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Age-related differences in CYP3A abundance and activity in the liver of the Göttingen minipig

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Abstract

In view of paediatric drug development, regulatory authorities often request safety studies in juvenile animals, including minipigs. Unfortunately, knowledge on the ontogeny of the biotransformation processes in animal models remains scarce and impedes a correct interpretation of the toxicity findings. 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 fetuses 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. The CYP3A inhibition by ketoconazole and testosterone and midazolam reduction of Luciferin-IPA metabolism in minipig liver microsomes substantiate that Luciferin-IPA is metabolized by CYP3A in minipigs. 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.

Introduction and background

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 (CYP) are important drug metabolizing enzymes, which facilitate the excretion of drugs from the body. Unfortunately, CYP enzymes can also be responsible for adverse drug reactions and drug-drug interactions. Maturation differences in CYP 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 homologues in Göttingen 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 homologues, e.g. nifedipine oxidation and testosterone 6 β -hydroxylation [13-16]. In addition, pigs and humans share anatomical and physiological characteristics, especially regarding the digestive and cardiovascular systems, which explains the high interest for this species as pharmacological and toxicological model [17].

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. Abundance of CYP3A was evaluated with an indirect enzyme-linked immunosorbent assay (ELISA) 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]. We evaluated the inhibitory effect of CYP3cide and ketoconazole and the interaction of the CYP3A substrates testosterone and midazolam on the metabolism of Luciferin-IPA.

Materials and Methods

Liver samples. Livers were obtained from healthy Göttingen minipigs. No CYP induction was performed. 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 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 group 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 fetuses 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), 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.

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, 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 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 (S9-fraction) 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 (Thermo Fisher Rockford, IL, 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 (Thermo Fisher Scientific, Rockford).

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 human liver microsomes (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, Rockford) and HLM (HMMC-H3A4-PL040, Life Technologies, Thermo Fisher Scientific, Rockford) 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, Taipei, Taiwan). The Pierce[®] Goat anti-rabbit horseradish peroxidase conjugated IgG (31460, Pierce Biotechnology, Rockford, USA) was used as secondary antibody. Peroxidase activity was detected with the QuantaBlu[™] Fluorogenic peroxidase substrate kit (Thermo Scientific, Rockford, USA), which is a fluorogenic substrate. Fluorescence was determined with the Ascent Fluoroscanner (Thermo Scientific, Rockford, USA) 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.

Incubation conditions for Luciferin-IPA in minipig liver microsomes. The kinetic studies were performed in non-treated NuncTM F96 MicrowellTM white Polystyrene plates (236205, Thermo Fisher Scientific, Rockford). 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-GloTM CYP3A4 Assay, V9001, Promega Corporation, Madison, USA) was generated from eight substrate concentration points (0.3125 – 40 µM). The estimated K_m was 3.66 ± 0.71 µM. To prevent substrate inhibition in the lowest age groups and to favour biotransformation of Luciferin-IPA by the porcine ‘CYP3A4 homologue’, 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.

In each incubation well, 1 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, NY, USA) and 1 µM Luciferin-IPA were co-incubated. The total incubation volume was 50 µL per well. Microsomal dilutions in 0.1 M KPO₄ buffer were pre-incubated at room temperature (RT) for 10 min. The Luciferin-IPA and the NADPH-regenerating system in KPO₄ 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, Madison, USA) 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, Madison, USA). 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, MA, USA) and HLM (HMMC-H3A4-PL040, Life Technologies, Thermo Fisher Scientific, MA, USA) were used as a positive control. Insect cell control supersomes (456201, Corning Incorporated, NY, USA), 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 MLM. Results from the Insect cell control supersomes were subtracted from the values obtained for the MLM, CYP3A4 Baculosomes[®] and HLM. 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.

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) were pre-incubated with a range of 8 CYP3cide [20] concentrations (0 – 1 µM) (PZ0195, Sigma-Aldrich, St. Louis, MO 63103, USA) 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 NuncTM F96 MicrowellTM 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.

