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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 postnatally. At one month of age, 60.5 and 75.4% of adult activities were observed for acetaminophen and dextrorphan 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 postnatally, as described in man.

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 (polyestrous 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 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 1. CYP1A, CYP2C, CYP2D and CYP3A isoforms in (mini)pig and human beings.

Göttingen minipig isoforms	Human isoforms	% of sequence identity of nucleotides	% of sequence identity of amino acids	Accession Number in NCBI databank
CYP2D21	CYP2D6	83.5%	78.3%	D89502
CYP3A22v1	CYP3A4	81.5%	75.0%	AB006010
CYP3A29v5	CYP3A4	82.5%	75.3%	AF424780
Domestic pig	Human			
CYP1A2	CYP1A2	85.0%	81.0%	NM_001159614
CYP2C33	CYP2C9	76.0%	64.0%	NM_214414

	CYP2C19	74.0%	63.0%	
CYP2C42	CYP2C9	85.0%	80.0%	NM_001167835
	CYP2C19	85.0%	81.0%	
CYP2C49	CYP2C9	83.0%	77.0%	NM_214420
	CYP2C19	84.0%	77.0%	
CYP3A39	CYP3A4	82.1%	75.9%	NM_214422
CYP3A46	CYP3A4	83.5%	77.8%	NM_001134824

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, USA). 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 localization 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.

Materials and methods

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 freezing of the liver. Samples from the left medial lobes (lobus hepatis sinister medialis) were taken and fixed in 4% (w/v) paraformaldehyde (24 h) in 0.01 M PBS (pH 7.4) at room temperature (RT) and routinely processed to paraffin blocks.

Isolation of liver microsomes

Porcine liver tissue was thawed on ice and washed with ice-cold homogenizing buffer (0.01 M potassium phosphate (KPO_4) buffer (451201, Corning Incorporated, NY, USA) 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 12,000*g* for 20 min at 4°C. The resulting supernatant (S9-fraction) 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 KPO_4 buffer containing 250 mM sucrose and Halt™ Protease Inhibitor Single-Use Cocktail (78430, Thermo Fisher Scientific, MA, USA)) and stored at -80°C until use. For each ml S9-fraction that was centrifuged, 150 µl of storage buffer was added to the microsomal pellet and homogenized. Total protein concentration was determined by the Pierce® BCA Protein Assay Kit with bovine serum albumin as a standard (23225, Thermo Fisher Scientific).

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 2mM 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 (V_{max}) and are routinely used to screen CYP450 activity in HLM at Janssen Research. In order to verify that HLM and minipig liver microsomes were functioning at V_{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, USA), 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 KPO_4 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 KPO_4 buffer were pre-incubated at 37°C for 5 min. The substrate mixture and the NADPH-regenerating system in KPO_4 buffer 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, USA) equipped with an electrospray ionization source operated in negative (OH-tolbutamide) or positive (1-OH-midazolam, dextrorphan, acetaminophen) mode. (Table 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). Results are expressed as single spheroids measurements (n = 8).

Table 2. Overview of mass transition for CYP450 probe substrates.

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

UGT activity assay

The protocol for the UGT-Glo^{TM} assay (V2082, Promega Corporation, Madison, WI, USA) was executed following the guidelines from the manufacturer. 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. $MgCl_2$ (M2670, Sigma-Aldrich, St Louis, MO, USA) and alamethicine (sc-200094, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 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, pH7.5) that was provided in the kit, was replaced by the more appropriate 0.1 M KPO_4 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 KPO_4 buffer (pH 7.4), 8 mM $MgCl_2$, 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 KPO_4 buffer (451201, Corning Incorporated), ultrapure water, alamethicine, UGT multienzyme substrate and microsomal protein were mixed and pre-incubated on ice. After 15 min, $MgCl_2$ 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).

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.

Statistical analysis

The Kruskal-Wallis (Mean Rank) 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, USA). 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, USA).

Results

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 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 3) Figure 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 3 Metabolite formation velocities of CYP450 substrates.

