A RE-ASSESSMENT OF THE RISK: BENEFIT ANALYSIS OF STATIN THERAPY DURING PREGNANCY: DO BENEFITS OF TREATMENT OUTWEIGH PUTATIVE REPRODUCTIVE RISKS

by

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Master of Science (2012)
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Abstract

An animal model has implicated elevated levels of tissue factor (TF), and resultant hypercoagulability and inflammation, as key factors in recurrent pregnancy loss (RPL) and has demonstrated that pravastatin is effective in treating this condition. In this study, we have re-evaluated the contraindication of statins during pregnancy. Evaluation has shown that while animal testing (at maternally toxic doses) and case reports of birth defects have led to the contraindication of statins during pregnancy, our controlled study, similar to previously published controlled studies, has failed to demonstrate increased fetal risks. As well, we demonstrated that transfer of pravastatin across the placenta is likely limited. While short term suspension of therapy during gestation is considered safe, extended time without therapy is detrimental to cardiovascular health. Coupled with a trend of elevated TF levels in women with RPL, reconsideration of the contraindication of statins is warranted based on appropriate risk: benefit assessment.

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Last but not least I could not have achieved any of my accomplishments without the loving encouragement of my husband, Adam.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABC: ATP-binding cassette transporters

ATP: Adenosine triphosphate

BCRP: Breast cancer resistance protein

C: Concentration

CI: Confidence interval

Cmax: Maximum concentration

CYP: Cytochrome P450

ET-1: Endothelin-1

FA: Fetal artery

FPP: Farnesylpyrophosphate

FV: Fetal vein

FVII: Factor VII

FVIIa: Activated Factor VII

FX: Factor X

FXa: Activated Factor X

G proteins: Guanine nucleotide-binding proteins

GGPP: Geranylgeranylpyrophosphate

GTP: Guanosine triphosphate

hCG: Human chorionic gonadotropin

HDL: High density lipoproteins

HMG-CoA: Hydroxymethylglutaryl-CoenzymeA

HR: Hazard ratio

IL: Interleukin

IFN: Interferon

IUGR: Intrauterine growth restriction

IVIg: Intravenous immunoglobulin

LAC+: Lupus anticoagulant positive

LDL: Low density lipoproteins

MA: Maternal artery

MDR: Multidrug resistance

MRP: Multidrug resistance protein

MV: Maternal vein

NA: Not available

NET: Norepinephrine transporter

OCTN1: Organic cation transporter-1

PARs: Protease activated receptors

P-gp: Permeability glycoprotein

pKa: Acid dissociation constant

PCOS: Polycystic ovarian syndrome

OAT: Organic anion transporters

OATP: Organic anion-transporting polypeptide

OR: Odds ratio

RPL: Recurrent pregnancy loss

RR: Relative risk

Sd: Standard deviation

SEM: Standard error of the mean

SERT: Serotonin transporter

sEng: Soluble endoglin

SLOS: Smith-Lemli-Opitz syndrome

sFlt-1: Soluble fms-like tyrosine kinase-1

SGA: Small for gestational age

SREBPs: Sterol regulatory element binding proteins

TF: Tissue factor

TFPI: Tissue factor protein inhibitor

TNF: Tumor necrosis factors

V: Volume

VACTERL: Vertebral, anal, cardiovascular, tracheo-esophageal fistula, renal, and limb defects

VEGF-1: Vascular endothelial growth factor

List of Appendices

1.1 Statement of the Problem

Recurrent Pregnancy Loss (RPL) affects 1-5% of couples trying to conceive (American Society for Reproductive Medicine., 2008). Defined as having experienced 2 or more consecutive miscarriages, RPL is understandably a traumatic experience. Current therapy is not regulated and is dependent on the specific treatment plan of the clinic. However, treatment has not been proven to increase live birth rates (Kaandorp, SP. *et al.*, 2010, Clark, P. *et al.,* 2010, Stephenson, MD. *et al.*, 1998, Christiansen, OB. *et al.*, 1995, Laskin, CA. *et al.*, 1997)

Recent animal studies have shown that elevated tissue factor levels are associated with pregnancy loss in mice (Redecha, P. *et al.,* 2009). These studies have illustrated the potential of statins for RPL treatment. Currently, statins are classified as contraindicated during pregnancy (FDA pregnancy X). This classification is largely based on the assumption that postponement of hypercholesterolemia treatment during gestation is not significantly detrimental, and theoretical risks of statins to the developing fetus outweigh cholesterol lowering therapy (Briggs, GG. *et al.,* 2008).

