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Gestational and Pregnane X Receptor mediated regulation of placental ABC drug transporters in mice.

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Running title: Impact of PXR on placental ABC drug transporters.

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List of non-standard abbreviations:

ABC : ATP Binding Cassette

PXR: Pregnane X Receptor

+/+ : homozygous wildtype animal

-/- : homozygous knockout animal

+/- : heterozygous knockout animal

GD: Gestational Day

PXR.1: Full length PXR

PXR.2: PXR splice variant

Abstract:

The ABC drug transporters in the placenta are involved in controlling the exchange of endogenous and exogenous moieties. PXR is a nuclear receptor which regulates the hepatic expression of several key ABC transporters but it is unclear whether PXR is involved in the regulation of these transporters in the placenta. This study explores the role of PXR in the regulation of placental drug transporters. The placental expression of *Mdr1a, Bcrp, Mrp1, 2* and *3* was examined in PXR knockouts (-/-), heterozygote (+/-) and wildtype (+/+) mice by quantitative PCR. The impact of PXR activation was examined in pregnant PCN-treated mice. As compared to controls, the basal expression of *Mdr1a, Bcrp, Mrp1, 2* was significantly higher in (+/-) and (-/-) mice. Alterations in the expression of mdr1a, bcrp and mrp1-3 between GD 10 and GD 17 was dissimilar between +/+ and -/- mice. While PCN treatment induced maternal and fetal hepatic expression of *Cyp3a11*; placental expression of transporters were not significantly changed. Overall, our results suggest a repressive role of PXR in the basal expression of several placental transporters and a tissue-specific induction of these target genes after PXR activation.

Introduction :

With an ever-increasing number of pregnant women receiving some medication, there is growing concern over drug safety and fetal outcomes. There is surprisingly scarce data on the disposition of drugs in pregnant women and the fetus. It is vital to understand the factors controlling fetal drug exposure and the single most important of these factors is the placenta. The placenta constitutes the main link between the maternal and the fetal systems during gestation. In addition to normal physiological functions, the placenta shields the fetus from toxins and other deleterious entities in the maternal blood. These functions are primarily performed by placental trophoblasts, originating from the fetal unit, expressing a number of key drug transporters (Vasiliou et al.,2009;Unadkat et al.,2004;Vähäkangas, & Myllynen,2009;Kolwankar et al.,2005). Alterations in transporter expression at the maternal-fetal interface can significantly influence exposure to many clinically important drug substrates resulting in therapeutic failure or detrimental effects to the fetus. Hence it is imperative to examine the impact of physiological and environmental influences on the regulation of placental transporters. A better understanding of these mechanisms may allow us to predict dietary, drug-drug or drug-disease interactions which contribute to altered fetal exposure or teratogenic risk during pregnancy.

The ATP-binding cassette (ABC) family of drug transporters, in particular, P-glycoprotein (PGP), Multidrug Resistance Associated Proteins (MRPs) and the Breast Cancer Resistance Protein (BCRP), are believed to perform an important aspect of this protective function by profoundly limiting the passage of xenobiotics into the fetal system. PGP and BCRP are expressed at the placental apical surface (Mao,2008;Ceckova-Novotna et al.,2006) along with MRP2 . MRP3 is believed to be expressed at the luminal side of the fetal capillaries. There is no consensus on the location of MRP1 in the placenta and this transporter has been reported on both

the apical and basolateral sides of the human placenta (Vähäkangas, & Myllynen,2009;Nagashige et al.,2003;StPierre et al.,2000). Fundamental studies establishing the importance of placental drug transporters have demonstrated increased fetal substrate accumulation and teratogenicity in PGP deficient mice (Lankas et al.,1998). Likewise, fetal exposure of the BCRP substrate glyburide is increased by two fold in BCRP deficient mice (Zhou et al.,2008). Downregulation of BCRP in placenta of endotoxin-treated rats has also been shown to significantly increase fetal exposure to glyburide (Petrovic et al.,2008). Thus, alterations in the expression of placental drug transporters can significantly impact fetal exposure and safety.

Pregnane X Receptor (PXR), a member of the superfamily nuclear receptor transcription factors, is believed to play a key role in the regulation of several major drug transporters and drug metabolizing enzymes in response to environmental factors (Cheng, & Klaassen,2006). PXR is activated by numerous clinically important xenobiotics, herbal products as well as endogenous steroids (Chang, & Waxman,2006). Indeed, largely due to increasing hormonal levels, the hepatic expression of PXR increases by about 20-fold during the perinatal period in mice (Masuyama et al.,2001). PXR-mediated induction of Cyp3a and several key ABC drug efflux transporters in liver such as *Mdr1, Mrp2, Mrp3 and Bcrp* has been demonstrated (Teng et al.,2003;Kliewer et al.,2002;Guo et al.,2002;Anapolsky et al.,2006). Likewise, PXR activation has been found to induce expression and activity of PGP at the blood brain barrier (Bauer et al.,2004;Bauer et al.,2006). This regulation may be tissue specific as PXR activation in mice has been reported to induce expression of target genes such as *Cyp3a11* in the liver and intestine but not in kidney (Cheng, & Klaassen,2006). However, the involvement of PXR in the regulation of placental drug transporters has not been established. Hence, given the importance of placental

ABC drug transporter expression on fetal safety and the potential impact of PXR activators on the expression of these transporters, the objective of this study was to elucidate the role of PXR in the regulation of several key ABC efflux transporters in placenta. This was achieved by examining the impact of PXR on the basal and inducible expression of drug transporters in the placenta and maternal liver of PXR wild type and knockout mice. Results from this study suggest a tissue specific role for gene regulation by PXR. The typical inductive effect seen in the hepatic tissues with PXR is absent in the placenta, and silencing of PXR in knockout mice leads to an elevated expression of major ABC drug transporters. These are novel findings underpinning the complex and varied way in which PXR influences its target genes in different tissues.

Materials and Methods:

Animals: Animal studies were conducted at the Division of Comparative Medicine, University of Toronto following protocols approved by the Animal Care Committee and conformed to the NIH "Principles of Laboratory Animal Care". PXR wildtype (+/+) C57/BL6 mice were purchased from Charles River Canada (Montreal, PQ). The PXR knockout (-/-) C57/BL6 mice were obtained with approval from Dr. Steven Kliewer (University of Texas, Southwestern Medical Center, Dallas, TX). PXR heterozygote (+/-) placentas were obtained by mating PXR -/- females with PXR +/+ males while the PXR wildtype (+/+) and knockout (-/-) placentas were obtained by mating homozygous parents.

For the purpose of obtaining timed pregnancies, the mice were paired overnight and the male removed the following morning. For the basal expression studies, the animals were sacrificed on gestational day (GD) 10 or GD 17 (corresponding roughly to mid-second trimester and late third trimester in human terms respectively), the placentas harvested, snap frozen in liquid nitrogen and stored at -80° C .

To elucidate the role of PXR on placental gene regulation, animals were dosed (50mg/ kg i.p.) for five days with the murine PXR activator, Pregnane-16alpha–carbonitrile (PCN; Sigma-Aldrich, Oakville, ON) as previously described (Teng, & Piquette-Miller,2005). Controls were administered corn oil vehicle. The treatment was started at GD 13 and the mice were sacrificed on GD 17. All tissues were snap frozen in liquid nitrogen, followed by storage at -80° C until analysis.

mRNA Determination: As previously described, total RNA extraction, RT and realtime PCR was conducted on the samples (Teng, & Piquette-Miller,2005). Total RNA extraction was

achieved using the QuickPrep RNA extraction kit supplied by Amersham Biosciences Inc. (Piscataway, NJ). cDNA was prepared from 1ug of RNA from each sample employing the First Strand cDNA synthesis kit (Fermentas, ON, Canada). Quantification of *Mrp1-3, Bcrp, Mdr1a, Oatp2 (Slco1a4), Pxr1, Pxr2, Cyp2b10 and Car* mRNA were carried out by real-time quantitative PCR using the LightCycler unit (Roche Diagnostics, Mannheim, Germany) with LC FastStart DNA Master SYBR Green I. All transcript levels were normalized to house keeping genes (*Gapdh & 18S*). We found no statistical difference in *Gapdh* transcript levels in the placentas of either genotype showing it to be a suitable normalizing gene. To minimize run variations, all samples were run concurrently. As the mRNA levels of *Mrp1* were below detection limits for the majority of the samples, this data is not shown. Primers used for *Mrp1-3, Bcrp, Mdr1a, Gapdh and Oatp2* have been previously described (Teng, & Piquette-Miller,2005) (supplemental Table 1).

Statistical Analysis: All studies were performed using n=6-8 mice per group with the exception of basal liver expression study which had 4 animals per group. Differences in the expression of key transporters between the three genotypes were determined by carrying out analysis of variance (ANOVA) with Tukey post hoc test and $p<0.05$ was considered significant. To assess the difference between the treatment and control groups in the PCN study, two tailed t-test was conducted and results were reported to be significant when $p<0.05$. Performing the non-parametric Man-Whitney U test yielded similar results. All statistics were carried out using GraphPad Prism v 4.0c (GraphPad Software, San Diego, CA.).

Results:

Impact of PXR on basal expression of placental transporters:

To examine the involvement of PXR on the basal expression of placental transporters near full term under normal physiological conditions, mRNA levels were measured in placentas isolated from PXR (+/+), (+/-) and (-/-) fetuses on GD 17. As illustrated in figure 1a, dramatic differences in the placental expression of several ABC drug transporters were detected between genotypes. Indeed, *Mdr1a, Bcrp*, *Mrp2 and Mrp3* mRNA levels in the knockouts were approximately 12-fold, 10-fold, 7-fold and 2-fold higher in the placenta of the PXR knockout mice as compared to values obtained in the wildtype ($p<0.05$). Interestingly, we observed intermediary transcript levels in PXR +/- placentas, thus showing a clear gradient in expression level in the three genotypes, akin to a dose related effect of PXR. As confirmation of reduced PXR expression, we measured the mRNA level of PXR in the +/- placental samples and found a 37% reduction in transcript level compared to the +/+ mice ($p<0.05$) as seen in figure 1 b. *Pxr* transcripts were not detected in the -/- placentas.

In order to establish whether the genotypic differences were pregnancy related, their expression were also examined in the hepatic tissues of the PXR -/- and +/+ mice at GD 17 and in non pregnant female mice (Figure 1 c). Non pregnant PXR -/- mice had 3.6, 3.2 , 3.9 and 1.9-fold higher transcript levels of *Cyp3a11* ($p<0.01$), *Mdr1a, Mrp2* ($p<0.05$) and *Mrp3* ($p<0.01$) respectively than their +/+ counterparts, while the levels of *Oatp2, Bsep and Ntcp* did not change. In pregnant animals, trends of reduced expression were seen as compared to the non-pregnant animals in both the genotypes for *Cyp3a11, Mrp2, Mrp3*, however the PXR -/- animals maintained higher levels of transcript for the PXR target genes.

