesis, it can be stated that the ADME of a drug in the Göttingen minipig significantly change during the first month of life. This information is key in view of the use of juvenile Göttingen minipigs in toxicity studies.

Following events were noted during the first month of life:

- CYP3A and UGT1A developed a mature distribution pattern in the liver and the small intestine, while Pgp expression already showed a mature pattern around birth
- CYP450 and UGT activities evolved from being nearly absent at birth to being prominently present by 28 days of age
- Gastric pH decreased significantly
- Relative organ weights were highest during the first week of life and decreased afterwards, mainly during the first month of life
- Absolute organ weights increased fast, which continued up to 3 to 4 months of age
- Body weight increased linearly, which continued up to 5 months of age

The finding that all these events, which largely influence the ADME of a drug in the body, occur simultaneously during these first four weeks of life, should be borne in mind when juvenile minipigs are used in in vivo studies. Consequently, when the main research question relates to metabolizing capacity only, in vitro studies may provide a more conclusive answer than an in vivo study. Nevertheless, the translational value of the juvenile Göttingen minipig as a preclinical model in paediatric drug development looks promising. The study results showed many similarities compared with the situation in man. Furthermore, anatomical and physiological characteristics, together with the reproductive capacities and the characteristics of the neonatal and juvenile offspring, at least favour the Göttingen minipig as a juvenile animal model. However, the choice of an animal model should be based on a case-by-case evaluation, rather than using one traditional animal model for all studies.
7.7  Future perspectives

Similar substrate specificity of CYP450 enzymes, but also similarity of effects of CYP450 inducers and inhibitors with man is pivotal for the predictive value of an animal model. The production of recombinant CYP450 enzymes from the Göttingen minipig and the related *in vitro* work will answer many questions to assess further the value of the juvenile and adult Göttingen minipig as a translational animal model for man. This work is currently ongoing in our group of Applied Veterinary Morphology.
7.8 References


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Age-related Differences in CYP3A Abundance and Activity in the Liver of the Gottingen 


8 Summary

Despite the fact that children are a very vulnerable part of society, they have been exposed to medical treatment that was authorized for adult use only, for decades. It is true that clinical trials in children are challenging, but the omission of their recruitment may seem unethical as well.

Chapter 1 describes the implementation of a legal context that promotes paediatric drug development, as well as the major hurdles related with it. Indeed, there is an ethical aspect, but there is also the complexity of a developing immature body. One of the key aspects for paediatric drug development is knowledge on the ontogeny of drug metabolizing capacity (drug metabolizing CYP450 enzymes and UGT enzymes). Not only in man, but also in animal models that are used in the preclinical phase of drug development. However, the choice of an appropriate animal model is key. We have described the juvenile Göttingen minipig as a promising animal model for paediatric drug development. Their major advantages are their many anatomical and physiological similarities with man, but also their advantageous reproductive capacities in view of juvenile studies.

The main aim of this thesis was to describe the ontogeny of some key determinants of drug metabolizing capacity in the juvenile Göttingen minipig, and to compare these results to what is known in man. Five more specific aims have been described in Chapter 2.

In Chapter 3, we have immunohistochemically localised P-glycoprotein and CYP3A in the small intestine and the liver of foetal, neonatal, juvenile and adult Göttingen minipigs. P-glycoprotein is an important drug efflux transporter and CYP3A enzymes represent a subfamily of the drug metabolizing CYP450 enzymes (Phase I-metabolism). Together, they determine largely the bioavailability of orally administered drugs. Therefore, knowledge on their developmental pattern is pivotal. P-glycoprotein appeared to be expressed already during late foetal period, without clear differences in abundance at later stages. CYP3A showed a clear postnatal increase in intensity. For both proteins, localisation was similar in the Göttingen minipig as in man. In conclusion, a similar spatio-temporal expression of both was present in the Göttingen minipig and in man.
In view of the major importance of CYP3A enzymes in human drug metabolism, we have further investigated the age-related pattern of two important members of this subfamily in Chapter 4. CYP3A7 is the most abundant CYP3A isoform in human foetal liver, while CYP3A4 becomes the most abundant isoform in adult liver. Around time of birth, a shift seems to occur between both isoforms. CYP3A4-like activity was clearly present in the Göttingen minipig liver, with absent or very low metabolism of a human CYP3A4 substrate in late foetal and neonatal, but that increased during further postnatal development. Furthermore, a high correlation was present between the CYP3A4-like activity and abundance data, obtained with an ELISA for detection of human CYP3A4 abundance. In contrast, a developmental pattern that correlates with that of CYP3A7 in man was not found in the liver of the Göttingen minipig by immunohistochemistry (Chapter 3), neither by activity assays with two human CYP3A7 substrates. (Chapter 4)

