Placental Pharmacology – Implications for Therapy in Pregnancy

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Medication use during pregnancy requires a balance between treating a condition in the mother, and minimizing potential risks in the fetus. In recent years, it has been estimated that between 50 and 70% of pregnant women in North America will take at least one prescription medication during their pregnancy. The decision to begin or continue treatment during pregnancy relies heavily on evaluating the risk-benefit ratio, and an important determinant in this risk assessment is estimating fetal drug exposure. The use of medications in pregnant women can be especially challenging, as there is very limited safety data in pregnancy. Recently, novel oral anticoagulants have been developed and approved for clinical use. However, the information regarding their fetal safety and placental transfer in humans is unknown. We used the \textit{ex vivo} placenta perfusion model to investigate anticoagulant (dabigatran, rivaroxaban, apixaban) transfer across the human placenta. Rivaroxaban and apixaban rapidly crossed the term human placenta, while dabigatran crossed the placenta to a lesser extent. The placenta perfusion results were adjusted to account for protein binding and pH differences between the mother and fetus. We also developed a pharmacokinetic model that adequately described the transplacental transfer of anticoagulants by using data from our experiments. While the placenta perfusion model can be technically challenging, its results strongly correlate with \textit{in vivo} placental transfer data. By evaluating the success rate of the perfusion model in our laboratory, we determined that establishing the fetal
circulation is an important stage of the protocol. This information can be used to create a focused training program to help increase the overall success rate of this model and productivity of the lab. Drug use in pregnancy is multifactorial, and placental transfer data is important in assessing which drugs can be used to treat the mother while protecting the unborn.
Acknowledgments

I would like to thank many individuals for their guidance, support and contributions during the course of my training.

First and foremost, I would like to express my profound respect and gratitude to Dr. Shinya Ito for his exceptional mentorship during my graduate studies. I thank him for his support and encouragement in pursuing my ambitions and career goals. I would like to acknowledge Dr. Gideon Koren for his continuous guidance and for providing me with countless opportunities.

I would like to thank Dr. Howard Berger for being a wonderful mentor and for the opportunity to collaborate together on many projects. I thank him for taking time to proofread my work, and for being a dedicated member of my graduate advisory committee. I would like to thank Dr. Micheline Piquette-Miller for her wise words and invaluable guidance during committee meetings. I wish to acknowledge Drs. Bhushan Kapur and Prateek Lala for their mentorship and enthusiasm in working with graduate students.

A very special thank you to Angelika Lubetsky for sharing her expertise of the placenta perfusion model and working tirelessly for successful experiments. I would like to thank Leonardo Pinto for all his contributions to these studies. I would especially like to acknowledge the staff in the Labour and Delivery Ward at St. Michael’s Hospital. Thank you to the obstetricians, residents, and nurses for all their help in facilitating tissue collection. I would like to acknowledge Dr. Katarina Aleksa, Ariane Mandel, Paula Walasek, Michael Leadley, and Hayley Craig-Barnes for their assistance with analytical methods in the laboratory. I want to thank Dr. Masanobu Takeuchi for introducing me to the exciting field of pharmacokinetic modeling, and taking the time to answer all my questions.

I would like to thank my fellow graduate students who have made this experience more than I could have hoped for. This talented group of people played an important role in making graduate school fun and I will always cherish the great conversations and meals we shared. I consider myself lucky for having shared the lab with you all and I couldn’t have imagined a better group of brilliant people. I am grateful for the friendships that will extend far beyond our time in the lab.
Last, but certainly not least, a very special thank you to my family and friends for their unwavering support, encouragement, and patience over the last few years. Thank you to my parents for being phenomenal role models and showing me what hard work and determination look like. They have been my cheerleaders every step of the way and I cannot thank them enough.
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<th>Description</th>
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<tbody>
<tr>
<td>AAG</td>
<td>$\alpha$-1 acid glycoprotein</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>APS</td>
<td>antiphospholipid antibody syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>B/U</td>
<td>bound/unbound ratio</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>CL</td>
<td>clearance</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>maximal plasma concentration</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DVT</td>
<td>deep vein thrombosis</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPI</td>
<td>enhanced product ion</td>
</tr>
<tr>
<td>F:M</td>
<td>fetal-to-maternal</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HIT</td>
<td>heparin-induced thrombocytopenia (HIT)</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>$K_{12}$</td>
<td>influx rate constant from maternal to placental compartment</td>
</tr>
<tr>
<td>$K_{21}$</td>
<td>efflux rate constant from placental to maternal compartment</td>
</tr>
<tr>
<td>$K_{23}$</td>
<td>efflux rate constant from placental to fetal compartment</td>
</tr>
<tr>
<td>$K_{32}$</td>
<td>influx rate constant from fetal to placental compartment</td>
</tr>
<tr>
<td>LMWH</td>
<td>low molecular weight heparin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MRP</td>
<td>multi-drug resistance associated protein</td>
</tr>
<tr>
<td>NOAC</td>
<td>novel oral anticoagulant</td>
</tr>
<tr>
<td>NSAID</td>
<td>nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OAT</td>
<td>organic anion transporter</td>
</tr>
<tr>
<td>OCT</td>
<td>organic cation transporter</td>
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<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PE</td>
<td>pulmonary embolism</td>
</tr>
<tr>
<td>pKa</td>
<td>log dissociation constant</td>
</tr>
<tr>
<td>t(_{1/2})</td>
<td>elimination half-life</td>
</tr>
<tr>
<td>TAFI</td>
<td>thrombin-activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>UFH</td>
<td>unfractionated heparin</td>
</tr>
<tr>
<td>UGT</td>
<td>uridine 5’-diphosphate glucuronosyl-transferase</td>
</tr>
<tr>
<td>UHPLC</td>
<td>ultrahigh-performance liquid chromatography</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>V(_d)</td>
<td>volume of distribution</td>
</tr>
<tr>
<td>V(_f)</td>
<td>volume of distribution in fetal compartment</td>
</tr>
<tr>
<td>V(_m)</td>
<td>volume of distribution in maternal compartment</td>
</tr>
<tr>
<td>V(_p)</td>
<td>volume of distribution in placental compartment</td>
</tr>
<tr>
<td>VTE</td>
<td>venous thromboembolism</td>
</tr>
<tr>
<td>ZPI</td>
<td>protein Z-dependent protease inhibitor</td>
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Chapter 1

General Introduction

1 Drug Use in Pregnancy

Medication use during pregnancy requires a fine balance between treating a condition in the intended patient, the pregnant woman, and minimizing potential adverse risks in the unintended patient, the fetus. Although a number of prenatal drug exposures are known to cause major malformations, there is limited information on the risks and safety for the vast majority of medications. With newer medications, there is typically no information about safety or efficacy in pregnancy, as clinical trials often specifically exclude pregnant women and ensure that women of childbearing potential do not become pregnant during the period of drug exposure. This is in contrast to recent trends indicating that the antepartum use of both over-the-counter and prescription medications have increased in the last 30 years. Retrospective database studies have estimated that between 50 and 70% of women in North America take at least one prescription medication during pregnancy (Kulaga et al. 2009; Mitchell et al. 2011). The decision to begin or continue treatment during pregnancy relies heavily on weighing the benefits of the drug to the mother against the potential risk to the fetus. An important determinant in this risk assessment is the estimation of fetal drug exposure, based on quantifying the amount of drug that crosses the placenta.

1.1 Transplacental Drug Transfer

During pregnancy, there are several physiological and anatomical changes that can alter the maternal and fetal pharmacokinetics of medications. These changes can impact the absorption, distribution, metabolism, and elimination of medications, and may affect their pharmacodynamic properties during pregnancy. Transplacental pharmacokinetics must also be considered in the context of fetal safety, as the placenta plays an integral role in fetal drug disposition.
1.1.1 Pregnancy-Related Physiological and Pharmacokinetic Changes

**Absorption**

Nausea and vomiting in the first trimester of pregnancy can decrease the amount of drug that is absorbed following oral administration. Therefore, oral medications should ideally be taken when nausea and vomiting are minimal. During pregnancy, gastric acid secretions are decreased and mucus secretion is increased, leading to an elevation in gastric pH (Gryboski & Spiro 1956; Loebstein et al. 1997). A change in gastric pH can increase ionization of weak acids and reduce their absorption. Weak bases will be primarily un-ionized, and absorbed more readily. In addition, slower intestinal motility can alter drug absorption and oral bioavailability. In late pregnancy, the gastrointestinal transit time is increased by 30–50% (Parry et al. 1970; Chiloiro et al. 2001). As well, increases in cardiac output and intestinal blood flow may increase drug absorption (Lees et al. 1967). After accounting for changes in gastric pH, intestinal motility, and cardiac output, it appears that pregnancy-related physiological changes may alter the rate of drug absorption.

**Distribution**

There are numerous physiological changes during pregnancy that can affect the distribution of drugs. Throughout pregnancy, there are marked increases in total body water, blood volume, and plasma volume. Blood volume begins to increase at 6–8 weeks gestation, and continues to increase until 32–34 weeks (Lund & Donovan 1967). The significant increases in extracellular fluid space and total body water will increase the volume of distribution (V_d) for hydrophilic drugs. This can lead to a decrease in the maximal concentration (C_max) for many drugs, if the dose is not adjusted. Maternal body fat increases by 3–6 kg, thereby increasing the V_d for lipophilic drugs (Lederman et al. 1997). These increases in total body water and fat content can affect the V_d of many drugs (Loebstein et al. 1997). The placenta and fetus can act as additional compartments for drug distribution, further increasing the apparent V_d for certain drugs.

Changes in plasma protein binding can affect the unbound fraction of a drug, and potentially alter its pharmacodynamics, as only the unbound form of the drug elicits a pharmacological effect. Throughout pregnancy, albumin concentrations decrease to approximately 70–80% of non-pregnant values in the third trimester (Krauer et al. 1984). While this decrease in albumin
has been attributed to dilution due to an increased plasma volume, another explanation is that this decrease is caused by a reduction in the rate of albumin synthesis or an increase in the rate of its catabolism (Frederiksen 2001). This is supported by the fact that levels of $\alpha$-1 acid glycoprotein (AAG) remain relatively stable during pregnancy (Piafsky & Woolner 1982; Krauer et al. 1984). Protein binding is important in the context of placental drug transfer, and this will be discussed in section 1.1.2.2.

**Metabolism**

Drug metabolism is altered in pregnancy, and these changes become more pronounced as the pregnancy progresses. The activities of many phase I cytochrome P450 (CYP) enzymes are increased in pregnancy, leading to increased clearance overall. The hepatic activities of CYP 3A4, CYP 2A6, CYP 2D6 and CYP 2C9 are all increased in pregnancy (Tracy et al. 2005; Hebert et al. 2008; Ryu et al. 2016). Due to increased clearance, many drugs will have sub-therapeutic concentrations, and may require an increased dose to maintain a therapeutic effect. It should be noted that some phase I enzymes, namely CYP 1A2 and CYP 2C19, have decreased activity in pregnancy (Brazier et al. 1983; Carter et al. 1986; Villani et al. 2006). Drugs that are metabolized by CYP 1A2 and CYP 2C19 may require smaller doses to minimize potential toxicity. Phase II enzymes are also altered in pregnancy, with the activity of uridine 5’-diphosphate glucuronosyl-transferases (UGTs) increasing by up to 300% in the third trimester (Pennell et al. 2004). The effect of pregnancy on enzyme activity varies with genotype, ethnicity, age, and certain disease states unrelated to pregnancy. In general, changes in drug metabolism and clearance may require dose adjustments for certain medications used in pregnancy. Following delivery, these doses may need to be re-adjusted, as enzyme activity returns to pre-pregnancy levels.

**Elimination**

Several renal changes occur during pregnancy, which can alter the elimination of renally-cleared drugs. Anatomically, the size of the kidneys increases during pregnancy, accompanied by a 60–80% increase in renal blood flow. As a result, the glomerular filtration rate (GFR) increases by 50% in the first trimester, and continues to increase throughout pregnancy. In some individuals, GFR begins to decrease in the last few weeks of pregnancy (Davison & Hytten 1974; Davison & Dunlop 1980; Odutayo & Hladunewich 2012). Studies using probe drugs have measured the
apparent activity of renal drug transporters, and found that the activity of P-glycoprotein (P-gp), organic cation transporter 2 (OCT2), and organic anion transporter 1 (OAT1) are all increased during pregnancy (Andrew et al. 2007; Hebert et al. 2008; Eyal et al. 2010). The overall increase in renal clearance can significantly increase the elimination rates of renally-cleared drugs, and drug dosages may need to be increased by 20–65% to maintain therapeutic levels (Anderson 2005).

1.1.2 Human Placenta: Structure and Function

1.1.2.1 Placental Development

Development of the placenta and fetus is a highly regulated process, which begins at the time of fertilization. After fertilization, the first three days of development occur in the fallopian tube, and on the fourth day, the morula enters the uterus. On the 5th day, the morula becomes a blastocyst, as a result of fluid accumulation and polarization of the cells. The blastocyst has an outer layer of cells, called trophoblasts, which will form the placenta and fetal membranes, and an inner cell mass, which will eventually form the embryo (Boyd & Hamilton 1970; Gude et al. 2004). The inner cell mass and outer layer of cells rapidly proliferate, and the zona pellucida surrounding the blastocyst is shed. Uterine secretions temporarily provide oxygen and metabolic substrates for the blastocyst until approximately day 6, when the blastocyst implants into the uterine lining (Aplin 2000). The uterine lining provides access to substrates, such as glycogen-filled stromal cells, which are necessary for fetal growth.

Implantation involves the movement of the blastocyst to usually the mid-to-upper anterior or posterior wall of the uterus, followed by adhesion and invasion. Trophoblasts rapidly proliferate and differentiate along two pathways to form either villous or extravillous cytotrophoblasts (Figure 1.1). Villous cytotrophoblasts ultimately form the outer cellular layer of syncytiotrophoblast, which is responsible for the transport of gases, nutrients and waste products, and the synthesis of peptide and steroid hormones. Extravillous trophoblasts (EVTs) eventually form structural components of the placenta, including columns, cell islands, and septa (Boyd & Hamilton, 1970). EVT have been shown to express high levels of human placental lactogen (hPL), and very little human chorionic gonadotropin (hCG) in vitro (Tarrade et al. 2001).
The syncytiotrophoblast extends into the endometrial epithelium and invades the connective tissue. As the trophoblast moves deeper into the endometrial surface (called decidua), vacuoles or empty spaces start to form. These vacuoles become confluent to form lacunar networks, and the lacunar space eventually becomes filled with maternal blood to form the intervillous space, thus establishing uteroplacental circulation (Aplin 2000). By the second week of placental development, a syncytiotrophoblast layer with a core of cytotrophoblast cells evaginates into the lacunar space to form primary mesenchymal villi. After further development, they become secondary villi by acquiring an inner core of embryonic mesoderm. By day 21, the embryonic mesoderm differentiates into blood vessels, which subsequently connect to vessels in the umbilical cord and embryo, thus forming tertiary villi (Boyd & Hamilton, 1970). Some villi are anchored to the maternal decidua, and others float freely in the lacunae.

At 4–5 weeks gestation, EVT forms columns as part of the cytotrophoblastic shell, which is at the feto-maternal interface. Proliferative trophoblast cells are found at the base of the columns, and invasive trophoblasts are at the distal portion of the columns. Invasive EVT are further categorized into interstitial EVT, which invade the decidua, and endovascular EVT, which remodel maternal blood vessels in the uterine decidua. Interstitial EVT promote the circumferential expansion of the placenta and recruitment of maternal arterioles, which ultimately results in growth of the villous region of the placenta (6). Interstitial trophoblasts become multinucleated and more rounded to form placenta bed giant cells (Boyd & Hamilton, 1970; Gude et al. 2004).

During the first trimester of pregnancy, the placenta differentiates and grows in a low oxygen environment. Endovascular EVT plug uterine spiral arterioles, and thus restrict uterine blood flow to the fetus (Rodesch et al. 1992). At 10–14 weeks gestation, the trophoblastic plugs are displaced and blood flows into the intervillous space to facilitate gas and nutrient exchange. The proportion of the placenta occupied by blood vessels increases throughout gestation to facilitate nutrient transport (Gude et al. 2004).

The three stages of human placental vascular development are: (1) vasculogenesis, (2) branching angiogenesis, and (3) non-branching angiogenesis. Approximately 21 days after conception, placental villi undergo vasculogenesis and blood vessels are formed (Risau 1997). Branching angiogenesis involves the formation of new branches from pre-existing vessels, resulting in an
increase in capillary density. There is a corresponding increase in end-diastolic blood flow velocity, which is likely due to decreased fetoplacental vascular impedance and increase fetal blood pressure (Hendricks et al. 1989). As metabolic demands of the fetus increase, there is an increase in placental growth and blood flow (Ahmed & Perkins 2000). Non-branching angiogenesis is the final phase of vascular development and this period begins at the start of the third trimester (~24–26 weeks gestation). There is longitudinal growth, looping, and coiling of the arteries, resulting in the formation of terminal villi (Kingdom et al. 2000). The exponential formation of terminal villi dramatically increases the surface area/volume ratio, and this is the primary site of gas and nutrient exchange.

**Figure 1.1. Trophoblast differentiation.** Reprinted from Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res. 2004; 114(5-6): 397-407. Copyright 2004 with permission from Elsevier.
1.1.2.2 Placental Anatomy

The placenta is the interface between the maternal and fetal circulations, and is responsible for nourishing and maintaining the developing fetus. The placenta is responsible for supplying the fetus with oxygen and nutrients, and clearing waste products from the fetal circulation. The functional unit of the placenta is a lobule, formally termed a cotyledon, and each cotyledon is perfused independently by maternal and fetal vessels. A typical human placenta is made up of 20–40 cotyledons (Syme et al. 2004).

The structure of a term human placenta is shown in Figure 1.2 (Serov et al. 2016). The placenta is connected to the fetus through an umbilical cord, which contains one umbilical vein and two umbilical arteries. The umbilical vein delivers nutrients and oxygen to the fetus, and the umbilical arteries carry deoxygenated blood from the fetus to the placenta. The umbilical arteries branch radially to form chorionic arteries, which end in capillary networks called villous trees (Syme et al. 2004). The intervillous space surrounds the fetal villous trees and contains a pool of maternal blood, which is supplied by maternal spiral arteries and carried away by uterine veins. Each cotyledon contains fetal villous trees suspended in maternal blood (Enders 1981), and this is the interface where maternal-fetal drug transfer can occur.

Each fetal villous tree is composed of fetal endothelial cells, villous stroma, and a trophoblast layer. The human placenta is haemochorial, where maternal blood is in direct contact with the trophoblast. The rate-limiting barrier to drug transfer across the placenta is a layer of polarized, multinucleated cells called syncytiotrophoblasts (Audus 1999; Cekovat-Novotna et al. 2006). Throughout pregnancy, the thickness of this trophoblast layer decreases, and the surface area for drug transfer increases to 12–14 m² (Enders 1981). Therefore, placental drug transfer at term may represent the highest fetal drug exposure compared to earlier gestational ages (van der Aa et al. 1998; Vahakangas & Myllynen 2006).
1.1.2.3 Factors Affecting Placental Drug Transfer

The rate and extent of drug transfer across the placenta depends on the physicochemical characteristics of the drug, including lipid solubility, molecular size, and degree of ionization. Protein binding in maternal and fetal plasma can restrict placental drug transfer, as only the unbound form of a drug can cross the placenta (Hill & Abramson 1988). Drug transfer is also related to the gestational age of the placenta, as the thickness of the trophoblast layer decreases
as the pregnancy progresses (Enders 1981). In addition, the expression of placental drug transporters and metabolic enzymes changes throughout pregnancy, and this can impact the amount of drug available to cross the placenta and reach the fetal circulation (Hutson et al. 2010; Iqbal et al. 2012).

Endogenous and xenobiotic compounds that are lipophilic and un-ionized, with a molecular weight less than 600 Da, can readily cross the placenta via passive diffusion (Syme et al. 2004). As a result, most small-molecule drugs tend to cross the placenta via diffusion, which is driven by the concentration gradient between maternal and fetal circulations (Audus 1999; Ala-Kokko et al. 2000). In general, molecules that are relatively lipophilic can dissolve in lipid membranes, and diffuse across the syncytiotrophoblast layer more readily than hydrophilic molecules. Because of this, molecular size does not heavily influence the diffusion of lipophilic drugs across the placenta, and their transfer is likely dependent on utero-placental blood flow (Giroux et al. 1997; Syme et al. 2004). Blood flow to the placenta increases during pregnancy from ~50 mL/min at 10 weeks to 600 mL/min at term. Molecular size can influence the placental transfer of hydrophilic drugs, as the rate of diffusion tends to decrease with increasing molecular size (Syme et al. 2004).

The extent of drug ionization in the maternal plasma is an important factor in the context of placental drug transfer, as only the un-ionized form of a drug can cross the placenta via diffusion (Syme et al. 2004). The log dissociation constant (pKa) of a drug can provide information about the degree of ionization in maternal and fetal plasma. The fetal circulation is slightly more acidic than the maternal circulation (7.35 vs 7.4, respectively), and this can affect the placental transfer for weak acid or basic drugs whose pKa is close to physiological pH (Reynolds & Knott 1989). For example, weak bases can become more ionized in the fetal plasma, resulting in ion trapping.

**Protein Binding**

Another important consideration in the disposition of drugs across the placenta is protein binding, as only the unbound form of the drug can equilibrate across the placenta. Two important binding proteins that bind a wide variety of drugs are albumin and α-1 acid glycoprotein (AAG). In general, albumin binds acidic, lipophilic drugs (Kragh-Hansen 1981), while AAG binds basic, lipophilic drugs (Piafsky & Woolner 1982; Paxton 1983). However, it should be noted that a specific drug may partially bind to albumin, AAG or other plasma proteins. Several factors can
influence the protein binding of a drug in the maternal and fetal plasma. These factors include the concentrations of plasma proteins in maternal and fetal plasma, the presence of competing endogenous or xenobiotic ligands, and saturation of drug-protein binding (Hill & Abramson 1988).

The concentrations of albumin and AAG differ in fetal and maternal plasma, and this can affect the transplacental transfer of drugs that bind these proteins. Throughout pregnancy, maternal albumin levels gradually decrease and fetal albumin levels tend to increase, with the fetal-to-maternal (F:M) albumin ratio increasing from 0.28 in the first trimester to 1.20 closer to term (Reboud et al. 1963; Krauer et al. 1984). As well, there is a 3-fold increase in the concentration of free fatty acids in the maternal circulation that can displace drugs from bound maternal albumin (Ridd et al. 1983; Nau et al. 1984). This can lead to increased binding of a drug to fetal albumin, which can, in turn, lead to increased placental drug transfer and possibly fetal drug accumulation. By comparison, AAG levels remain relatively constant in maternal plasma, and fetal levels gradually increase throughout pregnancy, with the AAG F:M ratio increasing from 0.09 in the first trimester to 0.37 at term (Krauer et al. 1984).

**Active Transport**

The placenta expresses numerous drug transport proteins that play an important role in limiting fetal drug exposure. Unlike diffusion, active transport requires energy, usually in the form of adenosine triphosphate (ATP) or via an electrical gradient generated by $[\text{H}^+]$, $[\text{Na}^+]$ or $[\text{Cl}^-]$ ions. Active transport in the placenta is mediated by transport proteins located on the brush-border apical and basolateral membranes of syncytiotrophoblast cells (Syme et al. 2004). Since active transport typically occurs against a concentration gradient, these transporters can concentrate nutrients in the fetal circulation (via influx) or prevent transfer of certain maternal drugs (via efflux).