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mRNA levels of *Car (*Constitutive Androstane Receptor), an important nuclear receptor which shares many overlapping gene targets with PXR, were not significantly different between PXR +/+ or -/-. Levels of *Hnf4α* mRNA, which is an important regulator of both PXR and CAR, were not significantly different in placentas isolated from PXR +/+ or -/- mice (Figure 2a).

Likewise, hepatic mRNA levels of *Car* were not statistically different between genotypes (Figure 2b). On the other hand, a significant increase in the hepatic expression of *Cyp2b10*, a classic target gene of CAR, was seen in the PXR knockout mice. As depicted in Figure 3, *Cyp2b10* levels in livers obtained from pregnant and non-pregnant PXR -/- mice were 6.8-fold and 2.8-fold higher than those seen in PXR +/+ mice ($p<0.05$), which suggests higher basal CAR activity in the PXR knockout mice.

Dissimilarity in the gestational regulation of placental transporters between wild type and knockouts was also detected. As depicted in Table 1, we observed 18 to19-fold decreases in mRNA levels of *Mrp3* and *Mdr1a* over the course of gestation in the wild type mice. In contrast, a pronounced increase in the expression of these genes was seen in the knockouts ($p<0.05$). Moreover, while mrp2 levels increased during the course of gestation in the placenta of all mice, a more pronounced induction was seen in the PXR (-/-) placentas as compared to wild type. While the placental expression of *Bcrp* decreased over the course of gestation in both genotypes, levels of *Bcrp* mRNA remained 4 to 5-fold higher in the placenta of the PXR (-/-) mice. Thus, in the absence of PXR, several of the major ABC drug transporters exhibit elevated expression levels relative to wild type mice over the course of gestation.

Impact of PXR Activation by PCN:

While it is well established that PXR activation plays an important role in the induction of *Cyp3a11* and several drug transporters in the liver, whether this influence extends to the placental transporters is unknown. Overall PCN treatment did not have a significant impact on expression of the ABC drug transporters in the placentas of PXR (+/+) wild type mice (Figure 4 a). Although there was a trend of higher levels of *Mrp2* in the PCN-treated mice, this failed to achieve a level of significance. Attempts to detect changes in placental *Cyp3a11* were unsuccessful as levels were below detection limit. However, we observed a 30 fold induction of *Cyp3a11* transcripts in the livers of the PCN-treated wild type mice confirming PXR activation with the PCN treatment protocol (*Fig 4 b*). PCN treatment did not significantly alter the expression of any placental transporters or hepatic *Cyp3a11* in PXR -/- animals ***(****Fig 4 c****)*** **.**To confirm that the PCN did in fact reach the placenta at sufficiently high concentration to activate PXR, we analysed the impact of PCN administration in the livers of fetuses from the treated and control PXR (+/+) mice. As compared to controls, a significant 1.75 to 2 fold induction of *Cyp3a11* and *Oatp2* mRNA levels were seen in fetal livers isolated from the PCN treatment group (Figure 5 a), providing evidence of PCN-mediated activation of PXR in the placental-fetal unit. Figure 5 b shows the absolute *Cyp3a11* basal transcript levels seen in the fetal and maternal liver in PXR +/+ animals at GD 17 .

PXR variants: In light of recent reports of opposing effects of PXR isoforms on their target genes, we examined the relative abundance of the full length active transcript *Pxr.1* and the major inhibitory isoform *Pxr.2* in the liver and the placentas of pregnant wild type mice at GD 17. No significant differences in the relative abundance of these isoforms were seen between placental and hepatic tissues (Figure 6 a). The full length transcript accounted for 80% and 84% of total *Pxr* in the liver and placenta, respectively.

Discussion:

To date, much research has demonstrated that ABC transporters play an important role in the pharmacokinetics and pharmacodynamics of many drugs. Depending upon their localization, alterations in the expression and activity of these transporters can result in therapeutic failure or toxicity. Of particular interest is the impact of ABC drug transporters in modulating transplacental transfer of their substrates. The placenta is an important barrier sequestering the fetus from xenobiotics circulating in the maternal system. Transporter expression and function in the placenta is of importance in determining the extent of fetal exposure to many clinically important xenobiotics. While early studies in the field have clearly demonstrated the crucial importance of the ABC transporters at the placental surface, data on the factors influencing their placental expression is limited. Many environmental factors exert their influence on these transporters through activation of nuclear receptors such as PXR. Interestingly, while the levels of PXR have been demonstrated to be elevated in the liver and ovaries during gestation, they remain relatively static in the placenta. While PXR activation has been shown to cause induction of several ABC drug transporters in the liver and in other tissues, such as the blood brain barrier and intestine, its influence on the placental drug transporters has not been examined to date.

Our results indicate that the PXR genotype had a great impact on the expression of several key ABC drug transporters in the placenta. As compared to wild-type mice with fully functional PXR, there was a pronounced increase in the expression of *Mdr1a, Bcrp, Mrp2* and *Mrp3* in the full term placenta of PXR null mice, with PXR heterozygote placentas expressing intermediate levels. Similar differences in the basal expression of PXR target genes were seen in hepatic tissues (Fig 1.b). Marked increases in the mRNA levels of *Mdr1a, Cyp3a11, Mrp2 & 3* were observed in the livers of the knockout mice as compared to the PXR +/+. This induction was

present regardless of the animals being pregnant or not, thus ruling out an elevated response in either genotype due to greatly increased circulating hormones seen in pregnancy. It is indeed very important to bear in mind that while significant changes in expression levels were seen with established PXR targets such as *Cyp3a11* and *Mrp* 2&3, increased basal expression was not detected for genes which are under predominant control of other nuclear receptors such as *Ntcp* (under RAR α control) and *Bsep* (under FXR control). Previous studies have also reported higher expression of *Mdr1a* and *Mrp2* in the liver and intestine of PXR null mice (Teng et al.,2003;Kliewer et al.,2002).

It is plausible that over-compensation by other members of the nuclear receptor family could be responsible for the differential expression between genotypes. One member of the nuclear receptor superfamily, the Constitutively Active Receptor (CAR), shares many structural and functional similarities with PXR (di Masi et al.,2009;Timsit, & Negishi,2007;Honkakoski et al.,2003). CAR and PXR each hetero-dimerize with Retinoid X Receptor (RXR) and have overlapping domains of genes on which they exert control. Indeed, both PXR and CAR have been shown to be involved in the regulation of CYP3A, MDR1, MRP2 and OATP2. Thus, in the absence of PXR, unhindered activity of CAR could lead to our observed increase in basal expression of the placental drug transporters. Moreover, CAR activation could be particularly enhanced as there is a dramatic rise in levels of the CAR activating estrogens during the course of gestation. While we found no significant differences in the transcript levels of CAR in the PXR knockouts in either the placenta or hepatic tissues, we observed significantly higher expression of *Cyp2b10* in the livers of both pregnant and non-pregnant PXR deficient mice. As *Cyp2b10* is primarily activated by CAR, this suggests an elevated basal activity and potential over compensatory role of CAR in the absence of PXR. An enhanced effect on CAR target genes

upon PXR activation has been previously reported in PXR null mice (Staudinger et al., 2003). Reduced competition for RXR, changes in the localization of nuclear transporter and increased availability of endogenous substrates or co-activators could all be putative pathways influencing genes under common control of PXR and CAR.

The impact of PXR activation on the regulation of transporters in placenta was further assessed in pregnant wild type and knockout mice. Activation of PXR has been shown to significantly induce the hepatic expression of *Cyp3a11, Oatp2* and several of the ABC drug transporters including *Mdr1, Mrp2, Mrp3 i*n non-pregnant mice (Anapolsky et al.,2006;Teng, & Piquette-Miller,2005). Likewise, PCN-mediated induction of several PXR gene targets has been reported in the intestine and blood-brain barrier (Cheng, & Klaassen,2006;Bauer et al.,2004). Interestingly, while a pronounced 30-fold elevation in the hepatic expression of *Cyp3a11* was observed in the livers of pregnant PCN-treated mice, confirming PXR activation, we did not observe any significant changes in the expression of the ABC drug transporters in the placenta. This is in stark contrast to the pronounced induction that is seen in the livers of PCN-treated mice (Teng, & Piquette-Miller,2005). The ability of PCN to reach the placenta at high enough concentrations to cause PXR activation was confirmed by the fact that we observed significant PCN-mediated induction of the PXR target genes, *Cyp3a11 and Oatp2*, in fetal livers. The lack of an inductive effect of PXR activation on the placental expression of several transporters, which are known to be regulated by PXR, points to a tissue specific role of the nuclear receptor. It is plausible that PXR-mediated regulatory effects may be different in placenta as alternative signaling pathways may be activated due to tissue-specific differences in nuclear receptor expression. There are numerous reports of dramatic, 20 fold increases in the levels of PXR transcripts in the liver of pregnant animals at full term. Indeed *Pxr* levels were 5 fold higher in

maternal liver as compared to the placenta in wild type mice on GD 17 (Fig 6 b). Therefore relative expression of PXR in placenta, as compared to liver could play a role in tissue differences in the regulation of target genes. On the other hand, tissue specific differences in endogenous substances, transcription co-factors or alternate isoforms could also be involved.

Tissue-specific differences in the relative expression of PXR isoforms in placental versus hepatic tissues were examined as recent studies have identified a number of PXR transcript splice variants which impose functional changes in gene target response (Lamba et al.,2005). PXR variants are by no means a rarity, and have been documented to constitute almost one third of all expressed PXR, with 15 isoforms reported in humans. Recent studies have begun to uncover the importance of looking at these variants, with two studies showing the repressive function of the main human isoform (PXR2) and its mouse counterpart (mPXR2). While both PXR 1 and PXR 2 bind to the same response element in target genes such as Cyp3a, PXR 2 has been found to be less flexible in its activation profile. More importantly, PXR2 has been found to repress the basal expression of CYP3a and MDR1. While PXR.1 is an inducer of genes such as Cyp3a, the PXR.2 isoform has been shown to have a dose-dependent suppressive effect on the basal expression of Cyp3a and Mdr1 (Lin et al.,2009;Matic et al.,2010). The precise function of this major PXR variant remains to be elucidated. Whereas a greater ratio of PXR.2 to PXR.1 in placenta could lead to changes in gene regulation after PXR activation, we found a similar relative abundance of the two isoforms in hepatic and placental tissues. Although this ruled out a potential dominant effect of PXR.2 in placenta, it is still plausible that other transcript variants could have a dominant suppressive role in the placenta. Alternatively, pregnancy related changes in systemic levels of steroidal hormones, which are known activators of several nuclear receptors including PXR and CAR, could dramatically alter gene regulation via PXR-mediated pathways,

particularly in hormone-sensitive tissues such as the placenta. Specifically, the nuclear receptors may be suppressed or maximally activated by pregnancy related hormones.

