PXR-Mediated Regulation of Placental Drug Transporters & Impact on Fetal Exposure to Lopinavir

by

Sarabjit Singh Gahir

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Abstract

Globally, there are close to 20 million women living with HIV. An increasing number of these women are of child bearing age. Current guidelines recommend treatment of all pregnant women with highly active antiretroviral agents to both maintain maternal health and to prevent the vertical transmission of the virus. Maintaining a balance between adequate levels of antiretroviral in the fetal system and preventing fetal toxicity are key to the success of this strategy. Despite increasing use, little is known about the transplacental accumulation of these agents.

ABC drug transporters at the placental surface are believed to play an important role in the protective function of the placenta. This thesis explored the involvement of Pregnane X Receptor (PXR), an established regulator of drug transporters in the liver and intestine, in the regulation of placental drug transporters and the impact of PXR genotype and associated differences in placental transporter levels on fetal drug accumulation of lopinavir, a key antiretroviral used extensively in pregnant women.

We examined the role of the nuclear receptor at the placental interface in PXR knockout and wildtype mice. Pegnenolone-16-α-carbonitrile (PCN) treatment failed to induce PXR and target genes in the placenta in contrast to the liver. Furthermore, an inverse relationship between placental PXR expression and the expression of Pgp, Mrp 1-3 and Bcrp was observed in the placental tissue.

PXR heterozygotes were bred in order to generate pregnant dams with varying expression of placental transporters in individual fetal units within the same dam. This model was used to study the impact of fetal genotype and placental transporter expression on fetal exposure to lopinavir within the same dam. A two fold higher fetal accumulation of lopinavir was observed the PXR null placentas as compared to the wild types. An inverse relationship was observed between the placental expression of Mdr1a and fetal exposure to lopinavir ($p<0.05$). Overall, the results establish the important role of drug transporters in controlling fetal drug exposure.

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"For a moment, nothing happened. Then, after a second or so, nothing continued to happen." ~ Douglas Adams.

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And so, as I am ending my journey as a graduate student, I can sincerely say that it has been a magnificent and satisfying experience. At the conclusion of this phase of my life, I can finally understand what Douglas Adams meant when he said, *"I may not have gone where I intended to go, but I think I have ended up where I needed to be."*

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List of Abbreviations

ABC:	ATP-Binding Cassette
AIDS:	Acquired Immunodeficiency Syndrome
ART:	Antiretroviral Therapy
BCRP:	Breast Cancer Resistance Protein
CAR:	Constitutive Androstane Receptor
DBD:	DNA Binding Domain
DHEA:	Dehydroepiandrostane
E3S:	Estrogen 3 Sulphate
F:	Bioavailability
FTC:	Fumitremorgin C
GD:	Gestational Day
HAART:	Highly Active Anti-Retroviral Therapy
HIV:	Human Immuno Deficiency Virus
i.p.:	Intra Peritoneal
i.v.:	Intravenous
LBD:	Ligand Binding Domain
LPV:	Lopinavir
MDR:	Multi-Drug Resistance
Mg/Kg:	Milligrams per Kilogram

MRP:	Multidrug Resistance Associated Proteins
MTCT:	Mother To Child Transmission
NBF:	Nucleotide-Binding Fold
NR:	Nuclear Receptor
NRTI:	Nucleoside Reverse Transcriptase Inhibitors
NtRTI:	Nucleotide Reverse Transcriptase Inhibitors
PCN:	Pegnenolone-16-A-Carbonitrile
PCR:	Polymerase Chain Reaction
P-Gp:	P-Glycoprotein
PI:	Protease Inhibitor
PXR -/-:	PXR Knockouts
PXR +/-:	PXR Heterozygotes
PXR +/+:	PXR Wild Type
PXR:	Pregnane X Receptor
RT qPCR:	Realtime Quantitative Polymerase Chain Reaction
RXR:	Retinoid X Receptor
SULT:	Sulfotransferases

Chapter 1 Introduction

1.1 Introduction

This thesis is a collection of work that takes a multi faceted look at the factors impacting the transport of xenobiotics across the placenta. The work is focused on a subset of the pregnant demographic, HIV-seropositive women who manage their pregnancies with highly active anti retroviral therapy (HAART). Thus, studies were carried out to understand the key molecular mechanisms behind placental drug transporter regulation. To better understand the pharmaco-therapeutic approaches employed to combat the threat of HIV during pregnancy, a study looking at trends in managing HIV positive pregnancies was conducted. Finally, with an aim of understanding how the placental drug transporters play a role in modulating fetal drug levels of the most commonly used antiretroviral agent, an in-vivo study was conducted in an animal model.

It is important to note that the role of placental transporters is not merely of academic interest, but rather very clinically relevant. Recent studies have revealed a highly variable prevalence of prescription medicine use during pregnancy ranging from just over 40% in Denmark to 93% in France. North American trends are moderate by comparison, with a recent Canadian study reporting 63% of all pregnant women being on some prescription medication during gestation. Furthermore, about 8% of the pregnancies were prescribed FDA category D or X medications - agents that have been shown positively in animal or humans to have potential for adverse reactions or fetal abnormalities (Daw et al. 2011). For us to be able to make safer therapeutic choices, we must improve our understanding of the factors affecting drug disposition at the placental barrier.

The subsequent section discusses the main concepts important to understand the results that follow. A brief introduction to the key ATP-Binding Cassette (ABC) transporters and nuclear receptors is followed by a synopsis describing the placenta- a site where numerous transporters are highly expressed. The introduction is rounded off by our current understanding of the HIV crisis, and the commonly used agents to combat it.

1.2 ABC Drug Transporters:

1.2.1 Definition:

The ATP-Binding Cassette (ABC) transporters constitute one of the largest gene families of membrane bound proteins involved in the active trafficking of their substrates across the cell membrane (Vasiliou, Vasiliou, & Nebert, 2009). Using ATP, these proteins can transport a staggeringly diverse range of endogenous and exogenous compounds including metabolic bi-products, lipids and clinically employed drugs against a concentration gradient.

Existing in both lower and higher life forms, these ancient genes have retained considerable homology across species. Comparison of the conserved ATP-binding domain across the family has led to their phylogenetic classification into seven subfamilies denoted from ABCA to ABCG (Hughes 1994). To date, 48 members of the ABC transporters have been identified in humans. Together, these genes constitute the largest group of transmembrane proteins (Dean, 2002).

1.2.2 Structure of ABC-Transporters:

ABC-transporters are characterized by the presence of a conserved ATP-binding domain, also known as the Nucleotide-binding fold (NBF), in addition to transmembrane domains. A typical protein consists of two NBFs (each with a Walker A, Walker B, and Walker C motifs) and transmembrane domains. Known exceptions to above described structural configuration are proteins such as the breast cancer resistance protein (BCRP, ABCG2), which being a half transporter, forms a functional homodimer. The NBFs are cytosolic in localization and serve as sites for energy generation, while the trans-membrane domains are believed to determine substrate specificity of the protein.

The transporters that have received the greatest attention from researchers are ones that have been implicated in multi-drug resistance (MDR) (Sharom, 2008). Indeed, a number of transporters are found to be over-expressed in cell lines displaying drug resistance, a fact that facilitated their discovery. Additionally, genetic anomalies in a number of these transporters is now associated with specific disease states ranging from certain neurological disease, metabolic disorders to altered drug response (reviewed by Borst & Elferink, 2002). Since we were exploring the key transporters important in drug-disposition, we focused on a subset of this super family. The transporters that are examined in the studies contained in this thesis are described in greater detail below.

Protein	Gene Name	Size (AA)	Membrane Localization	Tissue Localization	Important Substrate Classes (Selected)	Putative Physiological Role
MDR1, P-gp	ABCB1	1280	Apical	Liver, Pancreas, Kidney, Intestine, Placenta	Anticancer drugs; HIV protease inhibitors; Immunosuppressive agents; Antibiotics; Corticoids; Cardiac glycosides	Multidrug resistance
MRP1	ABCC1	1531	Basolateral	Ubiquitous	Daunorubicin, Mitoxantrone, Vinca alkaloids, Paclitaxel, Leukotriene C4	Cholesterol efflux and Inflammatory processes
MRP2	ABCC2	1545	Apical	Liver, Kidney, Gut, Placenta	Cisplatin, Vincristine, Daxorubicin, Protease inhibitors	Mutidrug resistance
MRP3	ABCC3	1527	Basolateral	Liver, Adrenals, Kidney, Gut, Placenta	Etopiside, Mitoxantrone	Bile acid secretion
BCRP	ABCG2	655	Apical	Placenta, Intestine, Brain, Liver, Kidney	Daunorubicin, Mitoxantrone, Methotrexate, AZT, Lamivudine	Toxin efflux, drug resistance

Table 1: A list of the main ABC drug transporters explored in this work, along with their basic bio-chemical and functional information.

1.2.3 P-glycoprotein (P-gp; ABCB1):

The archetypal ABC drug transporter, P-glycoprotein (P-gp) was the first discovered member of ABC family and remains one of the most widely studied drug transporter. This surface phosphoglycoprotein gained notoriety soon after its discovery in 1972 as a key factor responsible for drug resistance in cancer therapy (Dano, 1972; Juliano and Ling, 1976).

Encoded by the gene MDR1 (Abcb1) in humans [Mdr1a (abcb1a) and Mdr1b (abcb1b) in rodents], P-gp is a 1280 amino acid protein. Classified as an efflux transporter, this apically localized protein actively transports its substrates out of the cells, leading to a reduced accumulation of its substrates intracellularly. While initially identified as an over expressed factor in resistant tumor cells, P-gp is now known to be constitutively expressed in numerous tissues including the placenta, liver, the intestinal tract and the blood brain barrier (BBB) (Thiebaut et al., 1987; Cordon-Cardo et al. 1990)

Since the P-gp deficient mice have not been found to display any obvious physiological deficits and are viable, P-gp does not seem to be involved in any physiological roles vital to the sustenance of life (Schinkel et al. 1997). Interestingly however, Mdr1a knockout mice have been reported to develop colitis, pointing to a potential involvement in extruding toxins away from the gut epithelium (Panwala et al., 1998). It is also believed to play a role in protecting the tissues from systemically circulating toxins, although it's exact physiological role remains to be fully elucidated.

While the endogenous substrates of the transporters have not been conclusively identified, P-gp has been shown to transport a wide array of structurally diverse xenobiotics. The range of substrates includes both amphipathic and hydrophobic molecules; natural, semi-synthetic, and synthetic compounds. A large number of clinically employed agents such as anti-neoplastics (i.e. doxorubicin and members of the taxane family) were initially identified as P-gp substrates due to the resistance the over expression of the protein imparts. Other important drugs believed to be substrates of P-gp are the protease inhibitor (PI) class of antiretrovirals such as Lopinavir (LPV), cardiac glycosides such as digoxin, and a number of antibacterials amongst others.

Given the abundant expression of P-gp at sites determining drug disposition, and the wide range of clinically relevant substrates, P-gp can alter the pharmacokinetics of a number of therapeutics. Indeed, the influence of P-gp at all stages of the Absorption-Distribution-

Metabolism-Elimination paradigm of drug pharmacokinetics has been illustrated in a number of studies. P-gp at the intestinal can influence the absorption of substrates by extruding drug back into the intestinal lumen. Indeed, the absorption of Paclitaxel was found to be higher in P-gp null (P-gp -/-) mice as compared to wild type animals when administered orally. The bioavailability (F) of several HIV PIs has also been shown to be dependent on P-gp expression, with studies showing higher levels of PI in the systemic circulation in Mdr1a -/- and Mdr1a/b -/- mice as compared to wild type animals post oral administration (Sparreboom et al., 1997). P-gp has also been shown to modulate the intestinal metabolism of its substrates by increasing the systemic availability of the drug to intestinal enzymes such as Cyp3a (Watkins, 1997; Suzuki et al. 2000, Benet and Cummins, 2001). P-gp has been found to limit the brain concentration of substrates such as fexofenadine, saquinavir, and nelfinavir, with Mdr1a -/- mice showing elevated levels as compared to wild type mice (Tahara et al., 2005;.Kim et al., 1998). Reduced CNS concentrations of protease inhibitors such as saquinavir and nelfinavir as a result of altered P-gp levels has important implication for disease progression as the brain is a reservoir site for HIV. Another important site of P-gp expression is the placenta, the gateway to the fetal system. Here too, the role of P-gp in determining fetal concentration of its substrates has been demonstrated. The maternal to fetal transfer of indinavir and vinblastin have been shown to be increased in the presence of P-gp inhibitors in placental perfusion studies (Sudhakaran et al, 2008). More importantly, the protective role of placental P-gp was highlighted by Lankas et al in a study with a 100% incident of avermectin induced cleft lip in offsprings of Mdr1a -/- mice as compared to none in their wild type counterparts (Lankas et al.1998). Thus, P-gp can play an important role in the bioavailability, clearance and distribution of its substrates, thus impacting therapeutic outcomes in a variety of clinical settings.

The amount of drug transporters expressed at cell surfaces is highly variable, as the levels are constantly adjusted to influences both from within the biological system and the environment. P-gp levels are under the influence of a number of xenobiotics and physiological events such as inflammation and cellular stress. P-gp levels are up regulated by a number of drugs and dietary agents such as verapamil, midazolam, reserpine, St. John's Wort and clotrimazole. Inflammation, and pro inflammatory cytokines are known to down regulate the expression of the transporter. Alteration in P-gp expression is believed to occur primarily at a transcription level, with nuclear receptors playing an important role in its regulation. The nuclear receptors,

pregnane x receptor (PXR) and constitutive androstane receptor (CAR) are believed to be important regulators of P-gp in various tissues (Gieck et al., 2001; Burk et al., 2005).

1.2.4 Breast Cancer Resistance Protein (BCRP; ABCG2):

Discovered almost simultaneously by three independent groups in late 1990s, BCRP is a 72 kDa efflux transporter. It was cloned initially from the human cell line MCF-7/AdrVp (Adriamycin resistance breast cancer cell line), and subsequently identified in the placenta and mitoxantrone resistant colon cancer cell lines (Doyle et al., 1998; Allikmets et al., 1998; Miyake at al., 1999). Classified as a half transporter, this 655 amino acid protein forms 6 transmembrane domains, and requires homo-dimerization before it can function as an efflux transporter (Ozvegy, 2001).

In humans, BCRP is expressed to the highest degree in the placenta and stem cells, although it is also localized at the apical side of epithelial membranes in the intestine, the brain, liver and kidneys. Its high expression at stem cell surface, which is diminishes upon differentiation into pluripotent cells, along with high expression at the epithelial surfaces of sanctuary sites like the fetus and the brain point to a predominantly protective role. BCRP is capable of extruding both hydrophobic and hydrophilic substrates (such as conjugated metabolic end products) (Doyle and Ross, 2003; Allen et al, 1999; Merino et al, 2006). Although it is shares little structural similarity to P-gp and Mrp2, the substrate specificities of these transporters overlap to a significant extent.

The list of substrates transported by BCRP has expanded considerably over the years, and now includes cytotoxic chemotherapeutics such as methotrexate and mitoxantrone, as well as agents such as glyburide and statins (Gedeon, Anger, Lubetsky, Miller, & Koren, 2008; Gedeon, Behravan, Koren, & Piquette-Miller, 2006; Volk & Schneider, 2003). It is also implicated to be involved in the transport of environmental compounds such as dietary flavonoids, porphyrins and PhiP – a known carcinogen (Pavek et al, 2005; Mennone , Soroka, Harry & Boyer, 2010).

In addition to acting as substrates for BCRP, a number of drugs also inhibit BCRP. Common inhibitors include fumitremorgin C (FTC) and its derivative Ko143, GF120918 and a number of HIV PIs including nelfinavir, ritonavir along with kinase inhibitors such as imatinib (Weiss et al., 2007). (Noguchi, Katayama, Mitsuhashi, & Sugimoto, 2009)

Given its high expression in the placenta, a number of important studies have been conducted in perfused placental preparations. In one such study, Gedeon at al found that BCRP was

important in the transport of glyburide, an important hypoglycemic agent used in pregnant diabetics agent used during pregnancy. In this study, the transport of glyburide was increased in the presence of a BCRP inhibitor but not in the presence of a P-gp inhibitor (Gedeon et al., 2008). Using membrane vesicles obtained from term human placentas, BCRP was shown to effectively transport mitoxantrone (Kolwankar, Glover, Ware & Tracy, 2005). In-vivo studies have also found that inhibition of BCRP in P-gp knockout mice led to an increase in the fetal accumulation of topotecan (Jonker et al., 2000).

Physiologically, BCRP is believed to provide protection to the fetus from circulating xenobiotics (Hahnova-Cygalova, Ceckova, & Staud, 2011). Another putative role ascribed to the transporter is the regulation of steroidal hormone synthesis in the placenta. The placenta is an import site for regulating the levels of Dehydroepiandrostane (DHEA) and its sulfated metabolite (DHEAS). DHEA is a precursor to estrogen synthesis. Estrogen is metabolized to estrogen 3 sulphate (E3S), which serves as a reservoir of estrogen. Both of DHEA and E3S are substrates of BCRP, thus regulating the levels of these substrates by transporting them from the fetal side to the maternal blood, the transporter can regulate the rate of synthesis of estrogen (Imai et al., 2003; Grube et al., 2007). The fact that placentas derived from pregnancies with idiopathic fetal growth retardation exhibit lower BCRP levels than controls, suggests a possible role of BCRP in placental function and survival (Evseenko et al. 2007). The mechanism responsible for this down regulation has yet to be elucidated. On the other hand, BCRP-/- mice seem to show no fetal deficits in their offspring.

1.2.5 Multidrug Resistance Associated Proteins (MRP; ABCC):

Another important branch of the ABC drug transporter family are MRP transporters. The MRP transporters have been identified in numerous species ranging from humans, rodents, yeast and plants. MRP1 (ABCC1), a 192 kDa transporter with ubiquitous expression was the first MRP to be discovered and was cloned from a doxorubicin resistant lung cancer cell line (H69AR) in 1992 (Cole et al., 1992). A number of MRP genes have been since been shown to be encoded by the human genome.

While there are currently 12 members of the MRP family (reviewed by Keppler, 2011), this work focuses mainly on MRP1-3 given their substrate specificity and tissue distribution. A

distinguishing feature of the MRP family is the 13 amino acid long truncation between the Walker A and B motifs in the NBD as opposed to other ABC transporters. Furthermore, a number of key members of the MRP family (including MRP1, 2 and 3) possess an extra N-terminal trans-membrane domain (TMD0), and this domain is linked to the core region by a small linker (L0). The three isoforms of MRP (MRP 1-3), while similar in function and amino acid sequence, have distinct tissue distribution and membrane localization. The three genes share 21 splice junction sites. MRP 1 and MRP2 exhibit 48% sequence homology, while the sequence homology between MRP1 and MRP3 is 58% and 46% for MRP2 and MRP3.

At the time, following the discovery of MRP1, its substrate specificity was believed to be identical to P-gp, with minor differences in the affinity of agents transported by the two transporters. However, it was soon revealed that MRP1 preferentially transported organic anions. While MPR1 favors negatively charges species, such as xenobiotic conjugated to glutathione, glucuronate or sulphates, P-gp is more specific for positively charged or neutral, hydrophobic compounds (Leier et al., 1994; Jedlitschky et al., 1994; Deeley & Cole, 2006) . Despite the sequence being highly conserved across higher life forms, it is interesting to note that MRP1 exhibits species-specific substrate affinity. For example, in primates, MRP1 can shuttle drugs such as the anthracyclines (e.g. doxorubicin) while rodents and canine homologues do not transport the molecule. The same holds true for estradiol conjugates.

MRP1 is ubiquitously expressed, with highest levels observed in the lungs, testes, kidneys, heart and placenta. An interesting note about MRP1 expression pattern is the fact that within the organs of expression, it is normally distributed to regions with a high rate of proliferation or a specialized function (such as a physiological barrier). The placental syncytiotrophoblasts and the fetal capillaries are examples of the latter (St-Pierre, 2000).

Typically, ABC transporters thought to be important in cell defense are expressed on the apical aspect, as seen with P-gp, MRP2, BCRP etc. In the case of placental MRP1, early reports suggested apical expression, a number of subsequent reports have indicated towards a basolateral expression (Nagashige et al. 2003)

Although MRP1 -/- mice are viable, the protective role of MRP1 is highlighted as these animals are found to be more chemo sensitive. Furthermore, the tissues most sensitive to the toxicity are those normally associated with high MRP1 expression such as the testis and intestine (Kato et al., 2009; Tribull, Bruner & Bain LJ, 2003).

MRP2 (ABCC2):

First discovered in rat liver, this transporter was also called the hepatocellular canalicular multiple anion transporter or cMOAT. The protein is expressed as a functional membrane transporter in mammals and insects. The human MRP2 has 1545 AA residues.The MRP2 protein is found abundantly expressed in the hepatocyte canalicular membranes. It is also expressed on the apical surface of the proximal tubules of the kidney, the gall bladder, and the intestine. MRP2 is also expressed at sanctuary sites such as the blood-brain barrier and the placenta. Low levels of the protein in the testis and the lungs have also been reported.

Despite low sequence identity with mrp1, the two proteins share a similar list of substrates. While both transport GSH, glucuronidated or sulfated molecules, MRP2 has a higher affinity than MRP1 for bilirubin glucuronides. The substrates transported fall in a vast range of categories such as drugs, nutraceuticals, dyes, and toxic molecules. Some important drugs shown to be substrates of MRP2 include lopinavir, saquinavir, and benzylpenicillin.

The importance of MRP2 in normal physiological function can be appreciated by looking at cases where the expression or function varies from the norm. In Dubin-Johnson syndrome, the patients exhibit mutations in the ABCC2 gene. Decreased functional MRP2 leads to impaired biliary secretion of bilirubin conjugates, hyperbilirubinemia and jaundice. Other examples are seen in patients with 24C>T genetic polymorphism, and has been reported to be linked to toxicity associated with the xenobiotic mycophenolic acid. Similarly, the 1249G>A SNP has been linked with low systemic exposure and elevated clearance of talinol, possibly due to an increased expression of the gene.

MRP3 (ABCC3):

The last amongst the three MRPs to be discovered, MRP3 is a 1527 amino acid protein, with a 58% sequence homology with MRP1. Expressed primarily in the adrenal glands, kidneys, small intestine, pancreas and the gall bladder, it is less ubiquitous than MRP1.

However, similar to MRP1, MRP3 is also expressed on the basolateral membrane of polarized cells. The substrate affinity of MRP3 differs from the other members of the family. It transports a range of amphipathic anions and GSH as well as glucuronate conjugates.

1.3 Placental and Fetal CYPs:

In addition drug transporters, there has been interest in the expression of metabolic enzymes in the placental and fetal tissue as well. While a number of phase I and phase II metabolic enzyme transcripts have been reported in the placenta, their contribution to placental metabolism of xenobiotic has been not been clearly elucidated. Given the very low expression compared to the hepatic tissue, placental metabolism is believed to be very minor, with no appreciable impact on xenobiotic transport across the placenta (Hakkola et al, 1998; Prouillac and Lecoeur, 2010).

A number of members of the CYP family have been reported to be expressed in the placenta, including Cyp1a1, Cyp1a2, Cyp2d6, Cyp34, Cyp3a5 and Cyp3a7 (Hakkola et. al., 1996; Schuetz et. al., 1993; Pasanen et. al., 1997; reviewed by Prouillac and Lecoeur, 2010). However, the type and the extent of the enzyme expressed seems to be dependent on a number factors including the gestational age and maternal health status. As a generalisation, the expression of CYPs declines as gestation progresses. Furthermore, while the genes have been detected at the mRNA level (primarily in first trimester), very few have been confirmed at the protein level. Fewer still have been demonstrated to have any activity in the placenta (Prouillac and Lecoeur, 2010). Thus, given the very low expression of enzymes such as Cyp3a in the placenta, along with the dearth of evidence indicating activity in the placenta, indicates to minor, if any, contribution of placental metabolic pathways to the xenobiotic biotransformation pathways.

Similarly, while a number of Cyps have been detected in the fetal tissue their levels are much lower than the adult liver (upto 70% lower) (Pelkonen, 1980; Hakkola et. al.,1998). This translates to a much lower metabolic rates in the fetal livers. The two main Cyps in the adults liver, cyp3a4 and cyp3a5 mRNA have been reported in the fetal liver, however, low levels are detected at the protein level (Hakkola et. al., 1994; Wrighton et.al.,1990; Shuetz et. al., 1993). Furthermore, little is known about the extent of functional activity of these enzymes. The most important Cyp in the fetal liver is CYP3A7, accounting for approximately one thirds of the Cyp content (Kitada et. al., 1994; Shimada et. al., 1996). Interestingly, while it is a major fetal Cyp, its expression is much lower in the adult liver, with the balance shifting from CYp3a7 to Cyp3a4 post natally (Shuetz et. al., 1994; Lacroix et. al., 1997). The enzyme seems to play an important role in placental steroid metabolism (in the estriol synthesis) (Ryan et. al., 1980; Cresteil et. al., 1982). In conclusion, given the small size of the fetal liver, and lower enzyme levels expressed as compared to the adults, the metabolic contribution is thought to be minimal.

1.4 Nuclear Receptors:

One of the key mechanisms of the biological systems to respond to endogenous and exogenous stimuli are the transcription factors termed nuclear receptors (NRs). NRs constitute a gene superfamily comprising of 49 members responsible for regulating the expression of numerous target genes affecting a wide range of physiological functions such as growth, energy metabolism and drug disposition (reviewed by Huang, Chandra & Rastinejad, 2010). Over the last few years, investigations into the NR has greatly enhanced our understanding of the regulation of ABC drug transporters in responses to environmental or physiological stresses, with members such as PXR and CAR playing a key role.

Despite the wide range of ligands that interact with NRs, the basic structural features across members are consistent. Three major domains are shared by all NRs; namely the AF- 1 domain, the DNA binding domain (DBD) and the ligand binding domain (LBD). The AF-1 domain which is present at the amino acid terminus of the protein is responsible for constitutive, ligand -independent activation and is highly variable across the members of the family. The highly conserved DNA binding domain, which interacts with the DNA response elements (termed hormone response elements or xenobiotic response elements) of its target genes, is seen in all members, and it was this finding that enabled early researchers to screen cDNA

libraries and identify a large number of the known members of this superfamily. Towards the carboxylic acid terminus lies the ligand binding domain (LBD), a domain not as highly conserved as the DBD. Interestingly, the LBD of NRs has been found to be fairly flexible, a characteristic that enables them to accommodate structurally diverse ligands. Binding of endogenous or exogenous ligands to the LBD causes a conformational change in the NR. The conformational changes allow for binding of co-factors to the NR, causing translocation to the nucleus and activation of their target genes. In addition to a binding site, the LBD is also a domain for co-factor recruitment, dimerization and nuclear translocation.

1.4.1 Pregnane X Receptor (PXR; NR1I2):

The discovery of the murine homologue of PXR in 1998 by Kliewer et al, has greatly improved our understanding of the body's xenobiotic defense mechanisms (Kliewer et al., 1998). The human (Lehmann et al, 1998), rat (Zhang et al., 1999), canine and rhesus monkey (Moore et al., 2002) orthologs were identified shortly after. The human orthologue of PXR was previously termed as the steroid and xenobiotic sensing nuclear receptor (SXR) in humans. However, throughout this thesis, we will be refer to the gene as PXR for all species as this is the standard nomenclature for this gene.