Specific probe reactions are available to characterize human CYP450 activities. Many of these probe reactions appear to be specific for the porcine CYP450 orthologues as well. For this purpose, we have used phenacetin O-deethylation, tolbutamide 4-hydroxylation, dextromethorphan O-demethylation and midazolam 1’-hydroxylation to study the ontogeny of CYP1A2-, CYP2C9-, CYP2D6-, and CYP3A4-like activity in the livers of developing Göttingen minipig, respectively. The study design and results of this part of research are discussed in Chapter 5. Additionally, the ontogeny of UGT was investigated in the liver of the Göttingen minipig with a probe that is glucuronidated by several UGT1A and UGT2B enzymes in man. In short, all substrates were metabolized by the minipig liver microsomes, with the formation of at least the same metabolite as in man. All probe reactions showed a clear postnatal developmental pattern, which is in accordance with the situation in man. Maturation of phenacetin O-deethylation and dextromethorphan O-demethylation appeared to occur faster than that of tolbutamide 4-hydroxylation and midazolam 1’-hydroxylation.

The Göttingen minipig is gaining importance in toxicity studies, although the Beagle dog and the non-human primate, like the Cynomolgus monkey, remain the most common non-rodent model. Consequently, more background data are available for the dog and the NHP, which further promotes their use. Therefore, to further characterize the
Göttingen minipig, we have compiled data on organ weights, body weights and gastrointestinal pH from animals at different ages. Additionally, equations were generated to predict organ weight, based on body weights. Most organs followed a non-linear growth curve during the first five months of life, with the highest relative organ weights during the first week of life. These results are compiled and discussed in Chapter 6.

Finally, all results are compiled and discussed by answering five main questions in Chapter 7. The answers to these questions are summarized below.

1. P-glycoprotein, CYP3A and UGT1A could be detected with antibodies directed against human proteins. All proteins showed a similar spatio-temporal expression in the small intestine and liver of the Göttingen minipig compared with what has been described in man. In this view, the developing Göttingen minipig seems a promising translational animal model for man.

2. Five human probe substrates for CYP1A2, CYP2C9, CYP2D6, CYP3A4 and UGT were metabolized by porcine liver microsomes, with a clear postnatal increase in activity. More distinct developmental patterns were present for the individual substrates. Translation with human paediatric data can be made, but are challenging. Many factors hamper a clear estimation of the translational value of the Göttingen minipig. However, this holds for other animal models as well.

3. A postnatal developmental pattern for CYP3A4-like abundance and activity is clearly present in the liver of the Göttingen minipig. In contrast, a developmental pattern that correlates with that of CYP3A7 in man, was not detected in the Göttingen minipig. These results suggest that CYP3A7 is not present in this species.

4. The compiled morphometric data and gastrointestinal pH values are valuable for the characterization of the developing Göttingen minipig. Furthermore, such data can further strengthen the building of Göttingen minipig PBPK model.

5. The ontogeny of drug metabolizing enzymes is well documented in rodents. Many isoforms are present in many CYP450 subfamilies and they often show sex-related expression patterns. Whether rodents represent good translational models for human drug metabolism remains discussable. A non-rodent model, like the Beagle dog, NHP or Göttingen minipig may form a good
alternative. Based on our results, the Göttingen minipig seems a promising model. The position of the minipig among the other non-rodent model remains uncertain since very little research has been performed on the ontogeny of drug metabolizing capacity in the dog and in NHP.
9 Samenvatting

Kinderen vormen de meest kwetsbare groep binnen onze gemeenschap. Contradictorisch genoeg, werden én werden zij veelvuldig blootgesteld aan geneesmiddelen die enkel geregistreerd zijn voor gebruik bij volwassenen. Deze gebrekkige pediatrische registratie kan in hoofdzaak herleid worden tot een bestaande aversie om kinderen op te nemen in klinische studies. Het uitsluiten van kinderen uit dergelijke studies kan echter ook als onethisch beschouwd worden, aangezien gebrekkige kennis over de veiligheid en effectiviteit van geneesmiddelen kan resulteren in ongewenste en zelfs toxische reacties bij deze bevolkingsgroep.