As reported by others, our results demonstrated both pregnancy-related and gestational-related changes in the expression of drug transporters and Cyp3a enzymes. Hepatic expression of *Cyp3a11* have been shown to be reduced during pregnancy, a trend clearly seen in our wild type animals. Similarly, *Mrp3* levels are shown to be reduced in pregnancy, as seen in our data. However, we failed to see a down regulation in the levels of *Oatp2 and Mrp2* while it has been reported by other groups in pregnant rats. This could be due to species specific differences in regulatory pathways. Temporal changes in concentrations of progesterones (endogenous activators of PXR) and estrogens (endogenous activators of CAR) over the course of pregnancy could explain differences in the gestational-mediated changes of gene targets, such as the transporters, in the PXR genotypes. Indeed, although many target genes were below detection limit on GD10, changes in transporter expression with gestation progression to full term were markedly different in placentas isolated from PXR wild type or null mice. As compared to PXR wild type, a pronounced increase in the induction of *Mdr1a, Mrp2 and Mrp3* was seen in PXR null mice from GD10 (mid-gestation) to GD17 (near term). Moreover, levels of *Bcrp* remained much higher in the PXR (-/-) as compared to wild type. While studies looking at temporal changes in human placenta are rare, they have demonstrated significant differences in their expression throughout pregnancy. Similar to what we observed in the PXR (+/+) mice, reports indicate that the expression of MDR1 decreases at late gestation in human placenta (Sun et al.,2006). On the other hand, while we observed a decreased placental expression of *Bcrp* at full term, the placental expression of is increased in later gestational stages in humans (Yeboah et al.,2006). Our findings are in agreement with previously reported trend seen in rodent placenta

by a number of recent studies (Cygalova L et al., 2008; Kalabis et al., 2007; Yasuda S et al., 2005). The incongruity between the human and rodent expression levels can be due to a number of species-specific factors. While BCRP is expressed abundantly in humans and rodents, the expression profiles differ with humans having the highest levels in the placenta while in mice it is most abundant in the kidney. Additionally, in rodents, almost in sync with the falling placental levels of *bcrp,* there is seen an increase in the levels in the embryonic tissues (Hahnova-Cygalova L et al., 2010; Cygalova L et al., 2008). This could point to the waning reliance of the fetus on the placenta to sequester the fetus from xenobiotics, and explain the falling levels of placental levels as term approaches. Unfortunately, no corresponding human data are available and the exact reasons for such discrepancies need to be further explored.

As variation in placental transporters is clinically important, findings in the animal models are important given the similarity in both the type of placentas and the expression profiles of important drug transporters. Both the human and murine placentas are of the hemochorial type, in which the maternal blood is in direct contact with the placental (and hence fetal) trophoblast cells. Looking at the expression levels and temporal trends of major transporters, a lot of similarities exist between rodents and human. Whether, the similarity in transporter expression and placental structure translate into a corresponding impact on function is not well known.

Conclusion:

In a clinical setting, expectant mothers consume a wide array of xenobiotics. Recent studies have found that more than one third of all pregnant women consume at least one medication throughout the gestational period (Glover et al.,2003). A number of these agents are potential activators of PXR, an important transcription factor involved in the regulation of many hepatic

drug metabolizing enzymes and transporters. It thus becomes extremely important to better understand the influence PXR exerts over the ABC transporter expression as any alteration in the expression level can alter the fetal exposure to xenobiotics. Our results demonstrate a tissue-specific role of PXR in the regulation of the ABC-drug transporters. While in the liver, intestine and the blood brain barrier, PXR has been demonstrated to mediate the induction of the major ABC drug transporter genes, this inductive influence is absent in the placenta. Additionally, a possible repressive role for this nuclear receptor has also been uncovered, as illustrated by the elevated transporter expression in the knockout mice. Overall, the study clearly shows how little is still understood about the contrasting role that nuclear receptors such as PXR can play in modulating transporter expression in different tissues and highlights the need for further study in the field.

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Authorship Contribution:

Sarabjit Gahir: This author was involved in research design, conducting experiments, analysing data and manuscript preparation.

Dr. Micheline Piquette-Miller: This author is the corresponding author and was involved in research design, analysing data and manuscript preparation.

References:

Anapolsky A, Teng S, Dixit S, and Piquette-Miller M (2006) The role of pregnane X receptor in 2-acetylaminofluorene-mediated induction of drug transport and -metabolizing enzymes in mice. *Drug Metab Dispos***34:**405-9.

Bauer B, Hartz AM, Fricker G, and Miller DS (2004) Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood-brain barrier. *Mol Pharmacol***66:**413-9.

Bauer B, Yang X, Hartz AM, Olson ER, Zhao R, Kalvass JC, Pollack GM, and Miller DS (2006) In vivo activation of human pregnane X receptor tightens the blood-brain barrier to methadone through P-glycoprotein up-regulation. *Mol Pharmacol***70:**1212-9.

Ceckova-Novotna M, Pavek P, and Staud F (2006) P-glycoprotein in the placenta: expression, localization, regulation and function. *Reprod Toxicol***22:**400-10.

Chang TK, and Waxman DJ (2006) Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metab Rev***38:**51-73.

Cheng X, and Klaassen CD (2006) Regulation of mRNA expression of xenobiotic transporters by the pregnane x receptor in mouse liver, kidney, and intestine. *Drug Metab Dispos***34:**1863-7.

Cygalova L, Ceckova M, Pavek P, and Staud F (2008) Role of breast cancer resistance

protein (Bcrp/Abcg2) in fetal protection during gestation in rat. *Toxicol Lett*178:176-80**.**

di Masi A, Marinis ED, Ascenzi P, and Marino M (2009) Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Mol Aspects Med***30:**297-343.

Glover DD, Amonkar M, Rybeck BF, and Tracy TS (2003) Prescription, over-the-counter, and herbal medicine use in a rural, obstetric population. *Am J Obstet Gynecol***188:**1039-45.

Guo GL, Staudinger J, Ogura K, and Klaassen CD (2002) Induction of rat organic anion transporting polypeptide 2 by pregnenolone-16alpha-carbonitrile is via interaction with pregnane X receptor. *Mol Pharmacol***61:**832-9.

Hahnova-Cygalova L, Ceckova M, and Staud F (2010) Fetoprotective activity of breast cancer resistance protein (BCRP, ABCG2): expression and function throughout pregnancy. *Drug Metab Rev*

Honkakoski P, Sueyoshi T, and Negishi M (2003) Drug-activated nuclear receptors CAR and PXR. *Ann Med***35:**172-82.

Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JH, and Schinkel AH (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J Natl Cancer Inst***92:**1651-6.

Kalabis GM, Petropoulos S, Gibb W, and Matthews SG (2007) Breast cancer resistance protein (Bcrp1/Abcg2) in mouse placenta and yolk sac: ontogeny and its regulation by

progesterone. *Placenta***28:**1073-81.

Kliewer SA, Goodwin B, and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev***23:**687-702.

Kolwankar D, Glover DD, Ware JA, and Tracy TS (2005) Expression and function of ABCB1 and ABCG2 in human placental tissue. *Drug Metab Dispos***33:**524-9.

Lamba J, Lamba V, and Schuetz E (2005) Genetic variants of PXR (NR1I2) and CAR (NR1I3) and their implications in drug metabolism and pharmacogenetics. *Curr Drug Metab***6:**369-83.

Lankas GR, Wise LD, Cartwright ME, Pippert T, and Umbenhauer DR (1998) Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reprod Toxicol***12:**457-63.

Lin YS, Yasuda K, Assem M, Cline C, Barber J, Li CW, Kholodovych V, Ai N, Chen JD, Welsh WJ, Ekins S, and Schuetz EG (2009) The major human pregnane X receptor (PXR) splice variant, PXR.2, exhibits significantly diminished ligand-activated transcriptional regulation. *Drug Metab Dispos***37:**1295-304.

Mao Q (2008) BCRP/ABCG2 in the placenta: expression, function and regulation. *Pharm Res***25:**1244-55.

Masuyama H, Hiramatsu Y, Mizutani Y, Inoshita H, and Kudo T (2001) The expression of pregnane X receptor and its target gene, cytochrome P450 3A1, in perinatal mouse. *Mol Cell Endocrinol***172:**47-56.

Matic M, Corradin AP, Tsoli M, Clarke SJ, Polly P, and Robertson GR (2010) The alternatively spliced murine pregnane X receptor isoform, mPXR(Delta171-211) exhibits a repressive action. *Int J Biochem Cell Biol***42:**672-82.

Nagashige M, Ushigome F, Koyabu N, Hirata K, Kawabuchi M, Hirakawa T, Satoh S, Tsukimori K, Nakano H, Uchiumi T, Kuwano M, Ohtani H, and Sawada Y (2003) Basal membrane localization of MRP1 in human placental trophoblast. *Placenta***24:**951-8.

Petrovic V, Wang JH, and Piquette-Miller M (2008) Effect of endotoxin on the expression of placental drug transporters and glyburide disposition in pregnant rats. *Drug Metab Dispos***36:**1944-50.

St-Pierre MV, Serrano MA, Macias RI, Dubs U, Hoechli M, Lauper U, Meier PJ, and Marin JJ (2000) Expression of members of the multidrug resistance protein family in human term placenta. *Am J Physiol Regul Integr Comp Physiol***279:**R1495-503.

Sun M, Kingdom J, Baczyk D, Lye SJ, Matthews SG, and Gibb W (2006) Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation. *Placenta***27:**602-9.

Teng S, and Piquette-Miller M (2005) The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. *J Pharmacol Exp Ther***312:**841-8.

Teng S, Jekerle V, and Piquette-Miller M (2003) Induction of ABCC3 (MRP3) by pregnane X receptor activators. *Drug Metab Dispos***31:**1296-9.

Timsit YE, and Negishi M (2007) CAR and PXR: the xenobiotic-sensing receptors.

*Steroids***72:**231-46.

Unadkat JD, Dahlin A, and Vijay S (2004) Placental drug transporters. *Curr Drug Metab***5:**125-31.

Vähäkangas K, and Myllynen P (2009) Drug transporters in the human blood-placental barrier. *Br J Pharmacol***158:**665-78.

Vasiliou V, Vasiliou K, and Nebert DW (2009) Human ATP-binding cassette (ABC) transporter family. *Hum Genomics***3:**281-90.

Yasuda S, Itagaki S, Hirano T, and Iseki K (2005) Expression level of ABCG2 in the placenta decreases from the mid stage to the end of gestation. *Biosci Biotechnol Biochem***69:**1871-6.

Yeboah D, Sun M, Kingdom J, Baczyk D, Lye SJ, Matthews SG, and Gibb W (2006) Expression of breast cancer resistance protein (BCRP/ABCG2) in human placenta throughout gestation and at term before and after labor. *Can J Physiol Pharmacol***84:**1251-8.