With the potential of statins for RPL therapy and re-evaluation of harm of treatment cessation during pregnancy, it is important to determine the authenticity of putative risks, and if potential benefits of use outweigh risks.

1.2. Purpose and Study Objectives

The purpose of this study is to assess the safety of statins during pregnancy and to determine if the benefits of potential uses during pregnancy, in light of safety assessment, outweigh risks of exposure. The following 3 objectives were used in this study:

Objective 1: To determine if tissue factor levels are elevated in RPL patients compared to controls with healthy pregnancy histories and patients who are LAC+.

Objective 2: To follow-up with Motherisk callers who were exposed to statins during pregnancy to determine if statin exposure is associated with increased occurrence of birth defects.

Objective 3: To determine exposure of pravastatin to the fetus by assessing pravastatin transfer across a dually perfused term placenta.

1.3. Research Hypothesis and Rationale

The hypotheses matching each of the study objectives are as follows:

Hypothesis 1: Studies have attributed RPL to an increased level of tissue factor leading to hypercoagulation, which decreases blood flow and trophoblast proliferation. We hypothesize that, similar to patients who are positive for lupus anticoagulant (LAC+) and have had adverse pregnancy outcomes, pregnant and non-pregnant RPL patients will display high levels of tissue factor compared to controls.

Hypothesis 2: Initial case reports have illustrated teratogenic effects of statin exposure during pregnancy. However, controlled studies published subsequently have failed to confirm statins are major teratogens. We hypothesize that, similar to previously

published controlled studies, in our cohort of pregnant Motherisk callers exposed to statins the rate of major malformations will not be associated with birth defects compared to controls not exposed to known teratogens.

Hypothesis 3: Due to pravastatin's strongly hydrophilic properties, pravastatin is not expected to pass to the fetal side of the placenta via passive diffusion. However, due to pravastatin's potential to be transported via transport proteins, we expect pravastatin to enter fetal circulation, albeit in low concentration.

Chapter 2. Review of the Literature

2.1. Statins and Pregnancy

2.1.1 Mechanism of Action

Hydroxymethylglutaryl-CoenzymeA (HMG-CoA) reductase catalyzes the rate limiting step in the mevalonate pathway, the synthesis of mevalonic acid (Hampton, R. et al., 1996)(Figure 1). The mevalonate pathway is regulated through feedback inhibition. When the products of the pathway are low in the cell, transcription is activated by sterol regulatory element binding proteins (SREBPs). As well, when sterols and reaction intermediates are abundant, biosynthesis is decreased (Buhaescu, I. and Izzedine, H., 2007). Statins are able to occupy the active site on HMG-CoA reductase due to having a similar structure as HMG- CoA (Hill, JS. and Qiu, G., 2008). By occupying the active site on HMG CoA reductase, statins prevent the intended activating molecule from reaching the site and catalyzing the synthesis of mevalonic acid. As well, the interference of statins results in the increase in synthesis of microsomal HMG- CoA reductase and increase in LDL cell receptors, thus increasing clearance of LDL from circulation (Lennernäs H, and Fager G., 1997). An LDL reduction of 30% has been shown to correspond to a 30% decrease in cardiovascular events (Hill, JS and Qiu, G.,2008).

The effectiveness of statins ranges from 20-55% reduction in LDL. A 2003 six week randomized parallel group, open labeled multicentre study, recorded clinical differences among rosuvastatin, pravastatin, simvastatin and atorvastatin. Results of the

study revealed that, on average, rosuvastatin reduced LDL cholesterol levels 8.2% more than atorvastatin, 26% more than pravastatin, and 12-18% more than simvastatin (Jones, PH. *et al.,* 2003).

Relevant clinical pharmacology of the statins is described below, and summarized in Table 1.