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) were co-incubated with a range of 7 testosterone concentrations (0 – 50 µM) (T037, Sigma-Aldrich, St. Louis, MO 63103, USA), 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, St. Louis, MO 63103, USA) 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 NuncTM F96 MicrowellTM 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.

Mathematical and statistical analysis. For the ELISA-test, calibration curves were fitted and data were analyzed using the Masterplex[®] Readerfit 2010 software (Hitachi, San Francisco, CA, USA). The Kruskal-Wallis (Mean Rank) test was used to detect age- and gender-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 MLM was performed by a nonlinear regression analysis in GraphPad Prism Version 6.0 f (GraphPad Software, Inc., La Jolla, USA). Estimation of IC₅₀ values was performed by a non-linear regression analysis with a four-parameter logistic curve in GraphPad Prism Version 6.0 f (GraphPad Software, Inc., La Jolla, USA). Calculation of velocities of formation of D-Luciferin was performed in Microsoft Excel[®] (Version 14.3.1, Microsoft Corporation, Redmond, WA, USA). 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 genders. 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, USA). 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., La Jolla, USA).

Results

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. All 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 1), being significantly higher than in PND28 animals ($p = 0.001$). No gender-related differences were observed among PND 28 and adult animals ($p = 0.935$) (Figure 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.

Incubations with Luciferin-IPA. A statistically significant difference in CYP 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 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 $\pm$ 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 gender-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.

Inhibition of biotransformation of Luciferin-IPA with CYP3cide. CYP3cide inhibited biotransformation of Luciferin-IPA in both HLM and MLM from an adult sow. Metabolism of Luciferin-IPA in HLM was already inhibited for 90% at 0.03125 μ M CYP3cide, whereas this concentration only caused 7.4% inhibition in MLM. However, 0.125 and 1 μ M CYP3cide caused 47 and 92% inhibition in MLM, respectively (Figure 3).

Co-incubation of Luciferin-IPA with testosterone, midazolam or ketoconazole. Testosterone clearly showed a dose-related inhibition of D-Luciferin formation in the MLM, which was less pronounced in the HLM, whereas midazolam and ketoconazole resulted in an inhibition of the formation of D-Luciferin in both MLM and HLM (Figure 4).

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 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, which was originally developed to quantify human CYP3A4 [19]. Since cross-reactivity of the primary antibody with CYP3A5 and CYP3A7 was not evaluated, we consider these results as abundances of CYP3A isoforms in general. The obtained data are semi-quantitative and show abundances relative to the abundance of CYP3A in humans since no evidence exists that porcine CYP3A and human CYP3A4 bind in an identical way with the primary antibody. However, the amino acid sequences of CYP3A in Göttingen minipigs and in humans are highly similar, 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 the 'foetal' CYP3A7 isoform remains the dominant CYP3A enzyme until one year of age [2]. 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-homologue. The absence of a CYP3A7-homologue in the Göttingen minipig is unlikely as Hermann et al. detected some CYP3A7-like enzyme in foetuses at 100 days of gestation by Western Blotting. They also found a 9-fold higher CYP3A29 mRNA expression in the Day 1 animals compared to the foetuses [25].

We assessed the CYP3A activity by incubation of MLM with Luciferin-IPA. In humans, Luciferin-IPA is considered a highly specific substrate for CYP3A, with higher enzymatic efficiency for CYP3A4 compared to CYP3A7 and CYP3A5 [18, 26]. To promote binding of Luciferin-IPA to the minipig 'CYP3A4-homologue', the concentration of Luciferin-IPA in the incubation mixtures (1 μ M) was chosen below the estimated K_m (3.659 μ M). We showed that Luciferin-IPA was extensively metabolized by the MLM, supporting CYP3A activity. Additionally, pre-incubation of MLM with CYP3cide, which is a mechanism-based inactivator of CYP3A4, inhibited the metabolism of Luciferin-IPA [20]. This result was further substantiated by the inhibition of D-luciferin formation by testosterone, midazolam and ketoconazole. Ketoconazole is a typical CYP3A inhibitor and inhibits 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 MLM. Testosterone and midazolam are two commonly used CYP3A4 substrates in humans, and also in pigs they are metabolized by CYP3A [13-16, 28-30]. Co-incubation of Luciferin-IPA with testosterone or midazolam in the MLM, but not in the HLM, resulted in a reduction of the biotransformation of Luciferin-IPA. 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 6 β -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 MLM, although testosterone is a well-characterized CYP3A4 substrate in human beings.