Zhou L, Naraharisetti SB, Wang H, Unadkat JD, Hebert MF, and Mao Q (2008) The breast cancer resistance protein (Bcrp1/Abcg2) limits fetal distribution of glyburide in the pregnant mouse: an Obstetric-Fetal Pharmacology Research Unit Network and University of Washington Specialized Center of Research Study. *Mol Pharmacol***73:**949-59.

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Legend to Figures:

Figure 1 a: Basal mRNA expression of key transporters in the placenta of PXR wildtypes (PXR +/+), heterozygote (PXR +/-) and the knockout (PXR -/-) fetuses on GD 17. Levels were normalized to Gapdh and presented as % controls. {* $p<0.05$; ** $p<0.01$}. n=6-8

Figure 1 b: mRNA expression of *Pxr* in wildtype and heterozygote placentas at GD 17. Levels are normalized to Gapdh and presented as % wildtype levels. N= 6-8. . {* $p<0.05$}

Figure 1 c: Basal mRNA expression of key transporters in the liver of pregnant (GD 17) and non pregnant PXR +/+ and -/- mice. Levels were normalized to Gapdh and presented as % WT non-pregnant. {* $p<0.05$; ** $p<0.01$ compared to -/- pregnant , † $p<0.05$; ††$p<0.01$ compared to +/+ non-pregnant }. n=4

Figure 2 a: Expression of *Car* and *Hnf4 alpha* mRNA in the placental tissue of PXR WT and KO mice on GD 17. No statistically significant differences are observed between the two strains. Levels were normalized to Gapdh and presented as % WT. {* $p<0.05$; ** $p<0.01$}. n=6

Figure 2 b: Expression of *Car* mRNA in the maternal hepatic tissues of PXR WT and KO mice on GD 17. No statistically significant differences are observed between the two strains. Levels were normalized to Gapdh and presented as % WT. n=4

Figure 3: The expression of *Cyp2b10* mRNA in the livers of both pregnant (GD 17) and non pregnant PXR +/+ and -/-. Levels were normalized to Gapdh and presented as % controls. {* $p<0.05$; ** $p<0.01$}. n=4

Figure 4 a: Impact of PCN treatment on mRNA levels of ABC transporters in the placenta of PXR +/+ mice treated with 50mg/kg PCN (i.p.) daily for 4 days. Tissues were harvested on 5th day (GD 17) and the tissues analysed as described in the methods. PCN treatment did not significantly impact the placental transporters. Levels were normalized to Gapdh and presented as % controls. {* $p<0.05$; ** $p<0.01$}. n=6-8

Figure 4 b: Impact of PCN treatment on *Cyp3a11* mRNA levels in the maternal liver of PXR +/+ mice treated with 50mg/kg PCN (i.p.) daily for 4 days. Tissues were harvested on 5th day (GD 17) and the tissues analysed as described in the methods. Levels were normalized to Gapdh and presented as % controls. {* $p<0.05$; ** $p<0.01$}. n=4-6

Figure 4 c: Impact of PCN treatment on mRNA levels ABC transporters in the placenta of PXR -/- mice treated with 50mg/kg PCN (i.p.) daily for 4 days. Tissues were harvested on 5th day (GD 17) and the tissues analysed as described in the methods. PCN treatment did not significantly impact the placental transporters. Levels were normalized to Gapdh and presented as % controls. {* $p<0.05$; ** $p<0.01$}. n=6-8

Figure 5 a: Impact of PCN on fetal cyp3a and oatp2 (Slco1a4). Pregnant mice were treated with PCN (i.p.) as per the regimen described in the methods. On GD 17 the animals were sacrificed and tissues harvested. mRNA levels of cyp3a and oatp2 in the fetal liver were assesed using real time PCR as described. Both genes were upregulated in the fetal livers as compared to the controls (cyp3a by 75% and oapt2 by 100%).

Figure 5 b: : Basal mRNA expression of *Cyp3a11* in the liver and placenta of pregnant (GD 17) PXR +/+ mice. Levels were normalized to Gapdh and presented as % WT non-pregnant. n=4-6

Figure 6 a. The ratio of PXR.1 to PXR.2 mRNA in the liver and placental tissues of pregnant PXR +/+ mice on GD 17. The mRNA levels of PXR.1 and PXR.2 were determined by real time PCR. There was seen no significant diffrence in the relative abundance of the two forms of PXR

transcript between the hepatic and placental tissue. Levels were normalized to Gapdh and presented as % controls. {* $p<0.05$; ** $p<0.01$}. n=4-6

Figure 6 b: Basal mRNA expression of *Pxr* in the liver and placenta of pregnant (GD 17) PXR +/+ mice. Levels were normalized to Gapdh and presented as % WT non-pregnant. n=4-6

Tables:

Table 1: Temporal Transporter Expression During Gestation

Gene	Strain	GD 10	GD 17	Change
abc b1a (Mdr1a)	Wildtype	1950	100	↓ 19 fold
	Knockout	bdl	1333	↑
abc c2 (Mrp2)	Wildtype	bdl	100	↑
	Knockout	bdl	320	↑↑
abc c3 (Mrp3)	Wildtype	1800	100	↓ 18 fold
	Knockout	bdl	320	↑
abc g2 (Bcrp)	Wildtype	490	100	↓ 5 fold
	Knockout	1998	427	↓ 5 fold

Table summarizing the overall temporal trends in the placental transporter expression. The transporters were assessed at GD 10 and GD 17. All values normalized to WT levels on GD 17 as % controls.

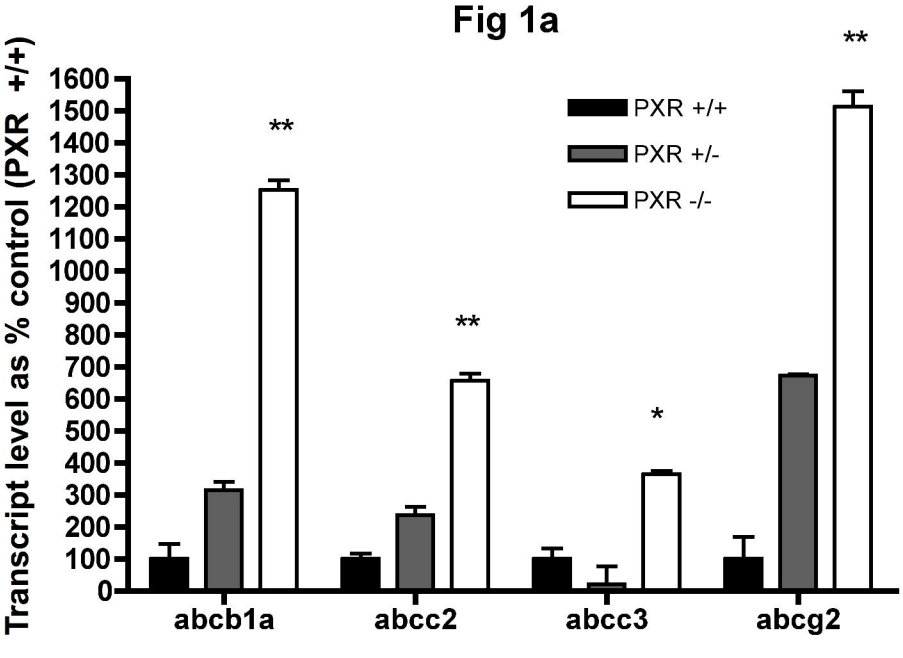
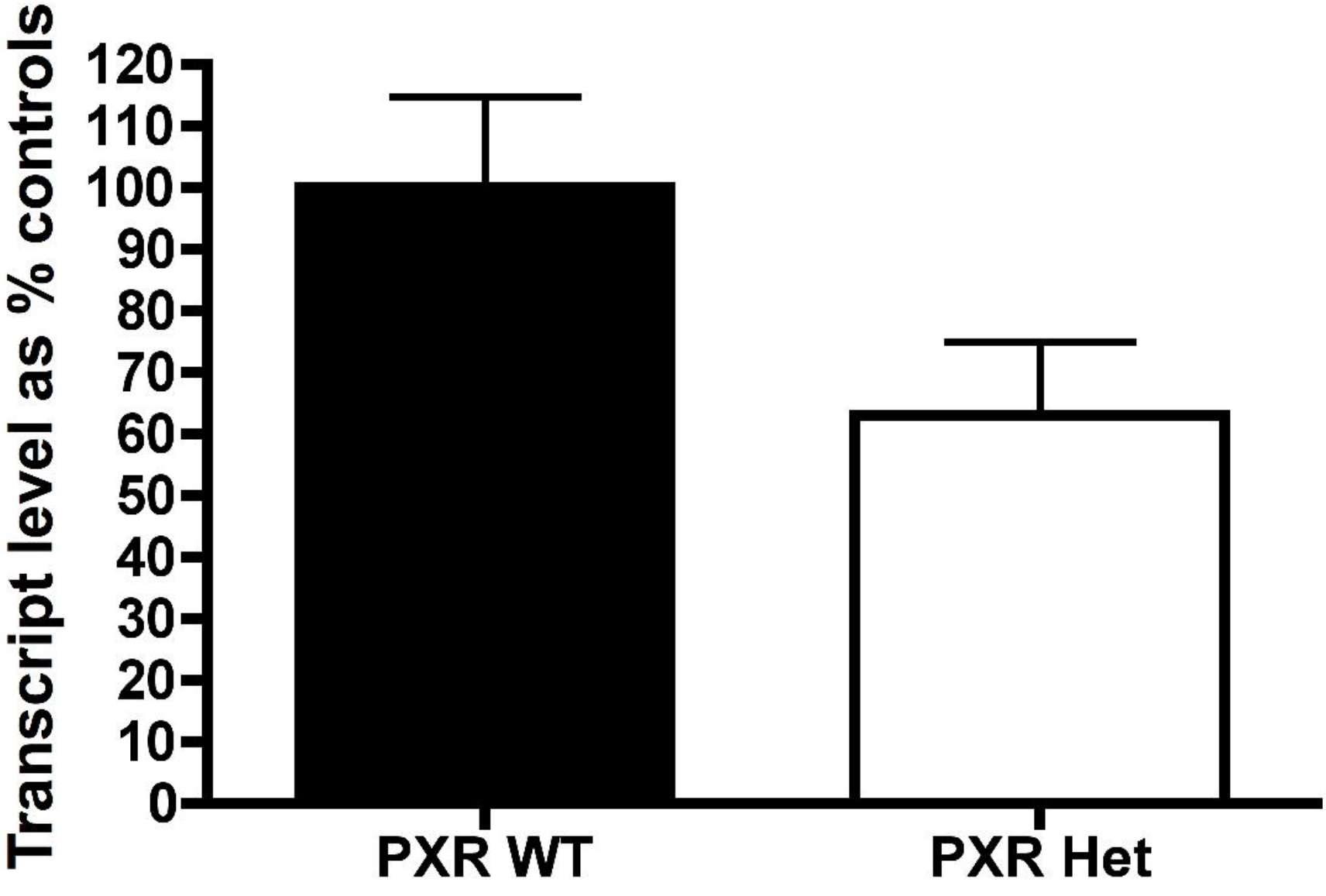