The placenta expresses several ATP-binding cassette (ABC) drug transport proteins, including P-glycoprotein (P-gp/ABCB1), breast cancer resistance protein (BCRP/ABCG2), and multi-drug resistance associated proteins (MRPs) (MacFarland et al. 1994; St-Pierre et al. 2000; Nagashige et al. 2003; Mathias et al. 2005; Meyer Zu Schwabedissen et al. 2005; Sun et al. 2006; Yeboah et al. 2006). These transporters can facilitate drug uptake or efflux depending on where they are expressed (Figure 1.3). P-gp and BCRP are expressed on the apical microvillous (maternal
blood-facing) membrane of the syncytiotrophoblast (Ceckova-Novotna et al. 2006; Hahnova-Cygalova et al. 2011), where they limit drug transfer across the placenta to the fetal circulation and play a crucial role in fetal protection against maternal toxins. In addition to xenobiotics, placental transporters have endogenous substrates such as amino acids, hormones, and vitamins (Ganapathy et al. 2000).

Pregnancy is a dynamic state in which the expression and activity of drug transporters changes as the pregnancy progresses. ABCB1 mRNA and P-gp protein are highly expressed in syncytiotrophoblasts of first trimester human placentas, but expression levels gradually decrease as the pregnancy progresses to term (Gil et al. 2005; Mathias et al. 2005; Sun et al. 2006). By comparison, studies have reported inconsistent findings on age-dependent changes in expression of human placental ABCG2 mRNA and BCRP protein. The levels of ABCG2 mRNA remain relatively stable in the human placenta from the first to third trimester (Mathias et al. 2005; Yeboah et al. 2006), and one study showed BCRP was more strongly expressed in term placentae than in the first trimester (Yeboah et al. 2006). Other studies have reported that mRNA levels for ABCG2 (BCRP) were much higher than those of ABCB1 (P-gp) in isolated primary term trophoblast cells (Serrano et al. 2007; Manceau et al. 2012).

### 1.1.3 Methods for Studying Transplacental Drug Transfer

The placenta plays a major role in determining fetal drug exposure, and the extent of exposure can be estimated by quantifying the amount of drug that crosses the placenta. Transplacental pharmacokinetic data can be useful in assessing fetal safety, but this data is usually very limited for new drugs. Due to ethical constraints, it is not possible to conduct controlled trials for new medications in pregnant women. For some medications, drug concentrations can be measured in cord blood from neonates exposed during pregnancy, but this requires mothers to receive the drug prior to delivery. Cord blood levels provide information for a single time-point, and are subject to high inter-individual variability. As well, placenta transfer in animal studies cannot always be extrapolated to humans because of species differences in the anatomical and biochemical structure of the placenta (Ala-Kokko et al. 2000). *In vitro* cell cultures and membrane vesicles are useful for studying mechanisms of placental transfer (passive diffusion vs. active transport), but this may not reflect the true *in vivo* environment as these models lack anatomic integrity and blood flow. The *ex vivo* placenta perfusion model resolves many of the issues associated with these other models. Table 1.1 outlines some of the key advantages and disadvantages of different methods used to study placental drug transfer.
Table 1.1. Advantages and disadvantages of various methods used to study placental drug transfer. Adapted from Giaginis et al (2011) and Hutson et al. (2011).

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vivo</em> human studies</td>
<td>• True <em>in vivo</em> measure in humans (accounts for whole-body pharmacokinetics in mother and fetus)</td>
<td>• Sampling is restricted until after delivery</td>
</tr>
<tr>
<td>(cord blood sampling at delivery)</td>
<td></td>
<td>• High inter-individual variability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cannot provide information regarding drug distribution in mother, fetus or placenta</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cannot study new drugs or toxic substances</td>
</tr>
<tr>
<td>Animal modes</td>
<td>• Studies can be performed throughout gestation</td>
<td>• Difficult to extrapolate to humans because of species-differences in placental structure</td>
</tr>
<tr>
<td></td>
<td>• Can measure drug distribution and accumulation in maternal and fetal tissues</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Low inter-individual variability (animals from same litter)</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> models</td>
<td>• Useful for studying transfer mechanisms (drug uptake, efflux, metabolism) throughout gestation</td>
<td>• Expression and activity of transporters and/or enzymes can vary between cultures</td>
</tr>
<tr>
<td>(membrane vesicles, primary cultures and trophoblast cell lines)</td>
<td>• Certain cell lines can form cellular monolayers (ie. BeWo cell line)</td>
<td>• Regulatory mechanisms may not be present in the preparation</td>
</tr>
<tr>
<td>Ex vivo placenta perfusion</td>
<td>• Closely resembles <em>in vivo</em> environment, as structural integrity and cell-cell organization are maintained</td>
<td>• Typically performed in term tissue, and cannot be used to study placental transfer in 1st/2nd trimester</td>
</tr>
<tr>
<td></td>
<td>• Can measure placental drug transfer over time</td>
<td>• Tedious procedure, time-consuming, high costs</td>
</tr>
<tr>
<td></td>
<td>• Samples can be collected from maternal, fetal, and placental compartments</td>
<td>• Low overall success rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• No standardization between perfusion laboratories (difficult to compare results)</td>
</tr>
</tbody>
</table>
**Ex vivo placenta perfusion**

The *ex vivo* dual perfusion of a single placental cotyledon is the only experimental model to study placental transfer of substances in organized human placental tissue, and may be able to predict fetal drug exposure more reliably than other experimental models (Hutson et al. 2011). A schematic representation of the placental perfusion model is shown in Figure 1.4. The placenta can be ethically studied outside of the human body, as tissue collection is non-invasive and causes no harm to the mother and newborn (Myren et al. 2007), and the placenta would otherwise be discarded as medical waste. The *ex vivo* placental perfusion model was originally described by Panigel *et al* (Panigel et al. 1967), and later modified to the form that is currently used in our laboratory (Schneider et al. 1972; Miller et al. 1985). The model has previously been used to study placental transfer of both endogenous and exogenous substrates, including amino acids, hormones, viruses, therapeutic agents, and drugs of abuse (Omarini et al. 1992; Muhlemann et al. 1995; Ala-Kokko et al. 2000; Malek et al. 2009; Hutson et al. 2011).

Following delivery, term placentae are collected and immediately transported to our on-site laboratory at St. Michael’s Hospital in Toronto, Ontario. A fetal artery-vein pair supplying a clearly defined cotyledon is identified and the corresponding maternal surface is checked for any signs of physical trauma. The fetal-artery vein pair is cannulated and the fetal circulation is established. The lobule is then clamped into a plexiglass chamber with the fetal side down and the excess placental tissue is removed. The perfusion chamber is placed in a water bath to maintain physiological temperature (37°C). The maternal circulation is established through the insertion of blunt-tipped needles into the intervillous space, roughly 2-3 mm below the decidual surface. Maternal venous samples are collected from multiple venous openings in the decidual plate.

The fetal and maternal flow rates are maintained at 2-3 mL/min and 12-15 mL/min, respectively, through independently-controlled roller pumps. Other perfusion laboratories use different flow rates (Mathiesen et al. 2010), and this can affect the time needed to measure transplacental transfer and reach steady state between the fetal and maternal circulations (Bassily et al. 1995; He et al. 2001). The circulations are equilibrated with 95%-O₂/5%-CO₂ (maternal) and 95%-N₂/5%-CO₂ (fetal) to mimic physiological conditions. Other perfusion laboratories use room air instead of 95% O₂/5% CO₂ to avoid hyperoxic toxicity (Mathiesen et al. 2010).

Once the maternal and fetal circulations are established, there is a control period where the blood is washed out and baseline parameters of placental viability are measured. After the control period, the drug of interest is added to maternal or fetal circulations to measure placental transfer in the maternal-to-fetal or fetal-to-maternal direction. The drug can also be added in equal concentrations to both circulations in order to measure drug accumulation. Closed (recirculating) perfusions can be used to evaluate drug transfer and maternal-placental-fetal drug distribution. Open (single-pass, non-recirculating) perfusions can be used to study drug clearance at steady state concentrations.

Several quality control parameters are monitored to ensure placental viability and integrity during the control and experimental phase of the placenta perfusion. These include arterial inflow pressure in the fetal circulation, pH, oxygen consumption, fetal oxygen transfer, glucose consumption, lactate production, and secretion of human chorionic gonadotropin (hCG) (Hutson et al. 2011). Antipyrine is typically added to the maternal circulation as a marker of flow-
mediated passive diffusion. Antipyrine can be used to standardize results between experiments and account for differences in flow rates and cotyledon size. The loss in volume (leak) from the fetal reservoir is considered to be the optimal measure of tissue viability and integrity. In general, a loss of 2–4 mL/hour from the fetal reservoir is accepted (Mathiesen et al. 2010).

The placenta perfusion model can be used to quantify kinetics and transfer from the maternal to fetal circulation by using the fetal-to-maternal drug concentration ratio (F:M). This parameter can be calculated at various time points to provide information about maternal-fetal drug distribution. The F:M ratio can also be used to identify when the experiment has reached steady state conditions, as depicted by a plateau on a F:M ratio-vs-time graph. In this context, steady state occurs where there is no net transfer from maternal-to-fetal and fetal-to-maternal circulations. The steady-state F:M ratio can be also used to categorize the extent of placental drug transfer: limited transfer (<0.1), transfer (0.1–1.0), or accumulation in the fetal compartment (>1.0) (Hutson et al. 2011).

A major limitation of the placenta perfusion model is that it cannot fully incorporate whole-body pharmacokinetic factors, including protein binding and drug elimination. Protein binding is especially important, as only the unbound form of a drug can cross the placenta. Other groups have added plasma proteins to the perfusion medium (Johnson et al. 1999; Schenker et al. 1999; Gavard et al. 2006; Hemauer et al. 2010), but it can be difficult to mimic exact physiological conditions as there are several proteins and endogenous factors that may influence binding in vivo. This may lead to discrepancies between perfusion data and in vivo samples collected at delivery.

In order to compensate for this, Garland (1998) developed an equation, later adapted by Hutson et al (2011), to predict the in vivo F:M ratio of total (bound plus unbound) drug concentrations at steady state. As seen in the following equation, the perfusion F:M ratio can be adjusted to account for protein binding and drug ionization in the maternal and fetal circulations,

\[
F: M = \frac{\% \text{ unbound}_M}{\% \text{ unbound}_F} \times \frac{1 + 10^{pK_a-pH_F}}{1 + 10^{pK_a-pH_M}} \times \frac{CL_{MF}}{CL_{FM} + CL_F}
\]
where % unbound\textsubscript{M} and % unbound\textsubscript{F} are the percentages of free drug in the maternal and fetal circulations \textit{in vivo}; pH\textsubscript{M} and pH\textsubscript{F} are the pH values of maternal and fetal blood \textit{in vivo}; pKa is the log dissociation constant for the drug; and CL\textsubscript{MF}, CL\textsubscript{FM}, and CL\textsubscript{F} are the maternal-to-fetal, fetal-to-maternal, and fetal clearances of the drug (Garland 1998). CL\textsubscript{F} is assumed to be negligible, and the perfusion F:M ratio at steady-state can be used to represent CL\textsubscript{MF}/CL\textsubscript{FM} (Hutson et al. 2011).

A systematic review of the placenta perfusion model evaluated its effectiveness in predicting placental drug transfer \textit{in vivo} (Hutson et al. 2011). In that study, fetal-to-maternal concentration ratios from perfusion experiments were compared with cord-to-maternal blood ratios collected at the time of delivery for 70 drugs. In general, the results from placenta perfusion experiments correlated well with the data \textit{in vivo}. After adjusting the perfusion fetal-to-maternal ratio of 24 drugs for protein binding and pH differences between the fetal and maternal circulation, there was a stronger correlation between the \textit{in vivo} results of cord-to-maternal drug concentration ratio and the perfusion experiments’ derived fetal-to-maternal ratio. The authors concluded that the perfusion model can be used to accurately predict placental transfer of small molecule drugs \textit{in vivo} (Hutson et al. 2011). In instances where information regarding placental transfer may not be available, the placenta perfusion model is a safe and ethical tool to predict placental transfer \textit{in vivo}.

1.2 Anticoagulation Therapy in Pregnancy

Coagulation is the process by which blood forms a clot and is an important part of hemostasis at a site of vessel injury. When a blood vessel is damaged, a platelet- and fibrin-containing clot is formed at the site of injury to stop the bleeding and allow for vessel repair to begin. This important role of blood coagulation necessitates that the response be quick, localized, and carefully regulated. Blood coagulation is initiated almost immediately after injury to the endothelial lining of a blood vessel. Proteins, such as tissue factor, are activated to initiate the aggregation of blood platelets to form a plug at the site of injury. The clotting process is further propagated by other coagulation factors in the plasma that respond in a complex cascade to form stable fibrin clots. These additional fibrin clots serve to strengthen the platelet plug. The clotting process is then terminated by antithrombotic control mechanisms, and as the injured tissue heals, the blood clot is subsequently removed by fibrinolysis (Furie & Furie 2005). The pathways of
fibrin clot formation and plasmin-induced fibrinolysis are linked and carefully regulated by each other (Lane et al. 2005). Blood coagulation disorders can occur when specific elements of the coagulation cascade are missing or dysfunctional, and can lead to increased risk of bleeding or thrombosis.

1.2.1 Overview of the Coagulation Cascade

The coagulation cascade is made up of two initial pathways: (1) the contact activation pathway, known as the intrinsic pathway, and (2) the tissue factor pathway, known as the extrinsic pathway (Figure 1.5) (Knesek et al. 2012). Activation of both pathways will eventually lead to the formation of a fibrin clot. In recent years, there has been a shift in understanding of the coagulation cascade, moving away from separate intrinsic and extrinsic pathways toward a more all-encompassing model (Hoffman & Monroe 2007). The coagulation cascade involves a series of stepwise activations of zymogens to active enzymes, resulting in significant response amplification and the formation of a fibrin clot. Coagulation factors are serine proteases, with the exception of Factor VIII and FV, which are glycoproteins, and factor XIII, which is a transglutaminase. The process of coagulation can further be divided into 3 phases: initiation, amplification, and propagation (Adams & Bird 2009).

The initiation phase begins with exposure of tissue factor (TF) to blood at a site of endothelial damage (Greer et al. 2004). TF is a transmembrane protein, and forms a catalytic complex with Factor VII. Once bound, Factor VII is rapidly activated to Factor VIIa. The TF:FVIIa complex activates Factor IX and Factor X (Osterud & Rapaport 1977; Mann et al. 2006). Factor X is subsequently activated to Factor Xa, and can then interact with its cofactor (Factor Va) to form prothrombinase complexes. These prothrombinase complexes can catalyze the generation of a small amount of thrombin (Butenas & Mann 2002). The Factor IX which is activated by the TF:FVIIa complex does not play an important role in the initiation phase of coagulation. The duration of the initiation phase is dependent on concentrations of the TF:FVIIa complex and tissue factor pathway inhibitor (TFPI) (Butenas & Mann 2002). TFPI neutralizes Factor Xa and the TF:FVIIa complex.

In the amplification phase, Factor IXa forms a complex with Factor VIIIa in the presence of calcium (Mann et al. 2006). The FIXa:FVIIIa complex plays an important role in amplifying the signal of the coagulation cascade, as this complex substantially increases Factor Xa production
(at a rate 50–100 times greater than the TF:FVIIa complex) (Butenas & Mann 2002; Mann et al. 2006). The increased levels of Factor Xa drive the accelerated production of thrombin. In the presence of calcium, the FIXa:FVIIIa and prothrombinase (FXa:FVa) complexes create a positive feedback loop, resulting in further thrombin generation (Mann et al. 2003). Thrombin enhances platelet aggregation, and activates FVIIa, by dissociating FVIII from its complex with von Willebrand factor (FVIII:vWF). Thrombin also catalyzes the conversion of Factor XI to Factor XIa (Monroe & Hoffman 2006; Adams & Bird 2009). These additional positive feedback loops result in increased amounts of thrombin generation in a short amount of time.

During the propagation phase, activated platelets aggregate at the site of injury to ensure localized thrombin generation. The increased thrombin production results in a ‘thrombin burst’ that leads to the conversion of fibrinogen to fibrin to produce a stable clot. Fibrin monomers coalesce into a fibrin polymer gel, and factor XIIIa covalently cross-links fibrin strands to form a stable fibrin network (Greer et al. 2004). The newly formed thrombin also activates thrombin-activatable fibrinolysis inhibitor (TAFI), which prevents plasmin-mediated fibrinolysis (Bouma & Mosnier 2003).

Fibrinolysis, or the breakdown of a clot, is an essential component in maintaining hemostatic equilibrium. Plasmin is an important mediator of fibrinolysis, and it acts by cleaving fibrin at specific lysine and arginine residues into soluble degradation products (Greer et al. 2004). Plasmin is produced from its precursor, plasminogen, via tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Adams & Bird 2009). These plasminogen activators are regulated by plasminogen activator inhibitors (PAIs), which are present at high levels in the blood. PAIs form inactivating complexes with tPA and uPA to limit plasmin generation (Sprengers & Kluft 1987).

The coagulation cascade is regulated at each level, and this is important in maintaining hemostasis. Tissue factor is regulated by tissue factor pathway inhibitor (TFPI), which neutralizes the activity of Factor Xa and inhibits the TF:FVIIa complex (Broze 1995; Dahlback 2000). Antithrombin is a serine protease inhibitor, and plays an important role in preventing coagulation by preferentially inhibiting free thrombin and Factor Xa. The anticoagulant actions of antithrombin can be enhanced by the administration of heparin (Dahlback 2000; Adams & Bird 2009). Protein C is activated when it binds to thrombomodulin in the presence of thrombin.
Activated protein C, along with its co-factor protein S, inactivates Factor Va and Factor VIIIa (Adams & Bird 2009). Protein Z inhibits Factor Xa via protein Z-dependent protease inhibitor (ZPI) (Corral et al. 2007). Vitamin K is an essential co-factor for Factors II, VII, IX and X, as well as Protein S, Protein C and Protein Z.

Figure 1.5. The coagulation cascade. Reprinted from Knesek D, Peterson TC, Markel DC. Thromboembolic prophylaxis in total joint arthroplasty. Thrombosis. 2012; 2012: 837896. Copyright © 2012 David Knesek et al (permission not required).

1.2.2 Maternal and Fetal Coagulation Systems

Pregnancy is classically a state of hypercoagulability, characterized by an increased level of many clotting factors, a decrease in the amount of natural anticoagulants, and a reduction in fibrinolytic activity in the blood (Dahlman et al. 1985; Bremme 2003; O'Riordan & Higgins 2003; Townsley 2013). These hemostatic changes become more pronounced as the pregnancy progresses (Bremme 2003) and are likely caused by natural hormonal changes that are associated with pregnancy (Sattar et al. 1999). Platelet counts decrease in healthy pregnancies, with the
maximal decrease occurring in the third trimester (Verdy et al. 1997; Boehlen et al. 2000). Levels of factors VIII, X, XII, von Willebrand factor, and fibrinogen increase by 20-200% during pregnancy (Stirling et al. 1984; Esmon 1993; Bremme 2003; Hellgren 2003; O'Riordan & Higgins 2003). Factor VII substantially increases throughout pregnancy, with levels up to 1000% at term (Dalaker & Prydz 1984; Bremme 2003). Factor XIII, which stabilizes fibrin, increases in the first trimester but reduces to 50% of non-pregnant levels at term (Persson et al. 1980). Factor II (prothrombin) and V levels are slightly changed early in pregnancy, but return to non-pregnant levels at term (Bremme 2003).

In terms of antithrombotic proteins, antithrombin and protein C levels remain unchanged during pregnancy, while protein S decreases (Kjellberg et al. 1999; Bremme 2003). Fibrinolytic activity is reduced as the levels of fibrinolytic inhibitors, thrombin-activatable fibrinolysis inhibitor (TAFI), plasminogen activator inhibitor-1 (PAI-1), and PAI-2 are significantly increased throughout pregnancy (Ku et al. 2003). The hypercoagulable state in pregnancy confers a survival advantage by minimizing blood loss after delivery, but it also predisposes pregnant women to higher risks for thromboembolism (Hellgren 1996; Thornton & Douglas 2010).

Maternal coagulation factors cannot cross the placenta to the fetal circulation (Cade et al. 1969). The fetal coagulation system is dynamic throughout gestation, and gradually evolves toward the neonatal and adult state. The synthesis of coagulation factors, such as fibrinogen, begins as early as 5.5 weeks, and fetal blood is able to form a clot at 11 weeks gestation (Zilliacus et al. 1966; Gitlin & Biasucci 1969). One study measured the concentrations of coagulation factors in healthy fetuses, ranging from 19 to 38 weeks gestation, and found that levels of coagulation factors typically increase with advancing gestational age. The levels of antithrombin, protein C, and protein S were also much lower, suggesting that fetal hemostasis maintains equilibrium between activators and inhibitors of coagulation (Reverdiau-Moalic et al. 1996). Compared to preterm and term neonates, levels of fetal plasma coagulation factors are generally much lower, and only factors V and VIII reach adult values at birth (Andrew et al. 1987, 1988, 1990; Reverdiau-Moalic et al. 1996). The neonatal coagulation system continues to develop through childhood, and matures to the adult form by late adolescence (Andrew et al. 1992).
During pregnancy, women are at an increased risk of arterial and venous thromboembolism. The risk for arterial embolism, which can result in stroke and heart attack, is 3-4-fold higher in pregnant women, compared to non-pregnant women of childbearing age (James et al. 2005, 2006a). The risk for developing venous thromboembolism (VTE) during pregnancy is increased by 4–5-fold, and increases by up to 20-fold in the postpartum period (Heit et al. 2005; Pomp et al. 2008). The increased risk for VTE is likely due to a decrease in venous capacitance and outflow, mechanical obstruction by the uterus, and possibly by decreased mobility (Macklon et al. 1997; Danilenko-Dixon et al. 2001; James 2009).

VTE, which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is one of the most common causes of maternal mortality in Canada and the United States (Chang et al. 2003; Lisonkova et al. 2011). A retrospective study found that VTE affected 28 per 100,000 pregnancies antepartum, and 65 per 100,000 postpartum (Simpson et al. 2001); another study reported that approximately 80% of VTE events in pregnancy are DVT, and 20% are PE (Heit et al. 2005; James et al. 2006b). Approximately 33% of pregnancy-related DVT events and 50% of PE events occurred in the postpartum period (Gherman et al. 1999; Ray & Chan 1999; Simpson et al. 2001; James et al. 2005).

Two important risk factors for the development of VTE include a history of thrombosis, and inherited or acquired thrombophilias. Additional risk factors include varicose veins, inflammatory bowel disease, urinary tract infection, diabetes, body mass index $\geq 30$ kg/m$^2$, increased maternal age ($\geq 35$ years), smoking, stillbirth, obstetric hemorrhage, preterm delivery, and caesarean section (Ginsberg & Hirsh 1998; Abdul Sultan et al. 2013; Sultan et al. 2013).

### 1.2.3 Current Therapies

As with many drugs prescribed during pregnancy, the use of anticoagulant therapy can be challenging because of the possibility for both maternal and fetal complications. Anticoagulant therapy may be required during pregnancy in women at high risk for venous thromboembolism, and in women with prosthetic or mechanical heart valves, atrial fibrillation, cerebral venous sinus thrombosis, and left ventricular dysfunction. In some cases, anticoagulants are used in
combination with aspirin for the prevention of recurrent pregnancy loss in women with antiphospholipid antibody syndrome (APS) (Bates et al. 2012).

Low molecular weight heparin (LMWH) and unfractionated heparin (UFH) are the preferred choices of anticoagulants for the prevention and treatment of thromboembolism in pregnant women, as they do not cross the placenta and are not associated with teratogenic effects (Forestier et al. 1984, 1987; Bates 2002; Greer & Nelson-Piercy 2005; Bates et al. 2012). LMWH and UFH bind antithrombin to induce a conformational change, which significantly accelerates antithrombin’s inactivation of factor Xa and thrombin, resulting in slower coagulation overall (Marcum et al. 1984; Klein et al. 2002). LMWHs are generally preferred over UFHs because LMWH has a higher bioavailability, longer plasma half-life, and more predictable pharmacokinetic response. In addition, LMWH has a lower risk of maternal heparin-induced thrombocytopenia (HIT) and heparin-associated osteoporosis compared to UFH (Bates et al. 2012). UFH is a less expensive alternative to LMWH, and may be preferred in later stages of pregnancy (closer to delivery) when rapid temporal control of anticoagulation is required (James 2011).