Hoofdstuk 1 beschrijft de wettelijke maatregelen die ingevoerd zijn om pediatrische registratie van geneesmiddelen te stimuleren. Tevens worden de moeilijkheden, die gepaard gaan met dergelijk onderzoek, belicht. Er is het ethische aspect, maar er is ook de complexiteit van een immatuur lichaam in ontwikkeling, dat dergelijk onderzoek bemoeilijkt. De capaciteit om geneesmiddelen te biotransformeren naar een meer wateroplosbare, en dus ook uitscheidbare metaboliet, is één van de belangrijke factoren die immatuur is bij jonge kinderen. Het spreekt voor zich dat het belangrijk is om de ontogenie van dergelijke metaboliserende enzymen (CYP450 enzymen en UGT enzymen) te karakteriseren. En dit niet enkel bij de mens, maar ook bij de proefdieren die betrokken zijn in de preklinische fase van geneesmiddelontwikkeling. De keuze van een geschikt diermodel is in deze context ook cruciaal. In deze thesis hebben we het juveniele Göttingen minivarken beschreven als een diermodel met potentieel in dergelijke context.

De hoofddoelstelling van deze thesis was de karakterisatie van de ontogenie van enkele belangrijke metaboliserende CYP450 en UGT enzymen in het juveniele Göttingen minivarken en deze te vergelijken met data bij de mens. Vijf specifieke doelstellingen werden beschreven in Hoofdstuk 2.

Hoofdstuk 3 beschrijft de immunohistochemische localisatie van het P-glycoproteïne en CYP3A enzymen in de dunne darm en lever van foetale, neonatale, juveniele en volwassenen Göttingen minivarkens. Het P-glycoproteïne is een belangrijke efflux transporter die opgenomen geneesmiddelen terug uit de cel kan pompen, terwijl CYP3A de belangrijkste CYP450 subfamilie vormt die verantwoordelijk is voor Phase I-
biotransformatie. Deze twee eiwitten werken samen en bepalen mee de biologische beschikbaarheid van orale geneesmiddelen. Gezien hun strategisch belang, was het interessant om hun ontogenie en localisatie te bestuderen in het Göttingen minivarken. Het P-glycoproteïne was reeds duidelijk aanwezig in de darm en lever van laat foetale dieren, zonder een duidelijk verschil in intensiteit van expressie bij oudere leeftijdsgroepen. CYP3A vertoonde wel een duidelijk postnataal ontwikkelingspatroon. Kort samengevat kan gesteld worden dat de spatio-temporale expressie van beide eiwitten gelijkaardig is bij de mens en het Göttingen minivarken.

De CYP3A subfamilie wordt beschouwd als de meest abundante, maar ook belangrijkste groep van geneesmiddel biotransformerende CYP450 enzymen bij de mens. De ontogenie van de isovormen CYP3A7 en CYP3A4 worden besproken in Hoofdstuk 4. CYP3A7 is de meest abundante CYP3A isovorm in de humane foetale lever, terwijl CYP3A4 de belangrijkste isovorm wordt postnataal. Bij nakende geboorte, daalt de hoeveelheid CYP3A7, terwijl de hoeveelheid CYP3A4 begint te stijgen. Ook bij het Göttingen minivarken was er duidelijke toename in postnataal metabolisme van een humane CYP3A4 probe. Deze resultaten correleren goed met de resultaten van een ELISA test voor bepaling van humane CYP3A4 abundantie. Er werden echter geen aanwijzingen gevonden voor het bestaan van een CYP3A7 ortholog in het Göttingen minivarken. Zowel het immunohistochemisch onderzoek (Hoofdstuk 3), als een activiteitsmeting met twee humane CYP3A7 probes bleken negatief.