Fig 1a
Transcript level as % control (PXR +/+)
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
PXR +/+
PXR +/-
PXR -/-
**
**
*
**
abcb1a
abcc2
abcc3
abcg2

Fig 1b


Transcript level as % controls
120
110
100
90
80
70
60
50
40
30
20
10
0
PXR WT
PXR Het

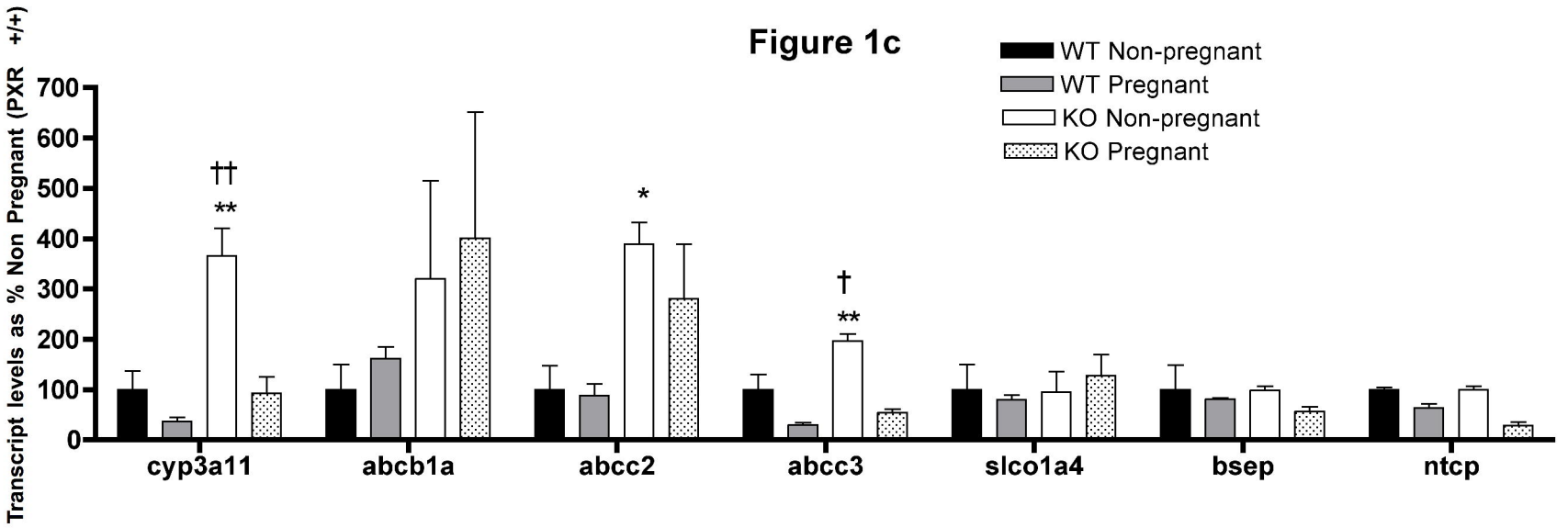


Figure 1c
WT Non-pregnant
WT Pregnant
KO Non-pregnant
KO Pregnant
Transcript levels as % Non Pregnant (PXR +/+)
700
600
500
400
300
200
100
0
††
**
*
†
**
cyp3a11
abcb1a
abcc2
abcc3
slco1a4
bsep
ntcp

Fig 2 a

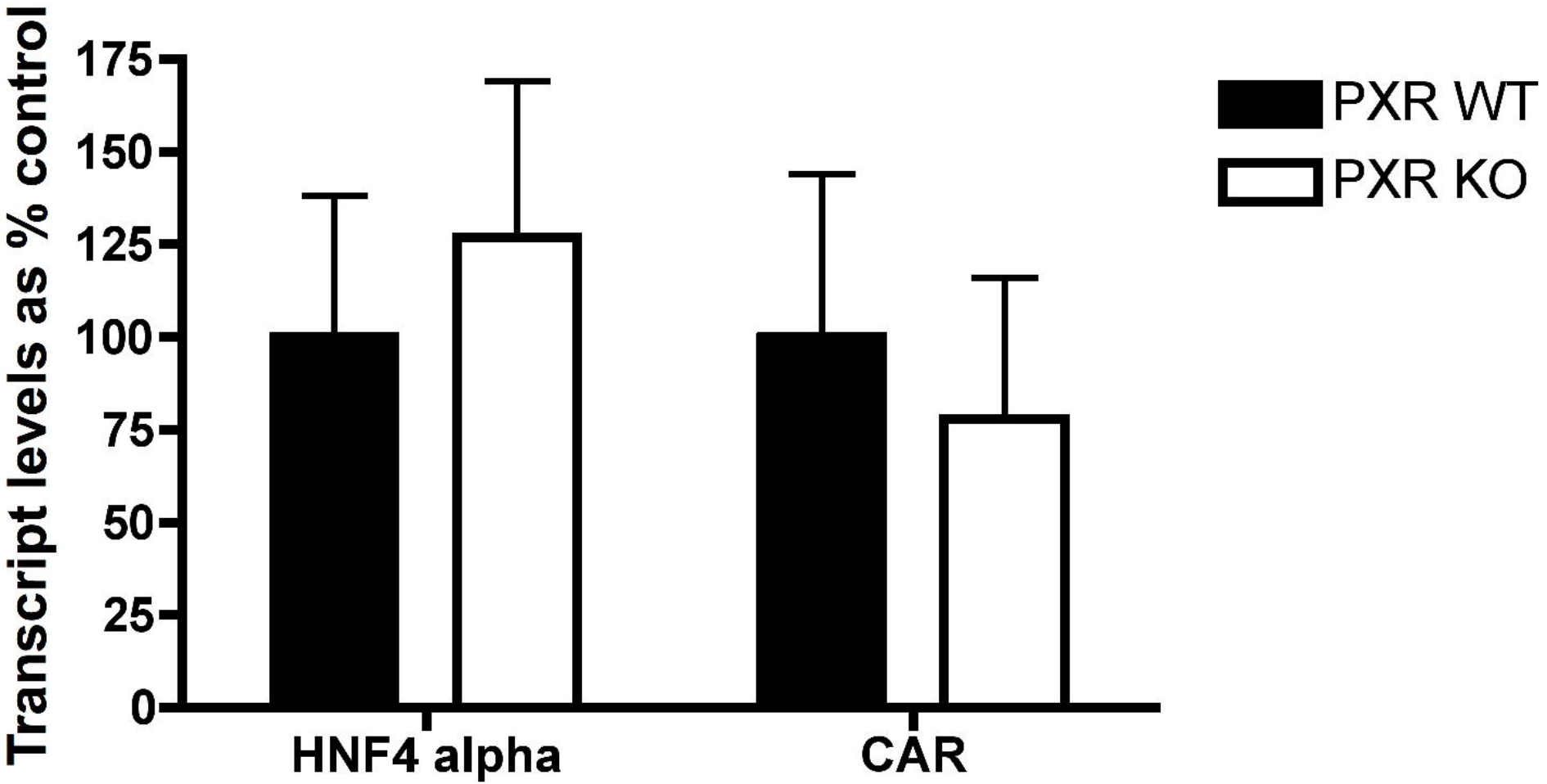

Transcript levels as % control
175
150
125
100
75
50
25
0
HNF4 alpha
CAR
PXR WT
PXR KO