In addition to high levels of expression in the liver and intestine, PXR has also been detected in the lungs, ovaries and the placenta (Masuyama et al., 2001; Kliewer 1998; Zhang 1999).. The expression patterns of PXR is not surprising as its phase II and III metabolic target genes are also expressed to a high degree in the same tissues. Named PXR due to its affinity to synthetic steroids, a wide array of xenobiotics also act as ligands for PXR. It is known as a promiscuous NR as its ligands encompass a range of natural and synthetic steroids as well as many clinically used therapeutics. PXR ligands include hormones and steroids such as progesterone, pregnelonone, corticol, aldosterone; vitamins including vitamin E, vitamin K2, retinol; bile acids; and drugs including rifampicin, phenobarbital, lovastatin, ritonavir, spironolactone. This promiscuity is seen as an indicator of the prominent role PXR plays as a xenosensor.

The key to PXRs ability to bind with a wide range of ligands lies in the characteristic shape of the LBD. The PXR crystal structure described by Watkins et al has revealed a large, extremely flexible and relatively amorphous shaped LBD (Watkins et al., 2001; Watkins et al., 2003). As a

result, a much wider array of molecules can be accommodated within the LBD. The LBD is not as well conserved for PXR as it is for other NR mammalian orthologs. There is a 77% amino acid identity between the human and murine LBD (Moore et al., 1998). The differences in the LBD between species is believed to be responsible for observed differences in substrate specificity between species. Indeed by humanizing mouse LBD by converting 4 amino acids to the corresponding human amino acids, groups have enabled the murine PXR to bind to ligands typically only recognized by hPXR (Xie et al., 2000).

The cellular localization of PXR is controversial, however an increasing number of studies point to it residing predominantly in cytosol (Kawana et al. 2003; Squires, Sueyoshi & Negishi 2004). Once it has been activated by the ligands, it is translocated to the nucleus, where it forms a heterodimer with retinoid x receptor (RXR) and this complex binds with a XRE sites in the promoter region of its target genes, thereby regulating transcription. The PXR/RXR complex can recognize an array of XREs including DR-3, DR-4, ER-6 and ER-8 motifs, which are found in the promoter region of numerous genes involved in metabolism and drug efflux.

The list of PXR target genes has been steadily growing over the last few years. It includes protein involved in all three phases of metabolism i.e. Oxidation (phase I), conjugation (phase II) and transport (phase III). Asides from CYP3A, the archetypal target gene for PXR, BCRP, OATP, P=gp and members of the MRP family are also regulated by PXR. For the purpose of the studies described in this thesis, we were interested in the transporters at the placental barrier. Amongst these, PXR is known to be involved in the regulation of MDR1, BCRP, OATP1A4 and MRP2.

Our understanding of the role PXR plays in regulating its target genes has been greatly aided by studies conducted in PXR knockout (PXR-/-) mice. Two main knockout colonies have been generated by independent research groups. Staudinger et al developed the PXR-/- model employed in this thesis by disrupting the DBD in the 5' region of the gene (Staudinger et al., 2001). In this model, the first exon has been replaced with a neo-resistance cassette, yielding a mRNA product that is truncated by approximately 200 basepairs as compared to the wild-type gene product. This truncated gene product is unable to bind with DNA, and hence is non-functional. Xie et al have also independently developed a PXR-/- model. In this colony, the gene

was rendered non-functional as a result of replaced exons 2 and 3 instead of exon 1 (Xie et al., 2000).

1.4.2 Constitutive Androstane Receptor (CAR; NR1I3):

Belonging to the same subgroup of the nuclear receptor superfamily as PXR, CAR was discovered in 1994 while screening of a cDNA library with a NR DBD-oligonucletide probe. The NR was initially christened the constitutively activated receptor due to it ability to bind to RXR and transactivate genes in the absence of any ligands in transfection assays. The NR is referred to the constitutive androstane receptor due to the two androstane metabolites that have been identified as the endogenous ligand for CAR. The primary site of expression for CAR is the liver, with a minor presence in the small intestine.

1.5 The Placenta:

The placenta is a unique organ of a limited lifespan, characterized by intimate apposition of the maternal and fetal systems. It is widely thought that placentation is one of the key evolutionary advantages that have ensured the reproductive success of vertebrates. This is due to both the protective and nutritive functions the placenta performs during the gestational period, ensuring the birth of an offspring fit for particular habitats. Indeed, a compromised placental structure and function are associated with reduced fetal survival and an increased risk for organic diseases later in life (Lewis, Cleal & Hanson, 2012).

There are several placental structures that have evolved in numerous mammalian species, possibly to best serve its purpose (reviewed by Enders & Blankenship, 1999). The simplest way of classifying the organ is on the basis of shape. There are four different classes based on the distribution of the chorionic villi. These are the diffuse placenta (with villi over the entire surface), the Cotyledonary placenta (with villi distributed into a disparate clumps), the zonary placenta (characterized by an intricate fold pattern) and the Discoid placenta (seen in rodents and humans). The discoid placenta is characterized by the villi limited to either a single or two discs. We observe a simplex, monodiscoid placenta in humans and rodents, while a duplex discoid placenta is observed in monkeys. Placentas with villi limited to sections of the placenta rather than the diffuse placenta type have a greater materno-fetal exchange surface area per unit

weight. Another important method of classification of placenta was proposed by Grosser, based on the number of layers of maternal tissue lost to the chorion during development. The number of layers separating the maternal and fetal blood has a very obvious impact on the physiological exchange between the two units. Again, this system of classification yields four distinct typed of placentae, i.e. the Epitheliochorial (no layers lost), the Syndesmochorial (the uterine epithelial layer is lost, and the maternal connective tissue is in direct contact with the chorion), the Endotheliochorial (the uterine epithelium and the maternal connective tissue are lost, with the chorion in contact with the maternal basal membrane) and the Haemochorial (characterized by the loss of all maternal tissue). Humans and rodents have a haemochorial type of placenta, in which chorion is in direct contact with the maternal blood (Ramsey, 1982).

It is important to note that the placental anatomy and physiology evolves as the gestation progresses. Upon implantation, the trophoblast layer differentiates into two types of cytotrophoblasts- the villous and extravillous cytotrophoblasts. While the extravillous cytotrophoblasts infiltrate the decidua attaching the placenta to the uterus, the villous cytotrophoblasts differentiate and fuse together to form syncytiotrophoblasts. Although feto-placental blood vessels develop inside the villi a week following implantation, there is limited maternal blood flow to placenta during the first trimester. Maternal blood flow begins to make its way to the intervillous space by the end of the first trimester and the placental barrier begins to function as venue of materno-fetal exchange. As gestation progresses further, the number of villi increases further, facilitating a better exchange between the two systems. The estimated surface area available at the materno-fetal interface at term is estimated to be approximately 13 m^2 on an average, with about 30% of the maternal cardiac output being directed towards the placenta.

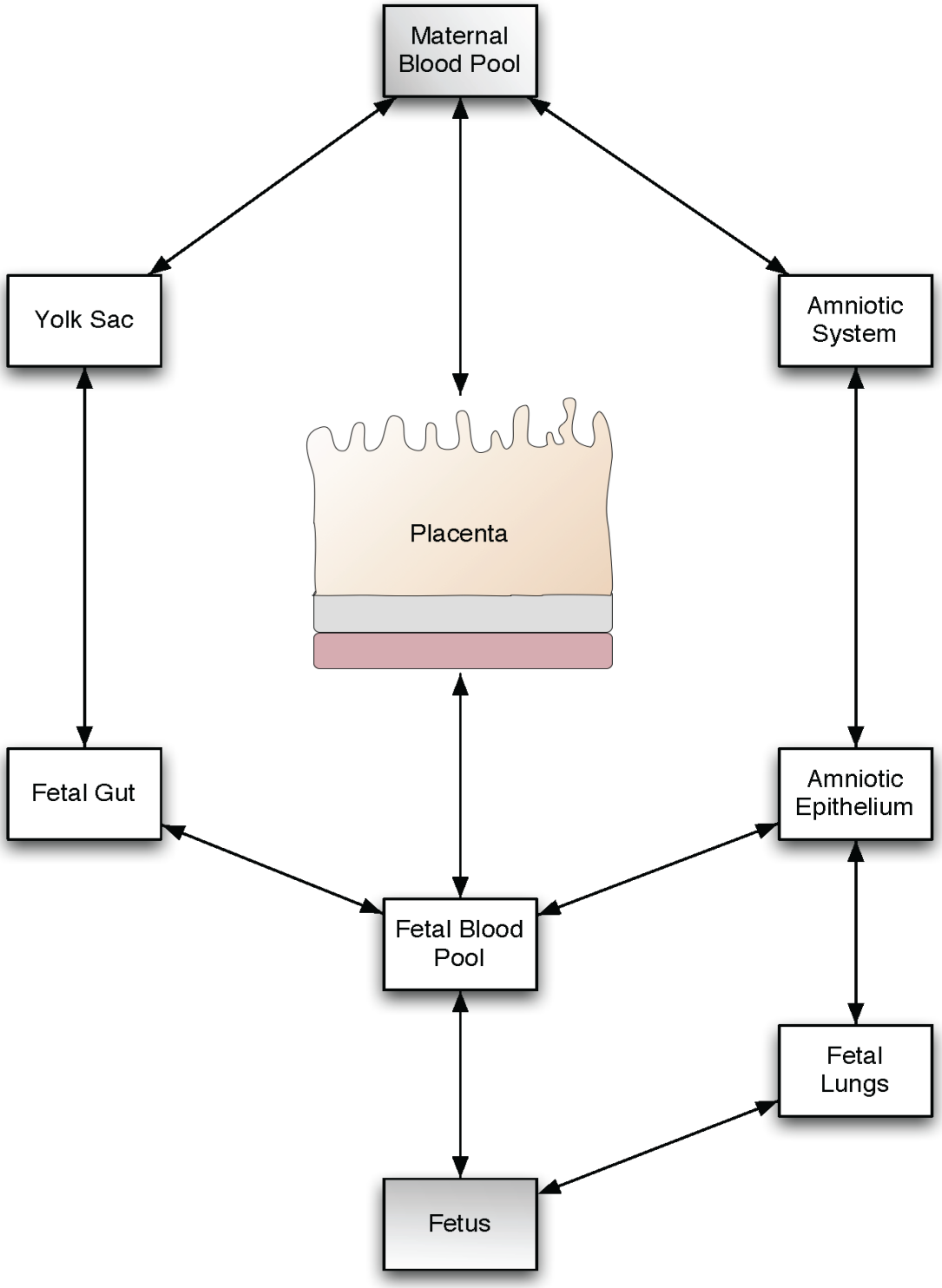


Figure 1: Diagrammatic description of the various routes for physiological exchange between the mother and fetus.

Although there are a few ways for physiological exchanges to occur, the placenta serves as the primary conduit for exchange of nutritional, metabolic and xenobiotic molecules both to and from the fetal circulation. The placenta is equipped with the tools to ensure the integrity of the fetal sanctuary is not violated. The first barrier in xenobiotic transfer from the maternal blood to the fetal system is the maternal facing apical syncytiotrophoblasts. With advancing gestation, this layer changes in thickness, thinning from 50 um in early phases of pregnancy top around 5 um towards term. Once past the apical syncytiotrophoblasts, endogenous and exogenous compounds are free to diffuse into the fetal system.

In addition to the anatomical barriers, one of the key modulators of this "gate keeper" function performed by the placenta are the ABC efflux transporters, especially when it comes to limiting the entry of xenobiotics into the fetal system. The placenta expresses a number of key ABC drug transporters including P-gp, Bcrp, and Mrp1-3 at various surfaces of the organ (Ni & Mao, 2011; Kozłowska-Rup & Czekaj, 2011; Staud et. al., 2010; Evseenko et. al., 2007). Thus, it is not unfair to hypothesize that the localization and amount of specific transporters at the materno-fetal interface will have an impact on the extent of fetal exposure to their substrates. Evidence supporting the important role that the placental transporters play in determining the fetal drug disposition has been accumulating over the last few years. Lankas et al illustrated the protective role of PGP at the placenta. This study found an increased risk of teratogenic effects of Avermectin (manifested as cleft palate) in Pgp -/- mice as compared to Pgp +/+ mice (Lankas et. al., 1998). Similarly, the fetal accumulation of a protease inhibitor saquinavir was found to be limited in Pgp null mice as compared to their wild type counterparts (Smit et al., 1999).

The expression of many of these transporters is highly variable within the placenta and is altered through the course of gestation as indicated in table 2. PGP levels have been found to decrease as pregnancy progresses (Sun et al., 2006). On the other hand, the levels of MRP2 increase as term approaches (Myer et al., 2005). Additionally, the levels of transporters are also believed to be altered in certain disease conditions, either due to the condition or the therapeutic intervention employed to correct the condition. There have been reports of elevated PGP levels in the placentae of HIV positive mothers compared to non-complicated pregnancies (Camus et al., 2006).

Since a change in placental transporter expression levels alters the extent fetal disposition of their substrates, it is vital to improve our understanding of the mechanisms that mediate this change. One avenue we decided to explore is the role of nuclear receptor PXR in the placenta. PXR is highly expressed in the placenta (Masuyama, et. al., 2001). Given the role that PXR

plays in the regulation of ABC drug transporters in the liver and intestine, the dramatically elevated levels of pregnanes (endogenous ligands for PXR) and the relative abundance of PXR in the placental tissue, we hypothesized that PXR plays an important role in regulating the expression of placental drug transporters.

Additionally, the functional units of the placenta are almost entirely derived from the fetal tissue (Lewis 2012; Ramsey 1982). For example, the chorion (responsible for fetal nutrition, respiration and excretion) is derived from the trophoblast layer of the blastocyst. Similarly the umbilical chord, the allantois, amnion and the yolk sac are also of embryonic derivation. Consequentially, it is the fetal genotype that plays a critical role in determining the genotype of the placenta.

1.5.1 Differences between the Mouse and Human Placenta:

Animal models provide researchers with convenient tools to study pregnancy related physiological phenomenon and disease conditions. The mouse model offers a very attractive avenue to explore these given the ability to produce transgenic and knockout strains, enabling us to target specific gene pathways. Furthermore, it offers the advantages of relatively low economic burden due to ease of housing in addition to our understanding of its physiology, ease of breeding and short gestational periods. These enable fairly quick data collection. While a very convenient model of human pregnancy, it is important to be cognizant of the similarities as well as differences between the structure and function of the placentae found in the two species.

As described earlier, both the human and mouse placentas are of the haemochorial type i.e. the trophoblastic tissue is in direct contact with the maternal blood. Despite this significant similarity, there exist some important differences between the two species as well. The differences amongst the two species begin from the time of deciduation, which involves the transformation of stromal cells of the uterus to yield decidual cells. While in humans deciduation begins preconception (Kliman, 2000), during late menstrual cycle, in mice the process is induced by implantation of the embryo (Enders and Welsh, 1993). Differences also exist between the two species as to when the definitive placental structure is apparent, with it being seen in human in early term (around day 21 of the gestation) (Malassiné, Frendo, and Evain-Brion, 2003). In humans, this process is initiated by blastocyst adhesion, followed by trophoblast proliferation and fusion to yield syncytiotrophoblasts. The chorionic villus is evident in the human placenta by day 21. The trophoblast cells in humans infiltrates the maternal tissues via two pathways, the first involves either interstitial invasion with the migration of trophoblasts to the decidual arterial wall or the differentiation of decidual and myometrial cells to yield multinucleated placental giant cells. The second route involves infiltration of the lumen and walls of the arteries by extravillous trophoblasts, thus resulting in extravascular invasion. This process is accompanied by the disappearance of the muscle layer and the replacement

of the endothelial cells by trophoblasts (Kaufmann et.al., 2003; Lim et. Al., 1997; Lyall, 2002).

In contrast, the murine placenta does not exhibit the definitive placental structure until mid-gestation. During the initial placentation, the trophectoderm differentiates via two distinct pathways. The first occurs via the generation of the extraembryonic ectoderm and exoplacental cone due to rapid proliferation of polar trophoblastic cells. The second pathway involves generation of primary trophoblastic giant cells brought about by the endoreplication of mural trophoblastic cells. As gestation progresses, around day 8, nutrient exchange is ensured by a vascularised zone that is generated by the migration of the embryonic mesoderm to the inner visceral endoderm giving rise to vascular cells and vitelline vessels. A distinctive chorioallantoinic placental structure becomes apparent around day 12 of the gestation, and is subdivided into a layer of maternal decidua, a junctional zone and a labyrinthine zone (Rossant and Cross, 2001; Downs, 2002). Trophoblastic giant cells limit the junctional zone to the maternal aspect. It is important to note that murine giant trophoblast cells are not analogous to their human counterparts, being generated by endoreplication, a process absent in the humans (Soares et. Al, 1996).

Another difference between humans and rodents is that the trophoblasts do not invade into the myometrium and the endovascular invasion occurs to a limited degree (Enders and Welsh, 1993). From the trophoblasts and its associated vasculature, the labyrinth is developed. Within the labyrinth, the trophoblasts further differentiate to yield three distinct layers, two layers of syncytiotrophoblasts layers in contact with fetal endothelium, and a single layer of cytotrophoblasts which is in contact with the maternal blood. Thus, three trophoblast layers separate the fetal system from the maternal blood, thereby yielding a haemochorial placenta. Hence, in the mouse, in addition to a delayed appearance of the definitive placental structure, the trophoblast invasion also occurs later and when it does, it happens in a spatially and temporally distinct manner.

Other aspects of differences between the human and murine placenta are the point during gestation when the fetal nutrition occurs directly via the maternal circulation, mediated by the trophoblasts and the arrangement of maternal and fetal circulation in the placentas. In the murine placenta, the labyrinthine structure enables a very efficient counter exchange between the maternal and fetal vasculature, which run parallel to each other. On the other hand, given the multivillous nature of the human placenta, we observe an intermediate between a concurrent and parallel flow, an arrangement considered to be less efficient than the architecture seen in murine placenta (Leiser and Kaufmann, 1994).

However, despite these developmental differences between the two species, the mouse model is still a very valuable tool to study placental drug disposition given major similarities. Importantly, the trophoblasts, the key cell type in the placenta, appear to follow the same pathway. In both cases, we observe an invasive pathway (trophoblast giant cells in the human placenta while trophoblastic glycogen cells in the mouse). While differences exist between two species in the number of trophoblast layers, studies looking at specific membranes in the two species indicate similarities between the

mouse and human placenta. The three layers of mouse placental trophoblasts are termed as layer I, II and III, with layer I in contact with the maternal blood spaces. This layer is thought to be intermittent and comprised of trophoblast giant cells and is believed to be highly permeable to most solutes. It is the syncytial layer, layer II, which is believed to be the first barrier to materno-fatal exchange (Enders and Blankenship, 1999). Electron microscopy has shown that alkaline phosphate in localized in layer II of the mouse, fitting well with its localization in the synctytiotrophoblast in the human placenta (Kusinskin et. al., 2010; Jones and Fox, 1976). Mouse trophoblast layer II and III are believed to be analogous to the human basal membrane of the syncytiotrophoblasts (Dilworth and Sibley, 2013). The exchange pathway in both species occurs via the syncytiotrophs. In addition, qualitatively similar expression in transcription factors and key drug transporters in both the human and murine placenta are observed. The placenta is an important site of expression for Mdr1a, members of the Mrp family and Bcrp in both humans and mice. Furthermore, in both the murine and human placentae, important nuclear receptors such as PXR and CAR are expressed. In conclusion, one must keep in mind both the key similarities and differences between the animal model and human placenta while interpreting results obtained with mouse placenta.

Gene	Species	Temporal Changes as Gestation progresses	Location
MDR1/ PGP/ ABCB1	Man	Decreased expression	Apical membrane
	Mouse	Decreased expression	
MRP2/ ABCC2	Man	Increased expression	Apical membrane
	Mouse	Increased expression	
BCRP/ABCG2	Man	Highly variable reports	Apical membrane
	Mouse	Variable, peaking at GD 15, decreasing towards term	

Table 2: Documented changes in ABC drug transporter levels during the course of gestation

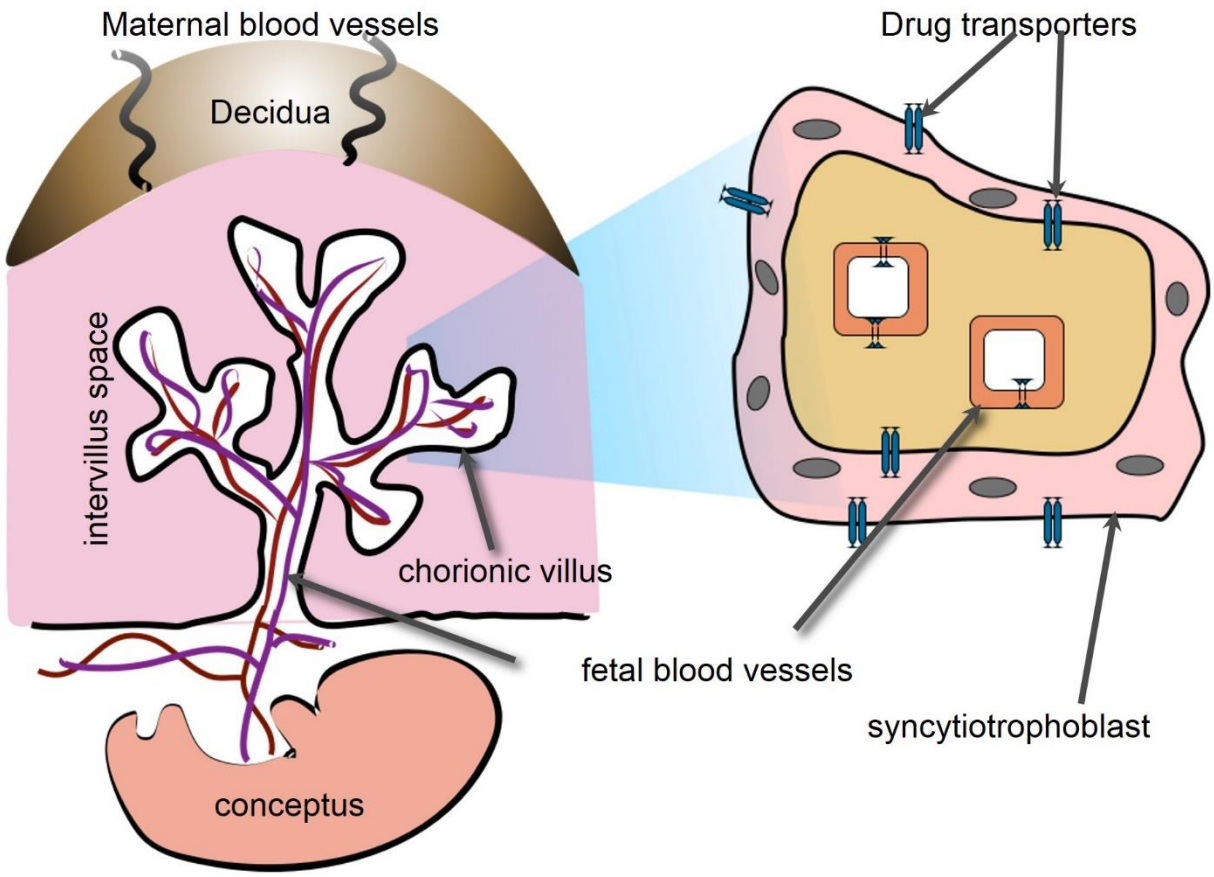


Figure 2: **Diagrammatic representation of the materno-fetal interface.**

1.6 Human Immuno Deficiency Virus (HIV):

At the forefront of the great health challenges that modern man has faced is the HIV. A retro virus, HIV has rapidly spread across the globe, presenting a formidable public health hurdle. HIV leads to an acquired deficit in cell mediated immunity. HIV infection leads to acquired immunodeficiency syndrome (AIDS), causing morbidity and mortality due to the host reaction to the virus rather than acting directly.

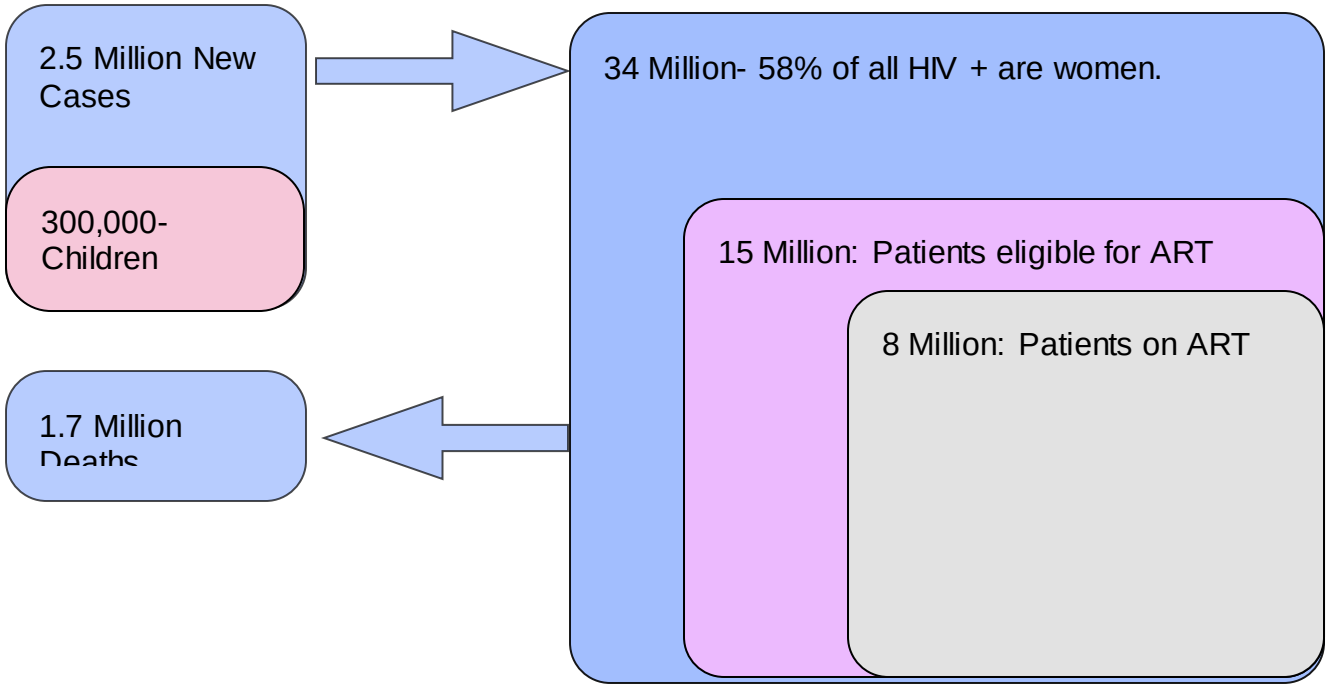


Figure 3: A diagrammatic representation of the global HIV epidemic.

While significant scientific progress has been made in treating the disease, it still remains a leading cause of deaths globally. As per the latest report by UNAIDS, there were 38 million people living with the virus. Despite a decline in new infections, there were still 2.5 million reported cases of new HIV infection and 1.7 million deaths due to the disease in 2012. Interestingly, women now form the majority of the total HIV patients globally. Close to 60% of all seropositive patients are females, a large proportion of whom are of child bearing age. The latest reports also indicate that 12% of all new cases of HIV were children (more than 300,000 annually). Most children acquire the virus through mother to child transmission (MTCT) during the pre- or peri-natal periods of the pregnancy (UNAIDS 2013).

Vertical transmission of HIV occurs when the fetal system is exposed to the systemically circulating virus in the maternal blood. Since the placenta is the only barrier between the two systems, it plays a critical role in preventing the vertical transmission. In the absence of intervention, the rates of vertical transmission are between 14-45%(John & Kreiss, 1996). MTCT of HIV can occur either in-utero, intrapartum or during breast feeding. There are a number of factors that influence the rates of vertical transmission including the stage of maternal disease, mode of acquisition, mode of delivery, viral phenotype and breast feeding behavior. In infants who are not breastfed, the most critical time seem to be the later half of the pregnancy and the intrapartum period. Indeed, in such cases, 95% of all infections occurred in the last trimester, with 65% occuring intrapartum (John & Kreiss, 1996).