Simvastatin is administered in its inactive hydrophobic lactone form ranging from 5-120 mg/day. When taken, it is hydrolyzed by cytochrome P450 3A4 (CYP3A4) into its active beta hydroxyacid form (Prueksaritanont, T. *et al.,* 1997). Simvastatin is highly extracted by first pass; since its site of action is the liver (Hamelin BA and Turgeon J., 1998), simvastatin exhibits low systemic exposure with a bioavailability of approximately 7% (Mauro VF., 1993). Simvastatin is also highly protein bound, at approximately 94% (Vickers, S. *et al.,* 1990). Simvastatin has an elimination half-life of 1.9 hours and peak plasma levels are observed within 2-4 hours after administered. 60% of the initial dose is eliminated in the bile, and 13% in the urine (Mauro VF., 1993). On average, a 20 mg dose of simvastatin has been shown to decrease LDL levels by 28% and to raise HDL levels by 14%. The 40 mg dose demonstrated an average decrease of LDL by 38% while raising HDL levels by 18% (Berger, GM. *et al.,* 1989).

Atorvastatin's starting dose is 10-20 mg/day and it can be given up to 80 mg/day (Jones P *et al.,* 1998). Peak plasma concentration is achieved at 1-2 hours after administration (Cilla, DD. *et al.,* 1996). Bioavailability of atorvastatin is 12%; its low bioavailability reflects extensive first pass effect by the liver (Corsini, A. *et al.,* 1999). Atorvastatin is highly protein bound at approximately 98 %. Atorvastatin is metabolized by CYP3A4 (Lennernas, H., 2003). 70% of atorvastatin activity is through its ortho and

para hydroxylated metabolites which exhibit HMG-CoA inhibitory effects equivalent to atorvastatin. Atorvastatin is eliminated primarily in bile. Plasma elimination half-life of atorvastatin is 11-14 hours (Cilla, DD. *et al.,* 1996) with its inhibitory effects lasting 20-30 hours due to active metabolites (Chong PH and Seeger JD.*.*,1997). In patients with hypercholesterolemia, a 2.5 mg dose of atorvastatin has been shown to decrease LDL by 22 %; a 40 mg dose exhibits a 58 % decrease (Cilla, DD. *et al.,* 1996).

Rosuvastatin is administered in its active form with a starting dose of 5 mg/day and maximum dose of 40 mg/day (Davidson, M.*,* 2004). Peak plasma concentration is observed 5 hours after administration. Bioavailability is approximately 20%. Rosuvastatin is 88% protein bound (Rosenson, RS*.,* 2003); 90 % of the parent drug is not metabolized. Its limited metabolism done by CYP 2C9 (Martin, PD. *et al.,* 2003). Elimination half-life of rosuvastatin is approximately 20 hours (McTaggart, F. *et al.,* 2001); active drug and metabolites are eliminated by renal (28%) and hepatic (72 %) routes (Martin, PD. *et al.,* 2003). In patients with hypercholesterolemia, rosuvastatin decreased LDL levels by 40% (Davidson, M. *et al.* 2002).

Fluvastatin's dosage ranges from 20-80 mg/day (Zavoral, JH. *et al.,* 1995). Fluvastatin reaches peak plasma concentration within one hour after administration (Tse, FL. *et al.,* 1992). Bioavailability of fluvastatin is 24 % with 99% protein binding (Hamelin BA and Turgeon J*.*,1998). Fluvastatin is metabolized primarily by CYP2C9 and by CYP2C8 and CYP3A4 (Fujino, H. *et al.,* 2004). Fluvastatin is primarily excreted as its metabolites with only 2% represented by the parent drug (Tse, FL. *et al.,* 1992). The elimination half-life of fluvastatin is less than 1 hour (Deslypere, JP*.,* 1994). In patients with hypercholesterolemia, 20 mg of fluvastatin resulted in a 25 % decrease in

LDL. The maximum dose, 80 mg, resulted in a 33% decrease in LDL (Zavoral, JH. *et al.,* 1995).

Lovastatin is administered in its inactive form. It is hydrolyzed by CYP3A4 into its active beta-hydroxy acid form (Jacobsen W. *et al.,* 1999) Systemic bioavailability of lovastatin is less than 5 % and is 95% protein bound (Igel, M. *et al.,* 2001). Half-life of lovastatin ranges from 1.1-1.7 hours (Moghadasian, MH*.., 1999*) and peak plasma concentrations are seen approximately 2 hours after administration (Sun, JX. *et al.,* 2002) The recommended dose of lovastatin ranges from 20-80 mg/day (McKenney JM*., 1988*). 10% of the oral dose is excreted in urine and 83% in bile (Duggan, DE.*et al.,* 1988). In hypercholesterolemia patients, a 20 mg dose of lovastatin decreased LDL concentrations by 25%, and the 80 mg/day dose decreased LDL concentrations by 45% (McKenney JM*.,* 1988).