Fig. 2 b

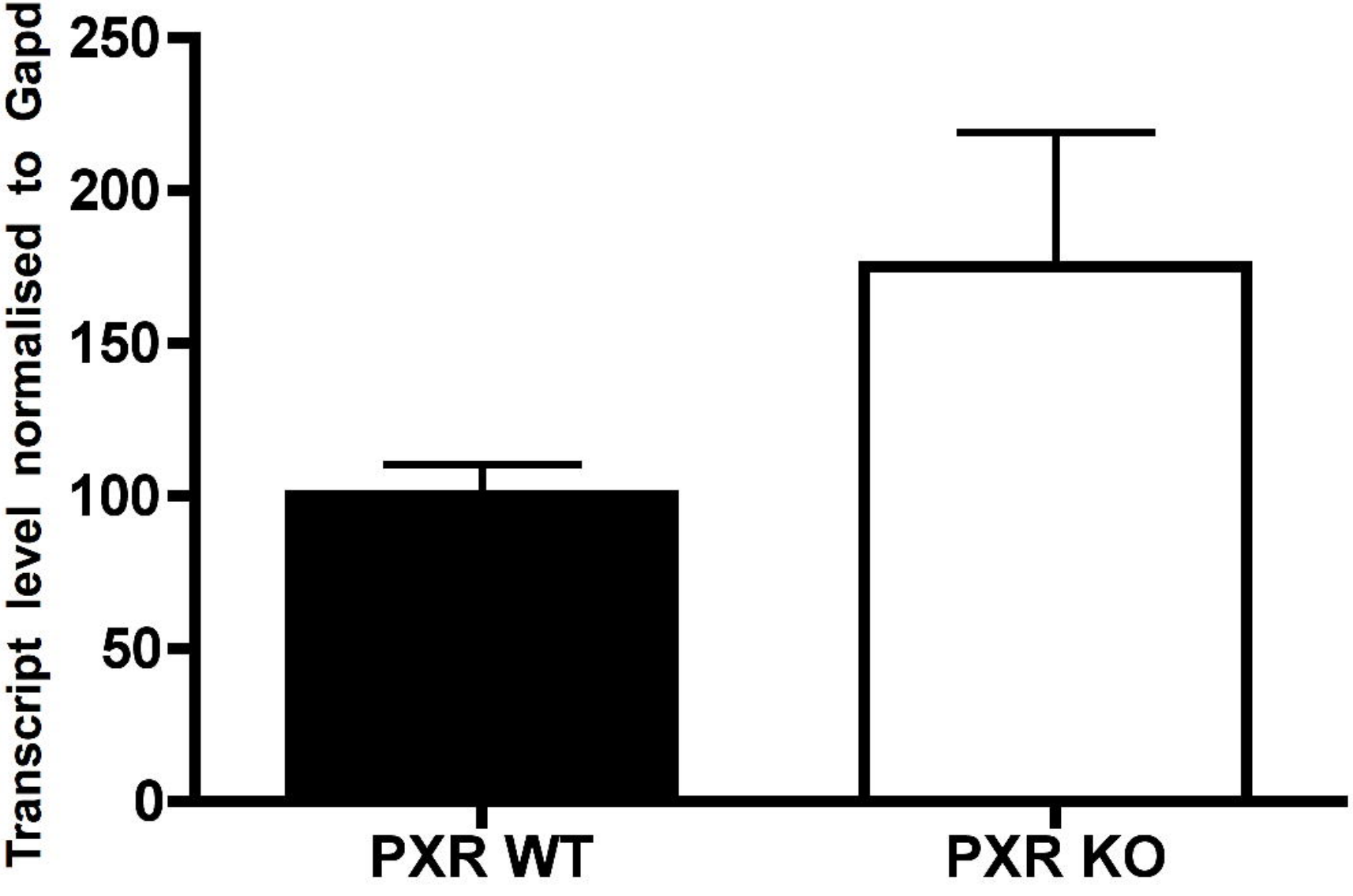

Transcript level normalised to Gapdh
250
200
150
100
50
0
PXR WT
PXR KO

Fig 3

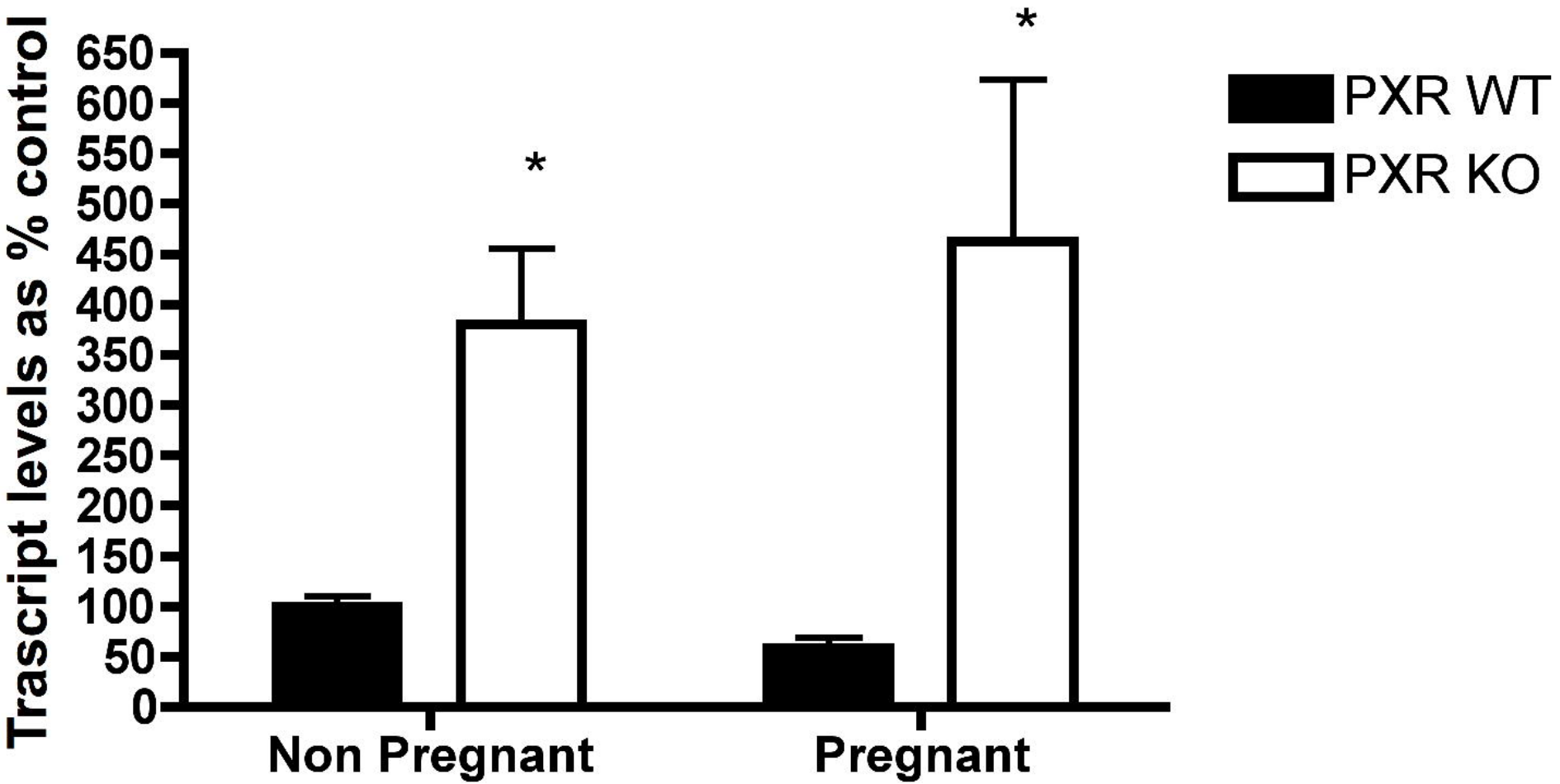

Trascript levels as % control
650
600
550
500
450
400
350
300
250
200
150
100
50
0
*
*
Non Pregnant
Pregnant
PXR WT
PXR KO

Fig 4a

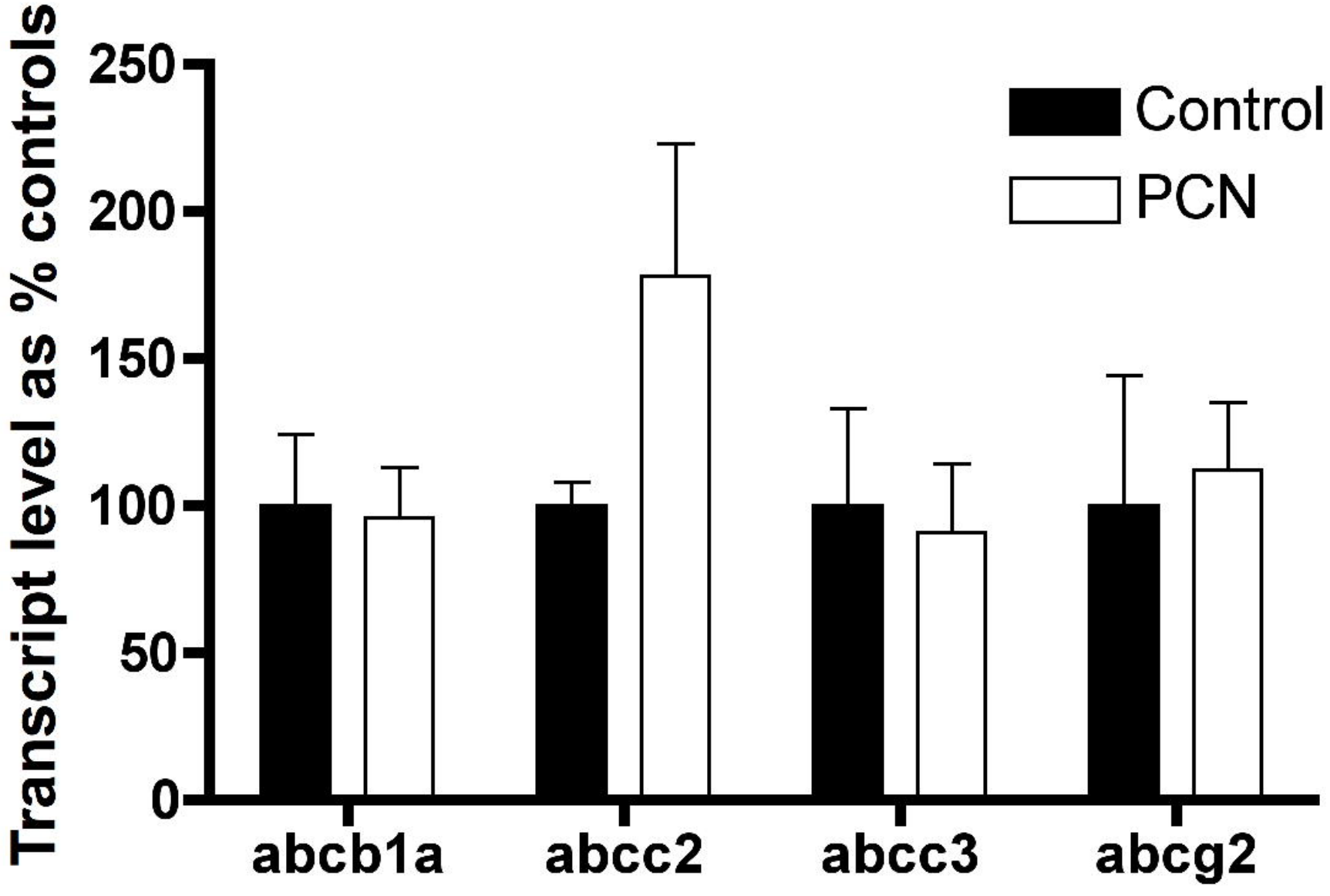

Transcript level as % controls
250
200
150
100
50
0
Control
PCN
abcb1a
abcc2
abcc3
abcg2