LMWHs and UFH are administered via subcutaneous injection, up to twice daily, and can result in pain at the site of injection. As a result of pregnancy-related physiological changes, both LMWH and UFH have shorter half-lives and lower peak plasma concentrations in pregnant women, compared to non-pregnant adults (Brancazio et al. 1995; Casele et al. 1999; Barbour et al. 2004). These changes may require the use of higher doses and more frequent drug administration.

Danaparoid sodium is a low molecular weight heparinoid, which may be used in pregnant women who develop HIT and require ongoing anticoagulant therapy (Bates et al. 2012). Danaparoid sodium is an effective antithrombotic agent that inhibits Factor Xa (de Valk et al. 1995). Animal studies and human case reports have shown that danaparoid sodium does not cross the placenta (Peeters et al. 1986; Henny et al. 1986; Greinacher et al. 1993; Lindhoff-Last et al. 2005), and it has a low cross-reactivity with UFH (Magnani 1993). Fetal toxicity has not been demonstrated with maternal use of danaparoid, but the quality of this evidence is low (Bates et al. 2012). Due to shortages, danaparoid was withdrawn from the US market in 2002, but it is still available in Canada, Australia, Japan and European countries.
Fondaparinux is a synthetic polysaccharide, which acts by inhibiting Factor Xa. Its structure is based on the active moiety of heparin. Using a dually perfused human cotyledon, fondaparinux did not cross the placenta from maternal to fetal circulation (Lagrange et al. 2002). Approximately 10% of maternal anti-Xa activity was detected in the umbilical cord plasma of five neonates whose mothers were treated with fondaparinux (Dempfle 2004). While there are reports of the use of fondaparinux during pregnancy (Parody et al. 2003; Mazzolai et al. 2006; Wijesiriwardana et al. 2006; Gerhardt et al. 2007), the information still remains very limited. The American College of Chest Physicians recommends limiting the use of fondaparinux during pregnancy to women with severe reactions to heparin who cannot receive danaparoid (Bates et al. 2012). Argatroban is a parenteral direct thrombin inhibitor, which requires continuous intravenous administration. Argatroban may be used in individuals with severe reactions to heparins who cannot receive danaparoid or fondaparinux (Bates et al. 2012). To date, only a few cases have described the use of argatroban in late pregnancy (Taniguchi et al. 2008; Young et al. 2008; Ekbatani et al. 2010).

The classical anticoagulant, warfarin, is contraindicated during pregnancy because of its associated embryopathy, characterized by nasal and limb hypoplasia, as well as congenital cardiac anomalies (McLintock 2013). The use of warfarin in pregnancy was also associated with early miscarriage, but studies could not determine if miscarriages were due to the use of warfarin itself or the underlying conditions for which warfarin was administered (Schaefer et al. 2006). Warfarin and other vitamin K antagonists freely cross the placenta, and have the potential to cause both fetal bleeding and teratogenic effects (Iturbe-Alessio et al. 1986; Ginsberg et al. 1989; Schaefer et al. 2006). Vitamin K antagonists inhibit the synthesis of vitamin K-dependent clotting factors, which include Factors II, VII, IX, and X, and the anticoagulant proteins C and S. These medications can cause fetal bleeding at any stage of pregnancy, but the risk for teratogenicity is highest with administration between 6 and 12 weeks gestation (Hall et al. 1980; Howie 1986; Iturbe-Alessio et al. 1986).

1.3 **Novel Oral Anticoagulants**

Recently, novel oral anticoagulants (NOACs) have been developed and approved for clinical use in non-pregnant adults. A major advantage is that these new medications can be prescribed at fixed doses without the need for routine monitoring or dose adjustment. Compared to vitamin K
antagonists, these novel anticoagulants have a faster onset of action, wider therapeutic window, and predictable anticoagulant effect. In general, there are no food interactions with these drugs, and only limited drug-drug interactions. Dabigatran (Pradaxa®), rivaroxaban (Xarelto®), and apixaban (Eliquis®) are three newer generation oral anticoagulants that act by directly inhibiting thrombin or factor Xa of the coagulation cascade. These novel oral anticoagulants are indicated for the prophylaxis and treatment of DVT and PE; and for the prevention of stroke and systemic embolism associated with nonvalvular atrial fibrillation. The chemical structures of these anticoagulants are shown in Figure 1.6 and their physicochemical and pharmacokinetic properties are summarized in Table 1.2.

Figure 1.6. Chemical structures of novel anticoagulants. Modified from Scaglione (2013).
Table 1.2. Physicochemical and pharmacokinetic properties of novel anticoagulants.
Adapted from Hellwig (2013) and Scaglione (2013).

<table>
<thead>
<tr>
<th></th>
<th>Dabigatran</th>
<th>Rivaroxaban</th>
<th>Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical dose (VTE prophylaxis)</td>
<td>110 mg twice daily</td>
<td>10 mg once daily</td>
<td>2.5 mg twice daily</td>
</tr>
<tr>
<td>Mechanism of action</td>
<td>Direct thrombin inhibitor</td>
<td>Direct factor Xa inhibitor</td>
<td>Direct factor Xa inhibitor</td>
</tr>
<tr>
<td>Size</td>
<td>628 Da (724 Da - prodrug)</td>
<td>436 Da</td>
<td>459 Da</td>
</tr>
<tr>
<td>LogP</td>
<td>3.8</td>
<td>1.5</td>
<td>1.83</td>
</tr>
<tr>
<td>Protein binding</td>
<td>34-35% bound (albumin)</td>
<td>90-95% bound (albumin)</td>
<td>87% bound (66% albumin, 9% AAG, 12% unknown)</td>
</tr>
<tr>
<td>Volume of distribution (Vd)</td>
<td>60–70 L</td>
<td>50 L</td>
<td>21 L</td>
</tr>
<tr>
<td>Primary mode of elimination</td>
<td>Renal</td>
<td>Renal</td>
<td>Fecal</td>
</tr>
<tr>
<td>Transporter interactions</td>
<td>P-gp (prodrug only)</td>
<td>P-gp, BCRP substrate</td>
<td>P-gp, BCRP substrate</td>
</tr>
</tbody>
</table>

AAG, α-1 acid glycoprotein; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein.

1.3.1 Dabigatran

Dabigatran acts as a direct, potent and competitive inhibitor of free and fibrin-bound thrombin (Huntington & Baglin 2003; van Ryn J 2008). Thrombin catalyzes the conversion of fibrinogen into fibrin during the coagulation cascade, and its inhibition prevents the development of thrombi. UFH and LMWH cannot inhibit fibrin-bound thrombin, and this has important implications because dabigatran can prevent fibrin-bound thrombin from continuing to trigger thrombus expansion, while UFH and LMWH cannot (Weitz et al. 1990; Maegdefessel et al. 2010).

Dabigatran is orally administered as the prodrug, dabigatran etexilate mesylate, which is rapidly converted to its active form by esterases in the plasma and liver (Stangier & Clemens 2009; Eisert et al. 2010). The absolute bioavailability following oral administration of dabigatran
etixilate mesylate is low at about 7% (Stangier & Clemens 2009), and maximal plasma concentrations \( (C_{\text{max}}) \) levels are reached within 0.5–2 hours. Dabigatran is partially bound to plasma proteins (35%) and its volume of distribution is 60–70 L, indicating moderate tissue distribution (Blech et al. 2008).

Dabigatran etixilate mesylate is not metabolized by CYP enzymes, and approximately 20% is conjugated with glucuronic acid and excreted via the biliary system (Ebner et al. 2010). The active drug, dabigatran, is the major circulating compound, and it is primarily excreted unchanged through the kidneys (Stangier & Clemens 2009). The average half-life \( (t_{1/2}) \) of dabigatran ranges from 8 to 10 hours after a single dose, and increases to 14–17 hours after multiple doses (Stangier et al. 2007). In cases of renal impairment, there are elevated plasma concentrations and a prolonged half-life for dabigatran (Stangier et al. 2010). The prodrug, dabigatran etixilate mesylate, is a substrate for P-glycoprotein, and therefore, co-administration with P-gp inhibitors or inducers may affect plasma levels of dabigatran (Eisert et al. 2010).

To date, there are no clinical trials evaluating the safety and efficacy of dabigatran in pregnancy in humans. Animal studies have investigated the potential teratogenic effects of dabigatran in rats and rabbits. There was an adverse effect on female fertility as noted by a decrease in implantation and an increase in pre-implantation loss at a dose of 70 mg/kg, which is 5-fold higher than the human dose. At higher doses (up to 10-fold higher than the human doses), there was a decrease in fetal body weight and viability in rats and rabbits. No major malformations were observed in fetal rabbits (Boehringer Ingelheim Canada 2016).

1.3.2 Rivaroxaban

Rivaroxaban is a direct, reversible, competitive inhibitor of factor Xa, which is an important component of both the intrinsic and extrinsic pathways in the coagulation cascade. As previously described, factor Xa directly converts prothrombin to thrombin via the prothrombinase complex, and its inhibition limits thrombin generation and the subsequent formation of a fibrin clot. Due to signal amplification in the coagulation cascade, one molecule of Factor Xa is able to generate more than 1,000 molecules of thrombin (Hoffman & Monroe 2007), making Factor Xa an ideal target for anticoagulation therapy (Spyropoulos 2007). Rivaroxaban can inhibit free Factor Xa, Factor Xa already bound in the prothrombinase complex, and clot-bound Factor Xa (Roehrig et al. 2005; Laux et al. 2007). As well, rivaroxaban has a high specificity for Factor Xa, as it does
not inhibit other serine proteases including thrombin, Factor VIIa, Factor IXa, Factor XIa, and activated protein C (Perzborn et al. 2005).

Rivaroxaban has a low water solubility and high membrane permeability, and it is rapidly absorbed following oral administration. It has a high oral bioavailability (80–100%) and $C_{\text{max}}$ levels are achieved 2–4 hours after administration (Kubitza et al. 2005b). There is very limited presystemic first-pass extraction, and rivaroxaban remains highly bound to plasma proteins (92–95%), with serum albumin as the predominant binding protein (Weinz et al. 2005). Rivaroxaban has an apparent $V_d$ of 50 L at steady state (Bayer Canada 2013).

Rivaroxaban is metabolized to inactive metabolites via CYP enzymes (primarily CYP 3A4 and CYP 2C8) (Lang et al. 2009). Approximately 66% of rivaroxaban is excreted via the kidneys, with the remaining 34% excreted in feces (Bayer Canada 2013). Rivaroxaban has a terminal half-life ($t_{1/2}$) of 5–9 hours in young individuals (Kubitza et al. 2005a, b), and this can increase up to 11–13 hours in the elderly as a result of age-related declines in renal function (Kubitza et al. 2008, 2010). Previous studies have shown that rivaroxaban is a substrate of the transporters P-gp and BCRP (Gnoth et al. 2011; Gong et al. 2013).

To date, there have been few reported cases of rivaroxaban exposure in human pregnancy. A case report described the use of rivaroxaban up to 19 weeks gestation. A male infant was born at 40 weeks gestation with no complications. The authors reported a small baby by weight, length, and cranial circumference ($13^{\text{th}}$, $30^{\text{th}}$, and $7^{\text{th}}$ percentiles, respectively), but attributed this to maternal smoking (Konigsbrugge et al. 2014). Recently, a case series was published including 37 women who were inadvertently exposed to rivaroxaban in the first and second trimesters. There was one case of a conotruncal cardiac defect in a woman with several co-medications and a previous fetus with a cardiac malformation in the absence of rivaroxaban exposure (Hoeltzenbein et al. 2016). These cases demonstrate the prospect that as prescriptions rates of novel oral anticoagulants (NOACs) continue to increase (Desai et al. 2014), the number of women with an unplanned pregnancy who are taking a NOAC will also likely increase.

Animal studies have examined the reproductive and developmental toxicity of rivaroxaban. Studies in rats and rabbits have shown reproductive toxicity related to the pharmacodynamic effect of rivaroxaban (ie. hemorrhagic complications). It should be noted that rivaroxaban was administered up to 38-fold (rat) and up to 89-fold (rabbit) higher doses than the typical
therapeutic exposure in humans. Even at the highest tested doses, there were no observed signs of teratogenicity. As well, rivaroxaban did not show any effect on female fertility at doses up to 200 mg/kg (Bayer Canada 2013).

1.3.3 Apixaban

Similar to rivaroxaban, apixaban is a reversible, direct inhibitor of factor Xa, and it can bind to free and prothrombinase-bound Factor Xa (Jiang et al. 2009). Apixaban is highly selective for factor Xa, and does not affect activated protein C, Factors IXa and VIIa, and thrombin (Pinto et al. 2007). Importantly, when compared to other NOACs (including dabigatran, rivaroxaban, and edoxaban), apixaban was associated with a relatively low risk of bleeding events while showing similar efficacy for the treatment of acute VTE (Mantha & Ansell 2015).

Following oral administration, apixaban is rapidly absorbed and \( C_{\text{max}} \) levels are achieved within 3–4 hours (Raghavan et al. 2009). Apixaban has an oral bioavailability of approximately 50% for doses up to 10 mg. Compared to dabigatran and rivaroxaban, apixaban has a lower volume of distribution at \( \sim 21 \text{ L} \), and remains highly bound to plasma proteins (87%) (Pfizer Canada 2015). The lower \( V_d \) is likely due to limited distribution in extravascular tissue, and not due to extensive plasma protein binding (He et al. 2011).

Apixaban is metabolized via CYP 3A4/5 with minor contributions from CYP 1A2 and 2J2 (Wang et al. 2010). The major circulating compound is unchanged apixaban and there are no active metabolites. Apixaban is eliminated via multiple routes, with \( \sim 50\% \) excreted unchanged in the feces and 25% excreted via the kidneys (Raghavan et al. 2009). Apixaban has an elimination half-life of approximately 8–9 hours (Frost et al. 2014). Using in vitro models, it has been shown that apixaban is a substrate for both P-gp and BCRP transporters (Zhang et al. 2013); plasma concentrations and the elimination half-life of apixaban may be altered if it is administered with inhibitors or inducers of these transporters.

Apixaban is a relatively new drug, and currently there are no clinical trials that have evaluated the safety and efficacy of apixaban in pregnancy in humans. Studies in mice, rats, and rabbits did not indicate direct or indirect effects of reproductive toxicity. There was an increased incidence of maternal bleeding when animals were administered levels of apixaban much higher than human therapeutic levels. There were no maternal or fetal deaths related to bleeding, and no
evidence of an increased risk of fetal malformations following *in utero* exposure to apixaban (Pfizer Canada 2015). A study in rats showed limited placental transfer of $[^{14}\text{C}]$-apixaban, with a peak fetal blood concentration that was approximately 35% of the maternal concentration (Wang et al. 2011).

### 1.4 Overall Rationale and Primary Objectives

Recent trends have shown that medication use in pregnancy has steadily increased in the last 30 years, and the decision to begin or continue treatment in pregnancy heavily relies on weighing the maternal benefits of the drug against the potential fetal risk. The use of newer medications in pregnancy is especially challenging, as there is typically no information about safety or efficacy in pregnancy. Clinical trials usually exclude pregnant women and ensure that women of childbearing potential do not become pregnant during the period of drug exposure. Several novel oral anticoagulants have been developed and approved for clinical use in recent years, and these medications have clear advantages over existing therapies. Dabigatran, rivaroxaban, and apixaban are three newer generation oral anticoagulants that can be used in the prevention and treatment of venous thromboembolism (VTE). However, information regarding fetal safety and placental transfer of these drugs is currently very limited. As prescription rates for novel oral anticoagulants continue to increase, the number of women with an unplanned pregnancy who are taking novel oral anticoagulants (NOACs) will likely increase.

*The main objective of this thesis was to investigate the transplacental kinetics of novel anticoagulants using the placenta perfusion model. By determining the rate and extent of placental drug transfer, we will be able to estimate fetal drug exposure. The proposed studies will focus on 3 anticoagulants (dabigatran, rivaroxaban, and apixaban), and placental drug transfer will be evaluated separately for each drug. The first objective of this thesis was to determine the rate and extent of the placental transfer of dabigatran. The second and third objectives were to examine the placental transfer of rivaroxaban and apixaban, respectively. Compared to rivaroxaban and apixaban, dabigatran exhibits more hydrogen bonding and has a higher polar surface area, which are characteristics that may reduce transport across the placental barrier. The large molecular weight and high lipophilicity of dabigatran may also limit its transfer. Based on its physicochemical properties, we hypothesize that dabigatran will have limited placental transfer compared with unbound rivaroxaban and apixaban.*
While the placenta perfusion model can be highly predictive of placental transfer \textit{in vivo}, a limitation of this model is that it cannot account for differences in protein binding between maternal and fetal plasma. A recent study has showed that placenta perfusion data can be mathematically adjusted in order to predict the fetal-to-maternal (F:M) ratio of total (bound plus unbound) drug concentration in plasma \textit{in vivo}. After adjusting for protein binding differences, the authors noted a stronger correlation between placental perfusion and \textit{in vivo} placental transfer (Hutson et al. 2011). Dabigatran exhibits low protein binding, while rivaroxaban and apixaban remain highly bound to plasma proteins \textit{in vivo}.

\textbf{The fourth objective of this study was to compare the placental transfer of three novel oral anticoagulants, after adjusting for differences in protein binding.} Since rivaroxaban and apixaban are highly bound to plasma proteins, we hypothesize that the F:M concentration ratios for these two medications will be altered after the adjustment. We also developed a pharmacokinetic model to estimate transplacental transfer parameters. We hypothesize that placenta perfusion data can be fitted to a pharmacokinetic model, and that this model will be able to predict transfer parameters.

The dual perfusion of a single placental cotyledon \textit{ex vivo} has been widely used among placental researchers to study transplacental drug transfer in organized human placental tissue. While this model can be highly predictive of \textit{in vivo} placental transfer, a major practical challenge of this model is the low overall success rate. This can result in high costs and longer time to complete a study examining placental drug transfer. Different perfusion laboratories report success rates between 5 and 20\%, and in general, 4-6 successful perfusions are ideally needed to establish transfer parameters. The method is technically challenging, and there are several criteria during the perfusion procedure that must be met in order to produce a successful experiment.

\textbf{The fifth objective of this thesis was to evaluate the success rate of the placenta perfusion model at several logistical stages throughout the experiment.} By examining the success rate at various checkpoints in the experiment, we will be able to identify stages in need of technical improvement. We hypothesize that establishing the fetal circulation will be an important stage in the perfusion protocol. We will also determine if the overall success rate of the placenta perfusion is similar between placentae obtained from vaginal and caesarean deliveries. We hypothesize that success rates will be similar between the two modes of delivery.
Chapter 2

Transfer of Dabigatran and Dabigatran Etexilate Mesylate across the Dually Perfused Human Placenta.

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\textbf{Contribution of this thesis author (P. Bapat):} I performed laboratory analysis of samples to measure drug concentrations and markers of placental viability with assistance from A. Lubetsky and K. Aleksa. I analyzed the data with assistance from J.N. Matlow and drafted the manuscript. All authors provided input and approved the final version of the manuscript.

\textbf{This work has been published:}

Transfer of dabigatran and dabigatran etexilate mesylate across the dually perfused human placenta.

2.1 Abstract

Objective: To assess the transplacental pharmacokinetics at term of the oral thrombin inhibitor, dabigatran, and its prodrug, dabigatran etexilate mesylate, to estimate fetal drug exposure.

Methods: Placentae were obtained with informed consent after cesarean delivery of healthy term pregnancies in Toronto, Ontario, Canada. The transplacental transfer of dabigatran and dabigatran etexilate mesylate was separately assessed using the ex vivo dual perfusion of an isolated human placental cotyledon. Dabigatran, at a concentration of 35 ng/mL, was added to the maternal circulation at the start of the experimental phase. Maternal and fetal samples were taken throughout the preexperimental (1 hour) and experimental (3 hours) phases for measurement of dabigatran and markers of placental viability. Separate placenta perfusions with dabigatran etexilate mesylate were conducted at an initial maternal concentration of 3.5 ng/mL. Dabigatran and dabigatran etexilate mesylate were measured using liquid chromatography-tandem mass spectrometry.

Results: There was slower transfer of dabigatran compared with antipyrine from the maternal-to-fetal circulation, and the median fetal-to-maternal concentration ratio of dabigatran was 0.33 (interquartile range 0.29-0.38) after 3 hours (n=3). The prodrug, dabigatran etexilate mesylate, had limited placental transfer as characterized by a fetal-to-maternal ratio of 0.17 (interquartile range 0.15-0.17) after 3 hours (n=3). Placental viability markers for all perfusions were within normal ranges.

Conclusion: This report provides direct evidence of the transfer of dabigatran and its prodrug across the term human placenta from the mother to the fetus. From a clinical perspective, these data suggest that, pending further study, dabigatran should not be used for anticoagulation of pregnant women, because the drug may have an adverse effect on fetal blood coagulation.
2.2 Introduction

Anticoagulant therapy often is prescribed in pregnancy for a variety of clinical indications. Common indications include: prophylaxis of venous thromboembolism in pregnancies at high or intermediate risk for thromboembolic disease, treatment of acute venous thromboembolism in pregnancy, management of pregnancy in women with artificial heart valves, and the prevention of pregnancy-related complications in women with inherited or acquired thrombophilias and hypercoagulable antibody syndrome (Royal College of Obstetricians and Gynaecologists 2009; Bates et al. 2012). Pregnancy itself is a hypercoagulable state (Dahlman et al. 1985; Bremme 2003; O’Riordan & Higgins 2003); thus, effective treatment for these women is critical (Fogerty & Connors 2009; James 2009; Che Yaakob et al. 2010; Greer 2012). The classic oral anticoagulant, warfarin, is contraindicated during pregnancy because it is associated with a severe embryopathy (Chan et al. 2000; Hassouna & Allam 2010; McLintock 2013). For this reason, unfractionated heparin or low-molecular-weight heparins are currently the drug of choice for most women requiring anticoagulation therapy during pregnancy (Bates et al. 2012). However, these are administered subcutaneously, resulting in patient discomfort, financial burden, and low adherence.

The need for effective and well-tolerated oral antithrombotic agents with more predictable pharmacokinetic profiles has led to the development of new agents that directly target thrombin (Huntington & Baglin 2003; Bauer 2011). Recently, the U.S. Food and Drug Administration approved the direct thrombin inhibitor, dabigatran etexilate mesylate, to reduce the risk of stroke and systemic embolism in patients with nonvalvular atrial fibrillation (Hankey & Eikelboom 2011). Dabigatran is orally administered as the prodrug, dabigatran etexilate mesylate, which is rapidly converted by a serum esterase (Stangier & Clemens 2009; Eisert et al. 2010). Unlike warfarin and heparin, direct thrombin inhibitors are able to inhibit both free and fibrin-bound thrombin (Huntington & Baglin 2003; van Ryn J 2008), potentially enabling more effective inhibition of coagulation (Weitz et al. 1990, 1998).