De vorming van een bepaalde metaboliet uit een probe kan gebruikt worden om een specifieke CYP450 isovorm te karakteriseren. Een aantal van dergelijke probe reacties werden ook geschikt bevonden om de porciene orthologen te bestuderen. In Hoofdstuk 5 beschrijven we het gebruik van phenacetine O-deethylatie, tolbutamide 4-hydroxylatie, dextromethorphan O-demethylatie en midazolam 1'-hydroxylatie om de ontogenie van de porciene CYP1A2, CYP2C9, CYP2D6 en CYP3A4 orthologen, respectievelijk, te bestuderen. Tevens werd een probe aangewend die gegluconideerd wordt door diverse humane UGT1A en UGT2B enzymen, met als doel een high-level inzicht te krijgen in de ontogenie van het porciene UGT. De ontogenie van de bestudeerde metaboliserende enzymen situeerde zich voor alle substraten postnataal, zoals beschreven bij de mens. De maturatie van phenacetine O-deethylatie en dextromethorphan O-demethylatie manifesteerde zich vroeger dan deze van tolbutamide 4-hydroxylatie en midazolam 1'-hydroxylatie.
Het Göttingen minivarken verwerft meer en meer naambekendheid binnen het preklinische milieu. Desalniettemin nemen de Beagle hond en niet-humane primaten toch nog de meest voorme rol in wanneer een niet-rodent model aangewezen is. Logischerwijs zijn er dus ook meer achtergrond data beschikbaar voor deze laatste species, wat hun gebruik verder stimuleert. In Hoofdstuk 6 hebben we dergelijke nuttige achtergrond data gecompileerd voor het Göttingen minivarken. Orgaan gewichten, lichaamsgewichten en gastrointestinale pH waarden werden bepaald bij diverse leeftijdsgroepen. Tevens, werden er mathematische vergelijkingen opgesteld die orgaan gewichten kunnen voorspellen op basis van lichaamsgewicht tijdens de postnatale ontwikkeling.

Tot slot, werden vijf vragen beantwoord in Hoofdstuk 7, die de resultaten van deze thesis compileren.

1. Het P-glycoproteïne, CYP3A en UGT1A werden succesvol gedetecteerd met antilichamen die gericht waren tegen de humane eiwitten. De gedetecteerde eiwitten vertoonden een gelijkaardige spatio-temporale expressie in the dunne darm en de liver van het ontwikkelende Göttingen minivarken.

2. De lever microsomen van het Göttingen minivarken metaboliseerden vijf humane probes voor CYP1A2, CYP2C9, CYP2D6, CYP3A4 en UGT. Het metaboliserende vermogen nam duidelijk toe met toenemende leeftijd. Isovorm-specifieke ontwikkelingspatronen waren aanwezig. Een translatie van de porciene data naar pediatrische gegevens werd gemaakt, maar blijft uitdagend. Dit geldt echter evenzeer voor andere diermodellen.

3. CYP3A expressie en activiteit zijn aanwezig in de lever van het Göttingen minivarken, die correleren met de ontogenie van CYP3A4 bij de mens. De resultaten van deze thesis suggereren echter dat er geen CYP3A7 ortholoog aanwezig is in het Göttingen minivarken.

4. De verzamelde morfometrische data en gastrointestinale pH waarden dragen bij tot de karakterisatie van het Göttingen minivarken als preklinisch juveniel model.

5. De ontogenie van geneesmiddel biotransformerende capaciteit werd reeds uitvoerig bestudeerd in de rat. Hieruit blijkt dat vele subfamilies meerdere isovormen bevatten die de ortholoog bijken te zijn voor slecht één isovorm bij de mens. Daarenboven, vertonen veel isovormen een uitgesproken
geslachtsgebonden expressie. Het is dan ook niet volledig duidelijk in hoeverre de rat de situatie in de mens goed nabootst. Er is slechts zeer weinig onderzoek verricht naar de ontogenie van geneesmiddel metaboliserende capaciteit bij de Beagle dog en niet-humane primaten. Bijgevolg is het moeilijk om een inschatting te maken over de superioriteit van één van de niet-knaagdier modellen. Desalniettemin blijkt het juveniele Göttingen minivarken een veelbelovend model te zijn.
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‘It takes two flints to make a fire.’ (Louisa May Alcott, °1832-†1888)

Ik start bij het begin.