Another vital factor in vertical transmission probability is the maternal viral load. This factor directly determines the net exposure of the pathogen that the unborn child is exposed to. Expectant mothers with detectable viral loads have been reported to be 2-3 times more likely to pass the virus to the child. Additionally, a high viral load also reduces the mother's immunity, compromising fetal safety. Reducing the viral loads in the maternal circulation will thus reduce the chances of MTCT (see European Collaborative Study 1992).

The rate of vertical transmission in HIV positive women is reduced to less than 5% when they receive antiretroviral therapy (ART) during gestation, delivery and breastfeeding. According to some estimates, HIV infections were prevented in more than 400,000 children as a result of ART between 2009-2011 (UNAIDS 2013). Thus, as our comfort with ARTs and the global

access to these medicines increase, the number of pregnant women on ART will continue to rise over the next few years. However, there is still very little known about the impact of in-utero exposure to ART on the fetus. Unless we improve our understanding of the mechanisms controlling the transplacental trafficking of these agents, we cannot be satisfied that an ideal treatment strategy has been achieved.

1.7 Antiretroviral Therapy:

First introduced in the 1980s, antiretrovirals have greatly reduced the mortality rates in HIV infected patients. Beginning in the mid 1990s, a combination approach, which aggressively targets the virus at various phases of its life cycle was introduced. Coined Highly Active Anti-Retroviral Therapy (HAART), this cocktail approach has become the standard of care in managing HIV-seropositive patients. HAART is comprised of a combination of at least three drugs (normally from at least two different classes) and has resulted in dramatic decreases in rates of AIDS, mortality and complications.

The main classes of Antiretrovirals are as follows:

- Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs and NtRTIs): These agents competitively inhibit the reverse transcription of the viral RNA. These agents impair integration into the host genome by providing “spurious” building blocks for reverse transcription.

- Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs): These agents inhibit the reverse transcriptase irreversibly by binding close to the catalytic unit of the reverse transcription enzyme. Used commonly in combination with NRTIs, treatment with NNRTIs is susceptible to development of resistance.

- Protease Inhibitors (PI): These agents disrupt virus assembly and maturation, by impeding the activity of viral protease. This leads to the generation of structurally compromised and non-infective viral particles.

- Integrase Inhibitors: The only approved agent in this class of Antiretrovirals is raltegravir. These agents prevent the provirus integration into the host genome.

- Entry and Fusion Inhibitors: These agents hinder with both the binding and fusion of the virus to the host cell membrane. The only approved fusion inhibitor is enfuvirtide binds to the attachment site of the HIV-1 virus. Entry inhibitors such as maraviroc bind and change the shape of CCR5 receptors, thus preventing entry of the virus into the host cell.

The history of antiretroviral use in Pregnancy:

The recommended first line HAART in pregnant seropositive women is a 3 drug combination of 2 NRTIs combined with a PI (frequently boosted with low dose ritonavir) or a NNRTI (AIDSinfo, 2013). In pregnant women, ritonavir boosted lopinavir is the first line PI while 3TC and ziduvudine are the first line NNRTI agents at present. There are currently more than 20 approved Antiretrovirals available in the US market. The first evidence underling the utility of antiretroviral during gestation came from a landmark study conducted in 1994. The Pediatric Aids Clinical Trail Group (PACTG) protocol 076 demonstrated that use of Zidovudine (AZT) prophylaxis during gestation (from week 14 to 34), infusion of i.v. AZT during labor and oral treatment of the neonate with AZT for 6 months post birth reduced the rate of vertical HIV transmission by more than 67% (Connor et al., 1994).

Further studies demonstrated the importance of viral load in determining the probability of MTCT. Reducing the viral load to less than 500 copies/mL was found to minimize the risk of vertical transmission while maintaining maternal health (Tubiana et al., 2010; European Collaborative Study, 1992). It was due to compelling data as these, along with the urgent need to minimize MTCT that eventually led to a push for HAART prophylaxis during pregnancy.

While the need to provide prophylactic care during pregnancy was accepted readily, the timing of initiation of the therapy has been debated at length. Studies such as the CIPRA-HT001 have suggested that it is most beneficial (from a mortality and morbidity point of view) to initiate ART before CD4 cell count falls below 200 cell/mm^3 (NIH, 2009) Based on these data, the WHO revised its guidelines recommending commencing ATR for the maintenance of the women's own health when the CD4 cell count falls below 350 cells/mm^3 regardless of gestational status (WHO HIV Guidelines, 2013). Most international health agencies follow a similar guideline, thus we are increasingly seeing the administration of antiretrovirals throughout the course of the pregnancy.

1.8 Interaction of Antiretrovirals, ABC Drug Transporters and HIV:

While much data has been generated about antiretrovirals and ABC drug transporters independently, many unanswered questions remain as to how these factors interact, particularly in the context of HIV. Clinically, an HIV patient consumes numerous antiretrovirals incessantly from the time of detection of his/her seropositive status. As we have seen, ABC drug transporters are expressed at all important epithelium membranes, and have been shown to be influenced by disease and environmental influences.

Based on numerous in-vitro and in-vivo findings, it has been established that many of commonly employed antiretrovirals are substrates, inducers or inhibitors of key ABC drug transporters. One of the most widely studied antiretroviral is saquinavir, a PI, first identified as a P-gp substrate in 1998 (Kim, Dintaman, Waddell & Silverman 1998). Studies conducted in P-gp overexpressing G185 cells, illustrated that co-administration of the P-gp inhibitor cyclosporine reversed their innate resistance to saquinavir. Furthermore, using a polarized epithelial transport assay, Saquinavir was shown to be a substrate for P-gp, inhibited the P-gp mediated efflux of rhodamine 123. The cellular efflux of saquinavir was also shown to be inhibited by P-gp inhibitors. Additionally, saquinavir has also been shown to be a P-gp inhibitor, along with other PIs such as lopinavir, ritonavir and nelfinavir (Tong et al., 2007; Drewe et al., 1999). Antiretrovirals can act to modulate the expression of transporters as well. Saquinavir has been established as a PXR ligand (Gupta et al,2008), thus capable of inducing PXR target genes such as P-gp, CYP3A and MRPs. Saquinavir is also a substrate and inhibitor of MRP2 (Williams et al. 2002).

Ritonavir, another important PI, that is commonly used as to improve the systemic availability of PIs, has also been established as a P-gp substrate. In addition, based on numerous in-vitro and in-vivo studies, ritonavir has been shown to be a very potent P-gp inhibitor. Furthermore, similar to saquinavir, it is a PXR ligand (Dussault et al., 2001), and imposes an up regulation in MDR1a mRNA levels (Perloff et al, 2001).

One of the most widely used PI for the management of HIV in seropositive pregnant women, lopinavir is a substrate and potent P-gp inhibitor. Using LS180 cells, lopinavir was found to activate PXR resulting in induction of CYP3A and P-gp (Gupta et al., 2008). Lopinavir is also

reported to be a substrate for murine Bcrp1, however this has not been confirmed clinically. In addition, lopinavir is a potent bcrp inhibitor along with being a substrate of MRP1 and 2 (Weiss et al., 2007).

Antiretroviral	PGP	BCRP	MRPs
Saquinavir	Substrate: Yes Inhibitor: Yes Inducer: Yes	Substrate: No Inhibitor: Yes Inducer: Yes	Substrate: Yes (MRP2) Inhibitor: Yes Inducer: Yes (MRP1, MRP2, MRP4 and MRP5
Ritonavir	Substrate: Yes Inhibitor: Yes Inducer: Yes	Substrate: No Inhibitor: Not Conclusive Inducer: ?	Substrate: Yes (MRP2) Inhibitor: Yes (MRP1) Inducer: No
Lopinavir	Substrate: Yes Inhibitor: Yes Inducer: Yes/?	Substrate: No Inhibitor: Yes Inducer: ?	Substrate: Yes (MRP1 & 2) Inhibitor: ? Inducer: ?
Indinavir	Substrate: Yes Inhibitor: Yes Inducer: No	Substrate: No Inhibitor: No Inducer:?	Substrate: Yes (MRP2) Inhibitor: Yes Inducer: ?
Darunavir	Substrate: Yes Inhibitor: Yes (weak) Inducer: Yes	Substrate: No Inhibitor: Yes (weak) Inducer: ?	Substrate: Not conclusive Inhibitor: ? Inducer: No
Nevirapine	Substrate: No Inhibitor: Yes Inducer: Not conclusive	Substrate: No Inhibitor: Yes (weak) Inducer: ?	Substrate: ? Inhibitor: Yes Inducer: ?
Zidovudine	Substrate: Not conclusive Inhibitor: No Inducer: Not conclusive	Substrate: Yes Inhibitor: Yes (weak) Inducer: ?	Substrate: Yes (MRP1-5) Inhibitor: No Inducer: Yes (MRP4, 5)

Table 3: A list of the major Antiretrovirals employed clinically and their relation with key ABC drug transporters found in the placenta.

Since antiretroviral agents are substrates of ABC drug transporters, changes in the expression and activity of the transporters can impact their pharmacokinetics and pharmacodynamics. In addition to factors such as non-compliance and drug resistance, suboptimal cellular penetration into sanctuary sites such as the brain and testis is believed to play an important role in failure of antiretroviral therapy. ABC drug transporters are expressed abundantly at a number of these sites and are believed to be one major cause of sub therapeutic intracellular concentrations. This is especially true for PIs, for which a high correlation between intracellular drug concentration and antiretroviral activity is documented (Bazzoli et al., 2010). It has been demonstrated that over expression of P-gp or MRP1 in T-lymphocyte cell lines leads to reduced accumulation of PIs (Srinivas, Middlemas, Flynn & Fridland, 1998). Over expression of P-gp and BCRP at cell surfaces have been shown to be directly related to PI and NRTI resistance respectively (Raffi, 2000; Wang et al., 2003).

In addition to impacting intracellular drug levels at target sites, the ABC drugs transporters can also exert their influence by altering the oral bioavailability and controlling the access of their substrates to sanctuary sites. Transporter expression at sanctuary sites such as the brain and placenta has important therapeutic outcomes for antiretroviral therapy. The importance of P-gp has been demonstrated using animals models. Greater amounts of saquinavir was found to accumulate in the fetal compartment of P-gp knockout mice as compared to wild type fetal units (Smit et al.1999). Inhibition of P-gp has also been demonstrated to increase trans-placental penetration of saquinavir in wild type mice and in human placental studies (Molsa et al., 2005). In human placental perfusion studies, the materno-fetal transfer of saquinavir was 6 times greater and 1.5 times greater for indinavir in the presence of P-gp inhibitors.

Finally, HIV infection itself has been associated with altered transporter expression levels. The mRNA levels of ABCB1, ABCC1, ABCC4 and ABCC5 have been found to be significantly higher in the PBMCs of HIV seropositive patients than healthy controls (Turriziani et al., 2008). Furthermore, the exposure to NRTIs seems to have an impact on the expression levels of ABCC4 and ABCC5 transcript levels, with lower levels reported in NRTI naive individuals

(Jorajuria et al., 2004). It is not entirely clear how much of the elevation is attributable to seropositive status as opposed to ART.

A number of reports have reported interaction between HIV seropositive status and P-gp expression levels, although the mechanism behind this interaction remains to be elucidated. In placentas obtained from HIV positive pregnancies, a significant increase of more than 3 fold was seen in placental MDR1 mRNA levels as compared to placentas obtained from HIV(-) women (Camus et al., 2006). The results were also reflected at the protein level, with a significant increase of more than 2 fold seen in placentas from infected mothers compared to the controls. Drug naive tumor cell lines exposed to HIV viral proteins have been shown to over express P-gp. This elevation of expression does not need any ART exposure, and is attributable to the presence of the HIV alone. In contradiction to this assertion are reports of significantly reduced P-gp expression in CD4+ NK cells obtained from HIV patients (Lucia et al., 1995). Similar decreases are observed in lymphocytes of HIV patients (Srinivas, Middlemas, Flynn & Fridland, 1998).

Thus, transporter expression patterns, disease and therapeutic intervention, all interact in a dynamic and complex way with each other. While the exact nature of this interaction is still poorly understood at the placental interface, its importance cannot be understated. Any significant change in the drug transporter level at the materno-fetal interface, whether it be due to normal physiological processes, drug induced or due to the circulating pathogen, can have a significant impact on therapeutic outcome. A change that disturbs the status quo, and moves in a direction that increases fetal drug exposure can result in potentially toxic levels crossing the placenta. Such scenarios can lead to unforeseen (in the true sense of the word, since very little safety data during pregnancy is available for the majority of Antiretrovirals) adverse events in the offspring. On the other hand, induction of transporter expression or function resulting in a pronounced decrease in fetal exposure to antiretrovirals can be equally troublesome. Adequate levels of antiretrovirals are required in both maternal (keeping the viral load at a minimum) and fetal systems (to combat any viral exposure), to prevent the viral transmission. If the levels in either the maternal or fetal system fall below the therapeutic threshold, appropriate efficacy may not be seen and could possibly resulting in vertical transmission of the virus to the offspring.

1.9 Project Rationale:

The AIDS epidemic has taken a tremendous socio-economic toll on society as a whole. The medical community has faced the challenge head on, combating and subduing the spread of the disease. Novel antiretrovirals have been developed at an astonishing speed, and our pharmaco therapeutic arsenal now contains a number of highly potent drugs. Encouraged by the effectiveness of the HAART approach, an ever increasing number of HIV patients are now receiving Antiretrovirals long term to manage their disease.

With effective and aggressive prophylaxis, the rates of vertical transmission of HIV from mother to child have been brought down to below 5%. The growing confidence in preventing MTCT, improved life quality and prolonged life span of infected patients has lead to an increasing number of HIV positive women choosing to bear children. While many of these drugs are substrates of the ABC dug transporters, there has been little research done looking at factors impacting the expression and activity if these transporters at the placenta- the gate keeper to the fetal system. ABC drug transporters have been recognized as key players in determining the disposition of their substrates, although bulk of the work has focused on major sites of absorption or sectretion. There is much uncovered ground when it comes to understanding the mechanisms behind placental transporter regulation and the role of these membrane proteins in determining fetal drug exposure to agents such as Antiretrovirals.

The placenta, has been shown to express two vital players- the nuclear receptor PXR and a number of important ABC drug transporters. It is important to note that a number widely used Antiretrovirals are substrates of P-gp, MRPs and BCRP; all of which are abundant in the placenta. The involvement of ABC drug transporters in trafficking Antiretrovirals across epithelial membranes has been established both in-vivo and in-vitro. For example, P-gp knockout mice have been shown to allow greater amounts of saquinavir and indinavir to make their way to fetal compartment as compared to their wild type counterparts. Oral bioavailability of lopinavir has been shown to be influenced by P-gp and MRP in animal studies. Moreover, a number of antiretroviral agents are PXR ligands and are capable of inducing the expression of these transporters.

The role of PXR in regulating the transporters of interest has been well established over the last few years. Numerous groups have shown the involvement of PXR in regulating P-gp, MRPs and

numerous other transporters in tissues such the liver, brain and the gut. However, no such studies have been conducted looking at placental PXR. This is surprising, given the moderately high expression of PXR in the placental tissue, and the high levels of circulating pregnanes, natural ligands to the NR seen during pregnancy. Given the established role of PXR as regulator of key transporters, we examined it role in the placenta. Employing PXR -/- mice, we were able to examine the role of PXR expression and activation on the regulation of its target genes in the placenta. Furthermore, we examined the impact of altered transporter expression on the fetal accumulation of the P-gp and Mrp2 substrate, lopinavir in-vivo in a PXR heterozygote model (PXR +/-). Our animal model, with a varying amount of placental expression of these transporters, allowed us to establish examine the role of these transporters in trans-placental transfer of lopinavir. Generating the placental units with varying transporter levels allowed us to control for confounding factors, and allowed us to examine the effect of altered transporter expression only at the site of interest.

Lopinavir was chosen being a first line PI used in HIV positive pregnant women and has been shown to be a substrate of P-gp and Mrp-2. This molecule allowed us to test out hypothesis regarding the importance of placental drug transporters in determining fetal drug exposure. It is vital to understand if changes in the expression of ABC drug transporters are indeed important enough to affect a therapeutically important alteration of the fetal penetration of their substrates. This will enable us to fine attain the fine balance needed to ensure safe and efficacious treatment. Additionally, understanding the mechanisms behind ABC transporter regulations, and how it relates to the established paradigm of NR mediated regulation is vital. It ultimately may enable us to predict potential transporter changes due to drug-drug, or drug food interactions and guide the next steps in this important area of exploration.

1.9.1 Hypothesis:

Our hypothesis is that:

- The placental ABC drug transporters are regulated in part by the nuclear receptor PXR.
- Changes in the placental expression of ABC drug transporters will alter the fetal exposure to their substrates.

1.9.2 Objectives:

Our main objectives were:

- To explore the role of PXR in the regulation of ABC drug transporters in placenta
- To explore the impact of PXR genotype and placental transporter expression on fetal exposure to a commonly used antiretroviral agent, lopinavir

Chapter 2 Gestational and Pregnane X Receptor mediated regulation of placental ABC drug transporters in mice.

This chapter is a reprint of “Gahir SS, Piquette-Miller M. Gestational and pregnane X receptor-mediated regulation of placental ATP-binding cassette drug transporters in mice. Drug Metab Dispos. 2011 Mar;39(3):465-71”

2.1 Abstract:

Purpose

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.

Methods

The placental expression of Mdr1a, Bcrp, Mrp1, 2 and 3 was examined in PXR knockouts (-/-), heterozygote (+/-) and wild type (+/+) mice by quantitative PCR. The impact of PXR activation was examined in pregnant PCN-treated mice.

Results

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.

Conclusions

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.

2.2 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 (Kolwankar, Glover, Ware, & Tracy, 2005; Unadkat, Dahlin, & Vijay, 2004; Vasiliou et al., 2009; Vähäkangas & Myllynen, 2009). 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 that contribute to altered fetal exposure or teratogenic risk during pregnancy.

The ATP-binding cassette (ABC) family of drug transporters, in particular, P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and 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. P-gp and BCRP are expressed at the placental apical surface (Ceckova-Novotna, Pavek, & Staud, 2006; Mao, 2008) 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 (Nagashige et al., 2003; St-Pierre et al., 2000) (Vähäkangas & Myllynen, 2009). Fundamental studies establishing the importance of placental drug transporters have demonstrated increased fetal substrate accumulation and teratogenicity in P-gp-deficient mice (Lankas et al., 1998) . Likewise, fetal exposure of the BCRP substrate glyburide is increased by 2-fold in BCRP-deficient mice (Zhou et al., 2008). Down-regulation of BCRP in placenta of endotoxin-treated rats has also been shown to significantly increase fetal

exposure to glyburide (Petrovic, Wang, & Piquette-Miller, 2008). Thus, alterations in the expression of placental drug transporters can significantly affect 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, and endogenous steroids (Chang & Waxman, 2006) . Indeed, largely due to increasing hormonal levels, the hepatic expression of PXR increases by approximately 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 (Guo, Staudinger, Ogura, & Klaassen, 2002) (Kliewer, Goodwin, & Willson, 2002) (Teng, Jekerle, & Piquette-Miller, 2003) (Anapolsky, Teng, Dixit, & Piquette-Miller, 2006). Likewise, PXR activation has been found to induce expression and activity of P-gp at the blood-brain barrier (Bauer, Hartz, Fricker, & Miller, 2004) (Bauer et al., 2006) . This regulation may be tissue-specific because 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 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.

2.3 Materials and Methods

2.3.1 Animals.

Animal studies were conducted at the Division of Comparative Medicine, University of Toronto (Toronto, ON, Canada), following protocols approved by the Animal Care Committee, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). PXR wild-type (+/+) C57BL/6 mice were purchased from Charles River Canada (Montreal, QC, Canada). The PXR knockout (-/-) C57BL/6 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, whereas the PXR wild-type (+/+) and knockout (-/-) placentas were obtained by mating homozygous parents.

For the purpose of obtaining timed pregnancies, the mice were paired overnight, and the males were 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), and the placentas were harvested, snap-frozen in liquid nitrogen, and stored at -80°C.

To elucidate the role of PXR on placental gene regulation, animals were dosed (50 mg/kg i.p.) for 5 days with the murine PXR activator, pregnane-16a-carbonitrile (PCN) (Sigma-Aldrich, Oakville, ON, Canada) as described previously (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.

2.3.2 mRNA Determination:

As described previously, total RNA extraction and reverse transcriptase and real-time PCR were 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 1 fg of RNA from each sample using a First Strand

cDNA Synthesis Kit (Fermentas Canada, Inc., Burlington, ON, Canada). Quantification of Mrp1–3, Bcrp, Mdr1a, Oatp2 (Slco1a4), Pxr1, Pxr2, Cyp2b10, and Car mRNA was performed by real-time quantitative PCR using a LightCycler unit (Roche Diagnostics, Mannheim, Germany) with LC FastStart DNA Master SYBR Green I. All transcript levels were normalized to housekeeping genes (Gapdh and 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. Because the mRNA levels of Mrp1 were below the detection limits for the majority of the samples, these data are not shown. Primers used for Mrp1–3, Bcrp, Mdr1a, Gapdh, and Oatp2 have been described previously (Teng and Piquette-Miller, 2005) (Appendix 2).

2.3.3 Statistical Analysis:

All studies were performed using n = 6 to 8 mice/group with the exception of the basal liver expression study, which had 4 animals/group. Differences in the expression of key transporters between the three genotypes were determined by performing analysis of variance with a 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 tests were conducted, and results are reported to be significant when $p < 0.05$. Performing the nonparametric Mann-Whitney U test yielded similar results. All statistics were performed using GraphPad Prism (version 4.0c; GraphPad Software Inc., San Diego, CA).

2.4 Results

2.4.1 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 Fig. 4A, dramatic differences in the placental expression of several ABC drug transporters were detected among genotypes. Indeed, Mdr1a, Bcrp, Mrp2, and Mrp3 mRNA levels in the knockout mice were approximately

12-, 10-, 7-, and 2-fold higher in the placentas of the PXR knockout mice compared with values obtained in the wild-type ($p < 0.05$). Of interest, 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 with that in the (+/+) mice ($p < 0.05$) as seen in Fig. 4B. Pxr transcripts were not detected in the (-/-) placentas.

To establish whether the genotypic differences were pregnancy-related, gene expression was also examined in the hepatic tissues of the PXR(-/-) and PXR(+/+) mice at GD 17 and in nonpregnant female mice (Fig. 4C). Nonpregnant 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, whereas the levels of Oatp2, Bsep, and Ntcp did not change. In pregnant animals, trends of reduced expression were seen compared with expression in the nonpregnant animals in both the genotypes for Cyp3a11, Mrp2, and Mrp3; however, the PXR(-/-) animals maintained higher levels of transcripts for the PXR target genes.

mRNA levels of Car [constitutive androstane receptor (CAR)], an important nuclear receptor that shares many overlapping gene targets with PXR, were not significantly different between PXR(+/+)or PXR(-/-). Levels of Hnf4a mRNA, which is an important regulatorof both PXR and CAR, were not significantly different in placentas isolated from PXR(+/+) or PXR(- /-) mice (Fig. 5A). Likewise, hepatic mRNA levels of Car were not statistically different among genotypes (Fig. 5B). 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 Fig. 6, Cyp2b10 levels in livers obtained from pregnant and nonpregnant PXR(- / -) mice were 6.8- 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 knockout mice was also detected. As depicted in Table 4, we observed 18- to 19-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 knockout mice ($p < 0.05$). Moreover, whereas mrp2 levels increased during the course of gestation in the placenta of all mice, a more pronounced induction was seen in the PXR(- / -) placentas compared with that in the wild-type placentas. Although 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 placentas of the PXR(- / -) mice. Thus, in the absence of PXR, several of the major ABC drug transporters exhibit elevated expres-sion levels relative to those of wild-type mice over the course of gestation. Impact of PXR Activation by PCN. Although it is well estab-lished 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 (Fig. 7A). Although there was a trend for 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 because levels were below the 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. 7B). PCN treat-ment did not significantly alter the expression of any placental transporters or hepatic Cyp3a11 in PXR(- /-) animals (Fig. 7C). To confirm that the PCN did in fact reach the placenta at sufficiently high concentration to activate PXR, we analyzed the impact of PCN administration in the livers of fetuses from the treated and control PXR(+/+) mice. Compared with control livers, a significant 1.75- to 2-fold induction of Cyp3a11 and Oatp2 mRNA levels was seen in fetal livers isolated from the PCN treatment group (Fig. 8A), providing evidence of PCN-mediated activation of PXR in the placental-fetal unit. Figure 8B shows the absolute Cyp3a11 basal transcript levels seen in the fetal and maternal livers 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 (Fig. 9A). The full-length transcript accounted for 80 and 84% of total Pxr in the liver and placenta, respectively.

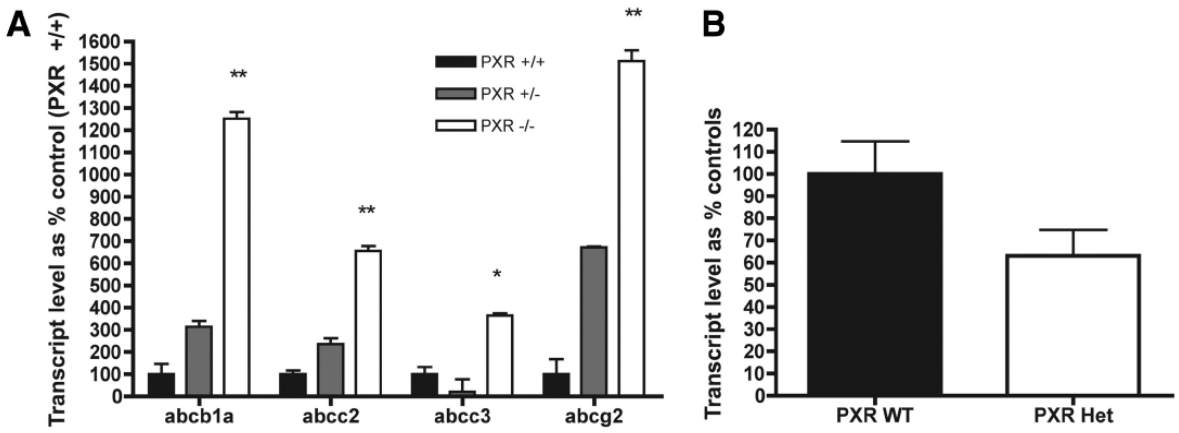


Figure 4A. Basal mRNA expression of key transporters in the placenta of PXR wildtypes (PXR +/+), heterozygotes (PXR +/-) and the knockout (PXR -/-) fetuses on GD 17. Levels were normalized to Gapdh and presented as % controls.{$p<0.05$;$p<0.01$}. n=6-8

Fugure 4B. mRNA expression of PXR in wild-type (WT) and heterozygote (Het) placentas at GD 17. Levels are normalized to Gapdh and are presented as percentage of wild-type levels. N=6-8; *$p<0.05$.