Although dabigatran offers a few of advantages over existing anticoagulants, there are currently no data regarding its placental transfer (Bates et al. 2012; Cutts et al. 2013). The aim of the present study was to assess the transplacental pharmacokinetics at term of dabigatran and its prodrug, dabigatran etexilate mesylate, by using the dual-perfusion human placental model.
2.3 Materials and Methods

The dual perfusion of a single placental cotyledon was previously described by Miller et al (1985) and adapted in our laboratory (Derewlany et al. 1991; Pollex et al. 2008). The present study was approved by the research ethics board at St Michael's Hospital in Toronto, Ontario, Canada. Placentae were obtained with informed consent after caesarean or vaginal delivery of uncomplicated term singleton pregnancies. We excluded placentae from women who were taking any medication during pregnancy or from women who had any disease during pregnancy including, but not limited to, hypertension, diabetes, thyroid dysfunction, cancer and infection (e.g., chorioamnionitis and TORCH infections [toxoplasmosis, other (syphilis, varicella-zoster, parvovirus B19), rubella, cytomegalovirus, herpes], or positive anti-HIV, anti-HBV, or anti-HCV serology). Immediately following delivery, the placentae were transported to the on-site laboratory in ice-cold heparinized phosphate-buffered saline. A fetal artery-vein pair supplying a well-defined cotyledon was identified, and the corresponding maternal surface of the cotyledon was examined to have an intact basal plate and no evident physical damage. If there were signs of physical damage, a different artery-vein pair and cotyledon were selected and examined. The ideal fetal-artery vein pair had minimal branching, and a cotyledon on the periphery of the placenta was preferred. The selected fetal artery-vein pair was cannulated, and independent maternal and fetal circulations were established within 30 minutes of delivery (Derewlany et al. 1991).

Perfusate consisted of 10.9 g/L M199 tissue culture medium containing 40,000 molecular-weight dextran (maternal 7.5 g/L; fetal 30.0 g/L), glucose (1.0 g/L), heparin (2,000 units/L), and kanamycin (100 mg/L). Antipyrine (1 mM) is added to the maternal perfusate as a flow-dependent marker of passive diffusion (Schneider et al. 1972). To mimic physiologic conditions in maternal and fetal blood, maternal and fetal perfusates were buffered to pH 7.4 and 7.35 (Reynolds & Knott 1989), respectively, by the addition of small volumes of sodium bicarbonate and hydrochloric acid (HCl).

A single-perfusion experiment consisted of a 1-hour preexperimental control phase followed by a 3-hour experimental phase. During both phases, flow rates were maintained at 2–3 and 13–14 mL/min in the fetal and maternal circuits, respectively. The maternal perfusate was equilibrated
with 95% O₂ and 5% CO₂ and the fetal with 95% N₂ and 5% CO₂, and the temperature of the circuits and perfusion chamber was kept at 37°C.

The fetal and maternal circulations were maintained until all residual blood was cleared out of the vessels. At this point, the maternal and fetal circuits were closed and replaced with 250 mL and 150 mL of fresh perfusate, respectively. To confirm tissue viability, maternal and fetal samples were taken every 15 minutes to analyze O₂ and CO₂ content, pH, glucose concentration, and lactate production using an on-site blood gas analyzer. Additional samples were taken from the maternal and fetal reservoirs every 15 minutes for analysis of human chorionic gonadotropin (hCG) secretion and antipyrine transfer. The integrity of the placenta was analyzed by monitoring fetal reservoir volume and fetal arterial inflow pressure. The perfusion was terminated if there was a loss in fetal reservoir volume greater than 4 mL/h or if fetal arterial inflow pressure deviated from 30 to 60 mmHg for an extended period of time. Before the experimental period began, the perfusates in the maternal and fetal reservoirs were replaced with fresh media, and the circulations were closed and recirculated.

In a closed-circuit experiment, dabigatran or dabigatran etexilate mesylate was added to the maternal circulation at a therapeutic concentration of 35 ng/mL or 3.5 ng/mL, respectively. Samples were taken from the maternal and fetal reservoirs for analysis of dabigatran transfer, O₂ and CO₂ content, pH, glucose consumption, and lactate production every 10 minutes for the first half hour and then every 30 minutes until the end of the 3-hour experimental period. Additional samples were taken directly from the maternal and fetal reservoirs every 30 minutes for analysis of hCG secretion and antipyrine transfer.

Perfusate samples were stored at -20°C until analysis. Antipyrine samples were assayed using an ultraviolet-visible recording spectrophotometer W-160 reading at 350 nm, and hCG samples were assayed using an enzyme-linked immunosorbent assay (ELISA) kit and a Biotek Synergy HT microplate reader at 450 nm.

Our method for extraction of dabigatran and dabigatran etexilate mesylate from perfusate was derived from Blech et al (Blech et al. 2008) and Delavenne et al (Delavenne et al. 2012). Briefly, 50 µL of perfusate sample was added into a 1.5-mL polypropylene tube along with 41.7 µL of 0.6 mg/mL of Dabigatran-d7 internal standard diluted in methanol:0.1 N HCl (90:10). A standard curve was also prepared at the following level: 0, 1, 5, 10, 25, and 50 ng by adding dabigatran
(0.7 mg/mL) and dabigatran etexilate mesylate (1 mg/mL) and 10 µL of the internal standard (0.6 mg/mL) with 900 µL methanol:0.1 N HCl (90:10). All samples were vortexed and centrifuged at 13,400×g for 5 minutes at room temperature. The supernatant was transferred to a 2-mL vial and 10 µL was injected on the ultrahigh-performance liquid chromatography (UHPLC).

The analysis of the samples was performed on an ABSciex QTRAP5500 and Shimadzu Nexera UHPLC system. Chromatography ran at a flow rate of 300 µL/min on a Kinetex XB-C18 column 50×2.1 mm, 2.6 micron with a gradient starting at 93% A (2 mM ammonium formate in ddH2O with 0.2% formic acid) and 7% B (2 mM ammonium formate in acetonitrile with 0.2% formic acid) ramping to 90% B at 0.6 minutes followed by isocratic elution at 90% B for 2.7 minutes. The total run time was 5 minutes, including reequilibration at the initial conditions. This method allowed for baseline chromatographic resolution of the dabigatran and dabigatran etexilate mesylate.

The mass spectrometer was operated in positive electrospray ionization mode with a source temperature of 500°C and an internal standard voltage setting of 5,000 V. Precursor-to-product ion mass transitions were established by standard infusions. Data were acquired in multiple reaction monitoring mode with mass transitions as follows: 472.1→289.2 m/z for dabigatran 1 (transition used for quantitation), 472.1→324.0 m/z for dabigatran 2, 472.1→306.1 m/z for dabigatran 3, and 478.2→292.0 m/z for dabigatran-d7 1 (retention time 0.47 minutes); and 628.2→289.1 m/z for dabigatran etexilate mesylate 1 (transition used for quantitation) and 628.2→434.2 m/z for dabigatran etexilate mesylate 2 (retention time 0.56 minutes). Data analysis and peak integration were performed using Analyst 1.6 software from ABSciex. Sample concentrations were calculated by plotting peak area ratios (analyte/internal standard) against calibration curves of extracted matrix spiked standards.

All data are presented as median and interquartile range (IQR) unless stated otherwise and comparisons between preexperimental and experimental phases were analyzed using a Wilcoxon sign rank test for nonparametric data.
2.4 Results

A total of six cotyledons from different placentae were perfused with dabigatran or dabigatran etexilate mesylate. The median weight of the six perfused cotyledons was 18.90 g (IQR: 12.26–24.69). Maternal and fetal flow rates were maintained at 14.10 (IQR: 13.98–14.38) and 2.62 (IQR: 2.09–2.90) mL/min, respectively. Throughout the experiments, measurements of placental viability and metabolic capacity remained within normal ranges (Table 2.1). The fetal arterial inflow pressure slightly decreased from the precontrol to the experimental period, yet remained within normal ranges. The rates of placental hCG production, glucose consumption, and oxygen consumption and delivery did not vary significantly between precontrol and experimental periods. Lactate production was constant during precontrol and experimental periods. Antipyrine equalized between the maternal and fetal reservoirs with a fetal-to-maternal (F:M) ratio of 0.87 (IQR: 0.82–0.92) after 3 hours. The rate of antipyrine disappearance from the maternal circulation was equal to the rate of appearance in the fetal circulation with values of 0.030 (IQR: 0.023–0.031) and 0.032 (IQR: 0.027–0.039) µmol/g/min, respectively (p=0.34). During all perfusions, fetal volume loss was never greater than 4 mL/hour and pH values remained within physiologic ranges.

Table 2.1. Measurements of placental viability and metabolic capacity during the perfusion experiments (n=6).

<table>
<thead>
<tr>
<th>Viability Parameter</th>
<th>Pre-control</th>
<th>Experiment</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal arterial inflow pressure (mmHg)</td>
<td>43.3 (32.5–45.6)</td>
<td>34.0 (30.9–38.8)</td>
<td>0.09</td>
</tr>
<tr>
<td>hCG production (mIU/g/min)</td>
<td>36.8 (15.3–108.8)</td>
<td>25.0 (8.2–55.4)</td>
<td>0.10</td>
</tr>
<tr>
<td>Glucose consumption (µmol/g/min)</td>
<td>0.27 (0.19–0.33)</td>
<td>0.21 (0.15–0.30)</td>
<td>0.58</td>
</tr>
<tr>
<td>Oxygen (nmol/g/min)</td>
<td>12.5 (11.2–14.8)</td>
<td>19.4 (15.9–30.3)</td>
<td>0.19</td>
</tr>
<tr>
<td>Transfer</td>
<td>376.1 (266.6–530.6)</td>
<td>399.3 (339.2–738.0)</td>
<td>0.14</td>
</tr>
<tr>
<td>Delivery</td>
<td>132.7 (104.5–231.1)</td>
<td>193.7 (124.5–363.0)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

hCG, human chorionic gonadotropin.
Data are median (IQR) unless otherwise specified.
After addition of dabigatran (35 ng/mL) into the maternal circulation (n=3), the rate of disappearance from the maternal circulation was faster over the first 30 minutes than the remainder of the perfusion (0.13 [IQR: 0.10–0.13; and 0.05 [IQR: 0.03–0.06] ng/mL/min, P<0.05; Figure 2.1A). After 3 hours, the median fetal concentration of dabigatran was 4.96 ng/mL (IQR: 4.54–7.08) and the median F:M ratio was 0.33 (IQR: 0.29–0.38). The transfer of dabigatran across the placenta was compared with antipyrine by comparing median fetal-to-maternal ratios (Figure 2.2A). Lines have been fitted through the first few data points, because transfer is expected to only occur in the maternal-to-fetal direction from 0 to 60 minutes. The F:M ratio for dabigatran increased at a slower rate as compared with that of antipyrine (0.0011 [IQR: 0.0009–0.0018] and 0.0052 [IQR: 0.0041–0.0061] min⁻¹; P<0.05).

When dabigatran etexilate mesylate (3.5 ng/mL) was added to the maternal circulation (n=3), there was limited transfer across the placenta, evidenced by a median fetal concentration of 0.19 ng/mL (IQR: 0.17–0.25) after the 3-hour perfusion (Figure 2.1B). The median F:M ratio of dabigatran etexilate mesylate was 0.17 (IQR: 0.15–0.17), suggesting that this prodrug's concentrations did not equalize across the placenta. Dabigatran concentrations were also measured in the maternal and fetal circulations, but levels were found to be negligible in these prodrug perfusions.
Figure 2.1. Dabigatran (A) and dabigatran etexilate mesylate (B) concentrations in maternal and fetal reservoirs during the 3-h experimental phase of the placenta perfusion after dabigatran (35 ng/mL, n=3) or dabigatran etexilate (3.5 ng/mL, n=3) was added to the maternal circulation. Data are shown as median and IQR at each time point.
Figure 2.2. Fetal-to-maternal ratios for antipyrine and dabigatran (A; n=3) and dabigatran etexilate mesylate (B; n=3) during the 3-h experimental phase of the perfusion. Regression lines have been fitted through the first few points (0-60 min) and extended to 180 min. Data are shown as median and IQR at each time point.
2.5 Discussion

The results of this perfusion study document that dabigatran crosses the term human placenta relatively slowly, reaching a F:M ratio of 0.33 after 3 hours. The selected dabigatran concentration of 35 ng/mL was within the therapeutic range but not high enough to theoretically cause drug saturation of the single placental lobule in our model. The F:M ratio is reported and it is expected that a similar F:M ratio would be achieved at higher therapeutic concentrations (150 ng/mL). Because dabigatran is administered as a prodrug, additional perfusions were conducted with the prodrug dabigatran etexilate mesylate. Dabigatran etexilate mesylate was found to have limited placental transfer, as evidenced by a F:M ratio of 0.16 after 3 hours. The results of the dabigatran perfusions are of greater clinical relevance, because this is the form that reaches the placenta through the maternal circulation, because dabigatran etexilate mesylate is rapidly cleaved to dabigatran through plasma esterase (Eisert et al. 2010).

Dabigatran is highly polar and exhibits hydrogen bonding, both of which are factors that can reduce transport across the placental barrier (Pacifici & Nottoli 1995). The relatively large molecular weight of dabigatran (molecular weight 628 Da) likely also limited its transfer, because molecules above 600 Da cross the placental barrier less readily (Pacifici & Nottoli 1995). In vivo studies have shown that P-gp may be involved in the efflux of dabigatran (Hartter et al. 2013). Taken together, the physicochemical and pharmacokinetic properties of dabigatran may be able to explain its limited placental transfer shown in the placenta perfusions.

The placenta expresses several efflux transporters, including P-gp and BCRP, and thus plays a major role in determining fetal drug exposure. However, the role of placental transporters was not analyzed in the present study. Future studies with the placental perfusion model or with cell culture preparations can determine a potential role of placental P-gp in the disposition of dabigatran across the human placenta.

A limitation of this model is that full-term placentae are used. As a result, it is not possible to extrapolate our results to earlier gestational ages when placental P-gp expression is higher. However, pregnancy is classically a state of hypercoagulability, and hemostatic changes become more pronounced as the pregnancy progresses (Dahlman et al. 1985; Bremme 2003; O'Riordan & Higgins 2003). There is a fivefold increase in the risk of developing venous thromboembolism during pregnancy and postpartum as compared with nonpregnant women of childbearing age.
(Pomp et al. 2008), suggesting that anticoagulant therapy may be required at later stages in pregnancy and closer to term.

In conclusion, the results of this perfusion study demonstrate that dabigatran crosses the term human placenta to some extent. Future studies will need to explore the role of placental drug transporters, especially P-gp, in affecting this process. From a clinical perspective, these data suggest that, pending further study, dabigatran should not be used for anticoagulation of pregnant women, because the drug may have an adverse effect on fetal blood coagulation.
Chapter 3

Rivaroxaban Transfer across the Dually Perfused Isolated Human Placental Cotyledon.

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\textsuperscript{2} Department of Pharmacology and Toxicology, University of Toronto, 1 King’s College Circle, Toronto, Ontario, M5S 1A8, Canada

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\textbf{Contribution of this thesis author (P. Bapat):} I was part of the collaborative team to design the experiment. I conducted the placenta perfusion experiments with technical assistance from L.S.R. Pinto and A. Lubetsky. I performed laboratory analysis of samples to measure drug concentrations and markers of placental viability with assistance from A. Lubetsky. I analyzed the data and drafted the manuscript. All authors provided input and approved the final version of the manuscript.

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3 Rivaroxaban transfer across the dually perfused isolated human placental cotyledon.

3.1 Abstract

Objective: The purpose of this study was to determine the rate and extent of rivaroxaban transfer across the term human placenta and determine whether passive diffusion was the primary mechanism involved in this transfer. Study Design: The transplacental pharmacokinetics of rivaroxaban was determined with the *ex vivo* placenta perfusion model. Rivaroxaban was added to the maternal or fetal circulation only (250 ng/mL). Additional experiments were conducted under equilibrative conditions with the addition of rivaroxaban to both the maternal and fetal circulations (250 ng/mL). Rivaroxaban concentrations were measured with the use of liquid chromatography-tandem mass spectrometry. Results: There was rapid transfer of rivaroxaban across the human placenta in both the maternal-to-fetal and fetal-to-maternal directions, as evidenced by transfer ratios of 0.69 (IQR: 0.58-0.73; n = 5) and 0.69 (IQR: 0.67-0.71; n = 2), respectively, after 3 hours. Under equilibrative conditions (n = 2), rivaroxaban concentrations remained relatively constant, which suggests that rivaroxaban crosses the placenta down a concentration gradient. Conclusion: This is the first direct evidence of rivaroxaban transfer across the term human placenta from both the mother-to-fetus and fetus-to-mother directions. Our results document that unbound rivaroxaban rapidly crosses the placental barrier via passive diffusion. However, because rivaroxaban is highly bound to plasma proteins (up to 95%), this suggests that the amount of unbound drug that may reach the fetus is likely much lower. Additional studies will need to explore its safety before administering rivaroxaban to a pregnant woman.
3.2 Introduction

Anticoagulant therapy is required in some high-risk pregnancies for the prevention and treatment of venous thromboembolism (VTE) in women at high or intermediate risk for thromboembolic disease; for the prevention and treatment of systemic embolism in women with mechanical heart valves; and, for the prevention of pregnancy-related complications in women with antiphospholipid antibody syndrome (Royal College of Obstetricians and Gynaecologists 2009; Bates et al. 2012). A retrospective cohort study reported that VTE affected 28 per 100,000 pregnancies antepartum, and 65 per 100,000 postpartum (Simpson et al. 2001). Although current guidelines recommend the use of unfractionated or low-molecular weight heparins (LMWH) (Bates et al. 2012), a recent open-label, randomized trial comparing antepartum prophylactic dalteparin (LMWH) with no dalteparin found that dalteparin did not reduce the occurrence of VTE, pregnancy loss, or placenta-mediated pregnancy complications, including severe pre-eclampsia, small-for-gestational-age infants, and placental abruption, in pregnant women with thrombophilia who were at high risk of these complications (Rodger et al. 2014). Similar to other drugs prescribed during pregnancy, the use of anticoagulant therapy can be challenging because of the possibility for both maternal and fetal complications.

Rivaroxaban (Xarelto®) is a new oral anticoagulant, with low solubility and high membrane permeability, which is increasingly being prescribed to treat deep vein thrombosis (DVT) and pulmonary embolism (PE) (Bayer Canada 2013). Following oral administration, rivaroxaban is rapidly absorbed and remains unchanged in plasma with no major or active circulating metabolites (Weinz et al. 2009). Rivaroxaban directly inhibits Factor Xa, which is an important component of both the intrinsic and extrinsic pathways of the coagulation cascade (Hoffman & Monroe 2007). Since factor Xa catalyzes the conversion of prothrombin (Factor II) to thrombin (Factor IIa) (Spyropoulos 2007), it is an ideal target for anticoagulation therapy.

The objectives of our study were to determine its transplacental pharmacokinetics at term, in order to estimate fetal drug exposure, and determine if passive diffusion is the primary mechanism involved in rivaroxaban transfer across the human placenta.
3.3 Materials and Methods

3.3.1 Ex vivo perfusion of human placental cotyledon

The dual perfusion of a single placental cotyledon has previously been described by Miller et al. (Miller et al. 1985) and adapted for use in our laboratory (Derewlany et al. 1991; Pollex et al. 2008). The study was approved by the research ethics board at St Michael’s Hospital in Toronto, Ontario, Canada, and mothers gave written consent prior to delivery. A single experiment consisted of a 1-hour control period, followed by a 3-hour experimental period. A full description of this method is presented in section 2.3.

3.3.2 Experimental period

Rivaroxaban was added to the maternal or fetal reservoir only, or simultaneously to both the maternal and fetal reservoirs at a therapeutic concentration of 250 ng/mL at the start of the experimental period. Samples were collected from the maternal and fetal reservoirs for analysis of rivaroxaban, O₂ and CO₂ content, pH, glucose consumption, and lactate production at 0, 10, 20, 30, 60, 90, 120, 150 and 180 min. Additional samples were taken directly from the maternal and fetal reservoirs every 30 minutes for analysis of hCG secretion and antipyrine transfer.

3.3.3 Sample analysis

Perfusate samples were stored at –20°C until analysis. Antipyrine concentrations were analyzed using a UV-visible recording spectrophotometer W-160 (Shimadzu, Tokyo, Japan) at 350 nm. HCG levels were determined using an ELISA kit (Alpha Diagnostic International, San Antonio, TX) and a Biotek Synergy HT microplate reader (Biotek instruments, Winooski, VT) at 450 nm.

Rivaroxaban analysis was performed by the Analytical Facility for Bioactive Molecules of the Centre for the Study of Complex Childhood Diseases at the Hospital for Sick Children, Toronto, Canada. A standard curve ranging from 0–500 ng/mL for rivaroxaban (Cedarlane, Burlington, ON, Canada) was prepared in maternal or fetal perfusate. Mobile phases were (A) 90% water and 10% acetonitrile and (B) 10% water and 90% acetonitrile, with both consisting of 5 mM ammonium formate (pH 3.2). All samples and standards were vortexed and centrifuged at 13 000 x g for 5 minutes at 4°C. 50 µL of standards or samples were added to 450 µL of the mobile phase A containing 10 ng/mL internal standard rivaroxaban-d4 (CacheSyn, Mississauga, Canada). Sample analysis was performed on an AB Sciex QTrap 5500 and Agilent 1290 UHPLC.
system. 2 µL of sample or standard were injected at a flow rate of 500 µL/min through a Kinetex XB-C18 column (50 x 3.0 mm, 2.6 µm)(Phenomenex, Torrance, CA, USA) isocratically in 1.6 min using a mobile phase composition of 60% A and 40% B.

The mass spectrometer was operated in positive electrospray ionization mode with a source temperature of 600°C and an internal standard voltage setting of 5,500. Precursor-to-product ion mass transitions were established by standard infusions. Rivaroxaban concentrations were acquired by multiple reaction monitoring mode using transitions 436.1→145 m/z and 436.1→231.1 m/z for rivaroxaban and 440.2→145 m/z and 440.2→235.25 m/z for rivaroxaban-d4. The range of rivaroxaban quantification was 0.5–500 ng/mL. Data analysis and peak integration were performed using Analyst 1.6 software from ABSciex. Sample concentrations were calculated by plotting peak area ratios (analyte/internal standard) against calibration curves of extracted matrix spiked standards.

Using the area under the curve (AUC) of rivaroxaban from the placenta perfusions, physiological rivaroxaban concentrations were simulated with 95% protein binding to estimate maternal and fetal exposure.

3.3.4 Statistical analysis

All data are presented as median and interquartile range unless stated otherwise and comparisons between pre-experimental and experimental phases were analyzed using a Wilcoxon sign rank test for non-normal data.

3.4 Results

A total of 9 cotyledons from different placentae were perfused with rivaroxaban and the physical parameters for the perfusions are shown in Table 3.1. All successfully perfused placentae were collected from caesarian deliveries. The (mean ± SD) maternal age was 33.0 ± 6.0 years, with a gestational age of 39.0 ± 0.4 weeks. The mean weight of the perfused cotyledons was 23.2 ± 7.9 g. Maternal and fetal flow rates were 14.1 ± 0.2 and 2.4 ± 0.3 mL/min, respectively. Measures of placental viability, integrity, and function remained within normal ranges and were not significantly different between the pre-experimental and experimental periods (Table 3.1). Lactate production remained constant for the duration of the perfusion. For all perfusions, antipyrine equalized between the maternal and fetal reservoirs after 3 hours with a final fetal-to-
maternal (F:M) ratio of 0.84 (IQR: 0.76–0.88; n=9), which is comparable to previous perfusion experiments (Annola et al. 2008; Myllynen et al. 2008; Matlow et al. 2013; Bapat et al. 2014). The rate of antipyrine disappearance from the maternal circulation was equal to the rate of appearance in the fetal circulation [0.025 (IQR: 0.015–0.031) vs. 0.023 (IQR: 0.014–0.029) µmol/g/min; \( p = 0.77 \)]. During all perfusions, fetal reservoir volume loss was never greater than 4 mL/hour and pH values did not deviate from physiological ranges.