Pravastatin is administered in its active form. Peak plasma concentrations are observed approximately 1-1.5 hours after administered (Hatanaka, T*.,* 2000). Absorption of pravastatin is 34% and bioavailability is 17% (Pan HY*.,* 1991), with 50% of the drug protein bound (Lennernäs, H. and Fager, G*.,* 1997). Pravastatin is extensively metabolized in the liver, its site of action (Hamelin, BA. and Turgeon, J*.,*1998). 20% is excreted in the urine and 70 % in bile (Singhvi, SN. *et al.,* 1990). Absorption half-life of pravastatin is between 1-2.5 hours in healthy patients. Pravastatin exhibits 'flip flop' kinetics in that it is more rapidly eliminated than it is absorbed (Hatanaka, T*.,* 2000). Due to its rapid elimination, no accumulation is observed following once or twice daily administration of pravastatin (Pan, HY*.,* 1991). Pravastatin is not extensively metabolized by the CYP isoenzymes. Rather, pravastatin undergoes sulfation,

isomerization, glucoronidation, oxidation and conjugation (Christians, U. *et al.,* 1998). Pravastatin dosage ranges from 5-40 mg/day (Andrews, TC. *et al.,* 2001). A 10 mg/day dose decreases LDL levels by 19 %; 20 mg/day decreased LDL by 24% (Jones, P. *et al.,* 1998); and the 40 mg/day decreases LDL levels by 34% (Shepherd, J. *et al.,* 2002).

Table 1: Summary of Pharmacokinetics

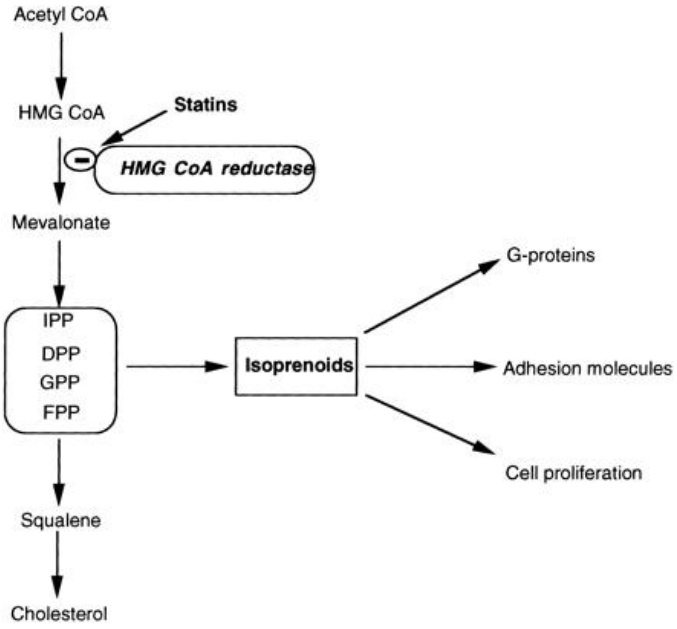
Statin	Dose range (mg)	Metabolism	LDL decrease (%)	Half-life (hours)
Simvastatin	5-120	CYP3A4	28-38	1.9
Atorvastatin	10-80	CYP3A4	22-58	14
Rosuvastatin	5-40	CYP2C9	40	20
Fluvastatin	20-80	CYP3A4	25-33	<1
Lovastatin	20-80	CYP3A4	25-45	1.1-1.7
Pravastatin	5-40	sulfation, glucuronidation	19-34	1-2.5