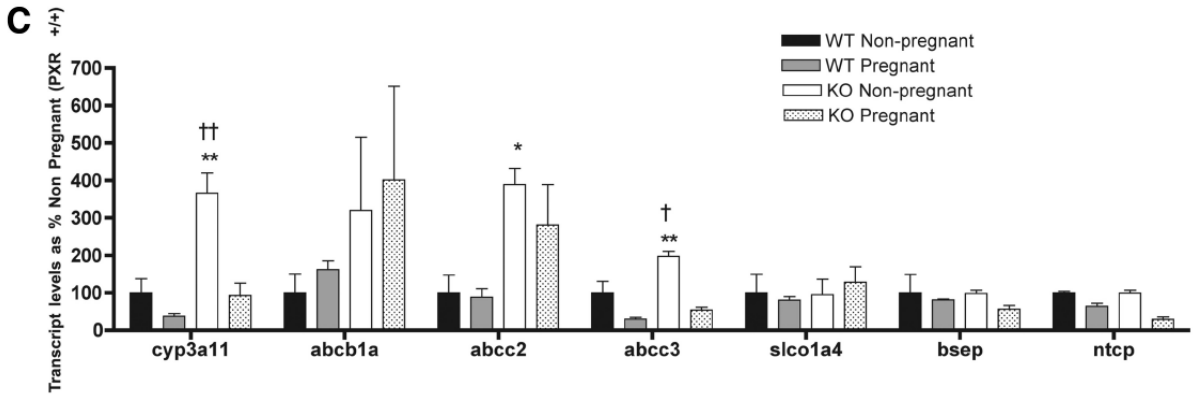


Figure 4C: Basal mRNA expression of key transporters in the liver of pregnant (GD 17) and non pregnant PXR +/+ and -/- mice. Levels were normalized to Gapdh and presented as % WT non-pregnant.{p<0.05;p<0.01 compared to -/- pregnant , † p<0.05;††p<0.01 compared to +/+ non-pregnant}. n=4

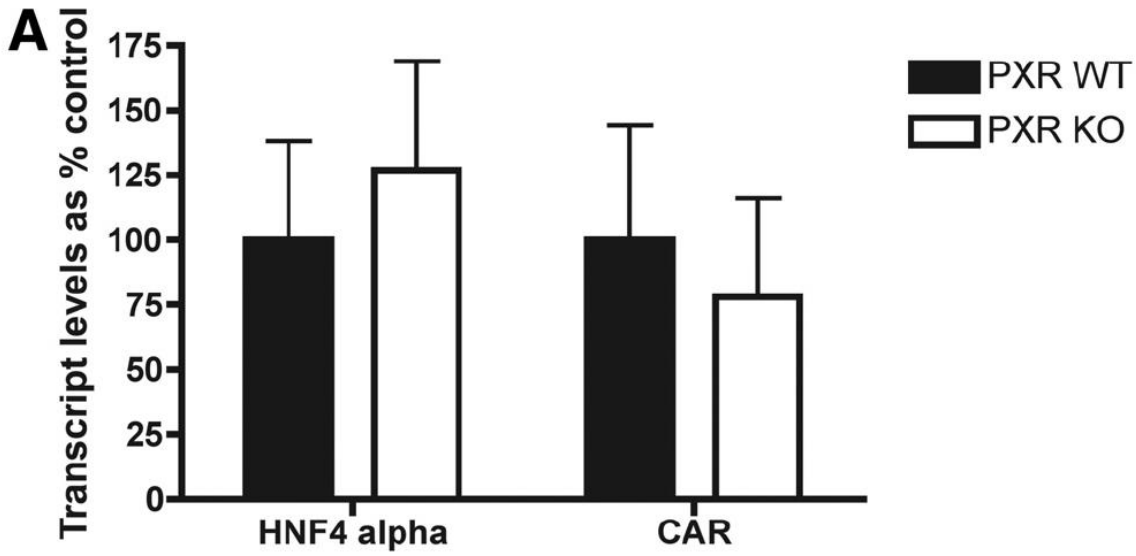


Fig 5A: Expression of CAR and HNF4 mRNA alpha in the placental tissue of PXR WT and KO mice on GD 17. No statistical significant differences are observed between the two strains. Levels were normalized to Gapdh and presented as % controls.{$p<0.05$;$p<0.01$}. n=6

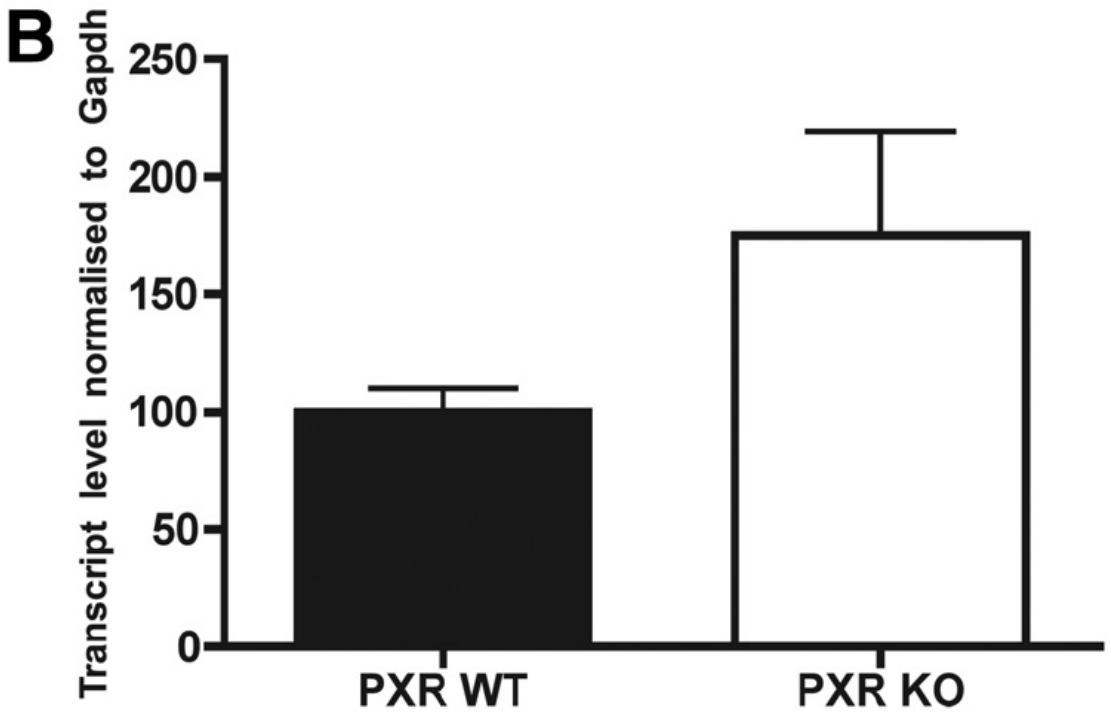


Fig 5B: Expression of CAR mRNA in the maternal hepatic tissue of PXR WT and KO mice on GD 17. No statistical significant differences are observed between the two strains. Levels were normalized to Gapdh and presented as % controls. n=4

Temporal transporter expression during gestation

This table summarizes the overall temporal trends in the placental transporter expression. The transporters were assessed at GD 10 and GD 17. All values normalized to wild-type levels on GD 17 as percentage of controls.

Gene	Strain	GD 10	GD 17	Change
abcb1a (Mdr1a)	Wild-type	1950	100	↓ 19-fold
	Knockout	bdl	1333	↑
abcc2 (Mrp2)	Wild-type	bdl	100	↑
	Knockout	bdl	320	↑ ↑
abcc3 (Mrp3)	Wild-type	1800	100	↓ 18-fold
	Knockout	bdl	320	↑
abcg2 (Bcrp)	Wild-type	490	100	↓ 5-fold
	Knockout	1998	427	↓ 5-fold

bdl, below the detection limit.

Table 5: 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.

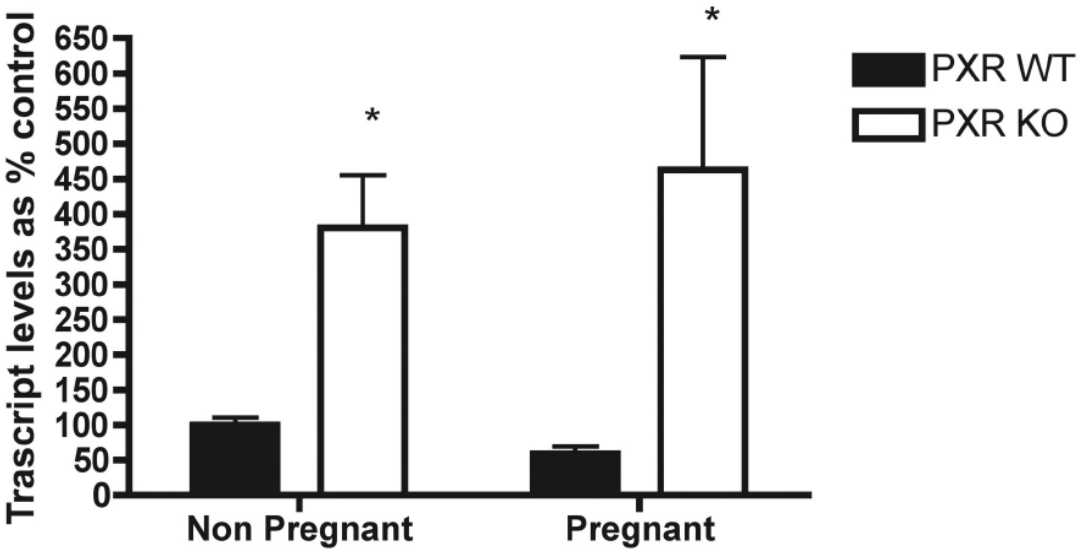


Fig 6: The expression of cyp2b10 mRNA in the livers of both pregnant (GD 17) and non pregnant PXR +/+ and -/-. Levels were normalized to Gapdh and presented as % controls.{p<0.05;p<0.01}. n=6

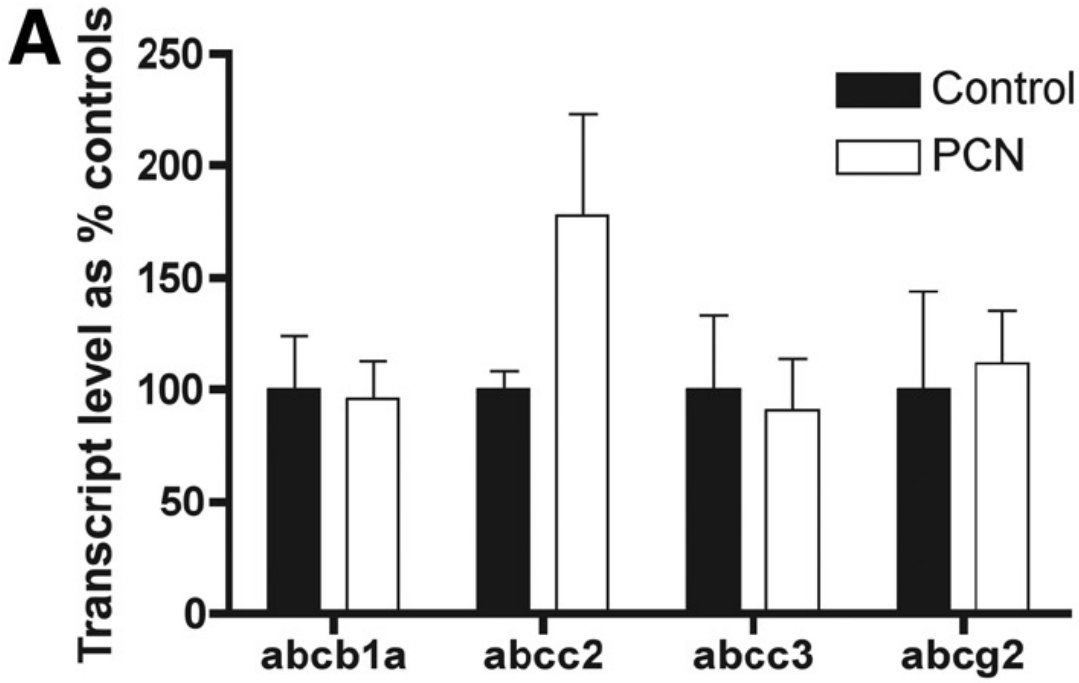
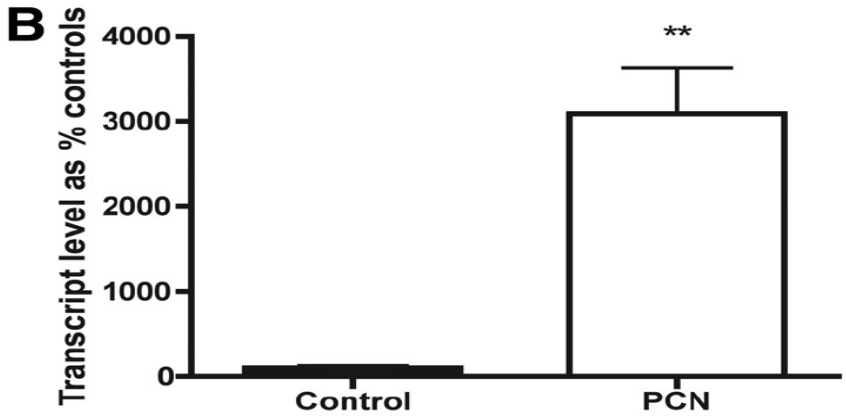


Figure 7A: Impact of PCN treatment mRNA levels in the placenta PXR +/+ mice treated with 50mg/kg PCN (i.p.) daily for 4 days. Tissues were harvested on 5th day (GD 17) and the tissues analysed as described in the methods. PCN treatment did not significantly impact the placental transporters. Levels were normalized to Gapdh and presented as % controls. {$p<0.05$;$p<0.01$}. n=6-8



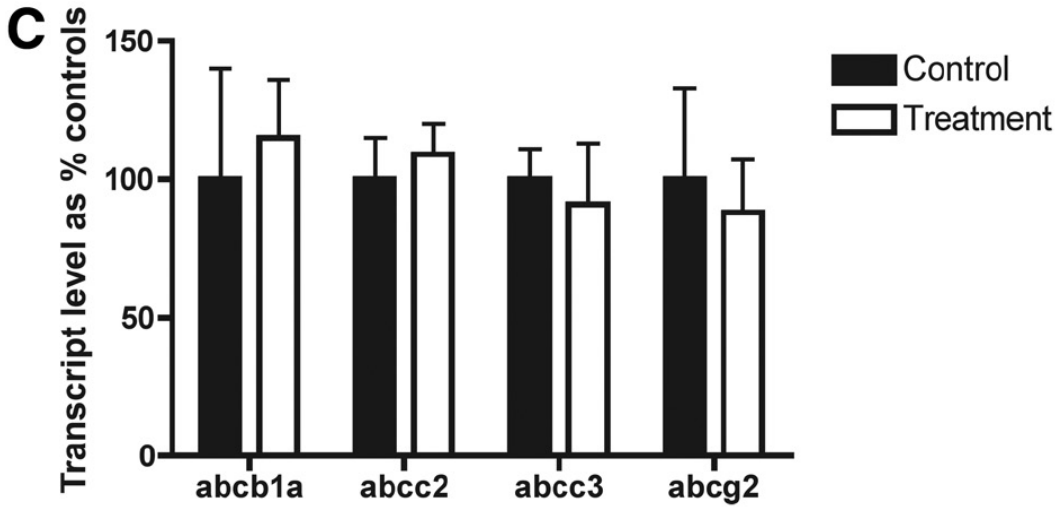


Figure 7 B: Impact of PCN treatment on CYP3a mRNA levels in the maternal hepatic tissue of PXR +/+ mice treated with 50mg/kg PCN (i.p.) daily for 4 days. Tissues were harvestedon 5th day (GD 17) and the tissues analysed as described in the methods. PCN treatment did not significantly impact the placental transporters. Levels were normalized to Gapdh and presented as % controls. ** $p<0.01$

Figure 7C: Impact of PCN treatment mRNA levels in the placenta 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.

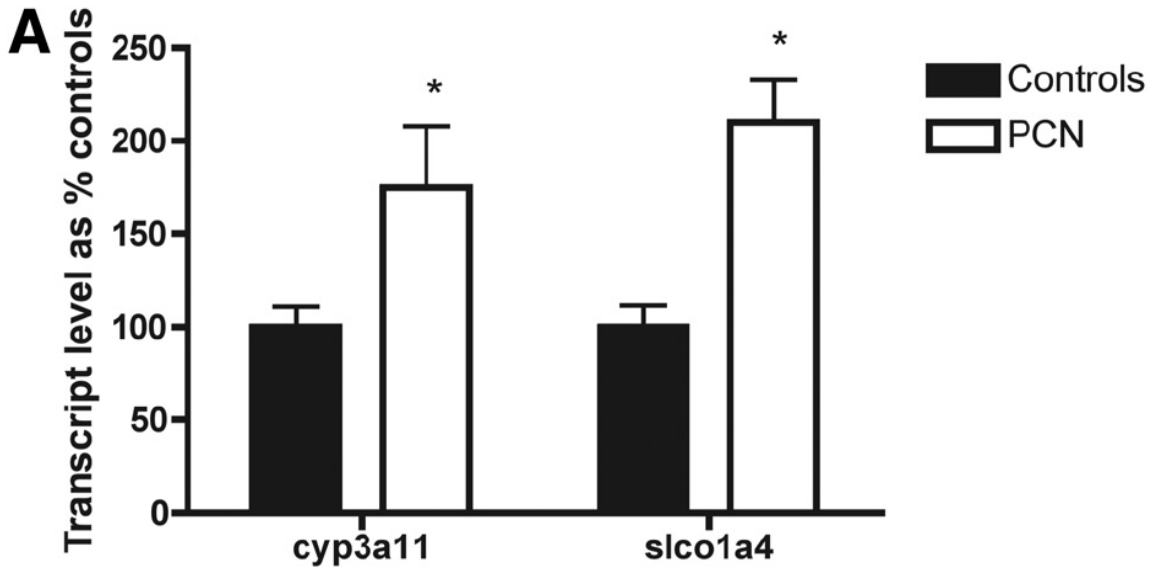


Fig 8 A: Impact of PCN on fetal cyp3a and oatp2 (Slco1a4). Pregnant mice were treated with PCN (i.p.) as per the regimen described in the methods. On GD 17 the animals were sacrificed and tissues harvested. mRNA levels of cyp3a and oatp2 in the fetal liver were assesed using real time PCR as described. Both genes were upregulated in the fetal livers as compared to the controls (cyp3a by 75% and oapt2 by 100%).

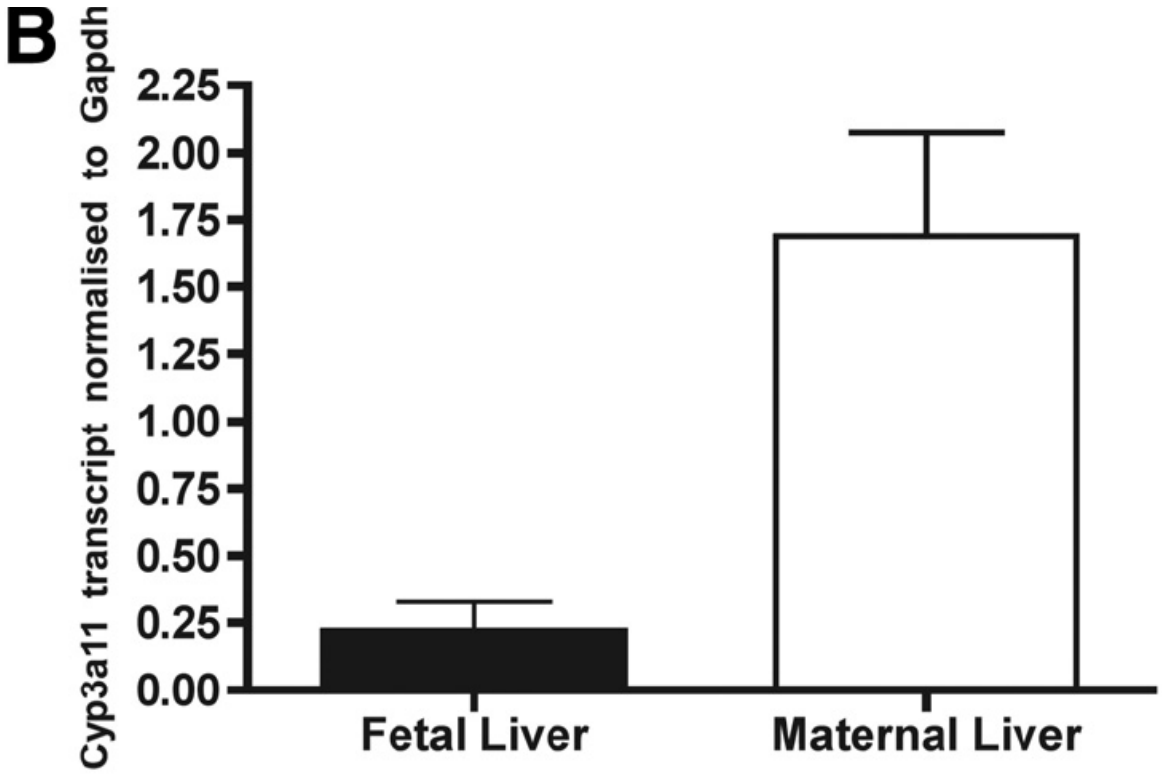


Fig 8 B: Basal mRNA expression of Cyp3a11 in the liver and placenta of pregnant (GD 17) PXR (+/+) mice.

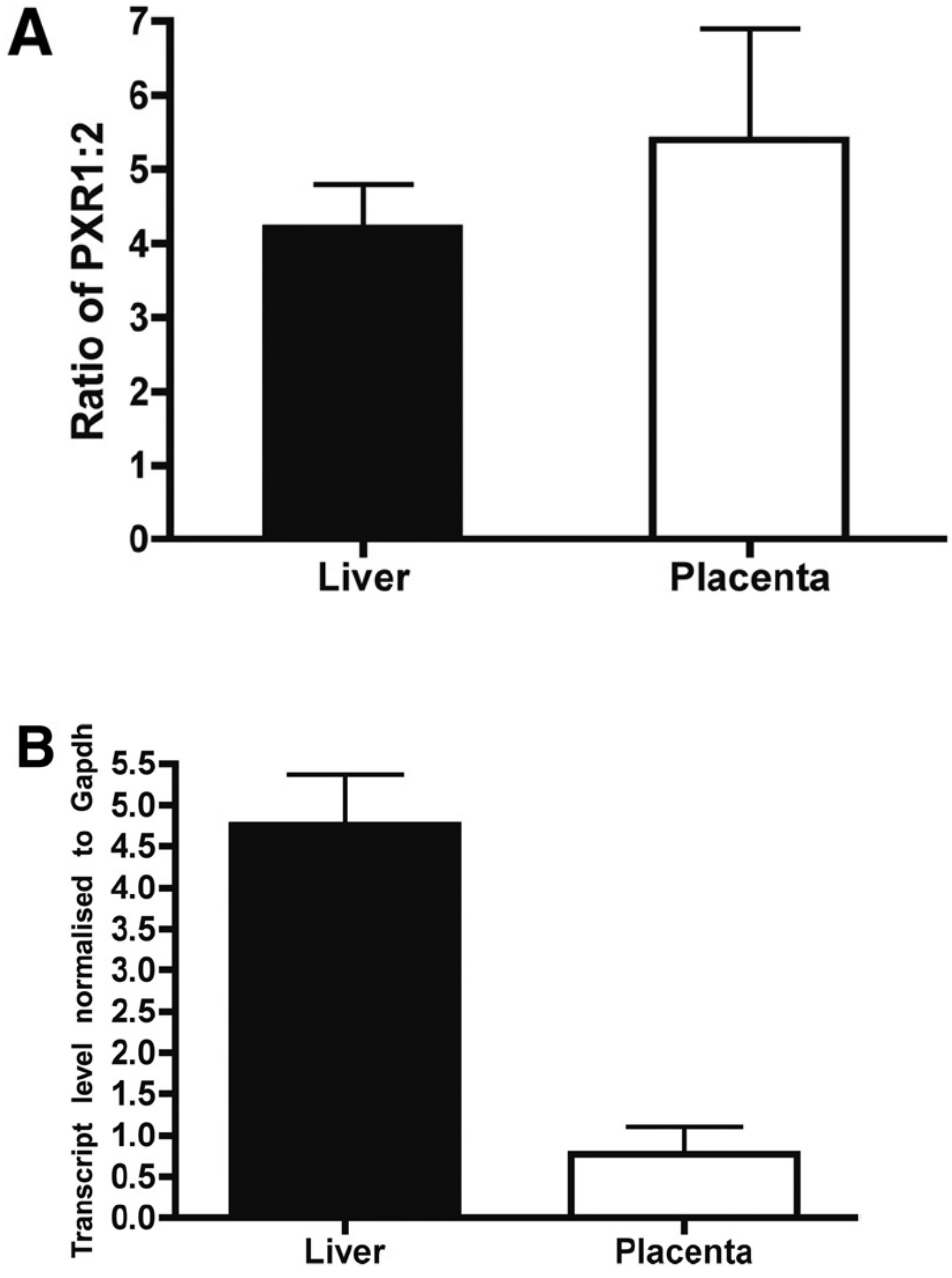


Fig 9 A. The ratio of PXR.1 to PXR.2 mRNA in the liver and placental tissues of pregnant PXR +/+ mice on GD 17. The mRNA levels of PXR.1 and PXR.2 were determined by real time PCR. There was seen no significant difference in the relative abundance of the two forms of PXR transcript between the hepatic and placental tissue. Levels were normalized to Gapdh and presented as % controls.{$p<0.05$;$p<0.01$}. n=6

Fig 9 B. Basal mRNA expression of Pxr in the liver and placenta (GD 17) PXR (+/+) mice.

2.5 Discussion

To date, much research has demonstrated that ABC transporters play an important role in the pharmacokinetics and pharmacodynamics of many drugs. Depending on 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. Although 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. Of interest, although the levels of PXR have been demonstrated to be elevated in the liver and ovaries during gestation, they remain relatively static in the placenta. Whereas 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. Compared with that in wild-type mice with fully functional PXR, a pronounced increase in the expression of Mdr1a, Bcrp, Mrp2, and Mrp3 was seen 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. 4B). Marked increases in the mRNA levels of Mdr1a, Cyp3a11, Mrp2, and Mrp3 were observed in the livers of the knockout mice compared with those of the PXR(+/+) mice. This induction was present regardless of the animals being pregnant or not, thus ruling out an elevated response in either genotype because of greatly increased circulating hormones seen in pregnancy. It is indeed very important to bear in mind that although significant changes in expression levels were seen with established PXR targets such as Cyp3a11 and Mrp2 and Mrp3, increased basal expression was not detected for genes that are under predominant control of other nuclear receptors such as Ntcp (under retinoic acid receptor a control) and Bsep (under farnesoid X receptor control). Previous studies have also reported higher expression of Mdr1a and Mrp2 in the liver and intestine of PXR-null mice (Kliewer et al., 2002) (Teng et al., 2003). It is plausible that overcompensation by other members of the nuclear receptor family could be responsible for the differential expression among genotypes. One member of the nuclear receptor superfamily, CAR, shares many

structural and functional similarities with PXR (Honkakoski, Sueyoshi, & Negishi, 2003) (Timsit & Negishi, 2007) (di Masi, Marinis, Ascenzi, & Marino, 2009). CAR and PXR each heterodimerize 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 because there is a dramatic rise in levels of the CAR-activating estrogens during the course of gestation. Although 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 nonpregnant PXR-deficient mice. Cyp2b10 is primarily activated by CAR, suggesting elevated basal activity and a potential over compensatory role of CAR in the absence of PXR. An enhanced effect of CAR target genes on 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 coactivators 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, and Mrp3 in nonpregnant mice (Teng & Piquette-Miller, 2005) (Anapolsky et al., 2006). Likewise, PCN-mediated induction of several PXR gene targets has been reported in the intestine and blood-brain barrier (Bauer et al., 2004) (Cheng & Klaassen, 2006). Of interest, although 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 result 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 livers of pregnant animals at full term. Indeed,

Pxr levels were 5-fold higher in maternal liver compared with those in the placenta in wild-type mice on GD 17 (Fig. 9B). Therefore, relative expression of PXR in placenta, compared with that of the 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 cofactors, or alternate isoforms could also be involved.