<table>
<thead>
<tr>
<th>Viability Parameter</th>
<th>Pre-experimental Period (median and IQR)</th>
<th>Experimental Period (median and IQR)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal arterial inflow pressure (mmHg)</td>
<td>41.2 (38.2–48.0)</td>
<td>43.7 (39.3–45.4)</td>
<td>0.57</td>
</tr>
<tr>
<td>hCG production (mIU/g/min)</td>
<td>132.7 (41.4–155.1)</td>
<td>116.6 (35.7–122.7)</td>
<td>0.09</td>
</tr>
<tr>
<td>Glucose consumption (µmol/g/min)</td>
<td>0.25 (0.18–0.25)</td>
<td>0.14 (0.13–0.16)</td>
<td>0.12</td>
</tr>
<tr>
<td>Oxygen Transfer (nmol/g/min)</td>
<td>4.82 (2.98–6.87)</td>
<td>5.67 (3.27–7.05)</td>
<td>0.38</td>
</tr>
<tr>
<td>Delivery</td>
<td>193.6 (124.89–199.46)</td>
<td>191.38 (123.80–195.68)</td>
<td>0.89</td>
</tr>
<tr>
<td>Consumption</td>
<td>16.08 (14.12–28.82)</td>
<td>14.97 (12.85–22.78)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

hCG, human chorionic gonadotropin; IQR, interquartile range.

After addition of rivaroxaban (250 ng/ml) to the maternal reservoir, there was rapid transfer of rivaroxaban from the maternal to fetal circulations (Figure 3.1). There was a biphasic decline in rivaroxaban concentrations in the maternal circulation, as characterized by a rapid decline in the first 30 min, and a slower decline for the remaining 150 min (-2.35 ng/ml/min vs -0.19 ng/ml/min; \( P<0.05 \)). After 3 hours, the median fetal concentration of rivaroxaban was 69.5 ng/ml (IQR: 67.9–84.7) and the median F:M ratio was 0.69 (IQR: 0.58–0.73). The F:M ratio of rivaroxaban were compared to those of antipyrine (Figure 3.2). Lines have been fitted through the first few data points (0–60 min), as transfer is expected to occur only in the maternal-to-fetal direction.
Figure 3.1. Rivaroxaban concentrations in maternal and fetal circulations during the 3-hour experimental phase of the placenta perfusion after rivaroxaban was added to the maternal circulation at a concentration of 250 ng/mL (n=5). Data are shown as the median and interquartile range at each time point. Simulated *in vivo* maternal and fetal concentrations are shown with 95% protein binding.
Figure 3.2. Fetal-to-maternal ratios for antipyrine and rivaroxaban during the 3-h experimental phase of the placenta perfusion (n=5). Regression lines have been fitted from 0–60 min and extended to 180 min. Data are shown as the median and interquartile range at each time point.
When rivaroxaban was added only to the fetal reservoir (250 ng/ml), there was rapid transfer from the fetal to maternal direction, with a final maternal-to-fetal ratio of 0.69 and final maternal concentration of 58.2 ng/ml. There was also a biphasic decline in rivaroxaban concentrations in the fetal circulation, similar to the pattern seen in perfusions where rivaroxaban was added to the maternal reservoir only (Figure 3.3).

![Rivaroxaban concentrations in maternal and fetal circulations](image)

**Figure 3.3.** Rivaroxaban concentrations in maternal and fetal circulations during the 3-h experimental phase of the placenta perfusion after rivaroxaban was added to the fetal circulation only at a concentration of 250 ng/ml (n=2). Data are shown as the mean and range at each time point.
During the perfusions where equal concentrations of rivaroxaban were added to the maternal and fetal reservoirs, the F:M ratio remained relatively constant over the 3-hour experimental period (Figure 3.4). The rate of rivaroxaban disappearance from the maternal and fetal reservoirs was similar at 1.51 and 1.86 ng/ml/min, and this was likely attributed to rivaroxaban loss within our model.

Figure 3.4. Rivaroxaban concentrations in maternal and fetal circulations during the 3-h experimental phase of the placenta perfusion after rivaroxaban was added to the maternal and fetal circulation at a concentration of 250 ng/ml (n=2). Data are shown as the mean and range at each time point.
3.5 Discussion

The results of this perfusion study show that rivaroxaban rapidly crosses the term human placenta, reaching a F:M ratio of 0.69 after 3 hours. The final F:M ratios of rivaroxaban and antipyrine are likely indicative of steady state, as these ratios remained relatively constant after 120 min (Figure 3.2), suggesting that net maternal-to-fetal transfer was no longer increasing.

The perfusions in which rivaroxaban was added to the fetal reservoir only were important to elucidate the role of the placenta in clearing the drug from the fetal circulation. The final transfer ratio in these perfusions was similar to that of the perfusions where rivaroxaban was added to the maternal circulation only, suggesting that rivaroxaban interacts with the placental barrier in a similar fashion in both directions across the placenta. Under equilibrative conditions, rivaroxaban concentrations remained relatively constant, suggesting that rivaroxaban crosses the placenta down a concentration gradient. The low molecular weight of rivaroxaban (436 Da) also supports the notion that rivaroxaban likely crosses the placenta via passive diffusion.

A limitation of the placenta perfusion model is that only full-term placentae are used. As a result, we cannot make generalizations to earlier gestational ages when placental structure is vastly different. Another important consideration with the placenta perfusion is that this model does not fully account for non-placental pharmacokinetic factors, including protein binding, clearance rate, and distribution in the maternal and fetal compartments. Protein binding is especially important since rivaroxaban is highly bound to plasma proteins (92–95% bound, mainly albumin) (Bayer Canada 2013). Differences in maternal and fetal plasma protein concentrations can affect the transplacental transfer of drugs that are highly bound. Throughout pregnancy, fetal albumin levels tend to increase, with the fetal-to-maternal albumin ratio increasing from 0.28 in the first trimester to 1.20 closer to term (Krauer et al. 1984). As well, the increased concentration of free fatty acids in the maternal circulation toward term can displace drugs for the binding spot on albumin (Ridd et al. 1983; Nau et al. 1984). This leads to increased binding of a drug to albumin in the fetal circulation compared to the maternal circulation, which can, in turn, lead to increased placental drug transfer and fetal drug accumulation since only the unbound form of the drug can equilibrate across the placental barrier. Assuming plasma protein binding of rivaroxaban at 95%, the fetal exposure to rivaroxaban is predicted to be only a small fraction of what is shown in the perfusion of the unbound drug (Figure 3.1).
Therefore, it is likely that fetal levels \textit{in vivo} will be substantially lower than the values obtained in our perfusion experiments, as a majority of the drug will remain bound to serum albumin in the maternal circulation. The proportion of rivaroxaban that is bound and unbound may be slightly different in maternal and fetal circulations, as compared to non-pregnant adults due to differences in albumin levels (Nau et al. 1984). Perfusion experiments that include proteins in the perfusion medium are technically challenging due to the high viscosity of the perfusate. As well, when trying to mimic physiological conditions, there are numerous proteins and endogenous factors that can affect binding \textit{in vivo} (Johnson et al. 1999; Schenker et al. 1999; Gavard et al. 2006; Nanovskaya et al. 2009; Hutson et al. 2011).

During the experiments, several parameters were measured to monitor placental viability and integrity. These parameters included arterial inflow pressure in the fetal circulation, pH, oxygen consumption, net fetal oxygen transfer, glucose consumption, lactate production, and secretion of human chorionic gonadotropin (hCG). As well, it has been suggested that fetal volume loss is the optimal measure of tissue viability (Mathiesen et al. 2010; Hutson et al. 2011). A loss up to 4 ml/hr from the fetal reservoir is typically accepted (Mathiesen et al. 2010). Antipyrine, which is a flow-limited marker that only undergoes passive diffusion, can also be used to measure tissue integrity. While the model cannot fully incorporate non-placental pharmacokinetic factors such as protein binding and liver metabolism, the placenta perfusion is the only experimental model that can be used to study human placental transfer of substances in organized placental tissue and may be able to more reliably predict fetal exposure compared to other experimental methods (Hutson et al. 2011). While animal studies can be very useful, they cannot always be easily extrapolated to humans because the placenta is the most species-specific mammalian organ (Carter 2007).

Previous studies have shown that rivaroxaban is a substrate for P-glycoprotein (P-gp) \textit{in vitro} and \textit{in vivo} (Lang et al. 2009; Gnoth et al. 2011). P-gp generally has a unidirectional effect on transcellular transport, and in the placenta, P-gp effluxes substrates from the fetal to the maternal side. Since the final transfer ratios were similar when rivaroxaban was added to the maternal or fetal circulation alone, it is unlikely that placental P-gp has a major role on the transplacental disposition of rivaroxaban at term.
A case report was recently published describing the use of rivaroxaban during the first and part of the second trimester of pregnancy (Konigsbrugge et al. 2014). A male infant was born spontaneously in the 40th gestational week without complications. The baby birth measurements were in the 13th, 30th, and 7th percentiles for weight, length, and cranial circumference, respectively, but the authors likely attributed the low growth percentiles to maternal smoking. Another study measured thrombin generation and activated partial thromboplastin time in adult plasma and neonatal cord samples with increasing concentrations of rivaroxaban (Novak et al. 2011). This article was useful in showing that rivaroxaban exerts similar hemostatic effects in neonatal cord and adult plasma.

In conclusion, this is the first direct evidence of rivaroxaban transfer across the human placenta. Our results document that unbound rivaroxaban rapidly crosses the term human placenta. However, since rivaroxaban is highly bound to plasma proteins (up to 95%) with an elimination half-life of 5–9 hours, the likely amount of unbound drug that may reach the fetus is relatively small. Future studies will need to explore safety before administering rivaroxaban to a pregnant woman.
Chapter 4

Examining the Transplacental Passage of Apixaban Using the Dually Perfused Human Placenta.

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Contribution of this thesis author (P. Bapat): I was part of the collaborative team to design the experiment. I conducted the placenta perfusion experiments with technical assistance from L.S.R. Pinto and A. Lubetsky. I performed laboratory analysis of samples to measure drug concentrations and markers of placental viability with assistance from A. Lubetsky and K. Aleksa. I analyzed the data and drafted the manuscript. All authors provided input and approved the final version of the manuscript.

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4 Examining the transplacental passage of apixaban using the dually perfused human placenta.

4.1 Abstract

**Background:** Apixaban is a novel oral anticoagulant that is increasingly being prescribed to women of reproductive age. However, information regarding its placental transfer is non-existent. **Objective:** To determine the rate and extent of placental transfer of apixaban, using the human placenta *ex vivo.* **Methods:** Placentae collected after caesarean or vaginal delivery of healthy term infants were perfused in the respective maternal and fetal circulation. At the start of the experiment, apixaban was added to the maternal circulation at a concentration of 150 ng/ml, and samples from maternal and fetal reservoirs were collected over 3 hours. **Results:** There was a rapid decline of apixaban in the maternal compartment, followed by emergence in the fetal compartment with a median fetal-to-maternal drug concentration ratio of 0.77 (IQR: 0.76–0.81) and fetal concentration of 39.0 ng/mL (IQR: 36.8–40.6) after 3 hours (n=5). The perfusion results were subsequently adjusted to account for differences in the concentration of plasma proteins in maternal and fetal blood, as apixaban remains highly bound to albumin and alpha-1 acid glycoprotein. After the adjustment, the predicted fetal-to-maternal ratio of total (bound plus unbound) apixaban concentrations *in vivo* ranged from 0.35 to 0.90. **Conclusions:** We conclude that unbound apixaban rapidly crosses from maternal to fetal circulation. We further predict that total apixaban concentrations in cord blood *in vivo* are 35-90% of the corresponding maternal levels, suggesting that apixaban could have a possible adverse effect on fetal and neonatal coagulation.
4.2 Introduction

Even in pregnancy, anticoagulant therapy is often indicated for women with conditions such as venous thromboembolic events (VTE), atrial fibrillation, and artificial heart valves (Royal College of Obstetricians and Gynaecologists 2009; Bates et al. 2012). In particular, VTE, including deep vein thrombosis (DVT) and pulmonary embolism (PE), are a major cause of maternal morbidity and mortality during pregnancy or after delivery. The risk for VTE in pregnant women is 4–5 times higher than the risk among non-pregnant women of the same age (Conti et al. 2014). Vitamin K antagonists are contraindicated in pregnancy because of their potential to cause adverse teratogenic effects (Hall et al. 1980; Iturbe-Alessio et al. 1986; Ginsberg et al. 1989; Schaefer et al. 2006).

Apixaban (Eliquis®) is a novel oral anticoagulant (NOAC) that is increasingly used for the prevention and treatment of venous thromboembolic events, including DVT and PE. Apixaban is also prescribed for the prevention of stroke and systemic embolism in patients with atrial fibrillation. The drug is rapidly absorbed with peak concentrations appearing 3–4 hours after tablet intake, and unchanged apixaban is the major component in human plasma with no active circulating metabolites (Pfizer Canada 2015). In human plasma, apixaban is highly bound to plasma proteins (87–93%) (Pfizer Canada 2015). Apixaban is a reversible, direct and highly selective inhibitor of free and prothrombinase-bound Factor Xa (Jiang et al. 2009). Factor Xa is activated via the intrinsic and extrinsic pathways of the coagulation cascade, and its inhibition prevents thrombin generation and the subsequent formation of a thrombus. Importantly, when compared to other NOACs (including dabigatran, rivaroxaban, and edoxaban), apixaban is associated with a relatively low risk of bleeding events while showing similar efficacy for the treatment of acute VTE (Mantha & Ansell 2015).

To date, no studies have examined the safety of apixaban in pregnant patients and its placental transfer in humans is currently unknown. As prescription rates for NOACs continue to increase (Desai et al. 2014), the number of women with an unplanned pregnancy who are taking NOACs will likely increase. Therefore, the objective of our study was to examine the disposition of apixaban across the term human placenta ex vivo, in order to estimate fetal drug exposure.
4.3 Methods

4.3.1 Dual perfusion of human placental cotyledon *ex vivo*

The dual perfusion of an isolated placental cotyledon *ex vivo* is a validated method to examine placental transfer of drug, as previously described by Miller *et al* (1985) and adapted for use in our laboratory (Derewlany *et al*. 1991; Bapat *et al*. 2014, 2015). This study was approved by the research ethics boards at St. Michael’s Hospital and The Hospital for Sick Children in Toronto, Ontario Canada, and this research was conducted in accordance with the Declaration of Helsinki. Women provided written consent prior to delivery at St. Michael’s Hospital, and term placentae were collected immediately following elective cesarean or vaginal delivery of healthy singleton pregnancies (>38 weeks). We excluded placentae from women who were taking any medication during pregnancy or from women who had any disease during pregnancy including, but not limited to, hypertension, diabetes, thyroid dysfunction, cancer and infection (e.g., chorioamnionitis and TORCH infections, or positive anti-HIV, anti-HBV, or anti-HCV serology). A single experiment consisted of a 1-hour control period, followed by a 3-hour experimental period. A full description of this method is presented in section 2.3.

At the beginning of experimental period, the maternal and fetal reservoirs were replaced with fresh perfusate, and apixaban was added to the maternal circulation at a concentration of 150 ng/mL, which is in the range of therapeutic plasma concentrations (Frost *et al*. 2013a, b). Samples were collected from the maternal and fetal reservoirs at 0, 10, 20, 30, 60, 90, 120, 150 and 180 min for measurement of apixaban levels, O$_2$ and CO$_2$ content, pH, glucose consumption, lactate production, antipyrine transfer and hCG secretion. Samples were stored at −20°C until analysis.

4.3.2 Sample analysis

Antipyrine concentrations were determined using a UV-visible recording spectrophotometer W-160 (Shimadzu, Tokyo, Japan) set at 350 nm. HCG levels were determined using an ELISA kit (Alpha Diagnostic International, San Antonio, TX) and a Biotek Synergy HT microplate reader (Biotek instruments, Winooski, VT) at 450 nm.

A standard curve ranging from 0 to 300 ng/mL for apixaban (AK Scientific Inc, Union City, CA, USA) was prepared in maternal or fetal perfusate. The internal standard, $[^{13}\text{C}_2,^2\text{H}_7]$-apixaban, was
added to each sample at 150 ng/mL (13C Molecular, Fayetteville, NC, USA). Sample analysis was performed on an AB Sciex QTrap 5500 and Shimadzu LC30-AD UHPLC system. Samples were injected (1 μL) at a flow rate of 0.6 mL/min through a Kinetex XB-C18 column (50 x 2.1 mm, 1.7 μm; Phenomenex, Torrance, CA, USA), using a variable gradient of distilled water (phase A) and acetonitrile (phase B), with both containing 0.1% formic acid. The gradient increased from 30% to 80% phase B during the first 1.2 min, decreased to 30% phase B for 0.1 min, and was then held at 30% phase B for 0.7 min, for a total run time of 2 min. The retention time was approximately 0.3 minutes. The mass spectrometer was operated in positive electrospray ionization mode with a source temperature of 600°C and an ion spray voltage of 5,300 V. Apixaban was detected in enhanced product ion (EPI) mode using transitions 460.2→443.2 m/z for quantification and 460.2→185 m/z for qualification. The internal standard was measured using transition 468.2→451.3 m/z. Data analysis and peak integration were performed using Analyst 1.6 software from ABSciex. The limits of detection and quantification for apixaban were 1.4 and 4.4 ng/mL, respectively. The intra- and inter-day coefficients of variation were both 10%.

4.3.3 Prediction of in vivo fetal-to-maternal (F:M) ratio of plasma drug concentration

Placenta perfusion data can be used to predict the fetal-to-maternal (F:M) ratio of total (bound plus unbound) drug concentration in plasma in vivo (Hutson et al. 2011). As seen in the following equation developed by Garland (1998) and later adapted by Hutson et al (2011), the in vivo F:M ratio at steady state can be predicted from the physiological differences in protein binding and pH between maternal and fetal circulations in vivo, drug ionization characteristics, and clearance parameters of the placenta perfusion experiments,

\[
F:M = \frac{\%\ unbound_M}{\%\ unbound_F} \times \frac{1 + 10^{pK_a-pH_F}}{1 + 10^{pK_a-pH_M}} \times \frac{CL_{MF}}{CL_{FM} + CL_F}
\]

where \(\%\ unbound_M\) and \(\%\ unbound_F\) are the percentages of free drug in the maternal and fetal circulations in vivo; pH_M and pH_F are the pH values of maternal and fetal blood in vivo; and CL_{MF}, CL_{FM}, and CL_F are the maternal-to-fetal, fetal-to-maternal, and fetal clearances of the drug obtained in the placenta perfusion experiments (Garland 1998). When using this equation, the
following assumptions were made as per previous analyses (Hutson et al. 2011): (i) only the free drug can cross the placenta passively, (ii) the free fraction is not concentration-dependent, (iii) only the unionized form of the drug can passively cross the placenta, and (iv) fetal clearance ($CL_F$) is negligible. The F:M concentration ratio of apixaban from the placenta perfusions at 180 min was used to represent the last term of the Garland equation (Garland 1998; Hutson et al. 2011). Because apixaban is unionized in physiological pH (Pfizer Canada 2015), the second term representing the ionization characteristics of the drug was assumed to be 1.

Unbound fractions (%) in the maternal and fetal circulations in vivo were estimated with the method developed by Hill and Abramson (1988). As described below, this method uses plasma concentrations of drug binding protein ($[P]$) and bound/unbound drug concentration ratio (B/U ratio) reported in non-pregnant adults to predict the B/U ratio in pregnant women and fetuses:

$$\left(\frac{B}{U}\right)_F = \left(\frac{B}{U}\right) \times \left(\frac{[P]_F}{[P]}\right) \quad \text{and} \quad \left(\frac{B}{U}\right)_M = \left(\frac{B}{U}\right) \times \left(\frac{[P]_M}{[P]}\right);$$

where (B/U) and [P] are bound/unbound drug concentration ratio and plasma concentrations of the binding protein in non-pregnant adults, respectively. Subscripts F and M denote fetal and maternal, respectively.

In non-pregnant adults, apixaban plasma protein binding is about 87%. Albumin accounts for the most of the binding (76%), and alpha-1 acid glycoprotein for about 10%. Binding protein for the remaining 14% is unknown (He et al. 2011). Because the Hill-Abramson method is valid under the assumption that the drug is bound only to a single protein, we used the following 2 different scenarios of apixaban plasma protein binding: (i) albumin is the sole binding protein; and (ii) alpha 1 acid glycoprotein is the sole binding protein. For albumin, $[P]_F / [P]$ is 0.866, and $[P]_M / [P]$ is 0.733. For alpha-1 acid glycoprotein, they are 0.37 and 1.0, respectively (Hill & Abramson 1988). Using these estimates, we derived “% Unbound” for maternal and fetal circulations as follows:

$$\% Unbound_M = 100 - \left[100 \times \left(\frac{B}{U}\right)_M\right] \quad \text{and} \quad \% Unbound_F = 100 - \left[100 \times \left(\frac{B}{U}\right)_F\right]$$
4.3.4 Statistical analysis

All data are presented as median and interquartile range (IQR) unless stated otherwise and comparisons between pre-experimental and experimental phases were analyzed using a Wilcoxon sign rank test for non-normal data. Any p-values less than 0.05 were considered to be statistically significant.

4.4 Results

Of the 25 screened and retrieved placentae, five cotyledons from five different placentae were successfully perfused with apixaban. Four of the perfused placentae were obtained from caesarian deliveries, and one from a vaginal delivery. The (mean ± SD) maternal age was 33.4 ± 6.5 years, with a gestational age of 39.6 ± 0.9 weeks. The weight of the perfused cotyledons was 24.21 ± 10.32 g. Maternal and fetal flow rates were 12.84 ± 0.40 and 2.63 ± 0.40 mL/min, respectively. Markers of placenta function and viability remained within normal physiological ranges and did not significantly change between the pre-experimental and experimental periods (Table 4.1). Lactate production remained also constant for the duration of the perfusion, indicating integrity of the perfused placentae. Antipyrine concentrations equilibrated across the placenta with a F:M concentration ratio of 0.96 (IQR: 0.86–0.99) after 3 hours, further indicating the integrity of the fetal capillary bed. During all experiments, pH values did not deviate from physiological ranges (maternal = 7.4; fetal = 7.35) (Reynolds & Knott 1989), and the volume loss in the fetal reservoir was never greater than 4 mL/hour. These parameters were all within the accepted ranges of the quality control measures.
Table 4.1. Markers of placental viability during the pre-experimental and experimental periods of perfusion (n=5).

<table>
<thead>
<tr>
<th>Viability Parameter</th>
<th>Pre-experimental Period (median and IQR)</th>
<th>Experimental Period (median and IQR)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG production (mIU/g/min)</td>
<td>161.3 (83.2–397.7)</td>
<td>108.0 (64.8–352.3)</td>
<td>0.69</td>
</tr>
<tr>
<td>Fetal arterial inflow pressure (mmHg)</td>
<td>38.4 (36.2–39.6)</td>
<td>37.3 (36.4–40.3)</td>
<td>0.47</td>
</tr>
<tr>
<td>Glucose consumption (µmol/g/min)</td>
<td>0.18 (0.10–0.18)</td>
<td>0.18 (0.12–0.21)</td>
<td>0.83</td>
</tr>
<tr>
<td>Oxygen Transfer</td>
<td>11.42 (7.73–16.27)</td>
<td>12.77 (7.24–15.07)</td>
<td>0.26</td>
</tr>
<tr>
<td>Delivery</td>
<td>253.7 (161.0–290.8)</td>
<td>236.7 (155.8–301.0)</td>
<td>0.34</td>
</tr>
<tr>
<td>Consumption</td>
<td>82.08 (56.31–87.01)</td>
<td>70.41 (47.81–91.50)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

hCG, human chorionic gonadotropin; IQR, interquartile range.

While the initial concentration in the maternal reservoir was 150 ng/mL, the maternal concentration at the start of the experiment was 108 ng/mL (IQR: 98.7–115 ng/mL), and this was likely attributed to apixaban loss within our system, such as binding to the placental tissue. Over the first hour of perfusion, apixaban concentrations further declined in the maternal circulation, and then remained relatively constant for the remaining 2 hours (Figure 4.1). At the end of the 3-hour perfusion, the median concentration of apixaban in the fetal circulation was 39.0 ng/ml (IQR: 36.8–40.6 ng/mL) and the F:M ratio was 0.77 (IQR: 0.76–0.81).
Figure 4.1. Apixaban concentrations in maternal and fetal circulations during the dual perfusion of an isolated placental cotyledon (n=5). Apixaban was added to the maternal circulation (150 ng/mL), and samples were collected from maternal and fetal reservoirs for 180 minutes. Data are shown as the median and interquartile range for each time point.