			Metabolite formation in pmol/min/mg MP (* or activity as percentage of activity of main CYP isoform) Mean ± Standard Deviation			
Age	Sex	N	Acetaminophen	4-OH-tolbutamide	Dextrorphan	1-OH-midazolam
84-86 DGA	M	4	13.1 ± 6.07	2.49 ± 0.71	95.2 ± 88.1	5.76 ± 6.31
	F	4	8.11 ± 2.45	2.43 ± 0.73	42.5 ± 73.1	2.01 ± 2.98
	All	**8**	**10.6 ± 5.04**	**2.46 ± 0.67**	**68.9 ± 80.1**	**3.88 ± 4.99**
108 DGA	M	4	7.43 ± 2.02	2.16 ± 0.95	150 ± 83.1	0.69 ± 0.19
	F	4	7.11 ± 0.37	1.82 ± 0.41	184 ± 65.7	0.80 ± 0.30
	All	**8**	**7.27 ± 1.35**	**1.99 ± 0.70**	**167 ± 71.7**	**0.75 ± 0.21**
Day 1	M	4	30.3 ± 32.0	2.90 ± 0.64	431 ± 329	2.74 ± 1.84
	F	4	46.1 ± 5.76	6.54 ± 2.52	582 ± 169	5.70 ± 5.35
	All	**8**	**38.2 ± 22.9**	**4.72 ± 2.59**	**507 ± 255**	**4.22 ± 4.03**
Day 3	M	4	87.6 ± 15.7	20.5 ± 6.66	3022 ± 856	20.8 ± 10.5
	F	4	67.8 ± 25.8	11.0 ± 5.66	1950 ± 905	17.1 ± 8.55
	All	**8**	**77.7 ± 22.4**	**15.8 ± 7.62**	**2486 ± 997**	**18.9 ± 9.08**
Day 7	M	5	171 ± 47.7	33.5 ± 20.0	4865 ± 1924	99.4 ± 37.9
	F	4	153 ± 90.0	15.7 ± 9.66	2577 ± 1833	53.3 ± 31.5
	All	**9**	**163 ± 65.2**	**25.6 ± 17.9**	**3848 ± 2136**	**78.9 ± 41.0**
Day 28	M	5	325 ± 90.7	48.1 ± 18.5	6430 ± 2573	295 ± 113
	F	5	946 ± 152	47.1 ± 7.70	10087 ± 1993	318 ± 117
	All	**10**	**636 ± 348**	**47.6 ± 13.4**	**8259 ± 2902**	**307 ± 109**
Adult	M	5	398 ± 196	173 ± 67.1	10390 ± 5105	738 ± 123
	F	4	1574 ± 318	104 ± 51.5	11408 ± 3868	687 ± 226
	All	**9**	**1051 ± 670**	**135 ± 66.0**	**10956 ± 4188**	**710 ± 179**
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 S1 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_{max}. (Table 3)

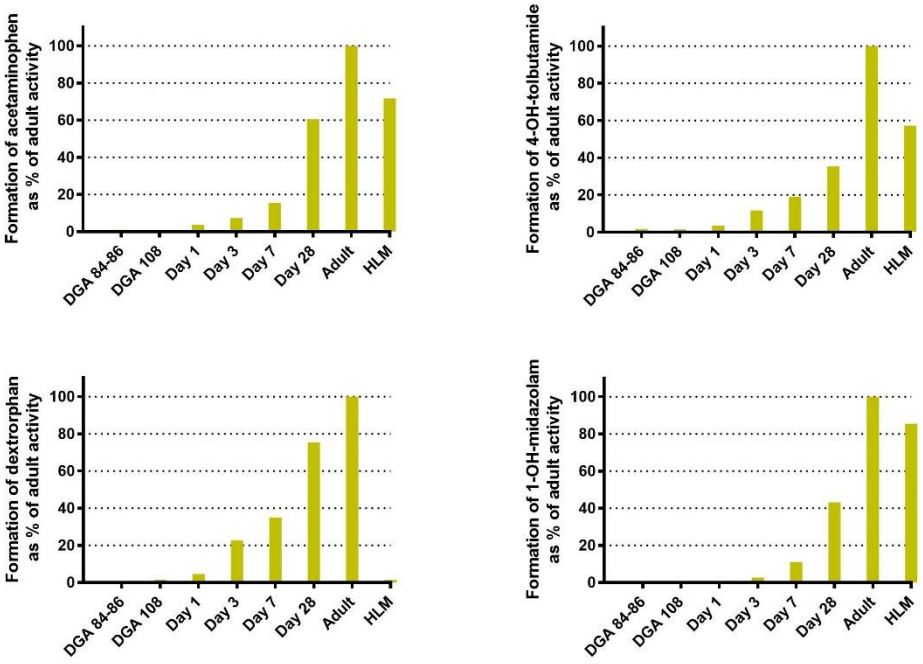

Figure 1. Mean formations of metabolites from CYP450 substrates as percentages of adult activities in the developing Göttingen minipig.