Tissue-specific differences in the relative expression of PXR isoforms in placental versus hepatic tissues were examined because studies have identified a number of PXR transcript splice variants, which impose functional changes in gene target response (Lamba, Lamba, & Schuetz, 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 (PXR.2) and its mouse counterpart (mPXR.2). Although 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. Of more importance, PXR.2 has been found to repress the basal expression of CYP3a and MDR1. Whereas 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 result 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. As an alternative, 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. In particular, the nuclear receptors may be suppressed or maximally activated by pregnancy-related hormones.

As reported by others, our results demonstrated both pregnancy-related and gestation-related changes in the expression of drug transporters and Cyp3a enzymes. Hepatic expression of Cyp3a11 has been shown to be reduced during pregnancy, a trend clearly seen in our wild-type animals. Likewise, Mrp3 levels are shown to be reduced in pregnancy, as seen in our data. However, we failed to see down-regulation in the levels of Oatp2 and Mrp2, whereas it has been reported by other groups in pregnant rats. This finding 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 gestation-mediated changes of gene targets, such as the transporters, in the PXR genotypes. Indeed, although many target genes were below the detection limit on GD 10, changes in transporter expression with gestation progression to full term were markedly different in placentas isolated from PXR wild-type or null mice. Compared with PXR wild-type mice, a pronounced increase in the induction of Mdr1a, Mrp2, and Mrp3 was seen in PXR-null mice from GD 10 (mid-gestation) to GD 17 (near term). Moreover, levels of Bcrp remained much higher in the PXR(-/-) compared with the wild-type mice. Although studies looking at temporal changes in human placenta are rare, they have demonstrated significant differences in gene 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, although we observed decreased placental expression of Bcrp at full term, the placental expression is increased in later gestational stages in humans (Yeboah et al., 2006). Our findings are in agreement with a previously reported trend seen in rodent placenta in a number of studies (Yasuda, Itagaki, Hirano, & Iseki, 2005) (Kalabis, Petropoulos, Gibb, & Matthews, 2007) (Cygalova, Ceckova, Pavek, & Staud, 2008). The incongruity between the human and rodent expression levels can be due to a number of species-specific factors. Although BCRP is expressed abundantly in humans and rodents, the expression profiles differ, with humans having the highest levels in the placenta, whereas in mice it is most abundant in the kidney. In addition, in rodents, almost in sync with the falling placental levels of bcrp, an increase in the levels in the embryonic tissues is seen (Cygalova et al., 2008) (Hahnova-Cygalova, Ceckova, & Staud, 2010). This result could point to the waning reliance of the fetus on the placenta to sequester it from xenobiotics and explain the falling placental levels as term approaches. Unfortunately, no corresponding human data are available, and the exact reasons for such discrepancies need to be further explored.

Because variation in placental transporters is clinically significant, findings in the animal models are important, given the similarity in both the type of placentas and the expression profiles of key drug transporters. Both 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.

In 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, Amonkar, Rybeck, & Tracy, 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 ABC transporter expression because 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. Although PXR has been demonstrated to mediate the induction of the major ABC drug transporter genes in the liver, intestine, and the blood-brain barrier, this inductive influence is absent in the placenta. In addition, a possible repressive role for this nuclear receptor has also been uncovered, as illustrated by the elevated transporter expression in the knockout mice. Overall, this 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.

Chapter 3 Management of HIV Positive Pregnancies In Ontario:Current Status

This chapter is a reprint of “Gahir S, Anger GJ, Ibrahim M, Read S, Piquette-Miller M. Management of HIV positive pregnancies in Ontario: current status. Can J Clin Pharmacol. 2009 Winter;16(1):e68-77.”

3.1 Abstract

Background

AIDS is one of the biggest health crises we face today. With nearly 20 million women infected with the virus that causes it, HIV, maternal transmission of HIV is increasingly becoming a serious concern and hindrance in stemming the proliferation of the disease. While an ever increasing number of pregnant women are being administered antiretrovirals to mitigate the vertical transmission of the virus, little is known about the changing trends in the type of agents used and the duration of therapy.

Objectives

This paper attempts to identify any changes in the pattern of HIV management in pregnant women for the period of time spanning 1998 to 2005.

Methods

Data from the charts of 183 patients were reviewed. A retrospective, longitudinal and cross-sectional patient chart review was employed to obtain data. Parameters such as therapeutic management of HIV, class of drugs used and duration of treatment were assessed to identify any evolving patterns over the course of the study.

Results

It was seen that over time, the number of women receiving adequate therapeutic interventions has steadily increased. We also identified evolving trends in terms of the classes of antiretrovirals employed and the duration of prophylaxis.

Conclusion

The strategies employed in the management of HIV positive pregnancies in Ontario, while evolving over time, were found to be in line with the guidelines in place. The information

delivered by this study might enable the medical community to assess the progress in dealing with this challenge thus far and further fine tune the current strategy.

3.2 Introduction:

World wide, there are approximately 20 million women of childbearing age infected with HIV. Women currently represent the fastest growing group of people with HIV in every region of the world, including Canada. An estimated 11,800 women are living with HIV/AIDS in Canada, a number that represents approximately 20% of the national total of HIV infected individuals and is 23% higher than 2002 values (P.H.A.O., Canada, 2006). In Ontario, women now account for 28.1% of all HIV infections, with the Greater Toronto Area (GTA) containing the highest concentration of cases (Remis et al, 2005) With an increasing number of these HIV sero-positive women becoming pregnant, the pharmacotherapeutic approach adopted by physicians and health care providers is paramount to the simultaneous prevention of disease progression and mother-to-child transmission (MTCT). The major factors governing vertical transmission of the HIV virus from mother to child can be broadly classified as maternal, obstetrical and neonatal. The single most important marker that can predict chances of MTCT is maternal viral load. The rates of perinatal viral transmission are proportional to the maternal plasma HIV RNA level (John et al, 2001; Thea et al., 1997; Magder et al., 2003). In addition to viral RNA levels, low counts of CD4 cells have been associated with higher incidence of MTCT.5 A Highly Active Antiretroviral Therapy (HAART) regimen during gestation offers further protection to MTCT, even in cases of low viral loads. HAART entails a minimum of three agents from at least two different antiretroviral drug classes and is the mainstay of most anti-HIV drug regimens. In terms of obstetrical considerations, pre-mature delivery (before 35 weeks), ruptured membranes and chorioamnionitis have all been found to contribute to a greater propensity for the virus to infect the child. Lastly, neonatal factors such as pre-term delivery and low birth weight are associated with in utero MTCT. Breast-feeding has also been implicated in viral transmission; although, this is more of a problem in resource limited settings rather than in Western countries where formula feeding is a viable alternative. Preventing vertical transmission of the virus is a vital target that must be achieved if we are to successfully combat the threat that the HIV/AIDS epidemic poses. Health and regulatory bodies have recognized this goal across the globe and this is reflected in the guidelines issued by these agencies.

The guidelines utilized by physicians and health care professionals within Canada vary to some extent but the majority adhere to the Canadian consensus guidelines for themanagement of pregnant HIV-positive women and their offspring (Burdge et al., 2003). Established by the Canadian HIV Trials Network Working Group on Vertical HIV Transmission, these guidelines provide detailed recommendations for preconception counselling as well as antenatal, intrapartum and postpartum care.

Briefly, according to the guidelines, preconception counselling should consist of ensuring HIVpositive women of childbearing age are properly educated on the prevention of unwanted pregnancy. Additionally, antiretroviral agents with potential teratogenic effects should be avoided when possible. Antenatal care should involve an individualized therapeutic regimen. It is recommended that HIV-positive pregnant women who have not previously received HAART should initiate a regimen consisting of two nucleoside analogues (e.g., zidovudine/AZT and lamivudine/3TC) in combination with either a protease inhibitor like nelfinavir or a non-nucleoside reverse transcriptase inhibitor such as nevirapine (Burdge et al., 2003). Treatment should be initiated 14 weeks into gestation if viral loads are sufficiently low and CD4 counts are in an acceptable range. For women already on HAART, it is recommended that their regimens be evaluated for teratogenic potential and adjusted accordingly. It is recommended that this latter group of women stay on their regimen for the entire duration of their pregnancy. Intrapartum care should consist of regular confirmation of viral load suppression. Patients should be informed of evidence to suggest that MTCT is reduced by approximately 50% when birth is via caesarean section as opposed to vaginal birth (Brockleburst, 1999). However, as studies comparing transmission rates seldom include women on HAART, it is recommended that caesarean section only be encouraged when viral loads are unsuccessfully managed (Burdge et al., 2003). Postpartum care recommendations for the mother include continuation of HAART once possible and contraception counseling. Postpartum care recommendations for the infant include an absolute avoidance of breast-feeding and the initiation of zidovudine within 6 to 12 hours of birth (maintained for up to 6 weeks). Immediate initiation of zidovudine/AZT and nevirapine therapy is recommended in the event that no therapy was prescribed to the mother during the antenatal or intrapartum periods. It is worth noting that U.S. guidelines formulated by the Perinatal HIV Guidelines Working Group in the United States are consistent with those of Canada (P.H.G.W., 2006)

The primary objective of this study was to examine the patterns employed in the management of HIV positive pregnancies in Ontario. In particular, we were interested in identifying the changing

patterns in therapeutic strategies and duration of treatment as well as comparing our observations to issued guidelines.

3.3 Methods:

This study examined patterns in therapeutic management of HIV sero-positive pregnant women in Ontario. Patient data was obtained from a database maintained by Motherisk (Sick Kids Hospital, Toronto, ON), a program with a mandate to provide counselling to both patients and practitioners on pregnancy-related topics ranging from drug safety and usage to disease risk. The University of Toronto Research Ethics Board approved the study. A retrospective, longitudinal and cross-sectional patient chart review was carried out to collect data. The data included information from HIV sero-positive pregnant women who were involved with the program from 1998-2005.

A detailed questionnaire was prepared to extract data from the patient charts. All patient identifiers were removed before the data were collected and analysed. The following data were collected: disease progression in mother at the time of pregnancy, incidences of infections and/or hospitalization during gestation, the details of therapeutic intervention including the class ofdrugs and duration of therapy, the mode of delivery, IV AZT administration during labor, gestational age at birth, weight of infant at birth and any complications post partum as well as newborn HIV status. All data were transcribed by the authors onto the questionnaire. While all available patient files for a particular year were examined, charts with missing data on drug usage, type of delivery and use of prophylactics during labor were excluded from the study. To assess the trends in drug use and period of prophylaxis, we performed a linear regression test on the data using GraphPad Prism (GraphPad Software, San Diego, CA.). Statistical analysis was done in consultation with Dr. Thomas Einarson (Leslie Dan Faculty of Pharmacy, University of Toronto).

3.4 Results

Patient files from 183 pregnant women were reviewed for the purpose of this study. The large majority of women were aware of their HIV status at the time of conception. The parameters

were analyzed on a year-to-year basis to enable identification of evolving patterns over the course of the study. With regards to ART (Antiretroviral Therapy) during the course of gestation, some clear changes were observed over the duration of the study (Fig. 10). While highly prevalent in 1998, the use of NRTIs monotherapy has seen a steady and steep decline with a decrease from 40% in 1998 to a complete absence in 2005. This trend was found to be highly significant ($r2 = 0.896$, $p<0.001$). Indeed the use of more than one class of antiretroviral drugs (i.e., HAART), has gained ground. The use of NRTI + PI, the traditionally favoured HAART cocktail, has also seen some changes. There appeared to be a downward trend in the use of this combination, especially from 2001 onwards; although the trend did not reach statistical significance ($p>0.05$). In the year 2001, this approach was employed to treat 61% of all analyzed cases while this number slipped down to 31% in 2005. The ground lost by the NRTI and NRTI + PI combinations was captured by NNRTI + NRTI combinations and accounted for approximately 38% of all ART in 2005. The trend towards the increasing use of NNRTI + NRTI was statistically significant ($p<0.05$; $r2= 0.69$). The period of ART during pregnancy was also examined (Fig. 11). There was a clear trend towards extending treatment duration with almost half of the patients receiving antiretroviral therapy throughout the course of pregnancy in 2005. This changing pattern reached levels of statistical significance and had a good correlation ($r2= 0.69$). The use of Antiretrovirals during the other sections of gestation was virtually unchanged. The administration of IV AZT during delivery and the mode of delivery were also tracked (Fig. 12). In recent years (from 2001 onwards), the strategy has had a very consistent with a high compliance rate (close to 80%). Additionally, the mode of delivery was tracked over the years (Fig. 13). The proportion of patients who delivered vaginally remained relatively steady from 1999-2005 (approximately 50%) while approximately one third elected to deliver by caesarean section. Data on the HIV status of the newborn was not well documented. While postnatal HIV testing was performed, data was not available to make accurate estimates about the extent of MTCT for the sample set. Of all the patient files available, only one case of vertical transmission was observed. However, due to the majority of the cases having incomplete HIV test result data, an accurate estimation of the success of this strategy could not be made. There was no change over the years in the gestational age of the newborn. However, there was a clear and statistically significant downward trend in the incidence of maternal complication during the course of gestation. The incidence of infections in the mother during pregnancy also went down, but failed to reach levels of statistical significance (Fig 14).

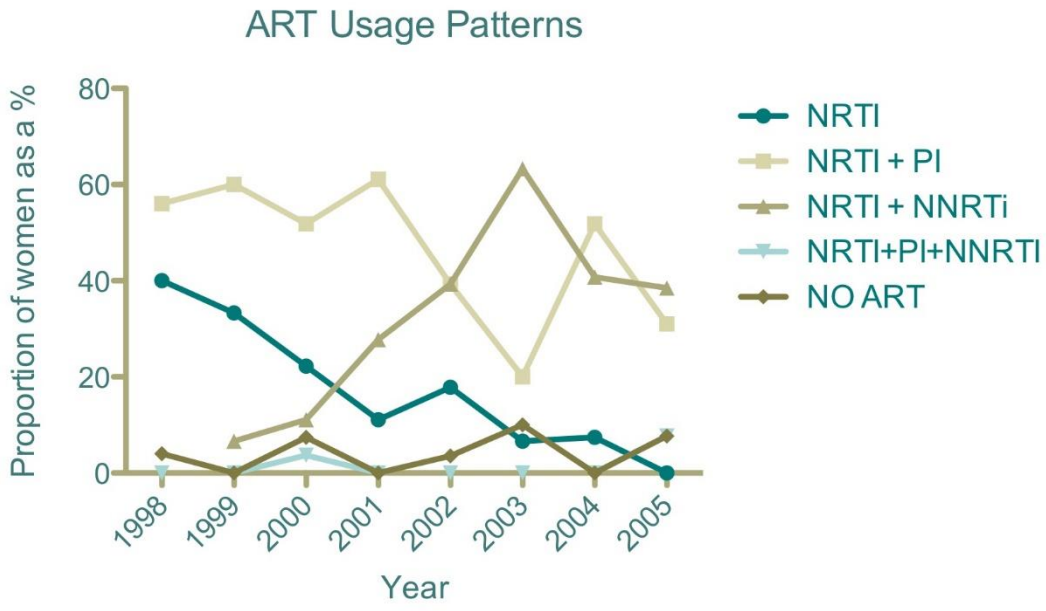


Figure 10: The evolving trend over the course of the study in the classes of anti-HIV drugs used to manage HIV-seropositive mothers.

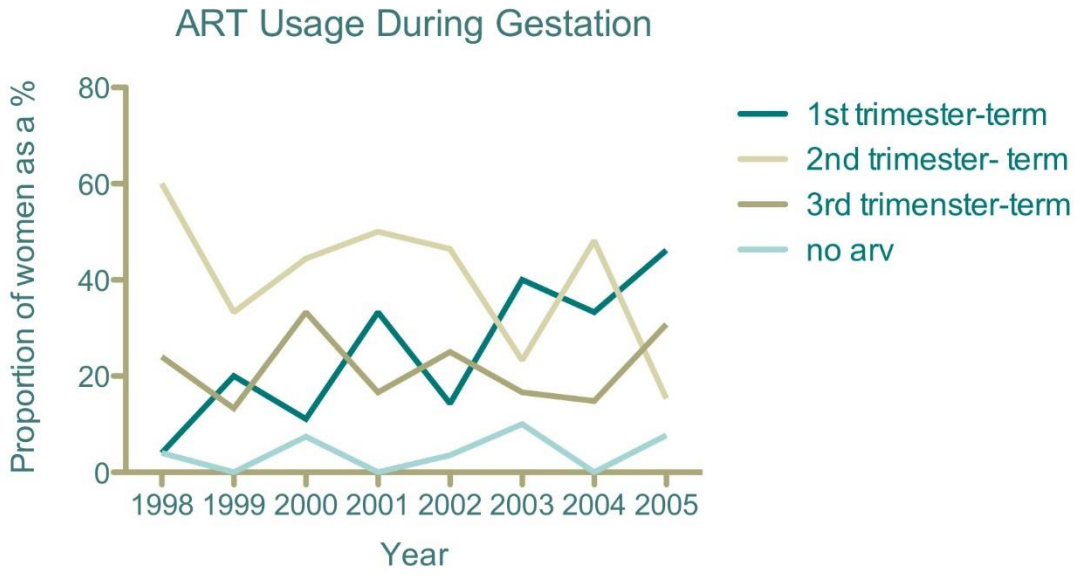


Figure 11: The pattern of initiation and duration of ART in pregnant HIV women.

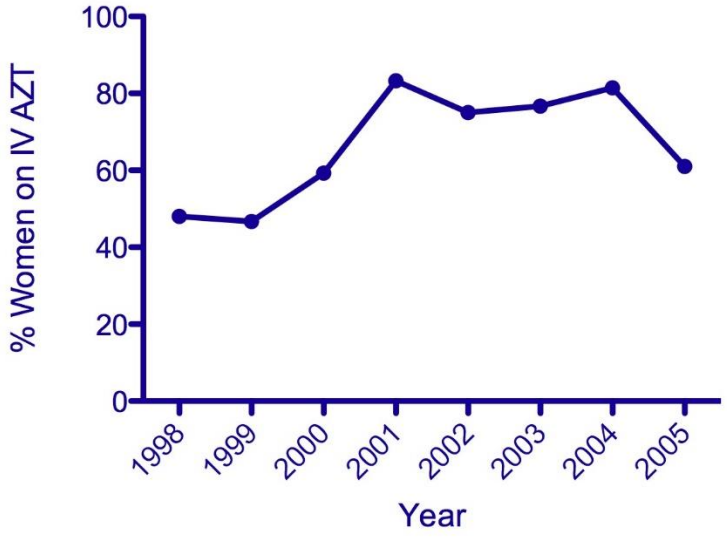


Figure 12: % women every year who received IV AZT during labor

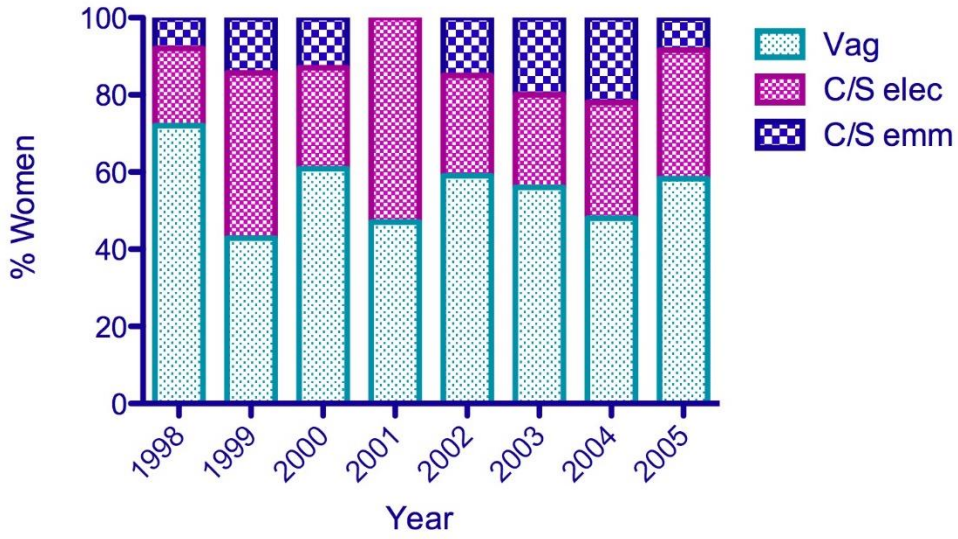


Figure 13: Breakdown of the type of delivery during the study period.

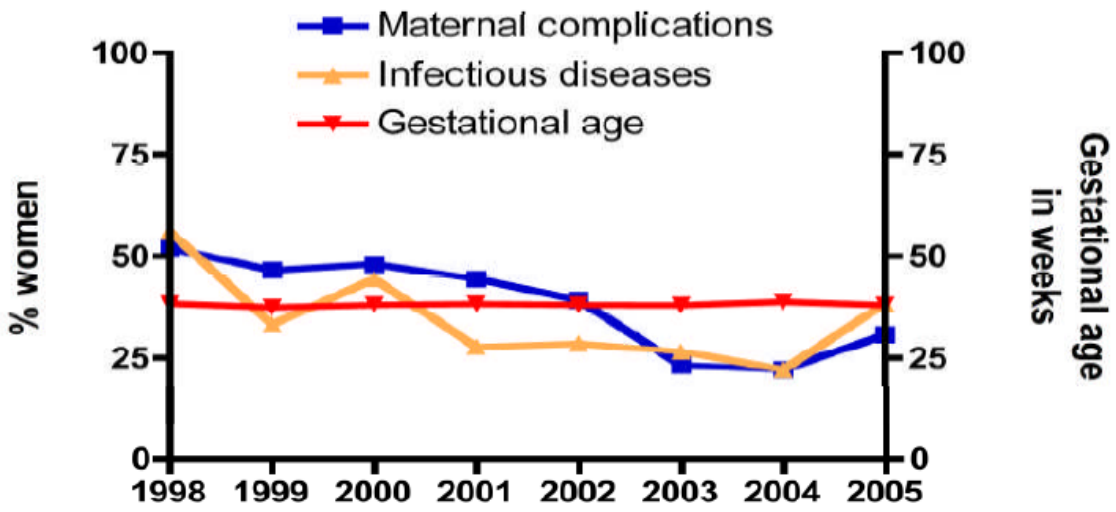


Figure 14: The incidences of maternal complications, infections and the averge gestational age in weeks.

Year	Patient files available	% Women outside the HAART umbrella
1998	25	44
1999	15	33
2000	27	30
2001	18	11
2002	28	21
2003	30	17
2004	27	7.4
2005	13	8
Total	183	

Patient files contributing to the results of this study according to year along with teh corresponding percentage that fell outside of the HAART umbrella. The mean number of patient files available for each year was approximately 23. In 1998, 44% of patients fell outside the HAART umbrella. This value dropped signficantly to 8% in 2005.

Table 5: Description of % women receiving medication as per the HAART recommendations.

3.5 Discussion

To examine if the therapeutic choices being made in the clinic are reflective of the dynamic changes in treatment guidelines, it is essential to retrospectively look at the strategies employed in managing HIV during pregnancy. In order to evaluate trends and current strategies in place, we examined the classes of drugs being used, the period of prophylaxis, the mode of delivery and use of IV infusion during labor.

MTCT is of growing concern globally as an increasing number of HIV sero-positive women are choosing to bear children. According to the latest estimates, more than 2 million HIV seropositive women become pregnant globally. With vertical transmission rates varying between 2% and 40%, there are more than 500,000 new child infections being reported each year (UNICEF, 2007). Current guidelines call for the use of HAART to effectively counter the HIV. This entails a drug regimen consisting of a minimum of three antiretroviral agents from at least two different classes of drugs. This strategy has been found to be the most effective in suppressing viral loads, which are directly correlated with rates of MTCT.

In 1998, the most popular combination prescribed was that of two NRTIs along with one PI. This was in line with that recommended within Canadian and international guidelines at that time. For approximately 4 years, until 2001, this cocktail enjoyed the confidence of prescribers asit was steadily employed in about 60% of all women on ART. However, from 2001 onwards there was a decline in the prescription of PIs in combination with NRTI. Instead, the combination of NRTI and NNRTI seemed to find favour. In fact, this particular combination saw a rapid surge in usage from 1998 to about 2003 when it was the most widely used combination. The reasons for this shifting preference for NNRTI over the traditionally established PIs are not entirely clear to the authors. However, as some recent reports indicate, NNRTIs have been found to offer several advantages over PI boosted regimens. The primary advantages include better CNS penetration and more “patient-friendly” dosage regimens. Additionally, NNRTI agents have been found to be better tolerated in patients than the PIs. It is also important to note that while more than 40% of treated women were not under the HAART umbrella in 1998 (many were receiving only NRTIs; this number fell rapidly from 1999 onwards. However, as recent as 2003, there were well over 10% of women receiving an ART regimen that did not meet the HAART requirements. Reasons for women still receiving suboptimal care need to be further explored and are a cause for great concern. Such high rates of inadequate treatment could possibly be

explained by factors such as inability of the patient to tolerate HAART cocktails and/or non-compliance on the part of the patient rather than physicians failing to comply with established guidelines.

Another important aim of this study was to examine the period of treatment during the course of gestation. The duration of prophylaxis and the point of initiation of ART during pregnancy are vital for both the efficacy of treatment and foetal safety. Keeping in mind that the agents being administered to HIV patients are highly toxic moieties, disrupting the normal cell machine,(Bialkowska et al, 2000; Chitnis, S., D. Mondal, and K.C. Agrawal, 2002; Oliverowt al., 2002; Gerschensonwt al., 2004; Poirier et al., 2004; Natl Toxicol Program Tech Rep Ser, 2006; Chan et al., 2007; Divi et al., 2007) these agents pose a very real risk to the developing foetus, especially during the early organogenesis phase (during the first trimester).

The issue is complicated by the acute shortage of data on the safety of these agents during pregnancy. Only a handful of trials have been conducted for the 16 or so Antiretrovirals commonly employed. The fact that the maternal-foetal interface is extremely permeable during early pregnancy, as the placenta is not fully developed, is also something that physicians have to consider during the management of an HIV sero-positive pregnancy. However, this is one of the rare medical conditions where the cost of erring on the side of caution carries an enormous price, both for the mother and the unborn child. The maintenance of the health of the mother should be of paramount importance while making decisions about initiation or discontinuation of therapy.

Upon examining the data pertaining to the period of ART treatment during gestation, we can identify some clear temporal trends. The most prominent trend observed over the course of the study was the steady increase in the percentage of women receiving therapy throughout the course of gestation. In the year 2005, approximately 46% of all women receiving treatment were treated throughout the gestational period and this was up from a mere 20% in 1999. This change in treatment pattern may be due to a number of factors. Firstly, it is possible that a large number of women were already on ART at the time of conception. Secondly, as per the guidelines, any woman whose viral load is not satisfactorily managed will stay on HAART, irrespective of the stage of pregnancy. Thirdly, the use of Antiretrovirals throughout the course of pregnancy could be asign of growing confidence in the safety of these agents during gestation.