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Curriculum Vitae Els Van Peer

PERSONALIA
Name: Els Van Peer
Date of birth: 17/10/1982
Address: Oostvaart 3, 9180 Moerbeke-Waas
Nationality: Belgian

WORKING EXPERIENCE
January 2017-present:
Academic researcher
Laboratory of Applied Veterinary Morphology

February 2011 - January 2017:
Academic assistant in Veterinary Sciences (6-year trajectory)
Laboratory of Applied Veterinary Morphology
This position comprises two main tasks, creating a variable job content:
- Research: preparing a doctoral thesis
- Teaching practical courses to the veterinary bachelor students (mainly anatomy)


October 2010 - January 2011:
Teaching assistant in Veterinary Sciences
Laboratory of Applied Veterinary Morphology
My task consisted of teaching practical courses to the veterinary bachelor students (anatomy and embryology).
July 2006 - September 2011:  
**Practitioner for companion animals**

At Veterinary practice ‘M. Lambie’ - Veterinary Practice ‘Mervet’ (4.5 years) - Veterinary Clinic ‘Het Binnenhof’

As a veterinary practitioner, extensive communication with colleagues and clients belonged to my daily routine. Goal-minded animal care, the willingness to learn and to provide good veterinary medicine formed part of my attitude.

**EDUCATION**

2003-2006: Master in Veterinary Sciences, University of Ghent, Belgium
2000-2003: Bachelor in Veterinary Sciences, University of Ghent, Belgium

**COURSES**

- Emtrain PhD Workshop 2015, 26-29th April 2015, Beerse, Belgium
- Laboratory Animal Science: certificate of experimenter Cat. C, February 2014, University of Antwerp
- Simcyp focused workshop: Predicting age-related changes to pharmacokinetics and drug-drug interactions including associated variability: linking this information to drug response in the paediatric population, 25-26th February 2013, Sheffield, UK
- FRAME/NADIR Training School in the Experimental Design and Statistical Analysis of Biomedical Experiments, 8-10th January 2013, University of Nottingham, UK
Bibliography

Publications in international peer-reviewed journals

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Van Peer E, Verbueken E, Saad M, Casteleyn C, Van Ginneken C, Van Cruchten S.  
Ontogeny of CYP3A and P-glycoprotein in the liver and the small intestine of the Gottingen minipig: an immunohistochemical evaluation.  
Oral presentations at international conferences

Van Peer E., Snoeys J., Jacobs F., Van Houdt J., Pijpers I., Casteleyn C., Van Ginneken C., Van Cruchten S.

**In vitro drug metabolism in juvenile non-rodent species: state-of-the-art.**
At the 44th Annual Meeting of the European Teratology Society, 11-14th September 2016, Dublin, Ireland

**Paediatric drug development: is the minipig a useful preclinical model?**
At the 8th Juvenile Toxicity Symposium, 7-8th April 2016, Beerse, Belgium

Van Peer E., Van Cruchten S.
**The juvenile minipig: a useful model for paediatric drug development.**
At the Salaam meeting Cost action, December 2015, Poznan, Poland

**Ontogeny of CYP3A in the liver of the Göttingen minipig.**
At the 7th Juvenile Toxicity Symposium, 23-24th October 2014, Beerse, Belgium

Van Peer E., De Vos M., Huyghelen V., Van Ginneken C., Van Chruchten S.
**Ontogeny of the drug efflux transporter P-glycoprotein in the small intestine of the pig: a preliminary investigation.**
At the 39th Annual Meeting of the European Teratology Society, 6th September 2011, Ghent, Belgium
Poster presentations at international conferences

Van Peer E., De Vos M., Huyghelen V., Willemen S., Van Ginneken C., Van Cruchten S.
Ontogeny of drug transport and metabolism in the pig small intestine: a preliminary investigation.
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