2.1.2. Pleiotropic Effects

In addition to the primary objective of statin therapy of lowering LDL cholesterol, the interference of the mevalonic acid pathway affects intermediates produced by the pathway (Figure 1.). Decreasing such intermediates, specifically isoprenoids, results in the potential of statins to be used in addition therapies which are known as pleiotropic effects (Hampton, R.., 1996). Significant isoprenoid reductions include FPP (farnesylpyrophosphate) and GGPP (geranylgeranylpyrophosphate). These lipid isoprenoids are significant posttranslational attachments of the Rho family of GTPases, such as Ras, Rac and Rap (Wang, CY. and Liu, PY., 2008). Indeed, in a 2004 double

blind, placebo controlled study (McCarey, DW, *et al.,* 2004) patients with active rheumatoid arthritis who received atorvastatin exhibited a decrease in their disease activity compared to the placebo group, a result of statin's anti-inflammatory effect. Outcomes from another study (Sena, A. *et al.,* 2003) showed that lovastatin exhibited anti-neuroinflammatory and disease reducing activity in patient with multiple sclerosis. Other potential uses of the pleiotropic effects include reducing hypertension (Ma, M *et al.,* 2012) and limiting cancer proliferation (Roy, M. *et al*, 2011, Riganti, C. *et al.,* 2011).

Figure 1: Inhibitory Effects of Statins on Cholesterol and Pleiotropic Effects



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2.1.3. FDA Pregnancy Classifications

In 1979, the FDA created categories to guide prescribing medications during pregnancy. In this system, drugs are classified into A, B, C, D or X. Medications classified into A are considered safe based on animal testing and human controlled studies. B medications have demonstrated safely in animal studies without controlled human studies, or, have demonstrated some adverse effects in animal testing but are considered safe based on controlled human studies. Medications classified into the C category are those without adequate animal or human data, and therefore recommended to avoid. Medications in the D classification have demonstrated risk in human pregnancies. However, their benefit may outweigh potential risks. The medications in the X class have demonstrated fetal abnormalities in human or animal testing and are therefore contraindicated (Sachdeva, P. *et al .,* 2009). This FDA labeling system has been criticized for its lack of clinical usefulness. It fails to provide adequate assessment regarding the risks and benefits of taking a specific drug while pregnant. The current categories rely heavily on animal data and do not account for timing of drug exposure or drug metabolism changes during pregnancy. Drugs in Categories B, C, and D are almost always assessed based solely on animal studies even though animal studies do not always translate into human risk (Law, R. *et al.,* 2010). The inability to interpret drug categories lead clinicians to look for additional information that often leads to hesitation in prescribing medications despite the potential harm the untreated condition may pose to the mother and/or fetus (Boothby, LA. and Doering, PL*.,* 2001).

2.1.4. Statins and their FDA Pregnancy Classification

Essential to embryonic and fetal proliferation, cholesterol is transferred across the yolk sac and placenta (Yoshida, S. and Wada, Y., 2005). It is an essential component of cell membranes as well as steroid hormones and bile acids. While fetal synthesis of cholesterol is essential during development, additional cholesterol is transferred from maternal circulation across the placenta. Placental ATP-binding cassette transporters A1 and G1 transport and regulate cholesterol levels; ABCG1 transports cholesterol to the fetus and ABCA1 effluxes cholesterol to maternal circulation (Aye, IL. *et al.,* 2010). Maternal cholesterol has been shown to increase during pregnancy. In a recent study (Ekhator, CN. and Ebomoyi, MI., 2012), cholesterol levels in pregnant patients were measured compared to non-pregnant women. It was found that LDL levels were significantly elevated by approximately 50 percent during gestation with the largest increase occurring during the first trimester and then decreasing as pregnancies progressed. In contrast, HDL levels decreased in the first trimester and then returned to control levels in the second trimester. The necessity of fetal synthesis of cholesterol is evident in Smith-Lemli-Opitz Syndrome (SLOS). SLOS is an autosomal recessive disorder of a deficiency in 7-dehydrocholesterol reductase essential for cholesterol synthesis (Porter, FD., 2008). SLOS affects approximately 1 in 20,000 to 70,000 births (Tint, GS. *et al.,* 1994). Outcomes of affected individuals range from stillbirths or neonatal deaths to congenital malformations and behavioural problems (Porter, FD., 2008). Despite their innate inability to synthesize cholesterol these neonates, even those born with severe cases, have measurable levels of cholesterol indicative of maternal transfer during gestation (Lindergaard, ML. *et al.,* 2008).