In order to predict the F:M ratio of total drug (bound + unbound) in vivo (Garland 1998; Hutson et al. 2011), two different scenarios were used as described in Methods: (i) albumin is the only binding protein; and (ii) alpha 1 acid glycoprotein is the only binding protein. The F:M ratio of total apixaban in vivo was predicted to range from 0.35 (scenario ii) to 0.90 (scenario i).

4.5 Discussion

The results of this placenta perfusion study are the first to document that unbound apixaban rapidly crosses the term human placenta from the maternal to the fetal circulation. Based on these ex vivo experimental data, we further predict that the steady-state apixaban concentration in cord blood in vivo is about 35-90% of the maternal level. As apixaban is primarily bound to
albumin, we speculate that the in vivo F:M ratio is likely closer to the albumin-based estimate of 0.90.

In instances where information regarding placental transfer may not be available, the placenta perfusion model is a safe and ethical tool to predict placental transfer in vivo. Apixaban is a relatively new drug and there are currently no published case reports describing its use in pregnant patients. A systematic review of the placenta perfusion results evaluated the model in predicting placental drug transfer in vivo (Hutson et al. 2011). In that study, F:M concentration ratios from perfusion experiments were compared with cord-to-maternal blood ratios collected at the time of delivery for 70 drugs. In general, the results from placenta perfusion experiments correlated well with the data in vivo. After adjusting the perfusion F:M ratios of 24 drugs for protein binding and pH differences between the fetal and maternal circulation, there was a stronger correlation between the in vivo results of cord-to-maternal drug concentration ratio and the perfusion experiments-derived fetal-to-maternal ratio. The authors concluded that the perfusion model can be used to predict placental transfer of small molecule drugs in vivo (Hutson et al. 2011).

In our analyses, the predicted ratio of F:M apixaban concentrations in vivo ranged from 0.35 to 0.90, suggesting that fetal apixaban exposure may be similar to the level of maternal therapeutic exposure. In general, a F:M drug concentration ratio can be used to categorize the placental transferability of drug: limited transfer (<0.1), transfer (0.1–1.0), or accumulation in the fetal compartment (>1.0) (Hutson et al. 2011). Although our prediction needs to be verified with in vivo data, we anticipate that apixaban concentrations in the cord blood will be in the vicinity of maternal therapeutic levels.

An important consideration with using any anticoagulant in pregnancy relates to the state of the coagulation system in maternal and fetal plasma. Pregnancy is classically a state of hypercoagulability, characterized by an increased level of many clotting factors, a decrease in the amount of natural anticoagulants, and a reduction in fibrinolytic activity in the blood (Dahlman et al. 1985; Bremme 2003; O'Riordan & Higgins 2003). More specifically, levels of Factor X increase in the early stages of pregnancy and remain elevated until 1 week after delivery (Hellgren & Blomback 1981). By comparison, fetal and neonatal levels of Factor X are markedly lower, ranging from 20–40 percent of adult levels (Andrew et al. 1990; Reverdiau-Moalic et al.
This suggests that fetuses and neonates may be more sensitive to the anticoagulant effects of apixaban. If intrauterine exposures to apixaban occur, coagulation monitoring of the infants may be necessary.

The therapeutic options are limited for pregnant women who require anticoagulation management. Unfractionated heparin (UFH) and low-molecular weight heparins (LMWH) do not cross the placenta and are not associated with an increased risk of teratogenicity or increased fetal bleeding (Bates 2002; Bates et al. 2012). For this reason, UFH and LMWHs are currently recommended for most women requiring anticoagulation therapy during pregnancy (Bates et al. 2012). However, parenteral administration poses significant challenges. We recently evaluated the placental transfer of two other novel oral anticoagulants: dabigatran and rivaroxaban using the same perfusion model (Bapat et al. 2014, 2015). Dabigatran and its pro-drug, dabigatran etexilate mesylate, had lower placental transfer as characterized by F:M ratios of 0.33 and 0.17, respectively (Bapat et al. 2014). Similar to apixaban, rivaroxaban concentrations in the maternal circulation rapidly declined in the first hour and remained relatively constant over the final 2 hours. The F:M ratio of rivaroxaban was 0.69 after 3 hours, and this transfer was most likely via passive diffusion (Bapat et al. 2015). Warfarin is another option, although it is teratogenic particularly at higher doses (Schaefer et al. 2006).

The placenta expresses numerous efflux transporters, such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), which can play a protective role in limiting fetal drug exposure (Iqbal et al. 2012). Using in vitro models, it has been shown that apixaban is a substrate for both P-gp and BCRP (Zhang et al. 2013), and thus, one could expect lower placental transfer, as exemplified in drugs such as glyburide and metformin (Kraemer et al. 2006; Pollex et al. 2008; Hemauer et al. 2010). A study in rats showed limited placental transfer of [14C]-apixaban, with a peak fetal blood concentration that was approximately 35% of the maternal concentration (Wang et al. 2011). The authors suggested that BCRP might play an important role in limiting placental transfer of apixaban to the fetus. However, due to its relatively small size and no ionization at physiological pH (Pfizer Canada 2015), it is likely that apixaban crosses the placenta extensively via passive diffusion (Pacifici & Nottoli 1995). Future studies are required to determine if human placental P-gp and BCRP are specifically involved in the distribution of apixaban in maternal and fetal circulations.
A limitation of this placenta perfusion model is that only full-term placentae are used. As such, we cannot make extrapolations about placental transfer in the first and second trimester. However, it should be noted that the need for anticoagulant therapy typically becomes more pronounced as the pregnancy progresses (Bates et al. 2012), suggesting that information regarding its placental transfer in later stages of pregnancy may be more clinically relevant. Another consideration with the placenta perfusion model is that it does not account for time-dependent dynamic changes of drug concentrations in the maternal and fetal compartments. Protein binding is also important as apixaban is characterized by relatively high plasma protein binding (87–93%) (Pfizer Canada 2015), and only the unbound form of a drug can cross the placenta (Pacifici & Nottoli 1995).

In conclusion, unbound apixaban rapidly crosses the placenta from the maternal to the fetal circulation in the human placenta perfusion model. We further predict that fetal apixaban concentrations in vivo at steady state are 35-90% of the corresponding maternal levels, raising a possibility for neonatal complications due to their hypocoagulability status. Future studies will need to explore safety before clinicians can consider the use of apixaban in pregnant women. As such, the recommendation still remains that women who become pregnant while taking apixaban should be switched to another anticoagulant such as LMWH or UFH.
Chapter 5

Prediction of human placental pharmacokinetics of novel anticoagulants using the *ex vivo* placenta perfusion and physiologically based models.

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**Contribution of this thesis author (P. Bapat):** I was part of the collaborative team to design the research study. I performed the *in vivo* prediction analyses, and developed the pharmacokinetic model with M. Takeuchi. I analyzed the data and drafted the manuscript. All authors provided input and approved the final version of the manuscript.
5 Prediction of human placental pharmacokinetics of novel anticoagulants using the *ex vivo* placenta perfusion and physiologically based models.

5.1 Abstract

**Background:** Our previous studies have provided information regarding the placental transfer of dabigatran, rivaroxaban, and apixaban using the dually perfused placenta *ex vivo*. A limitation of this perfusion model is that it cannot account for differences in protein binding between maternal and fetal plasma. The purpose of this study was to compare the placental transfer of three novel oral anticoagulants, after adjusting for differences in protein binding. As well, transplacental transfer parameters were estimated using a pharmacokinetic model. **Methods:** To account for the physiological differences in protein binding and pH between maternal and fetal circulation *in vivo*, the equation developed by Garland (1998) and later adapted by Hutson (2011) was used to predict the fetal-to-maternal (F:M) ratio of total (bound plus unbound) drug concentrations in plasma *in vivo*. A three-compartment model, consisting of maternal, placental, and fetal compartments was used to estimate transplacental pharmacokinetic parameters. **Results:** The predicted F:M ratios *in vivo* of dabigatran and rivaroxaban were 0.39 and 0.81, respectively. For apixaban, the predicted F:M ratio *in vivo* ranged from 0.35-0.90. The experimental and predicted F:M ratios were significantly different between dabigatran and apixaban. The concentration profiles of rivaroxaban, apixaban and dabigatran in the maternal and fetal reservoir were adequately described using a three-compartment transplacental pharmacokinetic model. **Conclusions:** In conclusion, the *in vivo* fetal levels of dabigatran, rivaroxaban and apixaban were predicted to be 39%, 80%, and 90% of corresponding maternal levels at steady state. We noted that *in vivo* estimates of the F:M ratio were 15% higher than what was shown in placenta perfusion experiments, and this was primarily attributed to plasma protein binding.
5.2 Introduction

Anticoagulants are often prescribed during pregnancy for the prevention or treatment of complications in women with venous thromboembolism (VTE), mechanical or artificial heart valves, and inherited or acquired thrombophilias (Royal College of Obstetricians and Gynaecologists 2009; Bates et al. 2012). Vitamin K antagonists, such as warfarin, are contraindicated in pregnancy because of an associated embryopathy characterized by nasal and limb hypoplasia, as well as congenital heart anomalies (Chan et al. 2000; Hassouna & Allam 2010). As well, vitamin K antagonists cross the placenta and have the potential to cause fetal bleeding and teratogenicity (Hall et al. 1980; Ginsberg et al. 1989; Schaefer et al. 2006). By comparison, low molecular weight heparins (LMWH) and unfractionated heparins (UFH) do not cross the placenta and are currently recommended for the prevention and treatment of VTE during pregnancy (Bates et al. 2012, 2016). As with any medication during pregnancy, the use of anticoagulants can be challenging due to the potential for maternal and fetal complications.

More recently, novel oral anticoagulants (dabigatran, rivaroxaban, apixaban) have been approved for use in non-pregnant adults for the prevention and treatment of VTE, including pulmonary embolism and deep vein thrombosis. These novel oral anticoagulants are reversible, direct inhibitors of thrombin or Factor Xa, and can be prescribed at fixed doses without the need for routine monitoring or dose adjustment. In general, there are no food interactions with these drugs, and only limited drug-drug interactions (Fox et al. 2012; Bauer 2013; Heidbuchel et al. 2013). To date, there is limited information regarding the use and safety of these drugs in pregnant women. Moreover, the information about placental drug transfer in vivo and fetal safety is unknown.

The decision to begin or continue treatment during pregnancy heavily relies on weighing the maternal benefits of the drug against the potential adverse fetal risks. An important determinant in this risk assessment is the estimation of fetal drug exposure, based on quantifying the amount of drug that crosses the placenta. By using the dually perfused human placenta ex vivo, our previous studies have provided information regarding the placental transfer of dabigatran, rivaroxaban, and apixaban (Bapat et al. 2014, 2015, 2016). A limitation of this perfusion model is that it cannot account for differences in protein binding between maternal and fetal plasma. However, a previous study has showed that placenta perfusion data can be adjusted in order to
predict the fetal-to-maternal (F:M) ratio of total (bound plus unbound) drug concentration in plasma \textit{in vivo} (Garland 1998; Hutson et al. 2011).

The purpose of this study was to compare the placental transfer of three novel oral anticoagulants, after adjusting for differences in protein binding. As well, we developed a pharmacokinetic model to estimate transplacental transfer parameters.

5.3 Methods

5.3.1 \textit{Ex vivo} placenta perfusion

The dual perfusion of an isolated term placental cotyledon \textit{ex vivo} is a validated method to study placental drug transfer, as previously described by Miller \textit{et al} (1985) and adapted for use in our laboratory (Derewlany \textit{et al} 1991; Bapat \textit{et al} 2014, 2015). This study was approved by the research ethics board at St. Michael’s Hospital, and women provided written consent prior to delivery. A single experiment consisted of a 1-hour control period, followed by a 3-hour experimental period. A full description of this method is presented in section 2.3. We previously performed placenta perfusion experiments to evaluate the transfer of dabigatran (Chapter 2), rivaroxaban (Chapter 3), and apixaban (Chapter 4).

5.3.2 Prediction of \textit{in vivo} fetal-to-maternal (F:M) ratio of plasma drug concentration

Using data from placenta perfusion experiments in Chapters 2 and 3, we predicted the F:M ratio of total (bound + unbound) drug concentration in plasma \textit{in vivo}. A full description of this prediction method is presented in section 4.3.3. Briefly, the following equation developed by Garland (1998) and later adapted by Hutson \textit{et al} (2011) was used to predict the \textit{in vivo} F:M ratio at steady state from the physiological differences in protein binding and pH between maternal and fetal circulations \textit{in vivo}, drug ionization characteristics, and clearance parameters of the placenta perfusion experiments,

\[
F: M = \frac{\% \text{ unbound}_M}{\% \text{ unbound}_F} \times \frac{1 + 10^{pK_a-pH_F}}{1 + 10^{pK_a-pH_M}} \times \frac{CL_{MF}}{CL_{FM} + CL_F}
\]

where \% unbound\textsubscript{M} and \% unbound\textsubscript{F} are the percentages of free drug in the maternal and fetal circulations \textit{in vivo}; pH\textsubscript{M} and pH\textsubscript{F} are the pH values of maternal and fetal blood \textit{in vivo}; and
CL_{MF}, CL_{FM}, and CL_{F} are the maternal-to-fetal, fetal-to-maternal, and fetal clearances of the drug obtained in the placenta perfusion experiments (Garland 1998). When using this equation, the following assumptions were made as per previous analyses (Hutson et al. 2011): (i) only the free drug can cross the placenta passively, (ii) the free fraction is not concentration-dependent, (iii) only the unionized form of the drug can passively cross the placenta, and (iv) fetal clearance (CL_{F}) is negligible.

In non-pregnant adults, dabigatran and rivaroxaban bind albumin at 35% and 95%, respectively (Weinz et al. 2005; Blech et al. 2008). Unbound fractions (%) in the maternal and fetal circulations in vivo were estimated with the method developed by Hill and Abramson (1988) as described in section 4.3.3. For the second term of the Garland equation, the maternal and fetal pH values were 7.4 and 7.35, respectively (Reynolds & Knott 1989), and a pK_{a} value of 12.4 was used for dabigatran (Blech et al. 2008). Since rivaroxaban is unionized at physiological pH (Bayer Canada 2013), the second term of the equation was assumed to be 1. The F:M concentration ratios from the placenta perfusions at 180 min were used to represent the last term of the Garland equation (1998).

5.3.3 Statistical analysis

All data are presented as the median and interquartile range (IQR), unless stated otherwise. Comparisons between different perfusion groups were analyzed with a Kruskal-Wallis test using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. Any p values less than 0.05 were considered statistically significant. All statistical analyses were performed in SPSS for MacIntosh Version 20.0 (IBM Corp., Armonk, NY, USA).

5.3.4 Model construction

As a simple structural representative of the experimental setup, a three-compartment model, consisting of maternal, placental, and fetal compartments, was used to estimate the following transplacental pharmacokinetic parameters (Figure 5.1); K_{12} (influx rate constant from maternal to placental compartment), K_{21} (efflux rate constant from placental to maternal compartment), K_{23} (efflux rate constant from placental to fetal compartment), and K_{32} (influx rate constant from fetal to placental compartment). V_{m} and V_{f}, representing the volumes of distribution in the
maternal and fetal compartments, were assumed to be 250 mL and 150 mL, respectively, reflecting the system setup. The model was described by the following differential equations:

\[
\frac{dX_m}{dt} = -X_m \times K_{12} + X_p \times K_{21}
\]

\[
\frac{dX_p}{dt} = -X_p \times K_{21} - X_p \times K_{23} + X_p \times K_{12} + X_p \times K_{32}
\]

\[
\frac{dX_f}{dt} = -X_f \times K_{32} + X_p \times K_{23}
\]

where \(X_m\), \(X_p\), and \(X_f\) are the amounts of the drug in the maternal, placental, and fetal compartments, respectively.

Compartment volumes were fixed at 250 mL (maternal) and 150 mL (fetal) as experimental constants. The parameters were estimated with a naïve average data approach using NONMEM (version 7.3), instead of population modeling with a nonlinear mixed effect method. The initial estimates were arbitrarily determined.
Figure 5.1. Three-compartment pharmacokinetic model of rivaroxaban, apixaban, and dabigatran transfer across the placenta. $V_m$ and $V_f$ represent the volumes of distribution in the maternal and fetal compartments; $K_{12}$, influx rate constant from maternal to placental compartment (h$^{-1}$); $K_{21}$, efflux rate constant from placental to maternal (h$^{-1}$); $K_{23}$, efflux rate constant from placental to fetal compartment (h$^{-1}$); $K_{32}$, influx rate constant from fetal to placental compartment (h$^{-1}$).

5.4 Results

5.4.1 Ex vivo placenta perfusion

A total of 13 cotyledons from different placentae were perfused with dabigatran (n=3), rivaroxaban (n=5) or apixaban (n=5) (Bapat et al. 2014, 2015, 2016). The distribution of the anticoagulants in the fetal and maternal circulations during the 3-hour perfusion is shown in Figure 5.2. After the 3-hour perfusion, the F:M drug concentration ratios (median and IQR) of dabigatran, rivaroxaban or apixaban were 0.33 (IQR: 0.29–0.38), 0.69 (IQR: 0.58–0.73), and 0.77 (IQR: 0.76–0.81), respectively (Bapat et al. 2014, 2015, 2016).
Figure 5.2. Fetal-to-maternal (F:M) drug concentration ratios for apixaban (n=5), rivaroxaban (n=5) and dabigatran (n=3) during the 3-hour experimental phase of the placenta perfusion. Data are shown as the median and interquartile range at each time point.

After adjusting the experimental F:M ratio to account for differences in plasma protein binding and pH in the maternal and fetal circulation, the predicted F:M ratios *in vivo* of dabigatran and rivaroxaban were 0.39 (IQR: 0.34–0.44) and 0.81 (IQR: 0.68–0.86). For apixaban, the predicted F:M ratio *in vivo* ranged from 0.35–0.90, as described in Chapter 4 (section 4.4). The experimental and predicted F:M ratios at 3 hours are shown in Figure 5.3.

A Kruskal-Wallis test was conducted to determine if there were differences in the experimental and predicted F:M ratios between drug groups. The median experimental and predicted F:M ratios were statistically significantly different between the different drug groups ($\chi^2 = 6.752$,
Pairwise comparisons were performed using Dunn’s procedure with a Bonferroni correction for multiple comparisons. This post-hoc analysis revealed statistically significant differences in median F:M ratios between dabigatran and apixaban (p=0.034), but not between dabigatran and rivaroxaban or rivaroxaban and apixaban.

Figure 5.3. Placenta perfusion and predicted fetal-to-maternal (F:M) drug concentration ratios for dabigatran (n=3), rivaroxaban (n=5) and apixaban (n=5) after 180-min perfusion. Placenta perfusion F:M ratios were obtained directly from experiments, and the predicted F:M ratios were calculated using the Garland equation (1998) with albumin as the sole binding protein. Data are shown as the median and interquartile range at each time point.
5.4.2 Model analysis

The concentration profiles of rivaroxaban, apixaban and dabigatran in the maternal and fetal reservoir were adequately described using a three-compartment transplacental pharmacokinetic model. The observed and simulated concentrations for the \textit{ex vivo} human perfusion model are shown in Figure 5.4, and the estimated transfer parameters of these drugs are presented in Table 5.1. The estimated rate constants for placental-to-maternal \((K_{21})\) and maternal-to-placental \((K_{12})\) transfer were higher for rivaroxaban and apixaban, compared to that of dabigatran. On the other hand, the rate constants for placental-to-fetal \((K_{23})\) and fetal-to-placental \((K_{32})\) transfer were higher in dabigatran than the other 2 drugs.

\textbf{Table 5.1. Pharmacokinetic parameters of transplacental transfer of rivaroxaban, apixaban and dabigatran.}

<table>
<thead>
<tr>
<th>Drug</th>
<th>(V_m) (L)</th>
<th>(V_f) (L)</th>
<th>(K_{12}) (h(^{-1}))</th>
<th>(K_{21}) (h(^{-1}))</th>
<th>(K_{23}) (h(^{-1}))</th>
<th>(K_{32}) (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rivaroxaban</td>
<td>0.25</td>
<td>0.15</td>
<td>1.37</td>
<td>2.69</td>
<td>0.52</td>
<td>0.49</td>
</tr>
<tr>
<td>Apixaban</td>
<td>0.25</td>
<td>0.15</td>
<td>4.28</td>
<td>3.10</td>
<td>0.30</td>
<td>1.20</td>
</tr>
<tr>
<td>Dabigatran</td>
<td>0.25</td>
<td>0.15</td>
<td>0.24</td>
<td>0.31</td>
<td>1.56</td>
<td>4.20</td>
</tr>
</tbody>
</table>

\(V_m\), volume of distribution in maternal compartment (L); \(V_p\), volume of distribution in placental compartment (L); \(V_f\), volume of distribution in fetal compartment (L); \(K_{12}\), influx rate constant from maternal to placental compartment (h\(^{-1}\)); \(K_{21}\), efflux rate constant from placental to maternal (h\(^{-1}\)); \(K_{23}\), efflux rate constant from placental to fetal compartment (h\(^{-1}\)); \(K_{32}\), influx rate constant from fetal to placental compartment (h\(^{-1}\)).
Figure 5.4. Concentration-time profiles for dabigatran, rivaroxaban and apixaban in maternal and fetal circulations. Lines represent the simulated drug concentrations, and circles represent mean drug concentrations from placenta perfusion experiments.
5.5 Discussion

The results of our study demonstrate the transplacental transfer of dabigatran, rivaroxaban, and apixaban in full-term human placentae, after adjusting for protein binding. In general, we noticed that our predicted in vivo F:M ratios were about 15% higher than the experimental F:M ratios, which highlights the importance of accounting for protein binding in the placenta perfusion. Other groups have added plasma proteins directly to the perfusate, but it can be difficult to mimic exact physiological conditions (Johnson et al. 1999; Schenker et al. 1999; Gavard et al. 2006; Nanovskaya et al. 2009; Hutson et al. 2011). The equation developed by Garland (1998) is an alternate method of accounting for differences in plasma protein concentrations and pH between mother and fetus, and has previously been used as a means of adjusting perfusion data to better predict in vivo placental drug transfer (Hutson et al. 2011).

Rivaroxaban and apixaban have similar physicochemical characteristics, in terms of molecular size (435 and 459 Da) and ionization, and this may explain their similar patterns of placental transfer shown in Figure 2. Dabigatran showed the least amount of placental transfer, likely due to its increased lipophilicity and relatively larger molecular size (628 Da).

Another important consideration in the disposition of drugs across the placenta is plasma protein binding, as the unbound form of the drug can equilibrate across the placenta. The concentrations of albumin and α-1 acid glycoprotein (AAG) differ in fetal and maternal plasma, and this can affect total drug concentrations (bound + unbound) in each compartment at equilibrium. Throughout pregnancy, maternal albumin levels gradually decrease and fetal albumin levels tend to increase, with the fetal-to-maternal albumin ratio increasing from 0.28 in the first trimester to 1.20 closer to term (Krauer et al. 1984). As well, there is a 3-fold increase in the concentration of free fatty acids in the maternal circulation that can displace drugs from binding to maternal albumin (Ridd et al. 1983; Nau et al. 1984). This can lead to increased binding of a drug to fetal albumin, which can, in turn lead to increased placental drug transfer and possibly fetal drug accumulation. By comparison, AAG levels remain relatively constant in maternal plasma, and fetal levels gradually increase throughout pregnancy, with the AAG F:M ratio increasing from 0.09 in the first trimester to 0.37 at term (Krauer et al. 1984). The major binding protein for dabigatran and rivaroxaban is albumin, and this may explain why the predicted F:M ratios in vivo were 15% higher than the ratio obtained from the experimental observations in our placenta
perfusion model of unbound drug transfer. The predicted in vivo F:M ratio for apixaban was estimated to be between 0.35 and 0.90, based on binding solely to AAG or albumin. We speculate that the in vivo F:M ratio is likely much closer to the albumin-based estimate of 0.90, as apixaban is primarily bound to albumin.