Fig 4b

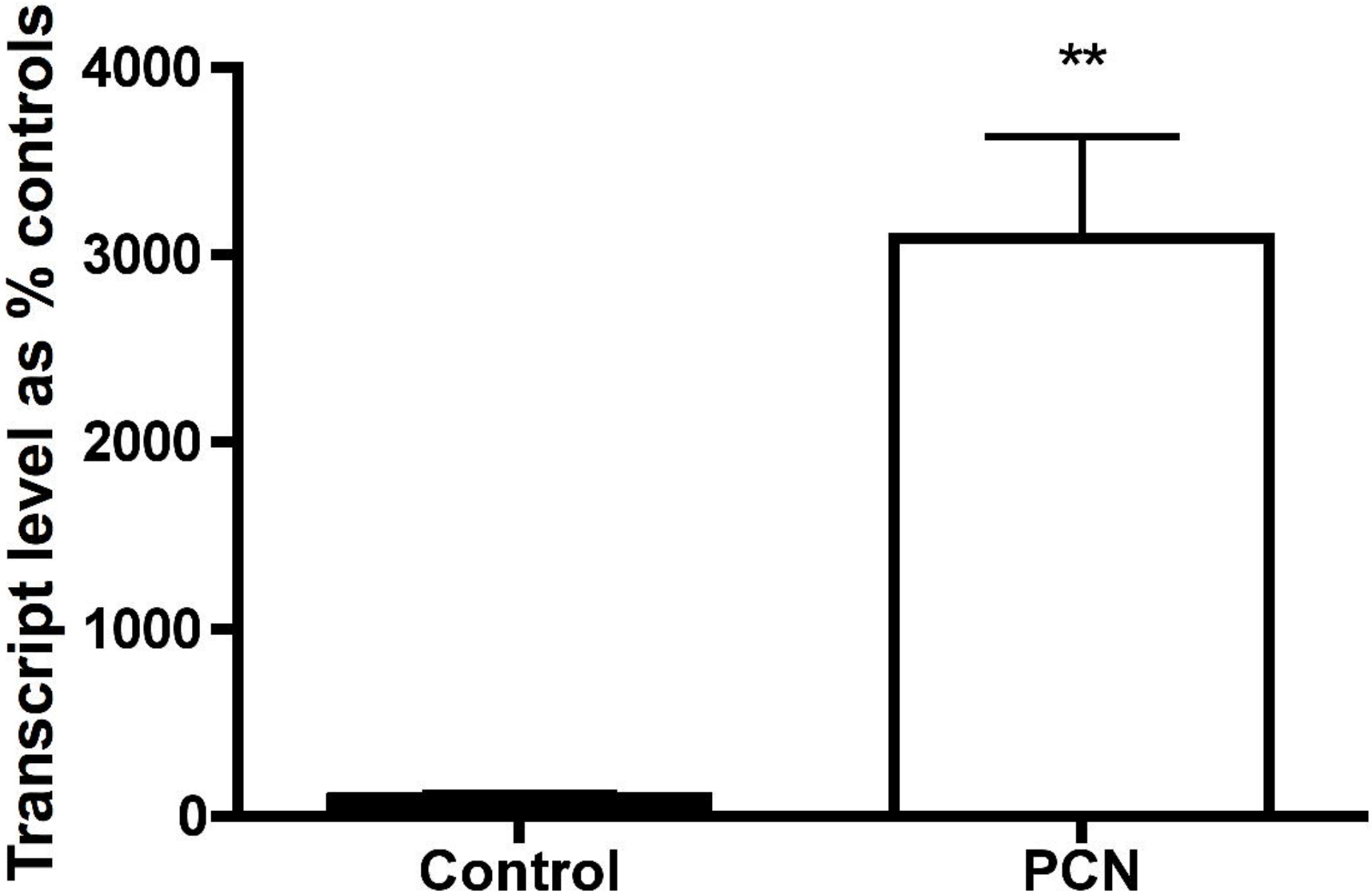

Transcript level as % controls
4000
3000
2000
1000
0
**
Control
PCN

Fig 4c

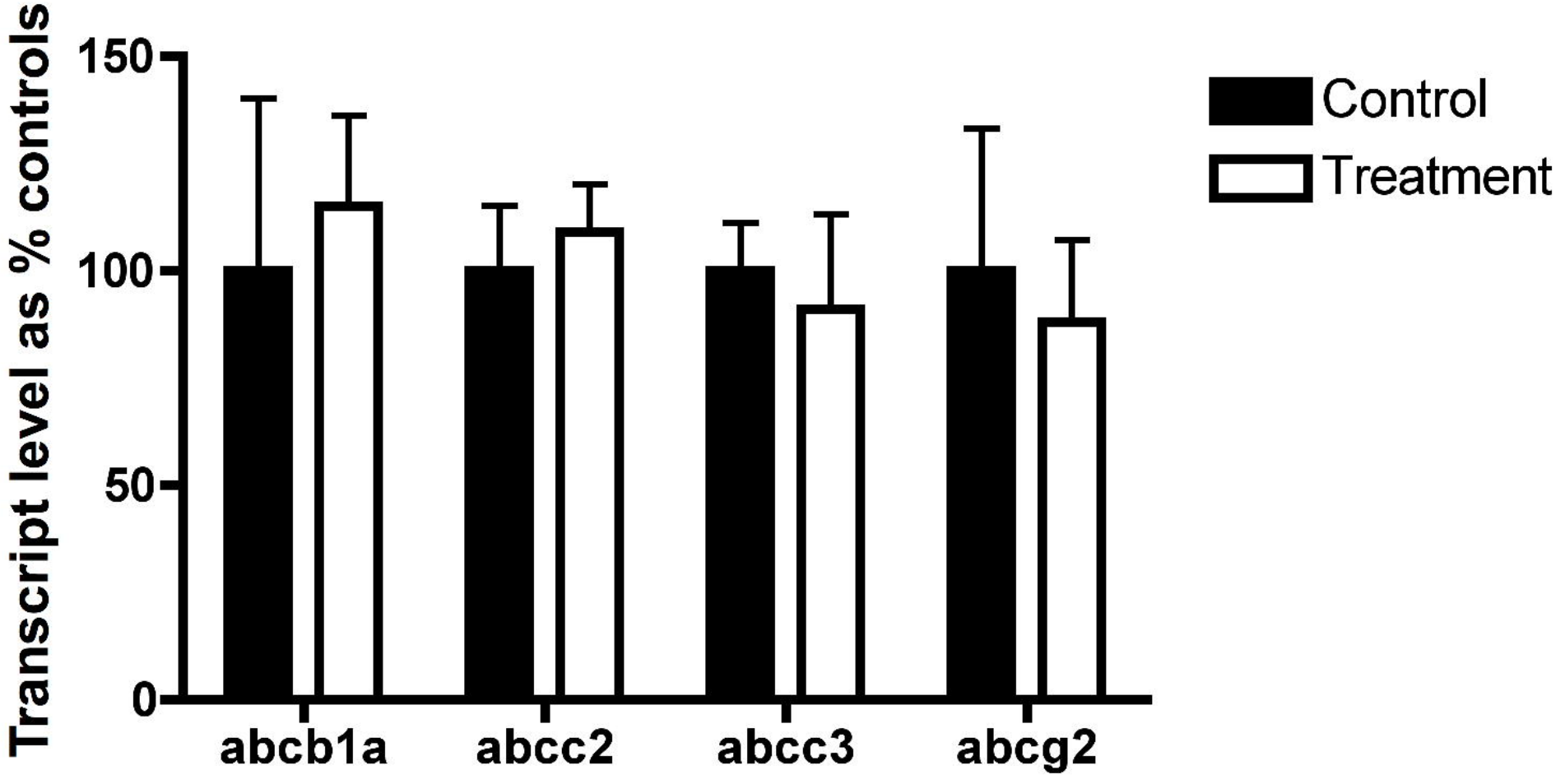

Transcript level as % controls
150
100
50
0
abcb1a
abcc2
abcc3
abcg2
Control
Treatment

Fig 5a

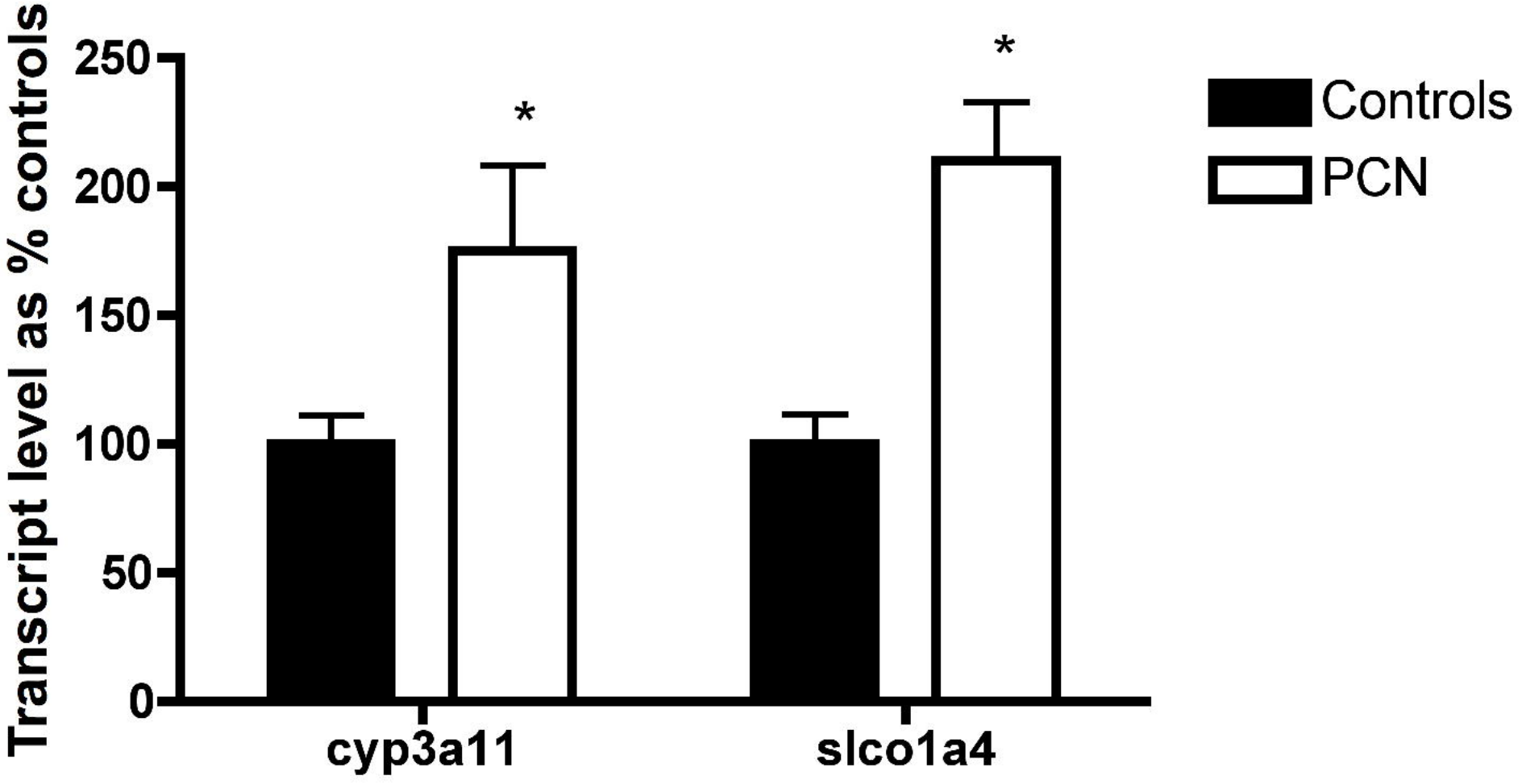

Transcript level as % controls
250
200
150
100
50
0
*
*
cyp3a11
slco1a4
Controls
PCN