While maternal cholesterol is known to be elevated during gestation, hypercholesterolemia during gestation has been shown to be associated with detrimental effects. High levels of LDL have been shown to be associated with an increase in preterm labour (Mudd, LM. *et al.,* 2012). In another study, maternal triglyceride levels were significantly elevated in women who experienced preeclampsia as well as in women with babies born small for gestational age (SGA) (Ziaei, S. *et al.,* 2012). In mice, maternal hypercholesterolemia was associated with increased activation of cholesterol synthesis in adult offspring (Goharkhay, N. *et al.,* 2008.). In a study of human fetal aortas, the aortas of fetuses born to hypercholesterolemic mothers exhibited elevated fatty streak formation compared to aortas from non-hypercholesterolemic mothers, suggesting a correlation between maternal hypercholesterolemia and fetal cardiovascular disease predisposition (Napoli, C. *et al.,* 1997). In the FELIC (Fate of Early Lesions In Children) study children born to hypercholesterolemic mothers had fatty aortic lesions which showed faster atherogenesis progression compared to children born to non hypercholesterolemic mothers (Napoli, C. *et al.,* 1999). While it is plausible to suggest that children born to hypercholesterolemic mothers have a genetic predisposition to atherogenesis and cardiovascular disease similar to their mothers, further animal studies have shown that cholesterol therapy during gestation leads to a decrease in atherosclerosis and cardiovascular risk in offspring (Napoli, C. *et al.,* 2000, Palinski, W. *et al.,* 2001, Elahi, MM. *et al.,* 2008). These studies suggest that an in utero hypercholesterolemic environment may initiate a predisposition to cardiovascular disease later in life.

The FDA has classified all statins as contraindicated during pregnancy (Briggs, GG. *et al.*, 2008). This classification is largely based on the rationale that suspending hypercholesterolemia therapy during pregnancy is not believed to have long term health effects to the mother. Moreover, since cholesterol is essential for fetal development, potential damaging effects may occur as a result of cholesterol lowering therapy (Briggs, GG. *et al.,* 2008).

2.1.5. Reproductive Outcomes in Animal Testing

Rosuvastatin crosses the rat placenta and is seen in fetal rat tissue at 3% of maternal concentration and in amniotic fluid at 20 % maternal concentration. Systemic exposure 10 times the maximum human dose (40 mg) resulted in decreased female pup body weight and delayed ossification (Gong, J*.,* 2003). In animal studies with simvastatin, no evidence of embryotoxic or teratogenic effects were observed (Wise, LD. *et al.,* 1990). In animal studies with atorvastatin, no reproductive or teratogenic effects were observed (Henck, JW. *et al.,* 1998, Dostal, LA. *et al.,* 1996) Developmental studies were conducted with lovastatin in mice at doses 10 and 47 times the recommended maximum dosage (80 and 400 mg/kg/day). At the 80 mg/kg dose, there was no increased occurrence of congenital malformations. At the 400 mg/kg dose, there was a slightly elevated occurrence of skeletal malformation compared to controls; however, it was still within normal range for the strain of mice. In rats at a dose of 800 mg/kg/day (103 times the maximum recommended dose based on weight) of lovastatin resulted in an increased occurrence of skeletal malformations (Minsker, DH. *et al.,* 1983, Kornbrust, DJ. *et al.,* 1989). However, it was found that this dose was maternally toxic and the effects on the fetus were a result of maternal toxicity (Lankas. GR. *et al.,* 2004, Wise LD. *et al.,* 2000).

Pravastatin was not found to be teratogenic at doses up to 1000 mg/kg in rats as well as up to 50 mg/kg in rabbits (Tanase, H. and Hirose, K., 1987, Tanase, H. and Hirose, K., 1987).

The safety of statins during human pregnancy has not been clearly determined. The statins are contraindicated as a group, not individually. Their contraindication has been justified based on the animal testing of one lipophilic statin, lovastatin. In animals, lovastatin had been shown to cause skeletal malformations at maternally toxic doses (Lankas, GR. *et al.,* 2004). In contrast, in animal testing of pravastatin, dosing up to 1000 mg/kg in rats and 50 mg/kg in rabbits (120 and 10 times the human dose, respectively) did not result in any teratogenic effects (Tanase, H. and Hirose, K., 1987, Tanase, H. and Hirose, K., 1987)