With regards to the mode of delivery, there are two factors to be kept in mind while considering this. Firstly, there have been published studies showing a lack of any benefit of an elective caesarean against MTCT in patients already on prophylactic ART, and a number of studies espousing its benefits in minimizing risk of vertical transmission (Arch Pediatr Adolesc Med, 2002; European Collaborative Study, 2005; The International Perinatal HIV Group, 1999, Kind et al. 1998; Shah 2006). Secondly, since it is an opt-in procedure, the majority of patients will understandably be reluctant to undergo surgery.

Together, the two factors can explain the low percentage of women opting to deliver via caesarean section when most of them have been on ART for a significant portion of the pregnancy. Thus, it is not surprising that over 60% of all women delivered vaginally and this has been the case throughout the study. Less than one third of all the cases examined deliver via elective caesarean section. The remaining women delivered via emergency caesarean section due to unforeseen complications. These figures remained more or less consistent across the study period. Of all the cases available, only one confirmed case of vertical transmission to the neonate was observed; encouraging as this is, it must be noted that a majority of the case files had incomplete or no data at all about the serological test done to test the newborn for the virus. Thus, this study is not able to fully address the efficacy of the changing patterns of drug therapy in terms of minimizing MTCT. However, it did show that the overall health of the mother during the course of pregnancy was better, as indicated by lower complications and infections.

The HIV/AIDS epidemic is uniquely characterized by a grave socio-economic impact in addition to tangible health effects on both the affected individual and the community as a whole. This has resulted historically in varied reactions from society, regulatory bodies and health professionals. Over the last few years, the face of AIDS has begun to change dramatically. What was initially believed to be a condition affecting only homosexual males has now spread to include a much greater proportion of the heterosexual populace. Furthermore, there has been a distinct and worrisome trend with the disease infecting an increasingly larger number of women as a percentage of all HIV sero-positive patients. In addition, an increasing number of HIV seropositive patients are choosing to bear children despite awareness of their HIV status. This may be explained by growing confidence in the efficacy of HAART in curtailing MTCT.

This new scenario has forced health and regulatory bodies to have a strategy specifically designed to address MTCT. As outlined, the guidelines call for rigorous ART with more than one

class of drug during the course of pregnancy along with some other recommendations such as IV AZT during delivery, elective caesarean section when necessary and the like. While HAART has proven to be a potent strategy to counter the virus, there remain some unanswered questions regarding these potent medications. For example, there is limited foetal safety data for these agents. Agents such as AZT and 3TC have been known to cause toxicities in mice foetuses following prolonged exposure during gestation (Zhang et al., 1998; Diwan et al., 1999; Walker et al, 2007) No such data on human foetal safety is available. Until this knowledge gap is bridged, the utilization of HAART to its maximal potential cannot be attained without some degree of risk.

In conclusion, the most effective weapon in our arsenal to counter MTCT is the judicious use of HAART. It is important to use the right combination of antiretroviral agents at the right dosage at the most appropriate time. In observing Ontario patients, we identified clearly evolving trends over time with respect to both the class of antiretroviral agents utilized and the period of prophylaxis. In addition to drugs, the mode of delivery can also play a role in determining the rates of vertical transmission of the virus for women with poorly controlled HIV. That being said, vaginal delivery seems to be the historically preferred mode and the trend we observed suggests that it will remain so in the future. It may be concluded that the management of HIV seropositive pregnancies in Ontario, while dynamic, is in line with the issued guidelines.

3.6 Acknowledgements

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Chapter 4 The Role of Placental Transporters and Fetal PXR Genotype in Determining the Transplacental Transport of Lopinavir.

Sarabjit S. Gahir and Micheline Piquette-Miller

(Manuscript in Preparation)

4.1 Abstract:

Purpose:

Lopinavir, a widely used drug for managing HIV positive pregnancies, is believed to be a substrate of drug transporters including Mdr1 (Abcb1) and Mrp2 (Abcc2) and is an inhibitor of Bcrp (Abcg2). Since we have previously shown the placental expression of key drug transporters to be linked to Pregnane X Receptor (PXR) genotype, we examined the impact of the expression of placental drug transporters and fetal PXR genotype on the transplacental transport of lopinavir.

Methods:

PXR +/- mothers bearing PXR +/+, PXR +/- and PXR -/- fetuses were generated as follows. PXR+/- females were mated with PXR +/- males thus generating PXR +/+, PXR +/- and PXR -/- fetuses. On gestational day 17, pregnant dams were dosed with 10 mg/kg lopinavir I.V. and sacrificed 30 minutes post injection. Concentrations of lopinavir in the maternal plasma and fetal tissue were measured by LC-MS/MS. The expression of transporters in placenta were determined by quantitative real time PCR. All fetal units were genotyped for PXR by PCR genotyping.

Results:

We demonstrated differences in the placental ABC drug efflux transporters as a function of PXR genotype. The levels of Mdr1a, Mrp2 and Bcrp mRNA in PXR +/+ placentas were significantly lower than those seen in the PXR -/- placentas. Furthermore, exposure to lopinavir for fetal units within the same dam was determined by the fetal PXR genotype. Concentration of lopinavir were two fold higher in the PXR +/+ fetal units as compared to their PXR -/- counterparts ($p<0.05$). A significant, linear correlation was observed between placental Mdr1a expression and fetal concentrations of lopinavir in the fetal tissue ($r=0.495$; $p<0.0016$) in that the fetal accumulation of Lopinavir was reduced dramatically with an increased Mdr1a expression. A

weaker linear correlation was observed between expression level of placental Mrp2 and fetal accumulation of Lopinavir ($r= 0.3$; $p=0.055$).

4.2 Introduction:

Globally, there are over 2 million children living with HIV, and according to WHO, most of these infections were preventable (UNAIDS, 2009) . The primary cause of childhood acquired HIV is believed to be mother to child transmission (MTCT) of the virus. Employing a strategy of aggressive, proactive prophylaxis when managing HIV-seropositive pregnancies by administration of Highly Active AntiRetroviral Therapy (HAART) has brought the rate of vertical transmission down to 1-2% from around 25% in the absence of these interventions (Sturt, Dokubo, & Sint, 2010; Van Dyke, 2011) . However, little is known about the factors controlling the transplacental trafficking of highly potent antiretrovirals.

The placenta is the sole gateway between the maternal and fetal systems, regulating exchange of both endogenous and exogenous molecules. This barrier site performs a critical role, limiting the access of drugs and xenobiotics (Prouillac & Lecoeur, 2010). (Syme, Paxton, & Keelan, 2004) The presence of several ATP Binding Cassette (ABC) drug transporters including Pgp, Mrps and Bcrp at the materno-placental interface is believed to be involved in the extrusion of a wide spectrum of drugs including antiretrovirals (Vähäkangas & Myllynen, 2009) (Ni & Mao, 2011) (Ni & Mao, 2010) (Tomi, Nishimura, & Nakashima, 2011) (Hutson 2011]). Indeed, the fetal accumulation of the protease inhibitor Saquinavir has been found to be elevated in PGP knockout mice models (Huisman et al., 2001) .

Lopinavir (LPV) is currently a first line protease inhibitor (PI) in managing HIV positive pregnancies (Navér et al., 2011) (Baroncelli et al., 2009; Janneh, Jones, Chandler, Owen, & Khoo, 2007) Several in-vitro and in-vivo studies have established that transporters such as PGP and MRP2 are involved in LPV absorption and disposition (Agarwal, Pal, & Mitra, 2007a; Gulati & Gerk, 2009; Kim et al., 1998; van Waterschoot et al., 2010). Inhibition or deficiency of PGP, which is encoded by Mdr1a and Mdr1b in rodents, has been shown to increase the oral bioavailability of LPV in addition to increasing its concentrations in the CNS. The placental transfer of protease inhibitors such as LPV has been shown to be highly variable in patients. It has been reported that the fetal cord to maternal plasma concentrations for LPV range from 0.05 to 0.34 (Marzolini et al., 2002) (Mirochnick & Capparelli, 2004) (Yeh et al., 2009). Given the established role of drug transporters in LPV transport, variability in transporter expression at the placental surface may play an important part in explaining the varying amount of LPV which reaches the fetal system.

Expression of drug transporters in the placenta has also been shown to be highly variable and are changing throughout gestation. Reports indicate that levels of P-gp decline while levels of Bcrp increase as gestation progresses (Gil, Saura, Forestier, & Farinotti, 2005; Sun et al., 2006; Yeboah et al., 2006) . The situation is further complicated by reports of disease-mediated alterations of drug transporters. In a study comparing the placental expression of PGP in HIV infected and uninfected women, dramatically higher PGP expression was seen in placentae of the infected women (Camus et al., 2006). Alterations in the expression of the key drug efflux transporters in the placenta could have a serious clinical impact. In the case of drugs such as antiretrovirals, a fine balance between efficacy and safety needs to be struck; given the interplay between PI concentration, viral load suppression, and MTCT on one hand and the potential for fetal drug toxicity on the other. Thus, it is important to be able to predict how environmental or pathophysiological influences can impact fetal drug exposure.

Nuclear receptors such as the Pregnane X Receptor (PXR) are known to be key regulators of ABC drug transporters such as Abcb1, Abcc2, Abcc3, and Abcg2 (Kliewer et al., 2002) (Teng & Piquette-Miller, 2008) (Anapolsky et al., 2006) (Teng et al., 2003). It is well known that PXR is activated by endogenous steroidal hormones and their metabolites, the levels of which are dramatically elevated during pregnancy. PXR is also activated by a number of dietary components, herbal remedies and clinically employed drugs (Chang & Waxman, 2006; Kliewer et al., 1998) . While PXR activation and the subsequent transporter induction at the liver and BBB has been shown to alter the bio-availability and CNS accumulation of many therapeutic agents, its role in determining fetal drug disposition is unclear and unexplored.

We have recently demonstrated a tissue-specific role for this nuclear receptor in the murine placenta (Gahir & Piquette-Miller, 2011) . Several PXR target genes (Pgp, Mrp1-3 and Bcrp) were found to be elevated in the placenta in PXR null mice, with dramatically higher levels of placental transporters in the PXR -/- mice as compared to PXR +/+ . These findings suggested that alterations of fetal PXR genotype could provide us with a unique murine model with clear differences in placental transporter levels within the same dam. Therefore, by breeding PXR heterozygotes (+/-), we generated a PXR +/- dam bearing PXR +/+, PXR +/- and PXR -/- fetuses and placentas (Figure 15) which allowed us to examine the impact of a range of transporter expression in placenta on substrate accumulation in the fetal units while maintaining a similar maternal physiological environment. This strategy enables us to delineate the

contribution of the placental transporters on LPV disposition without confounding maternal influences.

Using this model we explored the role of fetal PXR genotype and placental drug transporter expression on transplacental trafficking of LPV. Findings from this study have to potential to improve our understanding of the important role placental drug transporters play in fetal exposure of their substrates, and highlight the need to both monitor and modify maternal dosing regimens during pregnancy to ensure achieving the desired therapeutic outcome.

4.3 Materials and Methods:

4.3.1 Animals:

All animal studies were in accordance with the guidelines of the Canadian council of animal care. PXR heterozygote (+/-) animals were obtained by crossing PXR -/- females with PXR +/+ males. PXR wild type (+/+) 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) as described previously (Teng & Piquette-Miller, 2005). For the purpose of obtaining timed pregnancies, the PXR +/- male mice were paired overnight with PXR +/- female, and the male removed the following morning contingent to observance of a vaginal plug. On GD 17, pregnant PXR +/- animals were administered 10mg/kg LPV i.v. via tail vein (Lopinavir; USP, Rockville, MD.). LPV was dissolved in a ethanol:propylene glycol:5% dextrose solution (2:4:4 ratio). Animals were sacrificed at 30 minutes post injection and all maternal and fetal tissues were collected for analysis. The plasma was stored in -20º C, while the fetal and placental tissues were snap frozen and stored at -80º C till analysis.

4.3.2 mRNA Determination:

Expression of transporters were analysed in placental samples by real time PCR as previously described (Teng & Piquette-Miller, 2005). Total RNA extraction was achieved using the QuickPrep RNA extraction kit supplied by Amersham Biosciences Inc. (Piscataway, NJ). cDNA from 1ug of RNA employing the First Strand cDNA synthesis kit (Fermentas, ON, Canada). Quantification of mrp2, bcrp, and mdr1a and b 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. Mdr1b was very poorly expressed in the placenta of our murine model, with expression in most samples being below detectable limit. Hence, since we were unable to quantitatively compare expression levels between various genotypes, Mdr1b mRNA levels are not reported. All transcript levels were normalized to house keeping genes (cyclophyllin). The fetal units were genotyped for PXR by visualizing the placental PCR products on a 2% agarose gel.

4.3.3 Lopinavir detection in biological matrices:

LPV concentrations in the maternal plasma and fetal tissue samples were quantified using LC-MS-MS as previously described. Briefly, maternal plasma and fetal tissue samples were thawed to room temperature before processing. All fetal tissue samples were homogenized in glass tubes with deionized water. The plasma or fetal tissue homogenate was to tubes with pre-dried ritonavir (internal standard). Sample extraction was carried using the liquid-liquid extraction method. Samples were treated with 500 mM of sodium carbonate (50 uL), followed by a 1:1 solution of n-hexane and ethyl acetate. Extraction was completed by vortexing for 2 minutes, following which the organic layer was separated. Separation was achieved by centrifugation at 21000 g for 15 mins at 4° C. Supernatant was then transferred to a fresh tube (700 uL) and dried under nitrogen, followed by reconstitution with 80% methanol.

The system employed a CTC PAL autosampler unit (LEAP Technologies, Carrboro, NC) with a Agilent 1100 series pump (Agilent Technologies, Santa Carla, CA). The column used was a 50mm X 4.6mm, 5um Lichrosorb RP-8 (Phenomenex, Torrance, CA). An API 4000 triple quadrupole MS was set to the positive reaction monitoring mode (AB Sciex, Concord, ON, Canada).

The elution was achieved under isocratic conditions at a flow rate of 0.700 mL/min at room temperature. The mobile phase was 20:80 parts of 0.1% formic acid to 80% methanol. The internal standard used was Ritonavir. MRM transitions for LPV were m/z 629.3 to m/z 447.3 and for Ritonavir were m/z 721.3 to m/z 268, with the source temperature set to 500 C. All chromatograms were analysed using Analyst software, version 1.4.2 (AB Sciex).

4.3.4 Statistical Analysis:

Data generated from these studies were analyzed for statistical significance by carrying out analysis of variance (ANOVA) and significance was set to $p<0.05$. Linear correlation between various factors were assessed, and the Pearson r and absolute p-values are reported. All statistics were carried out using GraphPad Prism (GraphPad Software version 5.0c, San Diego, CA.).

4.4 Results:

4.4.1 The Impact of Fetal Genotype on Placental ABC Transporter Expression:

As compared to PXR +/+ placental units, we found approximately two to six fold differences in the placental mRNA expressions of Mdr1a, Mrp2 and Bcrp in fetal units with the PXR -/- genotype (figure 16). The expression of these transporters was significantly different in placentas obtained from PXR-/- fetal units as compared to PXR +/+ fetal units. ($p<0.05$). Intermediate levels of these transporters were seen in the fetal units with the PXR +/- genotype. While levels in the PXR +/- placentae were significantly different from the PXR +/+ fetal units ($p<0.05$), levels were not significantly different between the PXR +/- and PXR -/- units. This provided us with an animal model with varying placental expression of transporters within the same dam.

4.4.2 Impact of Fetal PXR Genotype on Fetal Accumulation of LPV:

We saw differences in the fetal LPV accumulation between the PXR +/+ and PXR -/- fetal units (figure 17). The fetal : maternal plasma LPV concentration was approximately two fold higher in the PXR++ fetuses compared to the PXR /- units ($p<0.05$). There was, however, no statistically significant difference observed between the PXR +/+ and the PXR +/- fetal units.

4.4.3 Association of LPV Fetal Accumulation with mRNA Expression of Placental Transporters:

4.4.3.1 MDR1a:

We observed a highly significant (p=0.0016) correlation between the Mdr1a transcript level and the amount of fetal accumulation of LPV (figure 18). As the transcript level of Mdr1a increased in the placental tissue, there was a clear trend towards reduced transplacental trafficking and fetal accumulation of LPV. A linear regression best fit the data, and the equation describing the data is y=15.38x+3.3.

4.4.3.2 Mrp2:

In placentas obtained from different PXR genotypes, higher transcript levels of Mrp2 temded to be associated with lower fetal concentrations of LPV (figure 19) However, this trend failed to reach significance (p=0.055) The data were best described with a linear regression fit, with the equation of the line of best fit being y=1.82x+14.

Interestingly, we found that placental expression of Mrp2 was highly and significantly correlated to the expression of Mdr1a (p<0.001) as shown in figure 20.

4.4.3.3 Bcrp:

No significant correlation (linear or polynomial) between BCRP expression and LPV fetal accumulation was detected in our study (figure 21).

4.4.4 Association of PXR Genotype with Fetal Weights:

We observed a wide range in the fetal weights during the study, with units ranging from approximately 500mg to 1000mg. We therefore determined whether there was any relationship between PXR genotype and fetal weights. No correlation between PXR genotype and feta weight was observed (figure 22). Additionally, PXR genotype does not seem to impact fetal survival. Fetal units of the three genotypes were obtained in a mendelian ratio from our cohort of dams.

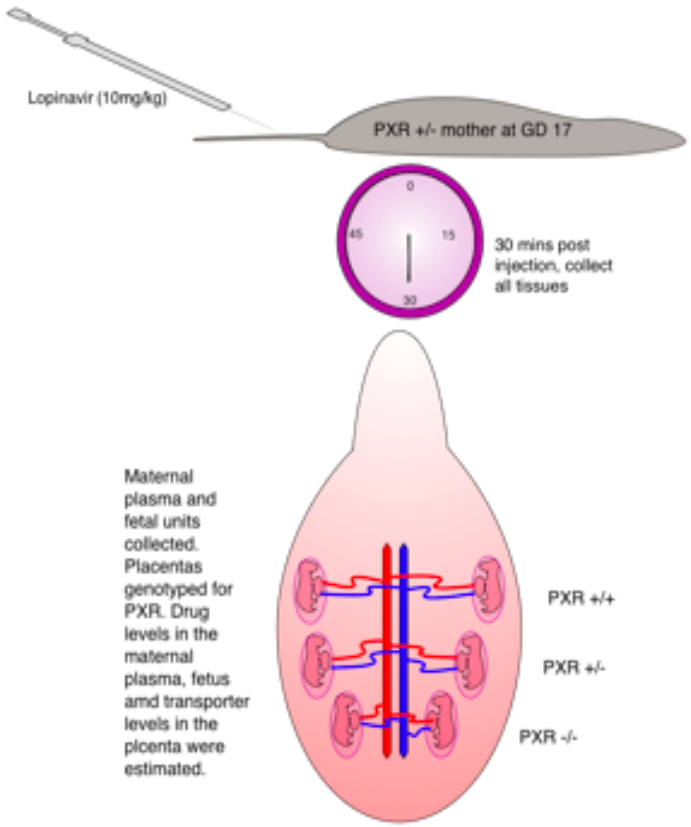

Figure 15: Scheme of Experiments

A Diagrammatic description of the experimental scheme.

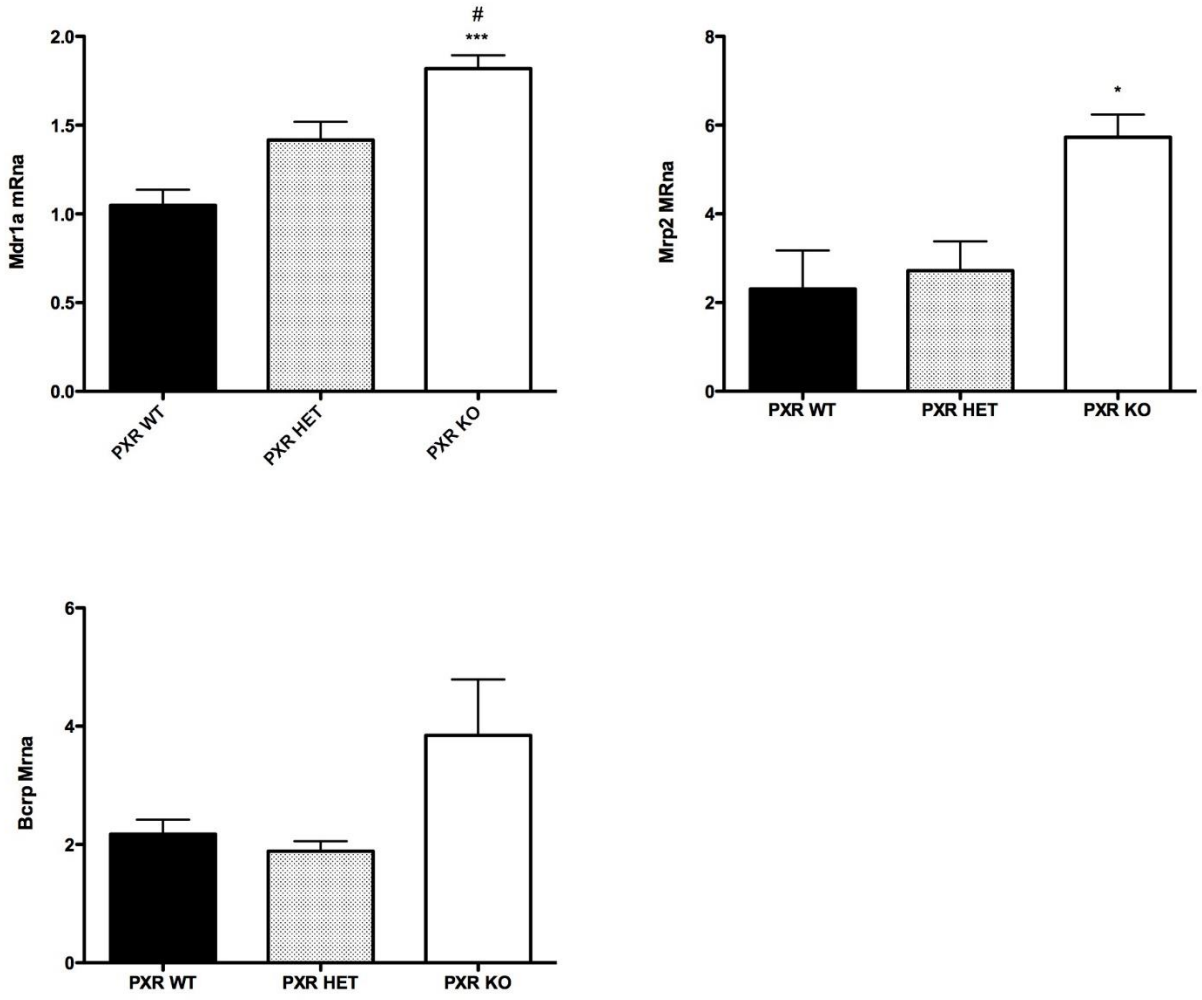

Figure 16: Basal mRNA expression of key transporters in the placenta of PXR wild-type [PXR WT], heterozygote [PXR HET], and knockout [PXR KO] fetuses on GD 17. Levels were normalized to Cyclophyllin , *p =0.05; , p*** =0.01 compared to PXR WT, # = P<0.05 compared to PXR HET. n>10

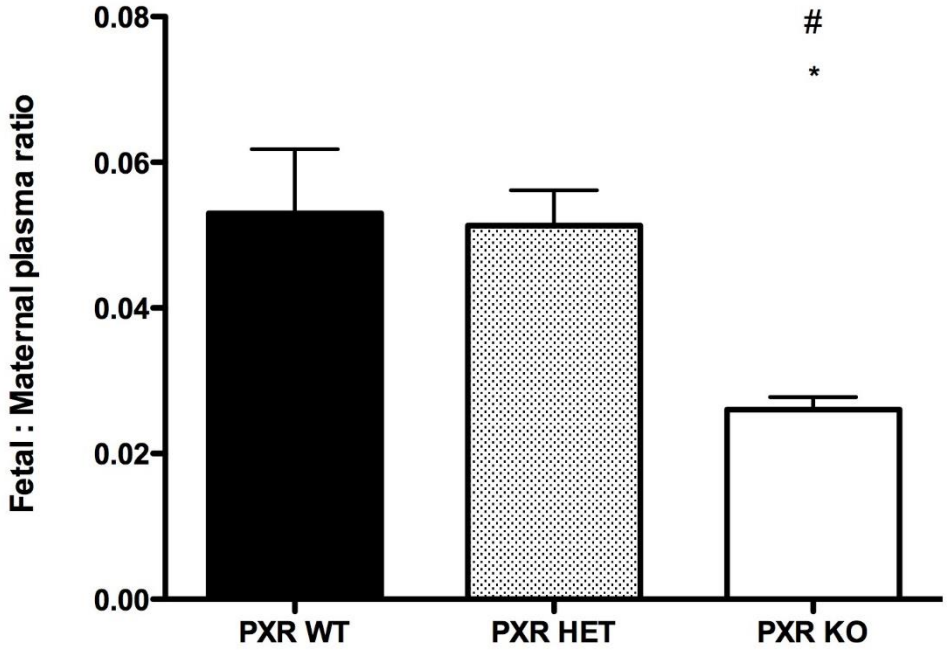

Figure 17:The impact of fetal PXR genotype on fetal accumulation of Lopinavir (10mg/kg) at 30 mins post i.v. injection. Fetal accumulation is estimated by the ratio of LPV in fetal homogenate per unit weight (ng/g): LPV concentration in maternal plasma (ng/ml). Data are represented as mean ratios, and error bars represent S.E. (n>10). (* $p<0.05$ compared to +/+, # $p<0.05$ compared to +/- fetal units).

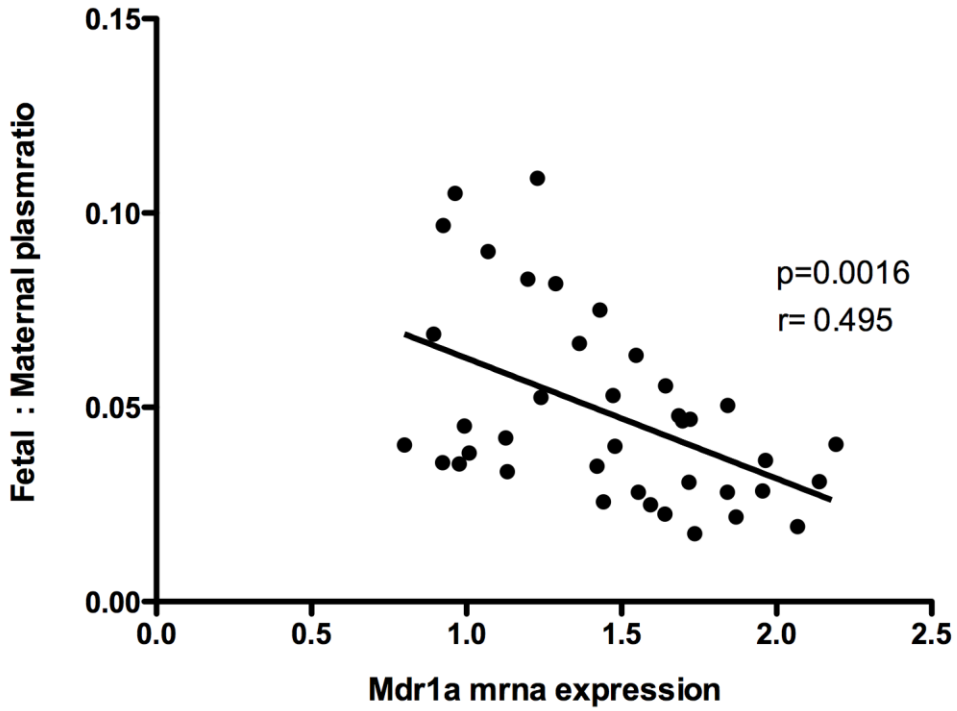

Figure 18:The impact of placental Mdr1a expression on fetal accumulation of Lopinavir (10mg/kg) at 30 mins post i.v. injection. Fetal accumulation is estimated by the ratio of LPV in fetal homogenate per unit weight (ng/g): LPV concentration in maternal plasma (ng/ml). n=39.