To date, there have been no reported cases of dabigatran and apixaban exposure in human pregnancy. A case report described the use of rivaroxaban up to 19 weeks gestation. A male infant was born at 40 weeks gestation with no complications. The authors reported a small baby for weight, length, and cranial circumference (13th, 30th, and 7th percentiles, respectively), but likely attributed this to maternal smoking (Konigsbrugge et al. 2014). Recently, there was a case series of 37 women who were inadvertently exposed to rivaroxaban in the first and second trimester. There was one case of a conotruncal cardiac defect in a woman with several co-medications and a previous fetus with a cardiac malformation in the absence of rivaroxaban exposure (Hoeltzenbein et al. 2016). Placental drug transfer of rivaroxaban was not measured in these cases. These cases reiterate the notion that as prescriptions rates of NOACs continue to increase (Desai et al. 2014), the number of women with an unplanned pregnancy who are taking a NOAC will also likely increase.

The three-compartment pharmacokinetic model adequately described the concentration-time profiles for NOACs in the placenta perfusion experimental system. This model estimated the influx and efflux rate constants between maternal, placental and fetal compartments. In particular, the efflux rate constants from the placenta to maternal compartment \((K_{21})\) were similar for apixaban and rivaroxaban at 2.69 and 3.10 h\(^{-1}\), which were higher than that of dabigatran (0.31 h\(^{-1}\)). Previous studies have shown that apixaban and rivaroxaban are substrates of the transporters, P-gp and BCRP (Gnoth et al. 2011; Gong et al. 2013; Zhang et al. 2013). As these transporters are expressed in the maternal interface of the placenta, where they move substances from the placenta to maternal circulation, the differences in the estimated \(K_{21}\) are consistent with their substrate status for these transporters. Interpretation of the other rate constants awaits further studies on expression, localization, and substrate specificity of other transporters in the placenta.

In conclusion, the in vivo fetal levels of dabigatran, rivaroxaban and apixaban were predicted to be 39%, 80%, and 90% of corresponding maternal levels at steady state. We noted that in vivo
estimates of the F:M ratio were 15% higher than what was shown in placenta perfusion experiments, and this was primarily attributed to plasma protein binding in vivo. Furthermore, we developed a pharmacokinetic model that was able to describe the concentration-time profiles of placental transfer of NOACs at term, and estimate transfer parameters. Our modeling approach provides another example of proof-of-principle demonstration, which will lay a foundation for physiologically-based pharmacokinetic modeling in pregnancy.
Chapter 6

Evaluating the success rate of the ex vivo dual perfusion of the human placenta.

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\textbf{Contribution of this thesis author (P. Bapat):} I was part of the collaborative team to design the research study. I collected the data from all placenta perfusion experiments, analyzed the data and drafted the manuscript. All authors provided input and approved the final version of the manuscript.
6 Evaluating the success rate of the ex vivo dual perfusion of the human placenta.

6.1 Abstract

**Background:** The dual perfusion of a single placental cotyledon *ex vivo* has been widely used among placental researchers to study placental transfer in organized human placental tissue. While this model can be highly predictive, a major challenge of this model is its low overall success rate, which can result in high costs and more time to complete a study. The method is technically challenging, and there are several criteria during the perfusion procedure that must be met in order to have a successful experiment. The objective of this study was to evaluate the success rate at several stages throughout the dual perfusion of an isolated placental cotyledon.

**Methods:** Placentae were collected with informed consent following caesarean or vaginal delivery of uncomplicated term pregnancies for a 3-hour perfusion of various test substances. Upon completion of each experiment, placentae were assigned one of six checkpoints corresponding to important stages of the placenta perfusion protocol. **Results:** 297 women provided written consent to participate in our study over a 20-month period, and of these, 251 placentae were collected. 99 placentae did not have an adequate fetal artery-vein pair for cannulation, and 113 placentae were prematurely terminated due to a fetal reservoir leak. The maternal circulation was established in the remaining 39 placentae, and of these, 25 placentae were successfully perfused for 3 hours. This corresponds to an overall success rate of 9.96%.

**Conclusion:** The results of our study show an overall success rate of 10% for the dual perfusion of an isolated human placental cotyledon *ex vivo*. By using the proposed checkpoints, we identified that setting up the fetal circulation is a critical stage in determining experimental success, and that this stage may be in need of technical improvement.
6.2 Introduction

The placenta is a complex organ that is responsible for nourishing and maintaining the fetus via the umbilical cord. The placenta is made up of vascular tissue in which oxygen and nutrients can pass from maternal to fetal circulation, while carbon dioxide and waste products can pass in the opposite direction (Syme et al. 2004). The placental functional unit is a lobule, termed cotyledon, and each cotyledon is independently perfused by maternal and fetal vessels. A typical human placenta is made up of 20-40 cotyledons (Syme et al. 2004).

The placenta also plays a major role in determining fetal drug exposure. It is possible to estimate total fetal drug exposure by measuring the amount of drug that crosses the placenta. Substances can cross the placenta via passive or active transport, as the placenta highly expresses transporters such as P-glycoprotein and breast cancer resistance protein (Ceckova-Novotna et al. 2006; Iqbal et al. 2012). Although animal studies are often conducted to study placental transfer of various compounds, it is difficult to extrapolate these results to humans because of species-differences in blood flow pattern, as well as biochemical and anatomical structure of the placenta (Leiser & Kaufmann 1994; Ala-Kokko et al. 2000). Also, animal studies are often performed at doses that are much higher than the relevant pharmacokinetic human doses (Brent 2004).

The dual perfusion of a single placental cotyledon *ex vivo* has been widely used among placental researchers to study placental transfer in organized human placental tissue (Hutson et al. 2011). The placental perfusion model avoids several issues that are associated with other placental techniques (e.g. ethical implications of *in vivo* human studies, physiological discrepancies in cell cultures) (Hutson et al. 2011). This model has previously been used to investigate the placental transfer of endogenous and exogenous substrates, such as amino acids, hormones, electrolytes, viruses, therapeutics, and illicit drugs (Omarini et al. 1992; Muhlemann et al. 1995; Ala-Kokko et al. 2000; Malek et al. 2009; Bapat et al. 2015). A systematic review of this model found that fetal-to-maternal drug concentration ratios from perfusion experiments matched well with umbilical cord-to-maternal blood ratios collected at the time of delivery. The authors concluded that the placenta perfusion model can reliably be used to predict placental transfer of small molecule drugs *in vivo* (Hutson et al. 2011).

A major challenge of this model is its low overall success rate, which can result in high costs and more time to complete a study. Different perfusion laboratories report success rates between 5
and 20% (Mathiesen et al. 2010; Grafmüller et al. 2013), and in general, 4-6 successful perfusions are ideally needed to establish transfer. The method itself is technically challenging, and there are several criteria during the perfusion procedure that must be met in order to have a successful experiment. The objective of this study was to evaluate the success rate at several stages throughout the dual perfusion of an isolated placental cotyledon. Information on the success rate at different stages can identify which stages of the protocol are in need of technical improvement.

6.3 Methods

A full description of the placenta perfusion method is presented in section 2.2. Term placentae were collected from vaginal or caesarean deliveries to examine the placental transfer of various medications (including those described in Chapter 2–4). Upon completion of each experiment, placentae were assigned one of six checkpoints (Figure 6.1), modified from Mathiesen et al (Mathiesen et al. 2010) and Karttunen et al (Karttunen et al. 2015). Depending on the stage at which the experiment was terminated, experiments were assigned to:

- **Checkpoint 0**: unable to obtain placentae after delivery
- **Checkpoint 1**: unable to set up successful fetal circulation (no adequate fetal artery/vein pair)
- **Checkpoint 2**: fetal circulation was established, and cotyledon was isolated and attempted to set up maternal circulation (fetal reservoir leak was greater than 4 mL/hr)
- **Checkpoint 3**: maternal circulation was established, and control period of perfusion was started
- **Checkpoint 4**: drug of interest was added and experimental period of perfusion was started
- **Checkpoint 5**: successful experiment with final antipyrine F:M ratio above 0.75 and fetal reservoir volume loss less than 4 mL/hr.

The amount of placentae (%) at each checkpoint was calculated in relation to the total number of placentae obtained, and our overall success rate was calculated. Maternal and gestational ages are presented as mean ± standard deviation, and differences between groups were determined using a student’s T-test. Any p-values less than 0.05 were considered as statistically significant.
Figure 6.1. Placenta perfusion protocol divided into various steps. Checkpoints represent the point at which an experiment was discontinued.
6.4 Results

In total, 297 women provided written consent to participate in our studies over a 20-month period, and of these, 251 placentae were collected from caesarean or vaginal deliveries. At the time of delivery, the mean maternal age was 34.2 ± 5.0 years and mean gestational age was 39.1 ± 0.7 weeks. The majority of placentae collected were obtained from caesarean delivery (85%).

46 placentae were not obtained due to scheduling issues (ie. if the delivery occurred outside of working hours), or if the participant was part of another research study requiring collection of the whole placenta (Checkpoint 0). If multiple deliveries occurred within a short time period, we were only able to collect one placenta as our currently laboratory setup can accommodate one placenta at a time, and the protocol requires the fetal circulation to be established within 15 minutes of delivery.

Of the 251 collected placentae, 99 did not have a suitable artery/vein pair and/or the maternal decidual plate was not intact, and the fetal circulation could not be established (Checkpoint 1). The fetal circulation was set up in a closed, recirculating configuration in the remaining 152 placentae. The experiment was prematurely terminated in 113 placentae primarily due to a leak in the fetal reservoir greater than 4 mL/hr, or if the fetal arterial inflow pressure deviated from 30–60 mmHg for an extended period of time (Checkpoint 2). The maternal circulation was successfully established in 39 experiments. At this point, both the maternal and fetal perfusates were replaced with fresh perfusate and the 1-hour control period was started. 12 placentae experienced a major leak (>4 mL/hr) during the control period and were immediately terminated (Checkpoint 3). In 27 experiments, there were no issues experienced in the control period. The maternal and fetal perfusates were replaced again and the drug of interest was added to start the experimental period. There was a leak in 2 of these experiments which were prematurely terminated (Checkpoint 4), and 25 placentae were perfused for the full 3-hour period (Checkpoint 5). This corresponds to an overall success rate of 9.96% (25 successful experiments from 251 collected placentae). A summary of this process is shown in Figure 6.2.
Figure 6.2. Flow diagram showing the number of placentae at each stage of the perfusion protocol.
The overall success rates for placentae collected from caesarean and vaginal and delivery were similar at 10.28% and 8.11%, respectively. While the mean maternal age was similar between caesarean and vaginal delivery groups, the gestational age was higher in the vaginal delivery group compared to caesarean delivery (39.7 ± 1.01 vs 39.1 ± 0.6 weeks, respectively; \( P = 0.004 \)).

After obtaining the tissue, the fetal circulation was established in 61\%(152/251) of collected placenta. This corresponds to 39\%(99/251) of collected placentae being discarded between Checkpoints 0 and 1. After passing Checkpoint 1, a large proportion of these placentae (74\%; 113/152) experienced a major leak and the experiments were terminated before the maternal circulation was established (between Checkpoint 1 and 2). Once the maternal circulation was established and the control period was started, approximately 70\%(27/39) of the placentae did not experience a leak. This corresponds to a 30\% termination rate between Checkpoint 2 and 3. After starting the 3-hour experimental period, 93\%(25/27) of placentae did not experience a major leak and were considered ‘successful’ experiments overall. The success rate at each checkpoint in relation to the total number of collected placentae is shown in Figure 6.3. The maternal and gestational ages were similar between ‘unsuccessful’ (Checkpoints 1-4) and ‘successful’ (Checkpoint 5) experiments.
Figure 6.3. Success rate (%) at each checkpoint of the placenta perfusion, calculated in relation to the total number of placentae collected (n=251).

6.5 Discussion

The results of our study show an overall success rate of 10% for the dual perfusion of an isolated human placental cotyledon ex vivo. While this rate was similar to reported success rates from other perfusion laboratories, we evaluated the success rate at various checkpoints throughout the experiment. The highest proportion of experiments was terminated between Checkpoints 0 and 2, suggesting that this stage is in need of improvement. After obtaining the tissue, the quality of the placenta was initially accessed by looking for a fetal artery/vein pair and signs of physical trauma and/or calcifications in the tissue. In many instances, there was an adequate fetal artery/vein pair, but the corresponding maternal side of the placenta had many tears and abrasions. Complex branching of the fetal arteries and veins also made it difficult to identify which artery/vein pair supplied a specific cotyledon.
After the fetal vessels were cannulated, a majority of experiments (75%) were prematurely terminated due to a major leak in the fetal reservoir (>4 mL/hr). The period between Checkpoint 1 and 2 includes several important steps such as cutting the surrounding areas of the cotyledon to remove excess tissue, clamping the cotyledon in the perfusion chamber and inserting maternal cannulae into the intervillous space to establish the maternal circulation. Previous studies have suggested that cutting the excess tissue and clamping the unperfused area surrounding the cotyledon can disrupt the closed fetal circulation (Miller et al. 2003; Myllynen et al. 2008; Mathiesen et al. 2009).

Only 25% of the placentae that were cannulated on the fetal side were cannulated on the maternal side as well. Given that one of the termination criteria is a fetal reservoir leak greater than 4 mL/hr, this suggests that cannulating fetal vessels and establishing a closed fetal circuit are important early steps in the experiment. Other perfusion laboratories accept fetal reservoir leaks from 2 to 4 mL/hr (Schneider & Huch 1985; Miller et al. 2003; Myllynen et al. 2003; Mathiesen et al. 2009). Using more stringent criteria for an acceptable leak (2 mL/hr) may help in identifying unsuccessful perfusions earlier, which can save time and valuable lab reagents. Interestingly, if an experiment reached the control period without any issues, the likelihood of having a successful experiment increased, as the proportion of terminated experiments decreased after Checkpoint 3 (see Figure 3). Once the 3-hour experimental period was started, the likelihood of a successful experiment dramatically increased to 93%.

In our study, the vast majority of collected placentae were obtained from caesarean deliveries. One explanation for this could be due to timing of delivery, as the majority of placentae were collected from scheduled caesarean sections. This allowed for appropriate planning and access to tissue during regular working hours. The timing of vaginal deliveries was highly variable and unpredictable in many instances. A previous study showed that placentae delivered vaginally displayed higher levels oxidative stress, inflammatory cytokines, and angiogenic regulators compared to placentae delivered by caesarean section (Cindrova-Davies et al. 2007). The authors suggested that a placenta subjected to labour does not reflect the normal in vivo state at a molecular level, and that a placenta from a caesarean section is a better representation of the functional term placenta. However, previous perfusion studies have shown that mode of delivery does not affect the transfer of antipyrine (Mathiesen et al. 2010; Mose et al. 2012) and the integrity of the placenta as measured by fetal reservoir volume loss during perfusions (Karttunen
et al. 2015). While the number of placenta collected from vaginal and caesarean deliveries differed, the overall rates of success were similar for both modes of delivery.

There are several challenges with the placenta perfusion model. Due to its low overall success rate, a large supply of placentae is needed complete a transplacental transfer study, which typically requires access to a large obstetric ward. Our perfusion laboratory was ideally located on the Labor and Delivery floor at St. Michael’s Hospital, and this minimized the time needed to transport the placenta to the laboratory after delivery. The obstetricians and nursing staff also need to be informed about this study in order to facilitate tissue collection. Additionally, researchers need to be trained with the technical skills and knowledge in order to generate reproducible results between experiments, and there can be high initial costs to set up a perfusion laboratory. Despite these challenges, the placenta perfusion is the only experimental model that can be used to study placental transfer of substances in organized human placental tissue (Hutson et al. 2011).

Furthermore, there is no standardized placenta perfusion protocol, making it difficult to directly compare results between laboratories. Most groups agree that measuring fetal reservoir volume loss and using a reference compound, such as antipyrine, are important when judging a successful experiment (Mathiesen et al. 2010; Karttunen et al. 2015). It has also been suggested that measuring oxygen transfer from maternal to fetal circulation is a good indication of the overlap between the circulations, and also indicates the oxygen state of the tissue (hypoxic vs hyperoxic) (Wier & Miller 1985). Differences in cannulation and perfusion techniques, and perfusate composition can affect the success rate, as well as the transplacental transfer of various compounds. A study by Mathiesen et al. showed that adding albumin to perfusate increased the placental transfer of benzo(a)pyrene in the placenta perfusion model (Mathiesen et al. 2009). By using the proposed checkpoints, we identified that the fetal cannulation is a critical stage in determining experimental success, and that this stage may be in need of technical improvement. Our lab currently uses metal cannulae fastened with surgical ligatures, while other groups have reported using umbilical catheters (Miller et al. 2003). If other perfusion studies report their success rates using these checkpoints, this can give insight into what changes can be made to improve the overall success rate of this model.
Chapter 7

7 General Discussion

7.1 Summary of Research Findings and Future Directions

Transplacental Transfer of Novel Anticoagulants

The use of newer medications in pregnant women is especially challenging, as there is typically no information about safety or efficacy in pregnancy. Clinical trials usually exclude pregnant women and ensure that women of childbearing potential do not become pregnant during the period of drug exposure. Several novel oral anticoagulants (NOACs) have been developed and approved for clinical use in recent years, and these medications have clear advantages over existing therapies. Dabigatran, rivaroxaban, and apixaban are three newer generation oral anticoagulants that can be used in the prevention and treatment of venous thromboembolism (VTE). However, the information regarding fetal safety and placental transfer of these drugs is currently unknown. As prescription rates for novel oral anticoagulants (NOACs) continue to increase, the number of women with an unplanned pregnancy who are taking NOACs will likely increase. We investigated the transplacental kinetics of novel anticoagulants using the placenta perfusion model.

• In the placenta perfusion model, unbound dabigatran crossed the term human placenta relatively slowly, reaching a fetal-to-maternal (F:M) ratio of 0.33 (IQR: 0.29–0.38) after 3 hours. Because dabigatran is administered as a prodrug, additional perfusions were conducted with its prodrug dabigatran etexilate mesylate. Dabigatran etexilate mesylate was found to have limited placental transfer, as evidenced by a F:M ratio of 0.17 (IQR: 0.15–0.17) after 3 hours.
• Dabigatran is highly polar and exhibits significant hydrogen bonding – both of which are characteristics that can reduce transport across the placental barrier. Therefore, the physicochemical properties of dabigatran may be able to explain its limited placental transfer shown in the placenta perfusions.
• In the placenta perfusion model, unbound rivaroxaban rapidly crossed the term human placenta, reaching a F:M ratio of 0.69 (IQR: 0.58–0.73) after 3 hours. This was likely
indicative of steady state, as this ratio remained relatively stable in the final hour of the experiment.

- Addition placental perfusions were conducted where rivaroxaban was added to the fetal reservoir only; these were important to understand the role of the placenta in clearing the drug from the fetal circulation. The final transfer ratio in these perfusions was 0.69 after 3 hours, suggesting that rivaroxaban interacts with the placent al barrier in a similar manner in both directions across the placenta. Under equilibrative conditions, rivaroxaban concentrations remained relatively constant, suggesting that rivaroxaban crosses the placenta down a concentration gradient via passive diffusion.

- In the placenta perfusion model, unbound apixaban rapidly crossed the term human placenta, reaching a F:M ratio of 0.77 (IQR: 0.76–0.81) after 3 hours. The placenta perfusion reached steady state, as concentrations in the maternal and fetal reservoirs remained relatively constant in the last hour of perfusion.

- Rivaroxaban and apixaban have very similar physicochemical characteristics, and therefore, it is likely that apixaban also crosses the term placenta via passive diffusion.

A limitation of this model is that only full-term placentae are used, and as such, we cannot make extrapolations about placental drug transfer in the first and second trimester of pregnancy. However, due to anatomical and physiological changes in placental structure, drug transfer at term may represent the highest level of fetal drug exposure compared to earlier gestational ages (Vahakangas & Myllynen 2006). Placentae were collected from healthy pregnancies, and women were excluded if they were taking any medications or if they had any illness during pregnancy. These exclusion criteria were set to ensure a large supply of placentae would be available for experiments. As well, if a woman had a major illness during pregnancy, the placenta was usually sent to the pathology department to be examined after delivery. This is an important limitation as certain disease states, including inflammation and infection, have an altered expression of drug transport proteins in the placenta (Wang et al. 2005; Camus et al. 2006; Mason et al. 2011; Petrovic et al. 2015), suggesting that healthy and disease-state placentae may be very different at the molecular and cellular level. In future studies, it would be interesting to determine if there are major differences in placental drug transfer between healthy and disease-state placentae. Given that certain disease states have an altered expression of placental drug transporters, it is possible that drugs that are substrates for these transporters will have an altered rate and extent of
placental drug transfer. It would be interesting to examine if certain disease states have altered placental transfer of drugs that cross the placenta via passive diffusion.

Placental metabolism can also affect the extent of fetal drug exposure. Compared to the liver, the activities of drug metabolizing enzymes in the placenta are relatively low (Hakkola et al. 1996). Despite this, placental metabolism can result in metabolites with toxic potential entering the fetal circulation system. The expression of these enzymes is depending on the developmental stage of the placenta (Myllynen et al. 2007). For phase I enzymes, CYP 1A1 is the predominant isoform present in the placenta, and it is expressed throughout gestation (Myllynen et al. 2009). CYP 1A2 mRNA has been detected in the first trimester placenta, but not at term. The functional activities of CYP 2C, CYP 2D6, and CYP 3A have not been reported (Syme et al. 2004; Myllynen et al. 2009). For phase II enzymes, UGTs are expressed throughout gestation, and have an important role in placental metabolic activity (Syme et al. 2004). Dabigatran is administered as the prodrug, dabigatran etexilate mesylate, which is rapidly converted to the active drug via plasma esterases. In the prodrug-only perfusions, the active drug was not detected in perfusate samples, indicating that the placentae did not metabolize and activate the prodrug. In our perfusion experiments with rivaroxaban and apixaban, we did not measure their respective metabolites. However, it should be noted that neither of these drugs have active metabolites.

During placenta perfusion experiments, many parameters were measured as markers of placental viability and quality control. These parameters included arterial inflow pressure in the fetal circuit, fetal reservoir volume (leak), and circuit flow rates, which were measured throughout the experiment. An on-site blood gas analyzer provided real-time measurements of O2 and CO2 content, pH, glucose, and lactate levels in the artery and vein of maternal and fetal circulations. Samples for antipyrine transfer and hCG secretion were analyzed after the experiment was complete. Prior to adding our drug of interest to the system, there was a 1-hour control period to ensure the perfusion system was working optimally, and to ensure that markers of placental viability were within acceptable ranges.

We noted a large variability between oxygen transfer, delivery, and consumption in the different sets of perfusions. While the oxygen data is corrected to account for the weight of the cotyledon, the physical surface area of the perfused cotyledon was not measured. Gas exchange occurs at the syncytiotrophoblast layer, and it is possible that certain cotyledons had a larger surface area.
for this layer. While it may not be feasible to measure the surface area of the syncytiotrophoblast layer in our perfused cotyledon, the correlation between cotyledon weight and surface area of the syncytiotrophoblast layer may require further evaluation. The physical surface area of the cotyledon may be a better representation of the surface area of the syncytiotrophoblast layer. As well, oxygen was delivered via two cannulas, which serve as maternal arteries. There could have been better oxygen and perfusate distribution if we increased the number of maternal cannulas. It is possible that rivaroxaban had an impact on the syncytiotrophoblast at the cellular level, and this may explain why oxygen consumption is much lower in placenta perfusions with rivaroxaban than those with dabigatran or apixaban. However, intra-experimental variability is an important concern, and major changes in viability markers between the control period and experimental period can indicate that the perfusion system may have malfunctioned, or that the perfused drug may be having an effect on the placental tissue. In our experiments, we did not find significant differences between the control and experimental periods for oxygen transfer, delivery, and consumption.