2.1.6. Cases and Studies of Statins in Human Pregnancy

In a 2004 summary of limited human case reports (Edison, RJ. and Muenke, M., 2004), 31 malformations were reported in 70 statin exposed pregnancies, justifying their category X rating. Only some lipophilic statins were found to be associated with teratogenic effects. Nevertheless, this causal relationship between birth defects and statin exposure has been strongly questioned. Critics of the report fail to see a pattern in the anomalies consistent with lipophilic statin use. Furthermore, the critics point out that a known abnormality of cholesterol biosynthesis, Smith-Lemli-Opitz syndrome, was not identified in the case reports. (Gibb, H. and Scialli, AR., 2005). Additionally, it is not known how many pregnancies were exposed since only spontaneously reported outcomes were published.

The available controlled studies will be reviewed below:

In a 2007 pharmacoepidemiological study (Ofori, B. *et al.,* 2007), the risk of congenital anomalies related to first trimester statin use was compared to women who stopped statin use prior to pregnancy and to women who used fibrates during pregnancy. Using the 'Medication of Pregnancy' registry, which covered patients from the Province of Quebec, the study identified 153 women who received a statin during the first trimester, 29 women who received a fibrate or nicotinic acid, and 106 women who received a statin 1 year to 1 month before conception. In the group of statin users, 64 live births were evaluable with a congenital anomaly rate of 3/64, 4.69%. In the fibrate/nicotinic acid users, 14 cases were evaluable and the congenital anomaly rate was 3/14, 21.43%. Finally, in the patients who stopped statin use before conception, the anomaly rate was 7/67, 10.45%. Significantly, pravastatin consisted of 20% of statin exposure during pregnancy. Despite this prevalence, there were no congenital anomalies associated with pravastatin among the live births.

In a 2008 prospective observational cohort study (Taguchi, N. *et al.,* 2008), the fetal toxicity of different statins was examined. In 64 cases of pregnant women taking statins compared to 64 pregnant women without exposure to known teratogens, there were no differences in the rate of major malformations of neonates. Similarly, there were no statistical differences in live birth rates, spontaneous abortions, therapeutic abortions, and stillbirth rates. The only statistically significant differences between the groups were the incidences of lower birth weights and earlier gestational age at birth in the statin group. The authors of the study suggest that maternal comorbidities might have been

responsible for these findings. However, the rate of neonatal health issues did not differ between the groups.

In a 2009 cohort study of statin exposure (McGrogan, A. *et al.,* 2009), 192 statin exposed pregnancies were identified. The authors compared the outcomes of the statin exposed group to 1943 control women matched for age and year of pregnancy. The rate of congenital malformations was 3% in both groups. However, there was an increased risk of abortions in the statin group. It is not clear if the cause of abortion was elective, spontaneous, or due to malformation in the fetus.

In a 2010 population based study (Colvin, L. *et al.,* 2010), risk of category D or X medications was studied based on prescriptions filled in Western Australia from 2002 to 2005. The authors identified 23 D or X category medications dispensed during pregnancy. Included in the medications dispensed were 33 cases of atorvastatin and 18 cases of simvastatin. Relative risk was calculated for birth defects compared to live births in women not dispensed a D or X category drug. For atorvastatin, the relative risk was 0.6 (0.1-4.6) and for simvastatin, relative risk was calculated at 1.2 (0.2-8.9).

The most recent controlled study was published in 2011 (Winterfeld, U. *et al.,* 2011). A multicentred, prospective study collected data from pregnancies exposed to statins from 1990 until 2009. Exposed pregnancies were compared to a control group of women requesting data from the same centre as the exposed group. The primary outcome was the occurrence of major birth defects. 249 women in the study's cohort were exposed to statins during pregnancy, compared to 249 controls. The rate of major birth defects was not found to be significantly different between groups with 3.6% in the exposed group versus 2.7% in the control group. Initially, miscarriage rates appeared

significant between groups (14.5% in exposed group versus 7.6% in control group). However, after correcting for maternal age and gestational age at recruitment, this difference became statistically insignificant (HR 1.36).