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 dextrorphan 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 2) Similarly, the highest correlation was found between D-luciferin formation, as a measure of CYP3A activity (previous study), and 1'-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 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 S2 in the Supplementary material. Sow 1, euthanized at 84 days of gestation, showed the highest 1'-OH-midazolam formation and secondly highest dextrorphan formation, while Sow 5, euthanized 6 months after parturition, showed the highest acetaminophen and dextrorphan formations.

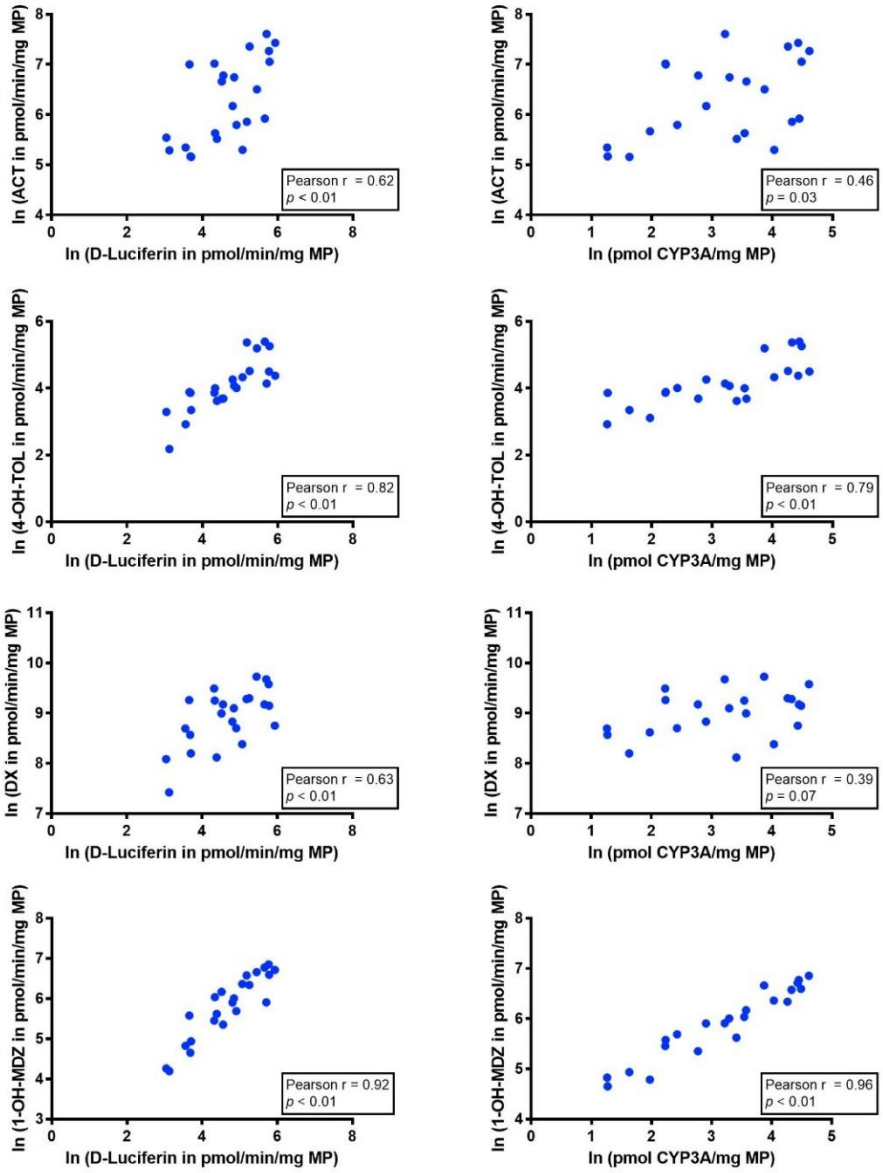

Figure 2 Correlations between CYP3A abundance and biotransformation of Luciferin-IPA *versus* biotransformation of CYP450 substrates. Calculation of correlation was performed on the ln-transformed data from PND7, PND28 and adult animals. Data on CYP3A abundance and D-Luciferin formation, as a measure 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), dextrorphan (DX) and 1'-OH-midazolam (1-OH-MDZ) formation have been made.