During pregnancy, hCG is critically important in maintaining the pregnancy, regulating blood flow to the fetoplacental unit, and delivering nutrients to the fetus (Cole 2010). In our placenta perfusion experiments, the secretion of hCG is an important marker of placenta viability. Between individual experiments, there was high variability in hCG secretion rates. Similar to oxygen parameters, hCG secretion is adjusted for cotyledon weight. It is possible that hCG secretion is not directly proportional to cotyledon weight, and that a different adjustment should be applied to the results. As well, hCG levels were measured using ELISA kits, and these kits can be subject to high variability. In the future, it is advisable to measure multiple samples using the same standards and quality control samples, and to ensure that all ELISA kits were from the same batch or lot number from the manufacturer. A previous study concluded that formic acid is placentotoxic as placental hCG secretion decreased between the control and experimental periods (Hutson et al. 2013). We did not have similar differences in our experiments, and cannot conclude if any of the studied NOACs are placentotoxic.

While the placenta perfusion model can be highly predictive of placental transfer in vivo, a limitation of this model is that it cannot account for differences in protein binding between maternal and fetal plasma. A recent study has showed that placenta perfusion data can be adjusted in order to predict the F:M ratio of total (bound plus unbound) drug concentration in
plasma *in vivo*. After adjusting for protein binding differences, the authors noted a stronger correlation between placental perfusion and *in vivo* placental transfer (Hutson et al. 2011). Dabigatran exhibits low protein binding, while rivaroxaban and apixaban remain highly bound to plasma proteins *in vivo*. We compared the placental transfer of these novel anticoagulants, after adjusting for differences in protein binding, and developed a transplacental pharmacokinetic model to estimate transfer parameters.

- After adjusting for differences in protein binding, our *in vivo* estimates of the F:M ratio were 15% higher than what was shown in placenta perfusion experiments. The *in vivo* fetal levels of dabigatran, rivaroxaban and apixaban were predicted to be 39%, 80%, and 90% of corresponding maternal levels at steady state. Since only the unbound form of the drug can cross the placenta, it is possible that our predictions are an overestimation of the fetal levels of rivaroxaban and apixaban, as these drugs remain highly bound to plasma proteins in maternal blood.

- We developed a pharmacokinetic model that was able to describe the concentration-time profiles of placental transfer of novel anticoagulants at term, and estimate transfer parameters. There was a higher efflux rate from the placenta to the maternal compartment for rivaroxaban and apixaban, and this may be explained by their interactions with placental drug transporters (P-gp and BCRP).

As with any medication in pregnancy, the use of anticoagulant therapy can be challenging because of the possibility for both maternal and fetal complications. It is important to weigh the maternal benefits of the drug against the potential fetal risks, and consider the potentially deleterious effects of untreated maternal illness. The use of anticoagulants is especially challenging because of the hypercoagulability status of the mother, compared to the hypocoagulable state of the fetus.

Pregnancy itself is a risk factor for VTE, and physiological changes in pregnancy suggest that women may require higher doses of an anticoagulant to achieve therapeutic levels of a drug. In this case, the fetus could be at increased risk for bleeding complications. The risk-benefit ratio is important to consider, as untreated VTE may lead to catastrophic outcomes. In fact, VTE, which consists of deep vein thrombosis and pulmonary embolism, is one of the most common causes of maternal mortality in Canada and the United States.
To date, there have been no reported cases of dabigatran and apixaban exposure in human pregnancy. Recently, there have been a few published case reports and a case series describing the use of rivaroxaban in the first and second trimesters (Konigsbrugge et al. 2014; Hoeltzenbein et al. 2016; Myers et al. 2016). Placental drug transfer of rivaroxaban was not measured in these cases. As prescription rates for novel anticoagulants continue to rise, the number of women who become pregnant while taking one of these medications will also likely increase. For this reason, it is important to study their placental transfer, in order to estimate the level of fetal drug exposure. To our knowledge, our studies are the first to examine the transplacental pharmacokinetics of dabigatran, rivaroxaban, and apixaban in term human placentae. We noted that rivaroxaban and apixaban have comparable patterns of placental transfer, and this is likely explained by their similar physicochemical characteristics, in terms of molecular size (435 and 459 Da, respectively) and ionization status at physiological pH. Dabigatran showed the lowest amount of placental transfer, likely due to its increased lipophilicity and relatively larger molecular size (628 Da).

Low molecular weight heparins (LMWH) still remain the gold standard for use in pregnant women requiring anticoagulation therapy (Bates et al. 2012). However, a recent open-label, randomized trial comparing antepartum prophylactic dalteparin (LMWH) versus no dalteparin found that dalteparin did not reduce the occurrence of VTE, pregnancy loss, or placenta-mediated pregnancy complications, including severe pre-eclampsia, small-for-gestational-age infants, and placental abruption, in pregnant women with thrombophilia who were at high risk of these complications (Rodger et al. 2014). It should be noted that LMWH do not cross the placenta in the second and third trimester (Forestier et al. 1984, 1987).

Many medications used in pregnancy are prescribed ‘off-label’, as drug manufacturers commonly exclude pregnant women from clinical trials when seeking to obtain approval from regulatory agencies. For commonly used medications, there are typically few (if any) controlled studies in pregnancy, and their use in pregnancy usually falls outside of the terms of the drug license. Medication use in pregnancy can depend on a variety of factors, such as the indication of the drug, the dose, route of administration, elimination half-life, duration of treatment, and the gestational age of the pregnancy. Case reports and case series can provide valuable, if anecdotal, information regarding pregnancy outcomes. Prospective, observational cohort studies can provide higher quality evidence to examine the safety and efficacy of a drug in pregnancy, but
these cannot achieve the same rigor and high quality of randomized controlled trials. For newer medications with no published case reports, animal studies can provide potentially useful information about reproductive and fetal toxicity. These animal studies should be conducted at doses that are comparable to human levels, but inter-species differences in placental structure must be considered. Information regarding placental transfer can help estimate fetal drug exposure, but this simply provides information for one piece of a very complicated puzzle.

The results of our placenta perfusion are considered to be ‘preclinical’, and perfusion data alone are not enough to make a recommendation about the use of a medication in pregnancy or change clinical practice guidelines. Future studies are needed to examine the safety and efficacy of these novel anticoagulants in pregnant women. As well, with more women becoming inadvertently exposed to novel anticoagulants in pregnancy, we urge clinicians to continue publishing these as case reports and case series. Data accumulating in large drug registries measuring pregnancy outcomes over the next few years will provide valuable insights. Another interesting area to consider is the use of these anticoagulants in lactating women, as the extent of NOAC excretion in breastmilk is not currently well defined. The risk for VTE is substantially higher in the postpartum period (Heit et al. 2005; Pomp et al. 2008), and therefore, women may require the use of anticoagulation therapy while they are breastfeeding.

We developed a pharmacokinetic model to describe the concentration-time profile of the placental transfer of novel anticoagulants using our perfusion data. While our model still needs to be validated, it allowed for the estimation of transfer parameters, which may be able to explain the observed pattern of drug transfer. Pharmacokinetic and pharmacodynamic models have widely been used to estimate drug disposition and clearance in non-pregnant populations. To date, only a few studies have used pharmacokinetic modeling to examine placental transfer and fetal safety. One study developed a pharmacokinetic model that used transfer parameters from placental perfusions and pharmacodynamic data from animal studies to quantitatively predict the human fetal toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs) (Shintaku et al. 2012). This study demonstrates that pharmacokinetic modeling is a powerful tool that can be used to examine fetal drug safety. In future studies, it may be interesting to combine animal data with our pharmacokinetic model of placental transfer to describe the whole-body pharmacokinetic profiles of the mother and fetus, and predict possible adverse effects of novel anticoagulants. Pharmacokinetic modeling may be another important tool that can be used in conjunction with
the placental perfusion model to provide safety data for new medications where no safety data in pregnancy is available.

**Evaluation of the Placenta Perfusion Model**

The dual perfusion of a single placental cotyledon *ex vivo* has been widely used among placental researchers to study placental drug transfer in organized human placental tissue. This model can be highly predictive of *in vivo* placental transfer, yet a major challenge of this model is its low overall success rate. This can result in high costs and longer time to complete a study examining placental drug transfer. The method is technically challenging, and there are several criteria during the perfusion procedure that must be met in order to achieve a successful experiment. We evaluated the success rate of the placental perfusion model at several stages throughout the experiment.

- We report an overall success rate of 10% for the dual perfusion of an isolated human placental cotyledon *ex vivo*, which is similar to reported success rates from other perfusion laboratories.
- By using the proposed checkpoints, we identified that cannulation of the fetal artery-vein pair is a critical stage in determining experimental success, and that this stage may be in need of technical improvement. Our lab currently uses metal cannulae fastened with surgical ligatures, while other groups have reported using umbilical catheters (Miller et al. 2003).
- While the number of placentae collected from vaginal and caesarean deliveries differed, the overall rates of experimental success were similar using placentae from either mode of delivery.

There are several challenges with the placenta perfusion model. Due to its low overall success rate, a large supply of placentae is needed to complete a transplacental transfer study, and this typically requires access to a large obstetrical ward. Our perfusion laboratory was ideally located on the Labor and Delivery ward of a tertiary care centre, and this reduced the time needed to transport the placenta to the laboratory after delivery. We were fortunate to have an enthusiastic obstetrical team who were well informed about this study, and helped to facilitate tissue collection in a timely and efficient manner. Additionally, researchers were trained with the technical skills and requisite knowledge, and were able to generate reproducible results between
experiments. Despite these challenges described above, the placenta perfusion is an important experimental model that can be used to study placental drug transfer (Hutson et al. 2011).

Currently, there is no standardized placenta perfusion protocol, making it difficult to directly compare results between laboratories. Differences in cannulation and perfusion techniques, and perfusate composition can affect the success rate, as well as the transplacental transfer of various compounds. Most groups agree that measuring fetal reservoir volume loss and using a reference compound, such as antipyrine, are important when judging a successful experiment (Mathiesen et al. 2010; Karttunen et al. 2015). One way to compare experimental data between laboratories would be to normalize the perfusion data of a specific drug to antipyrine. It has also been suggested that measuring oxygen transfer from maternal to fetal circulation is a good indication of the overlap between the circulations, and also indicates the oxygen state of the tissue (Wier & Miller 1985). Mathiesen et al. (2010) have also proposed a list of criteria to be included in perfusion publications and have recommended that each laboratory publish a detailed methods paper. Additionally, if other perfusion studies report their success rates using the proposed checkpoints, this can provide insight into potential changes that can be made to improve the overall success rate of this model. This can also be used to create a focused training program to help increase the overall success rate of this model and productivity of the perfusion lab.

The placenta perfusion model is currently used to evaluate the placental transfer of drugs that have already been approved by regulatory agencies for clinical use. In June 2015, the US Food and Drug Administration (FDA) implemented changes to drug labeling surrounding pregnancy and lactation. These changes were created to provide a more consistent format to present information about the risks and benefits of medications used during pregnancy and breastfeeding. The new rules replaced the product letter categories (A, B, C, D and X), which were formally used to classify the risk of using a medication during pregnancy. New drug labels will include 3 detailed subsections that describe risks within the real-world context of caring for pregnant women who may need medication. The new subsections will include information on medication use in pregnant women, lactating women, and men and women of childbearing potential. In particular, the Pregnancy subsection will be required to include relevant information, such as dosing and potential risks to the developing fetus. This section will also include information about a pregnancy registry. For many new medications, the Pregnancy subsection will not include a lot of information, as pregnant women are generally not included in
clinical trials, and women of childbearing potential must use contraception during the period of drug exposure. Nevertheless, with these changes, the FDA is recognizing the importance of studying medications in pregnancy, and we propose that placenta perfusion data be presented in the Pregnancy subsection of drug labels. Although the placenta perfusion cannot provide information about safety, it does provide important information regarding fetal drug exposure, which is one important consideration in fetal safety.
7.2 Overall Significance

Maternal health during pregnancy can be impacted by a large number of disease conditions, and these conditions are often treated using drugs with fetotoxic potential. Identifying which medications minimally cross the placenta is critical in being able to treat the mother while protecting the unborn. The ex vivo perfusion of the human placental cotyledon is a unique technique that separates the functions of the placenta from maternal or fetal influences. To our knowledge, this is the first ex vivo evidence of novel anticoagulant (rivaroxaban, dabigatran, apixaban) transfer across the human placenta. These results will increase our overall understanding of placental mechanisms involved in drug transfer, and will provide essential safety information for medication use during pregnancy. By mathematically adjusting the results to account for protein binding and pH differences between the mother and fetus, placenta perfusion data may accurately predict in vivo placental drug transfer. Although this perfusion model can be technically challenging, perfusion results strongly correlate with in vivo data. By evaluating the success rate of the perfusion experiments in our laboratory, we can determine which stages are most important and create a focused training program to help increase the overall success rate of this model and productivity of the perfusion lab.

As the use of medications continues to increase during pregnancy, it is important for medical scientists to examine the fetal risks and safety of various medications. This is a relatively understudied area of drug research, and in many instances, medications have very limited information about their use in pregnancy. Transplacental transfer studies, such as those presented in this thesis, can answer a very important question about the level of fetal drug exposure. While it is unethical to study drug safety in pregnant women, we can use a combination of in vitro and animal models, along with ex vivo placenta perfusions, to gain a better understanding about the use of a specific drug throughout pregnancy, and eventually make generalized conclusions and clinical recommendations. In terms of future drug discovery, we can use existing information to create new molecules, which are unlikely to cross the placenta, with the ultimate goal being to design medications that are safe to use in pregnancy.
Chapter 8
References


Appendices
Appendix A. Letter of Information and Consent Form

Letter of Information and Consent to Participate in a Research Study

Study Title: The Role of the Placenta in Fetal Pharmacology

Investigators:

On Site Principal Investigator:
Dr Howard Berger
Perinatalogist
Department of Obstetrics and Gynecology
St. Michael’s Hospital
Phone: (416) 867-7460 Ext. 8408
(Available Monday - Friday 9 am – 4 pm)

Off-Site Principal Investigator:
Dr Gideon Koren,
Director, The Motherisk Program
Division of Clinical Pharmacology and Toxicology
The Hospital for Sick Children
Phone: (416) 813-5781
(Available Monday – Friday 9 am – 4 pm)

Introduction:

Before agreeing to take part in this research study, it is important that you read the information in this research consent form. It includes details we think you need to know in order to decide if you wish to take part in the study. If you have any questions, please ask the study doctor or study staff to explain any words you don’t understand before signing this consent form. You will also have the opportunity to ask any additional questions on the day of surgery. Make sure all your questions have been answered to your satisfaction before signing this document.

All research is voluntary. You may also wish to discuss the study with your family doctor, a family member or close friend. It is important you know that this study is from the Hospital for Sick Children, and St. Michael’s Hospital is one of the sites where we are recruiting participants.

Conflicts of Interest

The principal investigators, Dr. Howard Berger and Dr. Gideon Koren, and the other research team members have no conflicts of interest to declare.
Sponsorship

The sponsor of this study is Dr. Gideon Koren and the Hospital for Sick Children. The funder of this research study is the Canadian Institutes of Health Research.

Background Information:

The placenta research laboratory at the Hospital for Sick Children is one of the few laboratories in North America currently studying drug transfer in the human placenta. The placenta is the special pregnancy tissue in the uterus that processes the mother's blood to give the growing baby nutrition and other important blood products. The placenta connects the developing baby to the uterine wall to allow nutrient uptake, waste elimination, and gas exchange via the mother's blood supply. In our lab, we employ a technique called “placental perfusion”. This unique technique separates the functions of the placenta from both the maternal and fetal influences. The use of this model will further our understanding of the transport and behavior of certain medications across the human placenta, throughout pregnancy.

Purpose of Research:

Pregnancy is a special state in which there are many physical changes that occur to both the fetus and the mother. While pregnant, some mothers need to take medication in order to maintain a healthy pregnancy. Some of these compounds can reach the growing baby by passing through the placenta. We would like to understand this process better.

It is very important for researchers and doctors to better understand how medications cross the placenta, so that in the future we may help women protect their unborn fetus from harm. This is a multi-centre study and participants are recruited at St. Michael’s Hospital and Mount Sinai Hospital. As part of this study, we would like to collect whole placentaes from 100 women at St. Michael’s Hospital, and 50 women at Mount Sinai.

Description of Research:

Normally, once your baby is born, the umbilical cord is clamped and the baby is separated from the placenta. The placenta is then delivered and thrown away.

If you agree to participate in this study, instead of the placenta being disposed of, we would like to use it as part of our study in our on-site laboratory. We will set up a placenta perfusion, which is a model that mimics the blood circulation systems for the mother and developing baby. We can use this model to study the transport of medications across the placenta without having to administer any medication to pregnant women. We will collect samples while using this model to determine if the study medication crosses the placenta, and these samples will be sent to the Hospital for Sick Children for further analysis. After our experiment, your placenta will be disposed of in the usual fashion according to the hospital ward’s guidelines.
No additional procedures or modifications to your care are required to assess the placenta after delivery. If your treating doctor decides that your placenta requires special testing after delivery as part of your hospital care, we will not collect it as part of this research study and you will be immediately withdrawn from the study.

**Potential Harms (Injury, Discomforts or Inconveniences):**

Collection of these samples will not affect your labour or the delivery of your baby. The whole placenta will only be assessed for research after it has been delivered. The assessment will be done at the time the placenta is routinely disposed. The collection carries no risk to you or your baby.

**Potential Benefits:**

There are no direct benefits from participating in this study. The results from this study may improve our understanding of drug transfer across the human placenta. In addition, we hope that the information obtained in this study will allow us to develop new treatment options for women during pregnancy while protecting their unborn fetus.

**Alternatives to Participation**

If you decide that you do not want to take part in this study, your choice will not affect the care that you or your family would otherwise receive. You will continue to receive the standard of care during your pregnancy and after you deliver your baby.

**Protecting Your Health Information:**

This consent form and all data collection forms will be held in the strictest confidence. To protect your anonymity, your name will not appear on any study files except for this consent form. Information from this study will be kept in a locked filing cabinet in the locked laboratories at the St. Michael’s Hospital and on a password-protected computer database in the research laboratories at the Hospital for Sick Children for three years and only study personnel and the Research Ethics Board office will have access to this information.

In addition to your placenta that will be used in our perfusion experiment, we will collect your age and the gestational age of your baby at the time of delivery. Following the experiment, your placenta will be securely disposed of as per standard hospital procedures. None of these research results will be placed in your medical records.

Once the study is complete, the results will be summarized and submitted to a medical journal for publication. The outcome of this study may be presented at conferences, scientific meetings and other public forums. It is important that you are aware that you will not be identified in any of these reports and your confidentiality will be maintained. No information that discloses your identity will be released or published without consent.
Experience in similar studies indicates that the greatest risk in this study to you is the unintentional release of information from your health records. The study personnel will protect your records and keep all the information in your study file confidential, including your name. The chance that this information will accidentally be given to someone else is small.

You may be contacted by a representative of the Research Ethics Board to ask questions about your experience with the recruitment and consent process or regarding your experience in the study, with a view to assuring and improving the quality of these processes.

**Potential Cost of Participation and Reimbursement:**

There are no costs associated with participating in this study. You will not be reimbursed for your participation in this study.

**Compensation for Injury:**

If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided to you in the same manner as you would ordinarily obtain any other medical treatment. In no way does signing this consent form waive your legal rights nor does it relieve the investigators or involved institutions from their legal and professional responsibilities.

**Participation and Withdrawal:**

Participation in any research study is voluntary. If you choose not to participate, you and your baby will continue to have access to customary care at St. Michael’s Hospital. If you decide to participate in this study you can change your mind without giving a reason, and you may withdraw from the study at any time without any effect on the care you and your baby will receive at St. Michael’s Hospital. Upon withdrawal from the study, it is within your rights to request the withdrawal of any of your data compiled up to that point.

Study personnel have the right to stop your participation in the study if your doctor orders additional testing for your placenta and thinks it is not in your best interest to continue with our study.

**New Findings or Information**

We may learn new things during the study that you may need to know. We can also learn about things that might make you want to stop participating in the study. If so, you will be notified about any new information in a timely manner. You may also be asked to sign a new consent form discussing these new findings if you decide to continue in the research study.
**Development for Commercial Gain:**

Research carried out on your samples by researchers at the Hospital for Sick Children, or their collaborators, may lead to the development of marketable treatments, devices, new drugs or patentable procedures. By participating in this study you will not benefit directly from any commercial products that may arise from this research.

**Research Ethics Board Contact:**

If you have any further questions about your rights as a research participant, you may contact the following individuals:

Dr. David Mazer  
Chair, Research Ethics Board  
St. Michael’s Hospital  
416-864-6060 ext 2557

Lorelei Nardi  
Team Leader, Research Ethics Office  
Hospital for Sick Children  
416-813-5718

**Further Questions:**

You have been given a copy of this information and consent form. If you have any questions about taking part in this study, you may contact Dr. Gideon Koren (The Hospital for Sick Children) at (416) 813-5781 or Dr. Howard Berger (St. Michael’s Hospital) at (416) 867-7460 Ext. 8408.
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title: The Role of the Placenta in Fetal Toxicology

Consent:

I acknowledge that the research study described above has been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of my right not to participate and the right to withdraw without compromising the quality of my medical care at St. Michael’s Hospital. As well, the potential risks, harms and discomforts have been explained to me and I also understand the benefits (if any) of participating in the research study.

I understand that I have not waived my legal rights nor released the investigators, sponsors, or involved institutions from their legal and professional duties. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. I have been assured that records relating to me and my care will be kept confidential and that no information will be released or printed that would disclose personal identity. I have been given sufficient time to read and understand the above information.

By signing this consent form, I give permission for my placenta to be used for research purposes after delivery. The placenta will be collected and will be processed at the time of delivery and used for the purposes outlined in the description of this research study.

I hereby consent to participate and will be given a copy of this consent form.

Participant’s Name (Please Print)  Participant’s Signature  Date

Name & Position of Person Obtaining Consent  Signature  Date

The Role of the Placenta in Fetal Pharmacology
Appendix B. Composition of M199 Culture Medium in Perfusate

<table>
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<th>Compound</th>
<th>Concentration (mg/L)</th>
<th>Compound</th>
<th>Concentration (mg/L)</th>
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<td>DL-Alanine</td>
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<td>L-Arginine • HCl</td>
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<td>DL-Serine</td>
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<tr>
<td>Polyoxyethylene sorbitan monooleate</td>
<td>20.0</td>
<td>DL-Tryptophan</td>
<td>20.0</td>
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<tr>
<td>Pyridoxal HCl</td>
<td>0.03</td>
<td>L-Tyrosine • 2Na</td>
<td>57.7</td>
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<tr>
<td>Pyridoxine HCl</td>
<td>0.03</td>
<td>DL-Valine</td>
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<tr>
<td>Riboflavin</td>
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<tr>
<td>Ribose</td>
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<tr>
<td>Thiamine HCl</td>
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<tr>
<td>Thymine</td>
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<tr>
<td>Vitamin A acetate</td>
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<tr>
<td>Xanthine • Na</td>
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<tr>
<td>Calcium chloride • 2H2O</td>
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<tr>
<td>Ferric nitrate • 9H2O</td>
<td>0.72</td>
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<tr>
<td>Magnesium sulfate (anhydrous)</td>
<td>97.7</td>
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<tr>
<td>Potassium chloride</td>
<td>400.0</td>
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<tr>
<td>Potassium phosphate</td>
<td>60.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium acetate (anhydrous)</td>
<td>50.0</td>
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<tr>
<td>Sodium chloride</td>
<td>8000.0</td>
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<tr>
<td>Sodium phosphate dibasic (anhydrous)</td>
<td>47.9</td>
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<tr>
<td>D-Glucose</td>
<td>1000.0</td>
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