The results from the biotransformation of Luciferin-IPA among different age groups reflected the results obtained by ELISA. This age-related pattern was observed for the metabolism of midazolam in male Camborough-29 pigs as well. The midazolam 1'-hydroxylation was very low at Day 1, was 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 (6 β -hydroxylation of testosterone) in the foetal liver, which increased to 30-40% and 100% of adult levels at one month and one year of age, respectively [5]. Blanco et al. studied midazolam as a substrate for CYP3A4/3A5 in HLM, but could not detect age-related differences from 0.5 years onwards [32]. These adult levels of CYP3A activity at one year of age were not detected by Stevens et al., who showed that CYP3A4 activity in children between 5 and 15 years was 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 gender 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 statistically 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 *in vitro* and *in vivo*, with a higher CYP3A4 activity in females compared to males [35-39]. The clinical relevance of this difference in CYP3A activity 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 humans. Shimada et al. found no gender-related differences in CYP3A abundance and activity in the liver microsomes from 30 Japanese and Caucasians, 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 6 β -hydroxylation ($r^2 = 0.724$) and for bufalin 5 β -hydroxylation ($r = 0.943$) [8, 9].

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. 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 MLM despite similar abundances. However, the biotransformation of Luciferin-IPA, the inhibitory potential of CYP3C and ketoconazole and the

reduction of Luciferin-IPA biotransformation by testosterone or midazolam in this study support the hypothesis that porcine CYP3A homologues 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.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1. Abundance of CYP3A in the liver of the Göttingen minipig.

Age	Gender	Number of	CYP3A abundance
		animals	Mean $\pm$ S.D.
Day 7	Female	N = 4	Below LLOQ
	Male	N = 3	4.07 $\pm$ 0.9
		N = 2	Below LLOQ
Day 28	Female	N = 5	19 $\pm$ 12.1
	Male	N = 5	20.8 $\pm$ 11.3
	Total	N = 10	19.9 $\pm$ 11.1
Adult	Female	N = 5	74.1 $\pm$ 29.5
	Male	N = 4	66.5 $\pm$ 17.3
	Total	N = 9	70.7 $\pm$ 23.8

Results from ELISA are presented as mean $\pm$ standard deviation ($\pm$ S.D.) and are expressed in ‘pmol/mg of microsomal protein’. These results are relative values, based on the signal to human CYP3A4. All foetal (84-86 and 108 days of gestation), PND1 and PND3 samples were below the LLOQ with 4 females and 4 males in each age group.

LLOQ, lower limit of quantification.

Figure 1. Gender-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 $\pm$ 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 gender-related differences in CYP3A abundance or activity were observed.

Figure 2. Formation of D-Luciferin in pmol/min/mg MP. The mean value of three replicates for each animal is represented by a dot. Upper and lower horizontal dotted lines represent the lower limit of quantification (LLOQ) and the lower limit of detection, respectively.

* Age groups differ significantly (only values above the LLOQ were used for statistical analysis): PND 7 and PND 28 ($p = 0.006$); PND 7 and adult ($p = 0.003$); PND 28 and adult ($p = 0.0003$)

Figure 3. The effect of various concentrations of CYP3cide on the biotransformation of Luciferin-IPA. Bullets represent percentage ratios of reaction velocity (incubations in duplicate) with pre-incubation of CYP3cide, divided by the control velocity without CYP3cide.

Figure 4. The effect of co-incubation of various concentrations of testosterone, midazolam or ketoconazole on the biotransformation of Luciferin-IPA. Bullets represent percentage ratios of reaction velocity (incubations in duplicate) with co-incubation of TST, MDZ or KCZ, divided by the control velocity without TST, MDZ or KCZ. In the upper (TST), middle (MDZ) and lower (KCZ) graphs, the IC_{50} values and their 95% confidence intervals are added.

Figure 5. 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.