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 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 S2) UGT activity was higher in the adult Göttingen minipig liver microsomes compared with HLM(050B) and HLM(040).

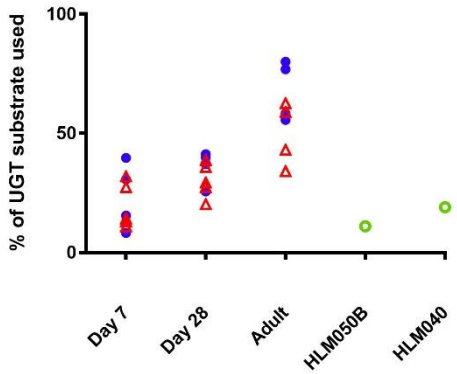

Figure 3. Consumption of the UGT multienzyme substrate in the developing Göttingen minipig. UGT activity is expressed as the percentage of consumed substrate. The mean value of two replicates for each animal is represented by a blue dot and a red empty triangle for male and female animals, respectively. HLM(050B) and HLM(040) are represented by a green circle.

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 ± 0.5). At 108 days of gestation, scores ranged from 1 till 3 (2.3 ± 0.6). At Day 1 and at Day 3, scores 2 or 3 were assigned (2.8 ± 0.4 and 2.9 ± 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 ± 0.8). For 28-day-old and adult animals, scores 4 and 5 were given (4.4 ± 0.5 and 4.6 ± 0.5, respectively). Figures 4, 5, and 6 represent images for scores 1, 3, and 5, respectively.

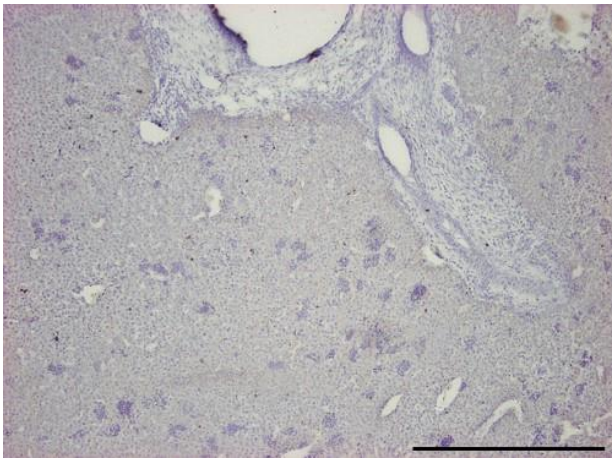

Figure 4. Score 1 in immunohistochemical detection of UGT1A. A mild staining among all hepatocytes is present in the liver of a foetus at 84-86 days of gestation. Scale bar 200 µm.

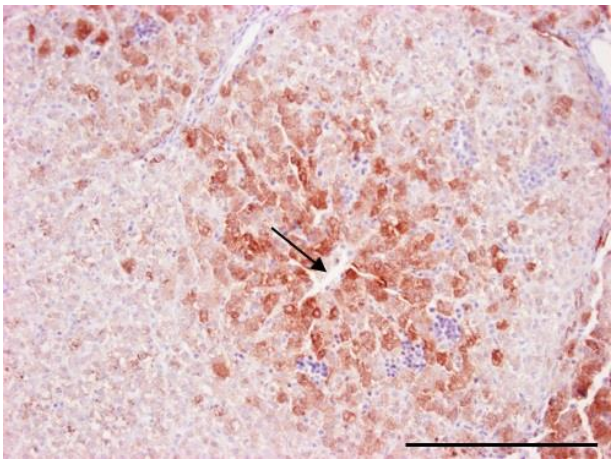

Figure 5. Score 3 in immunohistochemical detection of UGT1A. Small groups of more intensively stained hepatocytes appear close to the central vein of each lobule, though not generalized, in the liver of a 7-day-old minipig. The black arrow indicates the central vein. Scale bar 200 µm.