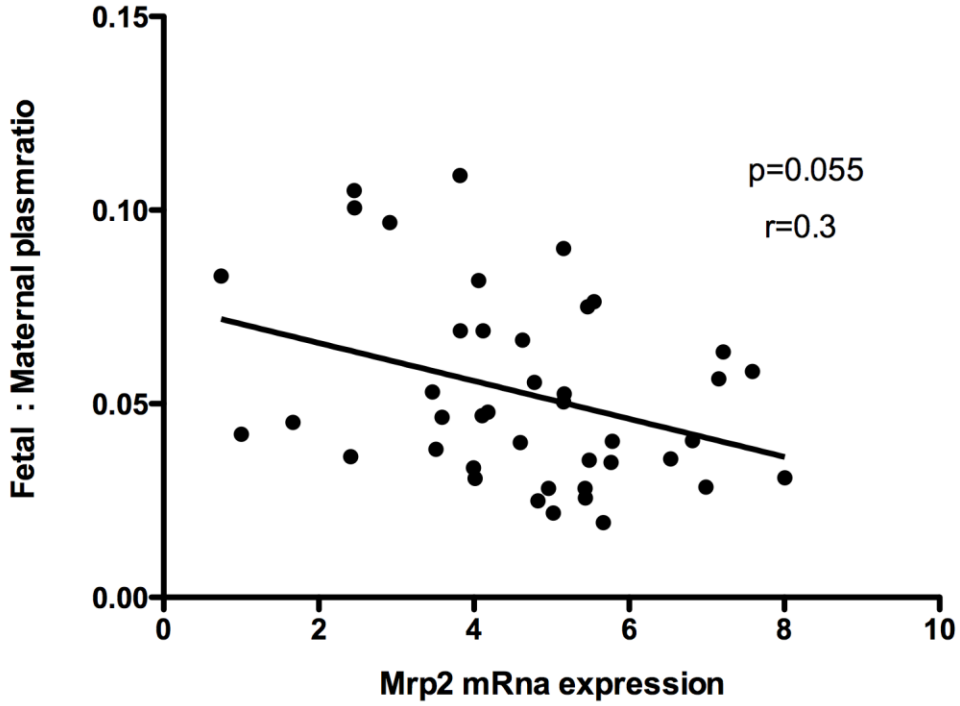

Figure 19:The impact of placental Mrp2 expression on fetal accumulation of Lopinavir (10mg/kg) at 30 mins post i.v. injection. Fetal accumulation is estimated by the ratio of LPV in fetal homogenate per unit weight (ng/g): LPV concentration in maternal plasma (ng/ml). n=39.

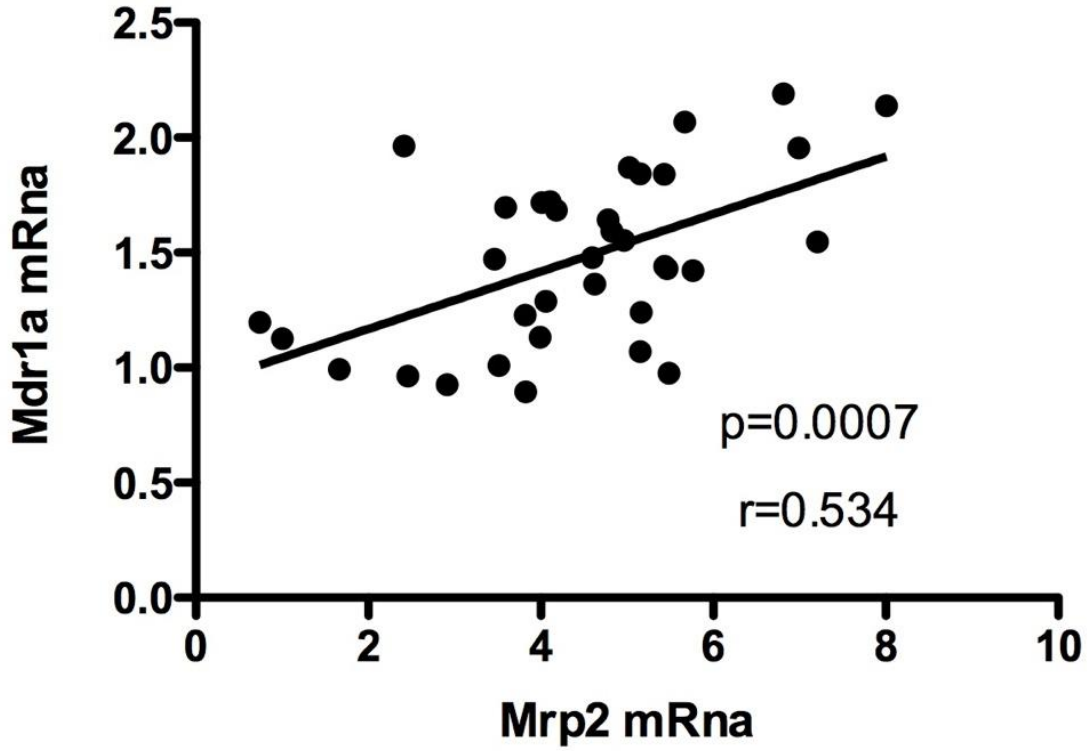

Figure 20: The correlation between placental mRNA expression of Mdr1a and Mrp2. Transcript levels were normalized to cyclophyllin. A significant (p=0.0007) and positive correlation was found. n=39

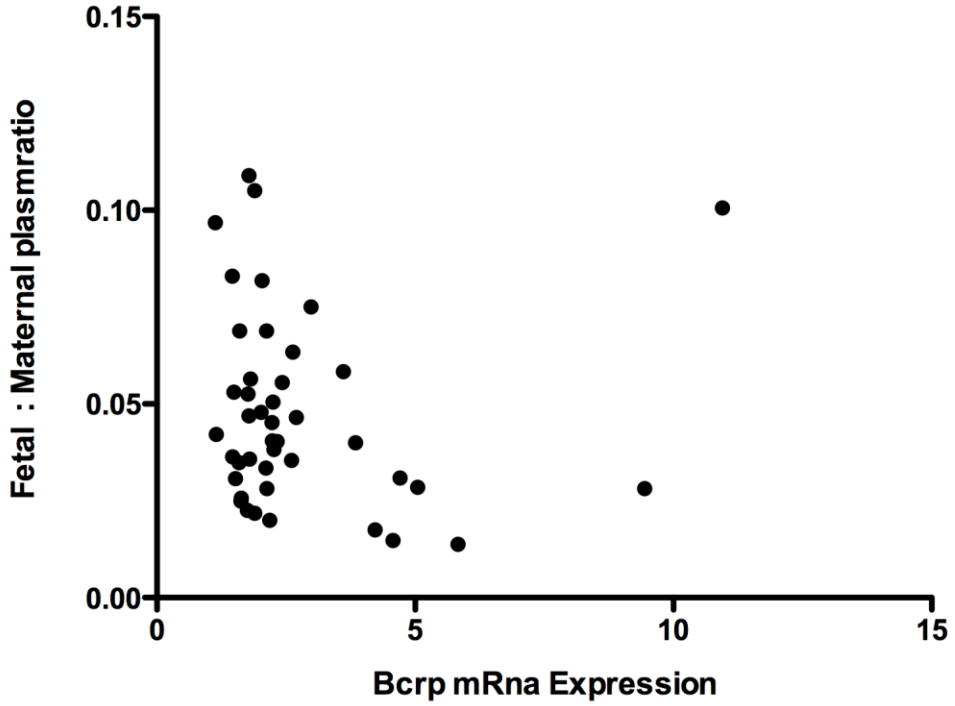

Figure 21: The impact of placental Bcrp expression on transplacental trafficking of Lopinavir (10mg/kg) at 30 mins post i.v. injection. Fetal exposure is estimated by the ratio of LPV in fetal homogenate per unit weight (ng/g): LPV concentration in maternal plasma (ng/ml). n=39.

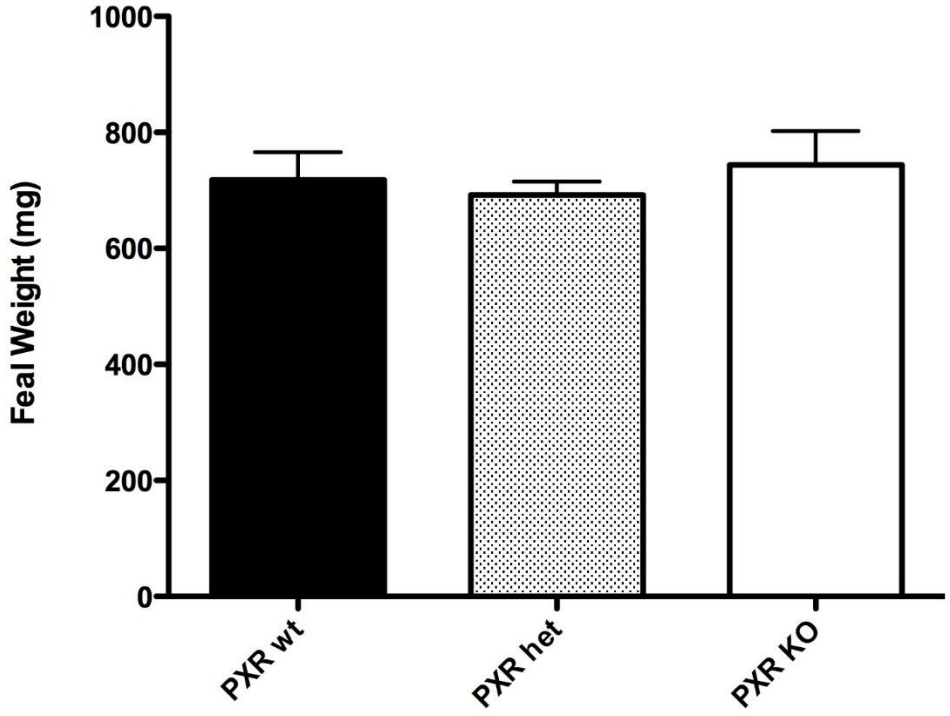

Figure 22: Relationship between the fetal weights and PXR genotype. The individual fetal weights (mg) were to be independent of the placental genotype. n=39

4.5 Discussion:

Many studies to date have reported low and highly variable placental transfer of protease inhibitors at delivery. While numerous studies in-vitro and ex-vivo studies have explored the role of placental transporters in drug transport, very little is still known about the impact that changes in transporter expression have on fetal drug exposure. Our findings demonstrate for the first time in-vivo, the impact of fetal PXR genotype on the placental drug transporter levels and the resultant impact on fetal drug exposure. In this study, we found that the expression of three key ABC drug transporters, Mdr1a, Mrp2 and Bcrp were significantly lower in fetal units with the PXR +/+ genotype as compared to the PXR-/- genotype. Additionally, we found that drug accumulation in the fetal units with the PXR +/+ genotype was approximately twice that seen in the PXR -/- genotype units. These findings indicate that placental expression of transporters can impact the fetal exposure to drug substrates.

In vitro and in vivo studies have previously demonstrated that LPV is transported by P-gp, which is primarily encoded by Mdr1a in murine placenta. Our model provided us with a four fold range in the expression of Mdr1a between PXR genotypes. With an increase in the transcription level of placental Mdr1a, we saw a distinct decrease in the fetal accumulation of LPV. Moreover, the fetal accumulation of LPV was significantly correlated to Mdr1a expression; illustrating the role of placental P-gp on fetal exposure to LPV. These findings are in line with data from a placental perfusion study done by Ceccaldi et al (Ceccaldi et al., 2009). Their study demonstrated that inhibition of PGP lead to an increased maternal to fetal placental transfer of LPV. More recently, placental Mdr1 expression has been implicated as one of the factors limiting the fetal exposure to LPV in a rat model of gestational diabetes (Anger & Piquette-Miller, 2011).

We found that the placental expression of Mdr1b was very low in our animals, with the majority of samples expressing the gene below detection limits. While P-gp is believed to be encoded by Mdr1a and Mdr1b, it has been demonstrated by a number of groups that in mice, it is Mdr1a that is the functional isoform in placenta (Lankas et al., 1998). Fundamental studies in mice show that the fetal exposure of Pgp substrates such as avermectin and digoxin are dependent on Mdr1a expression levels, with varying levels of Mdr1b failing to alter the transport of PGP substrates. Moreover, only placentas with reduced or no Mdr1a expression were found to be

susceptible teratogenicity with avermectin, highlighting Mdr1a as the vital and functional PGP in mouse placenta.

Mrp2 is another important drug efflux transporter localized on the apical surface of the placenta. The transporter has been shown to mediate the placental transfer of talinolol, a cardio selective beta-blocker. Similar to Mdr1a, lower fetal concentrations of LPV were observed in fetal units with higher placental levels of Mrp2, although this effect was not statistically significant. This points to a possible involvement of placental Mrp2 in determining fetal drug disposition. There is no consensus yet regarding the impact Mrp2 has on LPV disposition. In-vitro, Mrp2 has been shown to be involved in LPV transport at low concentrations (Agarwal, Pal, & Mitra, 2007b) . The placental permeability of talinolol was found to be increased in the presence of Mrp2 inhibitors (May et al., 2008). However, in assessing the impact of transporters on oral bioavailability of LPV, Waterschoot et al demonstrated that Pgp but not Mrp2 played a significant role in determining the systemic availability of LPV (van Waterschoot et al., 2010) . We found a strong correlation between the expression of Mdr1a and Mrp2 levels across our sample set. Hence, in the light of these facts, it is possible that the correlation seen between mrp2 and LPV fetal exposure is merely due to higher levels of Mrp2 evaluated in tandem with increasing levels of Mdr1a as a function of PXR genotype (figure 21), rather than an active role of Mrp2 in LPV transport.

While Bcrp is recognized as an important drug transporter (Robey et al., 2009) , and of great importance at the materno-fetal interface (Hahnova-Cygalova et al., 2011), it is not believed to be involved in LPV transport. In line with previous data, we failed to see any correlation between Bcrp expression and the fetal accumulation of LPV. However, the role of Bcrp in trans-placenta transport should be further explored given the wide range of drugs including Antiretrovirals it effluxes. Additionally, the whole complement of placental drug transporters becomes of vital importance when considering the clinical scenario where patients are administered more than one drug.

It is now recognized that PXR is a key regulator of a number of important drug transporters and metabolizing enzymes. As PXR is expressed to a considerable degree in the placenta, and has been shown to be activated by many clinically important agents and environmental factors, it is important to examine its role in the placenta. Recently, we found an inverse relationship between the PXR expression level and the placental expression of mdr1a, mrp2 and bcrp in

PXR +/+ and PXR -/- mice. As before, we found a difference in the placental expression of key ABC drug transporters depending on the PXR genotype (figure 16). This resulted in significant differences in LPV exposure between PXR +/+ and PXR -/- fetuses. These findings underline the important role PXR plays in determining the fetal exposure to xenobiotics by modulating the levels of placental drug transporters

Interestingly, we found that the difference in the expression of transporters between the three genotypes was not as great in this model as seen in our previous study. For instance, while we saw approximately 12 fold higher Mdr1a expression in the PXR-/- dams as compared to the PXR +/+ specimens, in the model using PXR+/- dams (Gahir & Piquette-Miller, 2011), this difference was muted to approximately a 2 fold differences between PXR +/+ and PXR-/- fetal units. Thus, the effect on transplacental transfer of LPV was not discernible between the PXR +/+ and the PXR +/- fetuses. This may be due to the fact that while in the current experimental design, we generated all three genotypes within a single PXR +/- mother, the previous study used employed PXR +/+ parents to generate PXR +/+ placentas, PXR -/- parents to generate -/- placentas and PXR-/- crossed with PXR +/- to generate PCR +/- placentas. Thus, the maternal environment to which the different PXR genotypic placentas are exposed were unique in the two studies and likely had an impact on the expression of target genes such as Mdr1a. Interestingly, PXR and numerous other nuclear receptors are believed to regulate the expression of sulfotransferases (SULT) (Sonoda et al., 2002) and may play a role in hormonal homeostasis. The steroidal hormones, including estrogen are known targets for SULTs. These hormones are implicated in the regulation of a number of ABC drug transporters (Kliewer et al., 1998). Therefore, altered levels of SULT in the maternal system could lead to an altered systemic hormone levels which can have an impact on the placental transporter expression. Additionally, we must keep in mind the potential changes in other transcriptional factors in PXR -/- animals.

In conclusion, given the recognized inter-individual (genetically or environmentally induced) and temporal fluctuations in their levels during gestation, the expression of placental transporters may have a pronounced impact on the outcome of pharmacotherapeutic interventions. The situation is further confounded by the fact that expression of drug transporters may be affected in both maternal and fetal tissues due to HIV infection and other co-morbidities. As a result, fetal drug exposure may be prone to variations throughout gestation, potentially leading to adverse outcomes. Our data demonstrate the importance of transporter expression in determining fetal exposure of drugs administered to the pregnant patient. Since the intent of pharmaco-therpeutic intervention can either be to treat the mother or the fetus, alteration in placental transporters

either lead to toxic accumulation in the fetus or a therapeutic failure due to inadequate transport of the drug across the placenta. A better understanding of the impact of key placental drug transporters will enable the design of a better, more effective and safer treatment strategy, and in-vivo studies are the first step in that direction. The current model can be used to study the in-vivo impact of placental transporters expression on the fetal exposure to a wide array of drugs, thus helping to bridge a critical knowledge gap in the area of drug usage in pregnancy.

5 Discussion

Since their discovery, ABC drug transporters have been shown to be important modulators of drug disposition. Expression and function of these membrane bound proteins impacts the bioavailability, distribution and clearance of their substrates. Their importance in determining the clinical outcome of therapeutic interventions has been underlined by their involvement in drug resistance to their substrates. More recently, there has been a growing interest in their role at important sanctuary sites, such as the blood-brain barrier, the testis and the placenta. ABC-drug transporters serve to maintain the integrity of these sanctuary sites, by limiting access to xenobiotic. A number of groups, including our lab, have shown altered levels of transporters can have an impact on the accumulation of their substrates. Furthermore, work stemming from several labs has shown that a number of ABC transporters expressed at various sites such as the liver, intestine and the brain are under the regulatory control of the nuclear receptor PXR. However, their role in the placenta and the mechanism controlling their expression at the materno-fetal barrier has not been fully understood.

5.1 PXR and Placental Transporter Expression and Regulation:

PXR is expressed in the placental tissue and levels remain fairly static during the course of gestation. Our studies have indicated that PXR genotype of the placenta has an impact on the expression of a number of key ABC transporters. When comparing the basal placental expression of Mdr1a, Bcrp, Mrp3 and Mrp3, we saw an increase in the expression of these transporters as we compared from PXR +/+ with PXR +/- and PXR -/- (placental transporter expression in PXR-/- > PXR +/- > PXR+/+). These transporters are very clinically relevant, actively shuttling a number of drugs including antiretrovirals across epithelial membranes. Thus, we found that the basal level of PXR expression is important in determining the expression of a number of drug transporters at the placenta.

This elevation was also seen in the liver, with levels of Mdr1a, Cyp3a11 and Mrp2 and 3 higher in the livers of the PXR -/- animals as opposed to the PXR+/+ mice. Further supporting the hypothesis that the elevation is due to PXR deficiency, we failed to see any difference in the

expression hepatic transporters not regulated by PXR, namely Bsep and Ntcp. Ntcp is shown to be controlled by RAR-alpha while Bsep is under the control of FXR.

The levels of steroid hormones are in a state of flux during the course of gestation, with levels increasing throughout till term. Since the circulating levels of steroidal hormones are elevated during pregnancy, and these hormones and their metabolites are known ligands for PXR, we explored if pregnancy had an impact on the transporter expression. We found the elevation in the transporter levels to be present regardless of the animal's pregnancy status, with the non-pregnant PXR -/- animals exhibiting consistently higher levels of the transporters in the liver. As seen in chapter 2, figure 4, Our findings are in agreement with data published looking at hepatic transporters in gestation. As with our data, Cy3a11 levels have been reported to be reduced during pregnancy, while Mrp3 has been shown to be elevated during the gestation state by other groups. However, while literature reports a suppression of Oatp2 and Mrp2 levels, we failed to see any such reduction in our model. While the reason for the conflicting results remains to be elucidated. One possible reason for the incongruence of the results could be species differences.

An important factor to consider when looking at NR regulatory pathways is compensation by closely related members. This is especially true for PXR, which shares a number of targets with the constitutively active receptor (CAR). Closely related both in structure and function, they act by hetero-dimerising with RXR. PXR and CAR exert their control over a common set of genes such as Cyp3a, Mdr1, Mrp2, Mrp3 and Oatp2. Given that we conducted our studies in pregnant PXR-/- animals, there are two factors that could increase the involvement of CAR mediated regulation

- the elevated hormonal levels during gestation, which are known activators of CAR, and
- the absence of PXR, leaving RXR free to bind with CAR.

We found no difference in the expression levels of CAR in either the placental or hepatic tissue in our animal model. However, we did observe higher levels of hepatic Cyp2b10- a marker for CAR activity- in PXR -/- mice. This finding points to a possible elevated activity of CAR in the knockout mice. Staudinger has previously reported an amplified effect of CAR activation in PXR -/- mice. The exact mechanism for the phenomenon is not yet fully understood. However, a

number of possibilities exist, such as reduced competition for RXR, a compensatory effect by CAR, and/or increased availability of both endogenous ligands and co-activators.

While understanding the molecular regulatory mechanism of drug transporters is undeniably important, we were also interested in the potential clinical implications of such changes. Taking advantage of the differences in basal expression of several transporters, we used the PXR knockout and wildtype mice to generate a novel model whereby differences in the placental expression of varying ABC drug transporters could be generated within the same dam. Breeding PXR +/- mice pairs, we were able to generate dams bearing pups, and hence placentas of all three possible genotypes. This led to placental units with varying amounts of transporter expression while maintaining a constant maternal environment for the fetal units. Indeed, we found that PXR genotype did in fact cause a variation is the placental transporter expression levels in this model- albeit the gradient effect seen was subdued as compared to the effect seen with homozygous mice.

Using one of the most commonly used PI in managing HIV positive pregnancies, we conducted an in-vivo study looking at fetal accumulation of Lopinavir in our model. We have shown for the first time that fetal accumulation of lopinavir is indeed dependent on drug transporter expression. Our data suggests that Mdr1a plays a key role in the placental transfer of LPV. Our data also suggest that Mrp2 but not Bcrp may also play a role.

Thus, the results of these studies clearly show the potential impact of placental drug transporters on the fetal drug exposure. The expression levels of drug transporters on the placental surface changes both in the natural course of gestation and in response to dietary, environmental or pathological influences. Therefore, one might anticipate a wide range of fetal exposure to drugs used during gestation, contingent upon the placental drug transporter at any given point during the pregnancy. This can lead to fetal exposure to drugs levels other than within the intended range and alter the safety and therapeutic outcomes of the therapeutic intervention. Data from this set of studies can help inform medical professionals in making better informed decisions when tailoring pharmaco-therapeutic regimens for HIV seropositive pregnant women. While the authors do not contend that the work can find direct clinical application, it does promise to guide further lines of enquiry when looking at the correlation between altering nuclear receptor and ABC drug transporter function and expression at the placental barriers.

5.2 Weaknesses:

As with all scientific endeavors, while the author is confident of the work presented in the thesis, there are always scope for improvement. Key weaknesses of the work are identified in this section.

The use of PXR knockout mice:

While knockout animal models present us with very powerful tools to examine gene regulation and function in an in-vivo setting, there are numerous alternatives available. One major downside of employing PXR knockout animal models is the unusual basal levels of a number of genes thought to be regulated by the nuclear receptor. Indeed, in our model, we did observe a marked increase in the basal expression of a number of key ABC drug transporters under the transcription control of PXR (Mdr1a, members of the Mrp family and Bcrp). This phenomenon has been observed previously by other researchers employing this animal model (Guo et. al., 2003, Staudinger et. al., 2003, Teng et. al., 2005). While the exact mechanism for this elevated expression has not been elucidated, it is possibly caused by an over-compensation by other closely related transcription factors in response to a loss of PXR. Since a number of these genes are determinants of drug absorption and clearance, we could see altered bioavailability and disposition of their substrates. In the case of the experiments performed as part of this thesis, this phenomenon enabled us to generate fetal units with a range of mRNA expression of key transporters within the same mother, thus providing us with an very useful tool to tease out the impact of altered expression if ABC transporters in the placenta on fetal exposure while exposed to the identical maternal drug concentrations.

A couple of other strategies could have been employed to instead of PXR knockout mice. One such technique involves modulation of protein activity, effected by inhibitors or antagonists. However, it is hampered by lack of specific, potent inhibitors which are nontoxic at effective doses in animals for a number of gene targets of interest. Alternatively, one could knock down specific genes using siRNA (small interfering RNA) or anti-sense nucleotides. Again, the method suffers from potential problems such as limited and unpredictable protein inhibition, and variability within animals in response to the treatment. On the other hand, our model ensures complete and sustained knockout of specific gene targets.

Use of Murine Placenta:

Studying the trans-placental trafficking of drugs is vital to answer questions the healthcare professional when treating pregnant women and/or the fetus. We employed a mouse model to answer questions regarding the regulation of placental transporters and their impact on lopinavir transport. While a very convenient tool, there are some shortcomings to the use of animal models, primarily the anatomical and functional differences between the murine and human placenta. This precludes the findings form in-vivo studies in animal models to being directly applicable to the clinical scenarios.

An alterative is the use of in-vitro models to explore transport of substrates across the placental barrier. One such method is the perfused placental cotyledon technique, a well validated method used by numerous groups. The model enables conducting studies either under closed or open condition to examine the extent of transplacental transport of xenobiotic and metabolites. Another alternative in-vitro method is the use of tissue culture, employing placental slices or villus explants, syncytiotrophoblastic monolayers, trophoblastic cell lines, or microvillous membrane vesicles. These methods enable the examination of transport systems and enzymes seen in the human placenta as opposed to the animal placenta. Furthermore, some of these techniques offer the advantage of working with a structurally intact placenta. However, all these techniques fall short in being able to account for the highly variable and dynamic physiological and biochemical variables seen in an in vivo setting. Furthermore, an in vivo model enables us to study temporal changes throughout pregnancy while this is not possible with in-vitro systems.

Conducting mechanistic studies exploring placental transporter regulation is, amongst other things, ethically impossible. The animal model enables us to target specific genes in the regulatory cascade in a viable animal and study the impact on the fetal transporter at various gestational stages. ALthough can estimate the fetal: maternal blood concentrations at term (at the time of placental expulsion), again we are restricted to a single gestational stage, and the probes we can study are limited. Furthermore, such scenarios do not afford us the fine control over possible when conducting these studies in the lab.

Thus, while significant differences exists between the mouse and human placenta, it offers us a useful tool to examine the mechanism being regulation of placental drug transporters in a dynamic, physiologically viable system at various stages of gestation. Furthermore, there is a

very high degree of similarity in the two placentas, anatomically as well as in the transporters expressed and the regulatory mechanism. Despite the advantages of this system, the results obtained from this study are mere guide posts for further clinical research and cannot be extrapolated to the patient population.