Fig 5b

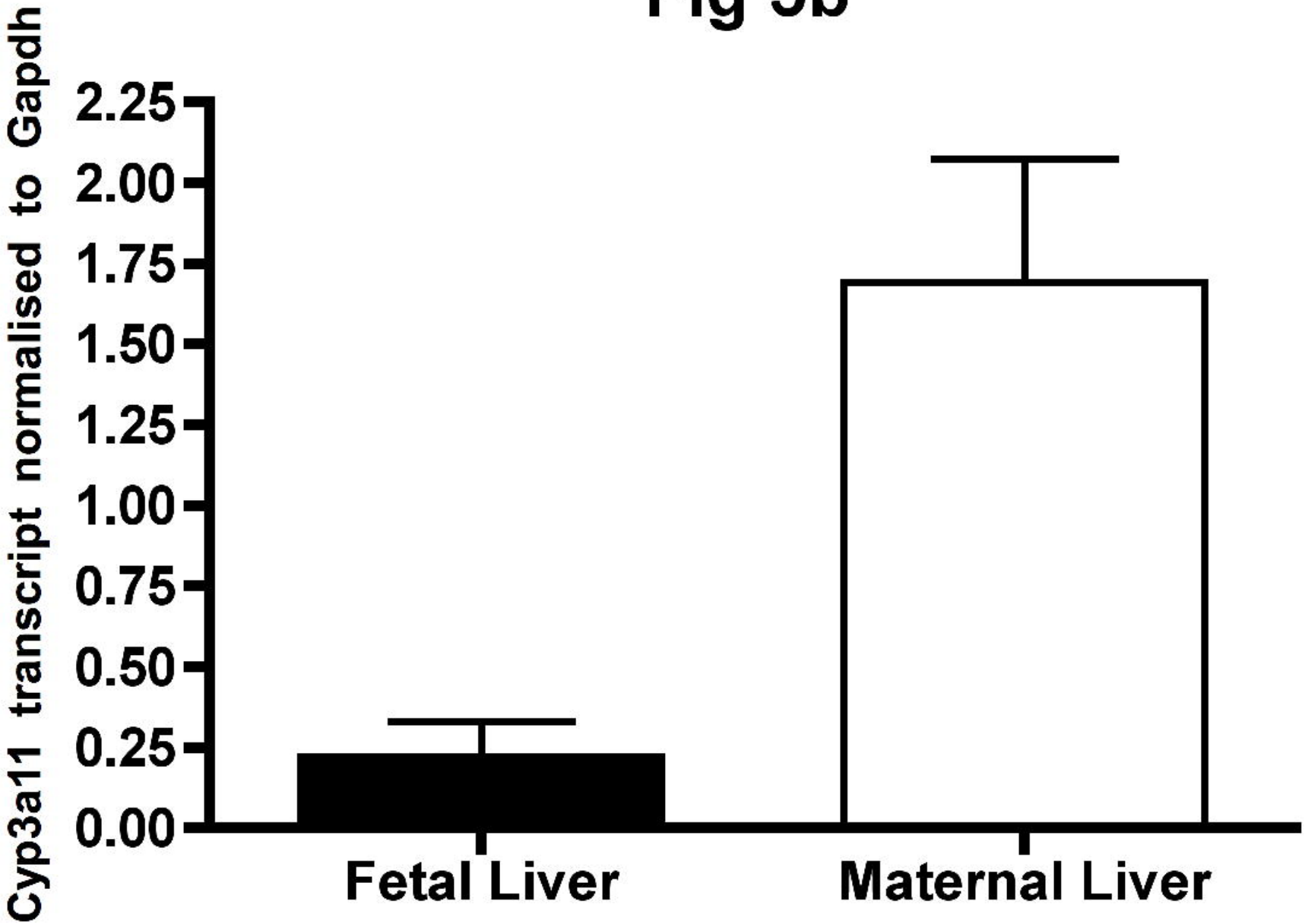

Cyp3a11 transcript normalised to Gapdh
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Fetal Liver
Maternal Liver

Fig 6a

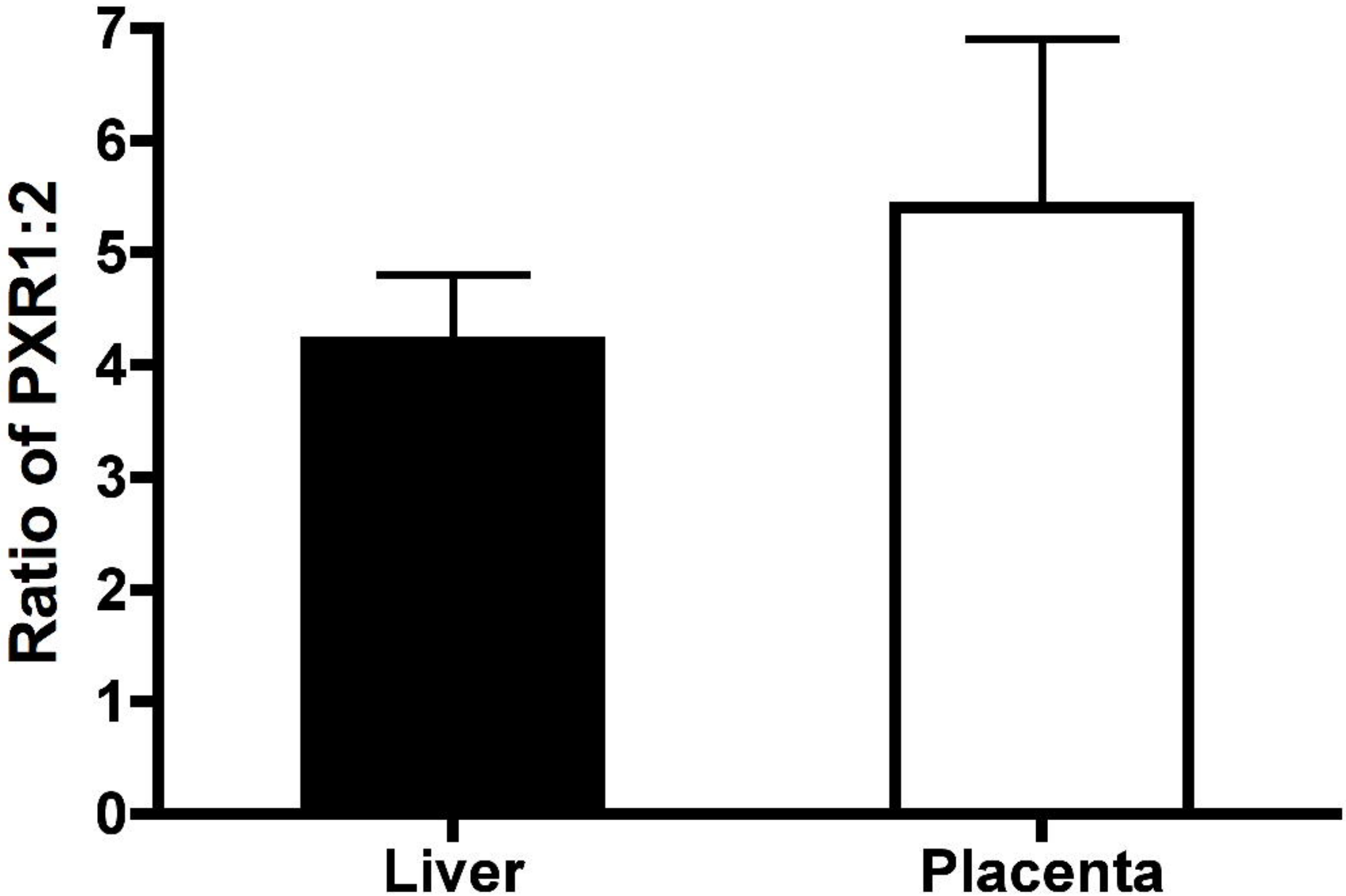
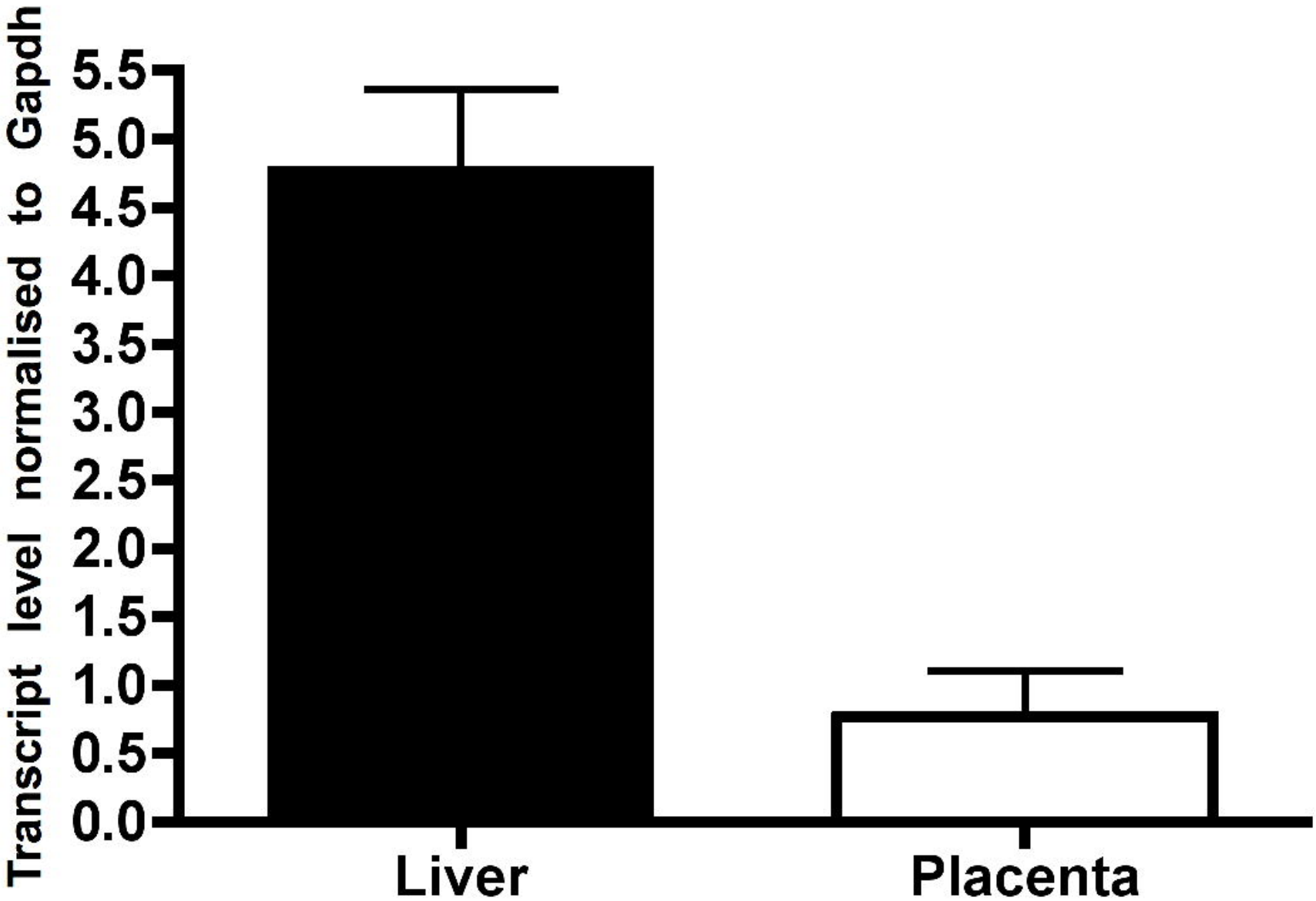


Fig 6b


Transcript level normalised to Gapdh
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver
Placenta