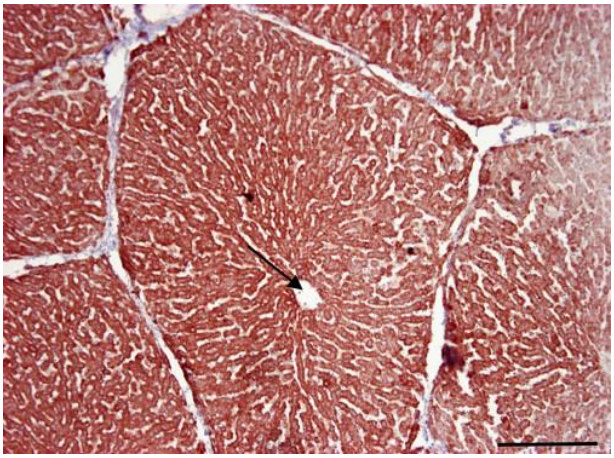

Figure 6. Score 5 in immunohistochemical detection of UGT1A. The hepatocytes of the entire liver lobules are intensely stained in the liver of an adult male minipig. The black arrow indicates the central vein. Scale bar 200 µm.

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 was highest in adult animals, but it was not significantly different from the activity in 28-day-old animals, already reaching 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 wells 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.

Supplementary tables

Table S1. *P* values for pairwise comparisons between two age groups.

		DGA 108	Day 1	Day 3	Day 7	Day 28	Adult
Phenacetin	DGA 84-86	0.093	0.009	**0.001**	**0.001**	**0.001**	**0.001**
	DGA 108		**0.0023**	**0.001**	**0.001**	**0.001**	**0.001**
	Day 1			0.009	**0.001**	**0.001**	**0.001**
	Day 3				0.016	**0.001**	**0.001**
	Day 7					**0.001**	**0.001**
	Day 28						0.142
Tolbutamide	DGA 84-86	0.248	0.006	**0.001**	**0.001**	**0.001**	**0.001**
	DGA 108		0.005	**0.001**	**0.001**	**0.001**	**0.001**
	Day 1			0.003	**0.001**	**0.001**	**0.001**
	Day 3				0.178	**0.001**	**0.001**
	Day 7					0.014	**0.001**
	Day 28						**0.001**
Dextromethorphan	DGA 84-86	0.036	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
	DGA 108		0.005	**0.001**	**0.001**	**0.001**	**0.001**
	Day 1			**0.001**	**0.0021**	**0.001**	**0.001**
	Day 3				0.149	**0.001**	**0.001**
	Day 7					0.003	**0.002**
	Day 28						0.111
Midazolam	DGA 84-86	0.401	0.294	0.003	**0.001**	**0.001**	**0.001**
	DGA 108		**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
	Day 1			0.003	**0.001**	**0.001**	**0.001**
	Day 3				0.007	**0.001**	**0.001**
	Day 7					**0.001**	**0.001**
	Day 28						**0.001**

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 S2. Individual velocities of CYP450 activities in sows.

Animal	Age	Euthanasia	Metabolite formation in pmol/min/mg MP				% of substrate used
			ACT	4-OH-TOL	DX	1-OH-MDZ	UGT MES
Sow 1	1yr 2 mo	84 DGA	1433	90.0	14454	**948**	**80.0**
Sow 2	1yr 4 mo	Day 8 after partus	1690	79.5	<u>6323</u>	823	76.9
Sow 3	2yr 9 mo	Day 30 after partus	1571	92.0	10931	566	<u>55.6</u>
Sow 4	2yr 7 mo	Day 30 after partus	<u>1157</u>	**193**	9402	731	58.3
Sow 5	2yr 5 mo	6 mo after partus	**2017**	<u>62.8</u>	**15930**	<u>368</u>	58.0

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 detrorphan; 1-OH-MDZ 1-OH-midzaolam; UGT MES UGT multienzyme substrate

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