Lack of Protein Expression Data:

In the studies described in this thesis, the changes in gene expression was measured using mRNA levels. Transcript levels were measured using quantitative, RT-PCR. This enabled us to accurately estimate the changes at the transcriptional level in response to gene knock down, genotype, and PXR activation on a number of genes in a highly sensitive and selective manner. While transcription is the initial step in gene expression, the function of a gene is determined by the translated protein. One possible criticism of this work is the lack of protein expression data. Protein expression data are important as it gives us an indication of a functional expression of the gene. Additionally, mRNA expression has been found to not necessarily mirror protein expression patterns. Similarly, transcripts levels are not always the best surrogates for functional activity of some transporters. Protein expression on the other hand has been demonstrated to correlate much better with enzyme and transporters activity.

However, protein levels were not determined in this work due to a couple of practical limitations. The studies were all conducted on murine placental tissue, which places limits on the amount of tissue available to work with. Given the minute amounts of tissue available, most of it being consumed in the mRNA extraction procedure and genotyping, insufficient amounts were remaining for protein analysis. Since each fetal unit is this work was treated as an individual, pooling tissues was not a viable option. Furthermore, western blotting is limited in its application due to a dearth of primary antibodies specific for a number of the proteins of interest.

Despite the lack of protein expression data, the functional activity of the proteins has been demonstrated in vivo using the lopinavir disposition assay. Lopinavir has been shown to be a substrate of PGP and MRP2. In the fetal accumulation study, a clear correlation between the mRNA levels and the amount of lopinavir accumulating in the fetal tissue is seen for mdr1a and abcc2. These findings are a clear indication of functional activity despite the absence of protein expression data.

5.3 **Future Studies:**

A number of potential areas for future work are listed below:

Determine what, if not PXR is involved in placental regulations. Explore other NRs:

As discussed before, there are a number of nuclear receptors that work in close concert to regulate a diverse array of target genes. For example, the cross talk between PXR and CAR in the regulation of hepatic metabolic and transport proteins is well established. While we have seen that PXR is not responsible for activating placental drug transporters, we cannot discount the involvement of other closely related nuclear receptors as regulators of placental drug transporters.

Furthermore, for PXR to be active, it needs to heterodimerise with its obligate partner, RXR. Any alteration in the intracellular localization of either of the NRs can impact the ability of PXR to regulate its target genes. Some groups have illustrated changes in intracellular localization of NRs such as RXR when treated with certain proinflammatory cytokines. A similar phenomenon in the placenta in the cellular: nuclear ratio of RXR and PXR in the presence or absence of PCN could help explain the lack of PXR activation seen in the placenta.

Can we replicate the result in an ex-vivo model, by administering transporter inhibitors:

Our data has clearly demonstrated the importance of altered levels of transporters in determining the fetal drug exposure of their substrates. However, using an animal model that relies on knocking out PXR in order to alter the transporter levels at placental interface has some limitations- namely the expression pattern of a number of transporters is coordinately altered. While this is a scenario very close to what is clinically seen, it makes it difficult to delineate accurately the importance of individual transporters in controlling the access of their substrates to the fetal compartment. An extension of the experiment described in this thesis would be to assess the influence of individual transporters on the fetal exposure of their substrates in the presence and absence of specific inhibitors.

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Appendices

Appendix1:

The Association of the Pregnane X Receptor and the Constitutive Androstane Receptor with Hepatic Gene Regulation during Inflammation in Mice.

Sarabjit S. Gahir , Carla M.J. Muytjens, and Micheline Piquette-Miller.

- This study is an extension of previous work conducted by Dr. S. Teng, and explores the role of PXR and CAR in mediating inflammation mediated changes in hepatic drug transporters. We intend to publish this study in the near future.

The Association of the Pregnane X Receptor and the Constitutive Androstane Receptor with Hepatic Gene Regulation during Inflammation in Mice.

Carla M.J. Muytjens[1], Sarabjit S. Gahir[1] and Micheline Piquette-Miller[1].

[1]Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

Address correspondence to: C.M.J. Muytjens, Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College Street, Toronto, ON, Canada, M5S 3M2.

ABSTRACT

Inflammation and pro-inflammatory cytokines suppress the expression of several hepatic transporters and metabolic enzymes and results frequently in cholestasis. The nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are pivotal in the regulation of several hepatic transporters, however their role during inflammation remains to be clarified. In the present study, the role of inflammation on PXR and CAR and on a wide range of hepatic transporters and drug metabolizing enzymes in wild type $PXR^{+/+}$ $CAR^{+/+}$, $PXR^{-/-}$ knockout mice and $PXR^{-/-}$ $CAR^{-/-}$ double knockout mice was studied. Inflammation induced by IL-6 caused a PXR and CAR dependent downregulation of BSEP, MRP2, and NTCP. The regulation OATP2 seems to be partly independent of PXR and CAR during inflammation. In summary, the results suggest a role of both PXR and CAR in the downregulation of several hepatic proteins during inflammation, and provides a potential target for novel therapy during cholestasis.

KEYWORDS Cholestasis, inflammation, PXR, CAR, drug transporters

Introduction

Under normal physiological conditions bile flows from the liver to the duodenum, however in cholestasis this bile flow is disrupted. Cholestasis is clinically defined by jaundice with a serum bilirubin greater than 50 µmol/L, pale stools, dark urine and generalized itchiness (Krell and Enderle, 1993). Bile functions in the digestion and absorption of lipids in the small intestine and hence is of influence on the absorption of fat soluble substances such as vitamins A, D, E and K. Furthermore, bile serves as the excretion route for bilirubin, and bile salts are known to be bactericidal (Trauner et al., 2010; Sung and Go, 1999). An obstruction of the bile flow, as seen in cholestasis, results in intraheptic accumulation of toxic bile constituents and consequently liver fibrosis (Stahl et al., 2008).

The biliary apparatus is a convergent system of canals that starts in the canaliculi, continues to the bile ducts and ends with the common bile duct (Esteller et al., 2008). Under normal physiological conditions, bile formation and secretion is mediated and maintained by the coordinated function of a set of membrane transporters located in the liver, and depends on the structural and functional integrity of the biliary tree. Hepatocytes generate primary bile in the canaliculi, while cholangiocytes modify the canalicular bile by secretory and reabsorptive processes as bile passes through the bile ducts (Forker et al., 1967; Alpini et al., 1994). In contrast to hepatocytes, where secretion is constant and poorly controlled, secretion by cholangiocytes is extensively regulated (Arrese et al., 2002; Alvaro et al., 1993).

Hepatocytes express a variety of drug transporters in the blood-facing sinusoidal and canalicular membranes forming efficient directional transport of molecules from the blood circulation to the bile (Nies et al., 2008). Uptake of bile acids into hepatocytes occurs mainly by the sodium taurocholate cotransporting polypeptide (NTCP). The canalicular export of bile salts is mediated by the bile salt export pump (BSEP) which constitutes the rate limiting step of hepatocellular bile salt transport and drives the enterohepatic circulation of bile salts (Stieger, 2011; Kosters and Karpen, 2008). Other hepatic transporter proteins, such as the ATP-binding cassette (ABC) transporters P-glycoprotein, multidrug resistance protein-2 (MRP2), organic anion transporter polypeptide (OATP) and breast cancer resistance protein (BCRP), have been

demonstrated to mediate the biliary excretion of drugs and their metabolites (Kusuhara and Sugiyama, 2010). In addition, MRP3, MRP4 and MRP5 have been found to be expressed in the sinusoidal membrane and mediate the sinusoidal efflux of drugs and their conjugated metabolites into the blood circulation (Kitamura et al., 2008).

Bacterial endotoxins have been shown to be a causative agent in inflammation induced cholestasis (Trauner et al. 1999a). Cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) are produced in response to the inflammatory stimulus, and are potent inducers of intraheptic cholestasis. The cholestatic effect of cytokines results mainly from the activation of intracellular signaling pathways that subsequently leads to an altered hepatobilairy transporter expression and function. Inflammation induced downregulation of various hepatic transporters involved in bile acid uptake and excretion results in impairment of bile formation and accumulation of bile acids and toxins in the liver and serum (Kosters and Karpen 2010). The inflammatory stimulus is known to have an effect on both bile acid-dependent and bile acid-independent fractions of bile flow (Geier et al., 2007). These cytokine induced effects are reversible and bile secretory function is restored upon disappearance of the inflammation injury (Trauner et al 1999b).

During the inflammatory response, both intraheptic cholestasis and decreased drug metabolism are frequently observed (Assenat et al., 2004). The suppressive effects of inflammatory stimuli are thus not limited to membrane bound transporters, but are seen in the cytochrome P450 (CYP) enzyme family as well. CYP constitutes a large and diverse group of enzymes whose main function is to catalyze the oxidation of organic substances. CYPs are highly expressed in the liver and are involved in multiple processes including drug metabolism, hormone synthesis, cholesterol homeostasis and vitamin D bioactivation and metabolic degradation (Lewis and Ito 2010; Tsuchiya et al., 2005; Pikuleva, 2006; Omdahl et al., 2001).

Recent studies have indicated that induction of hepatic transporters is mediated by activation of the pregnane X receptor (PXR) and Constitutive Androstane Receptor (CAR) (Kakizaki et al., 2009; Jonker et al., 2009; Stahl et al., 2008). PXR and CAR are both members of the superfamily of nuclear receptor transcription factors and are activated by numerous clinically

important xenobiotics, herbal products as well as endogenous steroids (Chang 2009; Mottino and Catania 2008; You 2004). CAR and PXR are expressed in multiple tissues and are highly expressed in the liver and intestine (Quatanani and Moore, 2005). Both PXR and CAR exert transcriptional regulation by binding to DNA response elements as a heterodimer with the retinoid X receptor (RXR). The biological and physiological consequences of PXR and CAR activation include drug metabolism, drug-drug interactions and the homeostasis of glucose, lipids, steroids, bile acids, bilirubin, cholesterol and bone minerals (Ihunnah et al., 2011; Sberna et al., 2010; Moreau et al., 2008).

The hepatic expression of both PXR and CAR decreases during the acute phase response and recent studies suggest that these decreased hepatic PXR and CAR levels are related to the observed downregulation of several hepatic transporters in inflammation (Staudinger et al 2001; Pascussi et al., 2000; Pascussi et al., 2003; Teng and Piquette-Miller, 2005). The combined role of PXR and CAR on gene regulation during inflammation however has not yet been fully established. The aim of this present study is to determine the impact of several inflammatory stimuli on the hepatic hepatic transporters and metabolic enzymes and the contribution of PXR and CAR to these changed. Elucidating the molecular mechanisms involved inflammation induced intraheptic cholestasis may represent potential novel targets for the treatment of cholestatic liver diseases as PXR and CAR are known to have a protective effect on cholestasis by activating both detoxification enzymes and transporters (Kakizaki et al., 2009).

Material and methods

Animals: The animal studies were conducted at the Division of Comparative Medicine, at the University of Toronto, following protocols approved by the Animal Care Committee and conformed to the NIH "Principles of Laboratory Animal Care". PXR and CAR wild type ($PXR^{+/+}$ $CAR^{+/+}$) C57/BL6 mice were purchased from Charles River Canada (Montreal, PQ, Canada). PXR knockout ($PXR^{-/-}$) C57/BL6 mice and PXR CAR double knockout ($PXR^{-/-}$ $CAR^{-/-}$) C57/BL6 mice were obtained from Dr. Christopher Sinal (Dalhousie University, Halifax, NS, Canada) with approval from Dr. Steven Kliewer (University of Texas, Southwestern Medical Center, Dallas, TX, USA). Animals were kept in a temperature-controlled facility with 12-h light/dark cycles and were fed a standard chow diet.

To study the influence of inflammation on hepatic gene regulation wild type $PXR^{+/+}$ $CAR^{+/+}$, $PXR^{-/-}$ and $PXR^{-/-}$ $CAR^{-/-}$ mice were injected with IL-1beta or IL-6 i.p. and were sacrificed 6 hours later. Cytokines including IL-6, which are secreted by macrophages and Kupffer have been characterized as a mediators of reduction in bile flow and organic anion excretion (Roelofsen et al., 1995; Whiting et al., 1995)

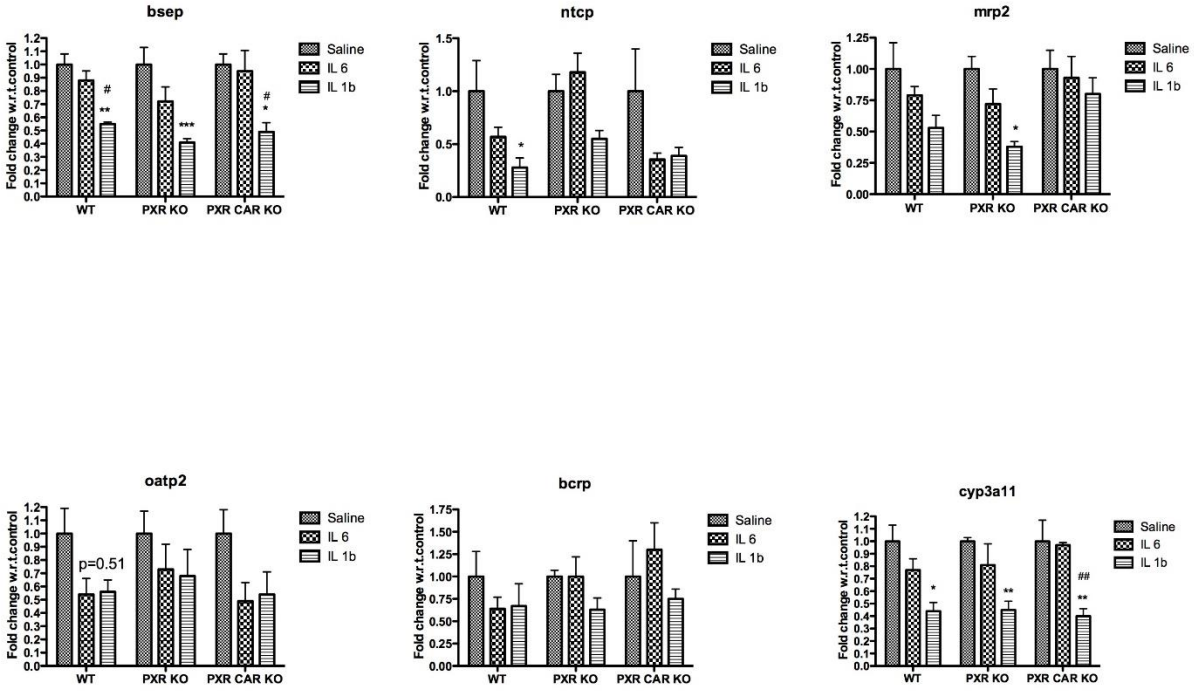
.

Determination of mRNA: As previously described, total RNA extraction, reverse transcriptase PCR and real time PCR were conducted on the hepatic samples (Teng and Piquette – Miller, 2005). In short, total RNA extraction was achieved using the QuickPrep RNA extraction kit supplies by Amersham Biosciences Inc. (Piscataway, NJ, USA). RNA concentration and purity were determined with the use of Nanodrop ND1000 (Nanodrop technologies, Wilmington, DE, USA). RNA underwent DNase treatment with the following protocol: 30' at 37°C, 10' at 75°C followed by the high capacity cDNA reverse transcriptase kit (Applied Biosystems, Streetsville, ON, Canada) to obtain cDNA according to the following protocol: 10'at 25°C, 120'at 37°C and 5'at 85°C. Quantification of mRNA was carried out by real-time quantitative PCR consistent with the following protocol: 2' at 50°C, 10' at 95°C and 50 cycles of 15'' at 95°C and 60'' at 60°C (HT7900, Applied Biosystems). The transcript level was normalized to housekeeping gene cyclophilin. There were no statistical significant differences in hepatic cyclophilin transcript levels of all three genotypes, showing it to be a suitable normalizing gene. Primers were synthesized by the DNA Synthesis Centre, Hospital for Sick Children (Toronto, ON, Canada). Data were analyzed via the standard curve method using SDS 2.2.1 software.

Statistical analysis: Studies were performed using n = 6 mice per treatment group. Differences between wild type $PXR^{+/+}$ $CAR^{+/+}$ versus $PXR^{-/-}$ and $PXR^{-/-}$ $CAR^{-/-}$ mice and between treatment groups were determined by either t-tests or one way ANOVAs and Scheffe post hoc test with $p<0.05$ considered to be statistically significant. Analysis was performed using PAWS statistics 18 (SPSS Inc. Chicago, IL, USA).

Figure 23:

Impact of IL-6 and IL-1β treatment on mRNA levels in the hepatic tissue of PXR +/+ (WT), PXR-/- (PXR KO) and PXR CAR -/- (PXR CAR KO). The tissues analysed as described in the methods. Levels were normalized to Gapdh and Cyclophylin and presented as % controls.



Results:

Comparison of cytokine IL-6 mediated effects in $PXR^{+/+}$, $PXR^{-/-}$ and $PXR^{-/-}$ $CAR^{-/-}$ mice:. The impact of PXR and CAR in the molecular mechanisms involved in the inflammation induced downregulation of hepatic transporters was determined in IL-6 treated wild type $PXR^{+/+}$ $CAR^{+/+}$ versus $PXR^{-/-}$ and $PXR^{-/-}$ $CAR^{-/-}$ mice. Treatment of wild type $PXR^{+/+}$ $CAR^{+/+}$ mice with IL-6 imposed a significant downregulation of Ntcp transcript levels 57% of the levels seen in the saline treated controls. Treatment with IL-6 did not result in a significant altered expression of MRP2, Bsep, Oatp2 or Cy3a11 compared to saline treated wild type $PXR^{+/+}$ $CAR^{+/+}$ controls, although the Oatp2 levels were reduced by approximately 50% in the IL-6 treated animals.

Administration of IL-6 in $PXR^{-/-}$ mice did lead to a significantly reduced mRNA expression of BSEP transcript levels of 70% of saline treated controls. No significant differences the mRNA expression levels were observed after IL-6 administration in $PXR^{-/-}$ mice for the other genes analysed

Administration of IL-6 in $PXR^{-/-}$ $CAR^{-/-}$ mice resulted in a significant downregulation of Ntcp mRNA transcript levels to 36% compared to saline treated controls. Treatment with IL-6 did not result in an altered mRNA expression of the other genes of interest compared to saline treated controls.

Comparison of cytokine IL-1β mediated effects in $PXR^{+/+}$, $PXR^{-/-}$ and $PXR^{-/-}$ $CAR^{-/-}$ mice:

The impact of PXR and CAR in the molecular mechanisms involved in the inflammation induced downregulation of hepatic transporters was determined in IL-1β treated wild type $PXR^{+/+}$ $CAR^{+/+}$ versus $PXR^{-/-}$ and $PXR^{-/-}$ $CAR^{-/-}$ mice. Treatment of wild type $PXR^{+/+}$ $CAR^{+/+}$ mice with IL-1β imposed a significant downregulation of Bsep, Ntcp and Cyp3a11. The treatment caused the transcript levels of Bsep, Ntcp and Cyp3a11 to fall to 55%, 28% and 45% of the control levels respectively. Treatment with IL-1β did not result in statistically significant altered expression of Oatp2 and Mrp2 compared to saline treated wild type $PXR^{+/+}$ $CAR^{+/+}$ controls.

Administration of IL-1β in $PXR^{-/-}$ mice did lead to a significantly reduced mRNA expression of BSEP, Cyp3a11, Mrp2 and Ntcp compared to saline treated controls. The treatment caused the transcript levels of BSEP, Cyp3a11, Mrp2 and Ntcp to fall to 41%, 45%, 38% and 55% of the control levels respectively. No significant differences in OATP2 mRNA expression levels were observed after IL-1β administration in $PXR^{-/-}$ mice.

Administration of IL-1β in $PXR^{-/-}$ $CAR^{-/-}$ mice resulted in a significant downregulation of Bsep, Ntcp and CYP3A11 mRNA transcript levels compared to saline treated controls. The treatment caused the transcript levels of BSEP, Cyp3a11, and Ntcp to fall to 49%, 40%, and 39% of the control levels respectively.Treatment with IL-1β did not result in an altered mRNA expression of Mrp2 and Oatp2 compared to saline treated controls.

Discussion:

Nuclear receptors are known to mediate the regulation of hepatic genes during normal physiology and metabolism and in adaptation to liver diseases (Arrese and Karpen, 2010). In this respect, cholestasis is frequently seen as a complication in patients with sepsis and extraheptic bacterial infections, and could be linked to inflammation induced decreases in hepatic transporter function (Trauner et al., 1999b; Moseley, 1997; Tănăsescu, 2004). Decreased hepatic PXR and CAR mRNA transcript and protein levels are associated with the downregulation of several hepatic transporters observed during inflammation (Staudinger et al., 2001; Pascussi et al., 2000; Pascussi et al., 2003; Teng and Piquette-Miller, 2005). The aim of this study was to investigate the role of nuclear receptors PXR and CAR in mediating changes in the expression of hepatic bile salt and acids transporters during inflammation.

While an extension of the studies conducted by Teng and Piquette-Miller, the inflammatory response seen in the animals during the studies described herein were very muted in response to the pro-inflammatory cytokines compared to results seen before by our lab and others. Despite administration of cytokine levels in line with the standard doses, the poor responsiveness of the animals can be due to a couple of possibilities. Firstly, there could be a difference in the potency of the administered cytokines themselves, since these are biologically

sourced agents. Batch to batch differences in potency differences, while uncommon, may occur and can possibly explain poor inflammatory response seen in the animals. Secondly, we failed to see any meaningful response to LPS treatment in our animals as compared to previously reported results. The development of endotoxin resistance, a phenomenon in which the inflammatory and fatal effects of LPS are mitigated as a result of prior LPS exposure, is another likely reason for the lack of response to LPS treatments. The latter is a more likely possibility given that the age of the mouse colony and potential for repeated exposure in the animal holding facility.

The biliary secretion of bile acids is crucial for multiple liver functions including digesting fatty nutrients and driving bile flow. Furthermore, impaired bile flow results in an accumulation of bile acids, and subsequently leads to inflammatory liver injury. Multiple hepatic transporters are involved in processes to avoid toxicity due to bile acid over-accumulation (Wang et al., 2010). In this respect, basolateral transport systems are responsible for translocating molecules across the sinusoidal membrane, whereas active canalicular transport systems are responsible for the biliary excretion of drugs and metabolites (Chandra and Brouwer, 2004). The bile flow is mediated by the major canalicular transporters BSEP, MRP2 and BCRP. The protein activity of these transporters depends on the expression regulation and localization at the canilicular membrane (Mottino and Catania, 2008).

The effects of inflammation on BSEP mRNA expression and the involved molecular mechanisms seem to be dependent on the presence of nuclear receptors PXR and CAR. The downregulation observed in BSEP mRNA expression after IL-6 administration seems to be mediated via the nuclear receptors PXR and CAR. However, the exact role of the nuclear receptors PXR and CAR in BSEP regulation remains to be determined. Other studies also observed a significant downregulation in hepatic BSEP in mice challenged with LPS, partly through FXR dependent mechanisms (Jahnel et al., 2009; Lickteig et al., 2007; Wagner et al., 2003; Plass et al., 2002). It is therefore possible that the regulation of BSEP involves multiple other nuclear receptors besides PXR and CAR.

MRP2 functions in the terminal excretion and detoxification of endogenous and xenobiotics organic anions, particularly in the unidirectional efflux of substances conjugated with

glutathione, glucuronate or sulphate (Nies and Keppler, 2007). In the present study IL-6 and IL-1beta did not exert a significant effect on the regulation of MRP2.

In hepatocytes, the vectorial transport of bile acids from blood to bile is ensured by multiple transporters including NTCP and OATP2 (Alrefai and Gill., 2007). Administration of IL-6 did not exert an effect on hepatic NTCP mRNA transcript levels. Previous research has shown that OATP2 is regulated by a complex interacting network of hepatocyte nuclear factors and several nuclear receptors including PXR and CAR (Geier et al., 2007). The role of inflammation on hepatic OATP2 regulation remains to be determined in the present study.

Overall, nuclear receptors play a pivotal role in the regulation of genes responsible for the metabolism and transport of bile acids. Both nuclear receptors PXR and CAR require RXR for heterodimerization up activation. The posttranscriptional regulation of RXR during the acute phase response by phosphorylation and nuclear export also represents a common denominator for a coordinated downregulation of other nuclear receptor heterodimer target genes (Feige et al., 2005). Furthermore, PXR and CAR share a functional redundancy with the nuclear receptor FXR, so the effect of inflammation on the hepatic transporters and metabolic enzymes are most likely an interplay between various members of the nuclear receptor family. Likewise, compensatory changes in several hepatic transporters could occur in knock out models and during disease states in order to preserve homeostasis, and hence have an influence on the observed results.

A common theme of all bile acid-activated receptors is their ability to counter-regulate effector activities of cells of the innate immune system establishing that signals generated by these receptors and their ligands function as a braking signals for inflammation in entero-hepatic tissues. Bile acids are known to act as inflammagens, and directly activate signalling pathways in hepatocytes that stimulate production of proinflammatory mediators (Allen et al., 2011). The ability of bile acid-activated receptors to integrate metabolic and inflammatory signaling makes them particularly attractive targets for intervention in immune-mediated diseases (Fiorucci et al., 2010). Furthermore, the extreme flexibility and versatility of nuclear receptors opens the prospect of regulating their transcriptional activity by ligands, post-transcriptional modifications

and co-receptors (Moreau et al., 2007). In this respect, administration of specific CAR or PXR ligands could result in a coordinated stimulation of major hepatic bile acid and bilirubin metabolizing and detoxifying enzymes and hepatic efflux systems (Wagner et al., 2005). Therapeutic approaches targeting nuclear receptors in cholestasis may stimulate these adaptive changes and open a new perspective for the treatment of cholestatic liver diseases (Wagner et al., 2010). The present study provides a first step in elucidating the impact of PXR and CAR in the molecular mechanisms involved inflammation induced changes in hepatic transporter regulation and further research may lead to the discovery of potential novel targets for the treatment of cholestatic liver diseases.

Apendix 2:

List of Primers

Gene	Forward Sequence (5' – 3')	Reverse Sequence (5'-3')
Gapdh	CCATCACCATCTTCCAGGAG	CCTGCTTCACCACCTTCTTG
Mrp1	TGAGTGTGCAGAAGGTGGAG	ACCCGCGTGTAGTCCATTAT
Mrp2	CTGAGTGCTTGGACCAGTGA	CAAAGTCTGGGGGAGTGTGT
Mrp3	CGCTCTCAGCTCACCATCAT	GGTCATCCGTCTCCAAGTCA
Bcrp	GGCGGAGGCAAGTCTTCGTTG	TGGGCAGGTTGAGGTGCTCCAT
Mdr1a	CCCATCATTGCGATAGCTGG	TCCAACATATTCGGCTTTAGGC
Oatp2	CCTTAAAGCCAACGCAAGAC	CACTCCTGCACAGACCAAAA
Bsep	GTTCAGTTCCTCCGTTCAAA	AAGCTGCACTGTCTTTTCAC
Ntcp	ACACTGCGCTCAGCGTCATTC	GCCAGTAAGTGTGGTGTCATG
Car	CACAGCGGCGGGCAGAGAAA	AGGCCGGAGGCTTGAACTGC
Cyp2b10	CCCAGTGTTCCACGAGACTT	GGTGCCGACAAAGAAGAGAG
Cyp3a11	CGCCTCTCCTTGCTGTCACA	CTTTGCCTTCTGCCTCAAGT
Pxr1	GACGCTCAGATG CAAACCTT	TGGTCCTCAATAGGCAGGTC
Pxr2	CCTGCAGTGTTCCACAGT GGCTGT	CCGCGCAGCTGCAGAGAGAT
Pxr	GTTCCTGATTCTTCAAGGTGG	TCTTCCTCTTGATCAAGGCC