In addition to the few controlled studies, there have been case reports published reporting statin exposures during pregnancy. While a few case reports have illustrated birth defects, limited information can be extrapolated from these reports and it is comforting that these reports have not shown specific patterns of malformations (namely VACTERL). As well, case series studies have shown malformation rates of exposed babies to be similar to expected population rates (Pollack, PS. *et al.,* 2005, Petersen, EE. *et al.,* 2008, Yaris, F. *et al.,* 2004, Freyssinges, C. and Ducrocq, MB*.,* 1996, Teelucksingh, S. *et al.,* 2004, Vagt, A. *et al.,* 2000, Seguin J and Samuels, P*.,* 1999). Furthermore, in a 2007 systemic review of statin use in the first trimester of pregnancy, the rate of congenital anomalies in women who used statins during pregnancy was not statistically different compared to the general population (Kazmin, A. *et al.,* 2007).

As shown above, in animal testing, lovastatin was the only statin to exhibit teratogenic effects, albeit at maternally toxic doses. This result, together with questionable negative effects described by case reports, has resulted in a tainted view of statin use during pregnancy. While there is no doubt regarding the essential role of cholesterol during fetal development, maternal hypercholesterolemia has been illustrated to be detrimental to the offspring as well as the mother. Suspension of cholesterol therapy begins while planning a pregnancy. This stage may, in reality, be longer than desired. Coupled with suspension during gestation, breastfeeding and perhaps planning a future pregnancy, this may result in suspension of treatment for years, sometimes much

of a woman's childbearing years. This time off of therapy can have serious ramifications for the woman's long term health.

The debate about adverse reactions to lipophilic statin use continues, but case studies and analysis of pravastatin use in pregnancy fail to report any teratogenic effects. Yet all drugs in the statin family have been grouped as a whole with respect to their teratogenic potential.

The hydrophilicity of pravastatin is believed to prevent its transport to extra-hepatic spaces, including the embryo (Edison, RJ. and Muenke, M., 2004). The FDA category X rating has not been questioned due to lack of data involving statins in pregnancy and because long term treatment of hypercholesterolemia is not affected by temporary discontinuation during pregnancy.

While all statins are currently contraindicated in pregnancy, this is due to the limited animal data and the known potential benefits not outweighing the potential harm. Traditionally, statins are prescribed as a cholesterol lowering agent and this benefit, perhaps, does not justify their use in pregnancy as a short discontinuation has not been perceived as a major medical risk. However, recent evidence (Kusters, DM. *et al.,* 2010) suggests that in childbearing years, including preconception, when cholesterol lowering therapy is stopped - pregnancy, breastfeeding and subsequent pregnancies may lead to many years without essential hypercholesterolemia therapy. This, taken together with the evidence that maternal hypercholesterolemia has detrimental effects on the developing fetus warrants re-evaluation of statin therapy during pregnancy.

2.2 Statins for Gynecological and Obstetrical complications

In addition to conventional statin treatment, there is emerging evidence that a variety of obstetrical and gynecological complications may benefit from the pleiotropic effects of statins.

2.2.1. Endometriosis

In endometriosis, excessive angiogenesis and invasion of endometrial endothelial cells occurs with elevated inflammatory cytokines. Current treatment for endometriosis is surgery, but statins are being investigated based on their anti-inflammatory effects. They have been shown to decrease endothelial cell invasion through decreasing Rho/Rac activity inhibiting excessive cellular growth (Sokalska, A. *et al.,* 2010, Sokalska, A. *et al.,* 2012) and decrease inflammatory cytokines elevated in the disease (Bruner-Tran, KL*.,* 2009, Oktem, M. *et al.,* 2007).

2.2.2. Polycystic Ovarian Syndrome

Statins show potential for treating polycystic ovarian syndrome (PCOS). PCOS patients exhibit chronic inflammation, elevated androgen levels and endothelial dysfunction (Rashidi, B*.,* 2011). In two randomized controlled studies, atorvastatin and simvastatin decreased androgen concentration compared to controls on placebo. Simvastatin decreased levels lower than the standard therapy, Metformin. (Sathyapalan T. *et al.,* 2012, Banaszewska, B*.,* 2011). In two additional randomized controlled studies, patients exposed to simvastatin and atorvastatin had decreased proinflammatory markers in addition to decreased androgen levels (Rashidi, B*.,* 2011, Raja-Khan N*.,* 2011). Despite potential for treating endometriosis and PCOS, patients planning a pregnancy

with these conditions are eliminated from exploring statin therapy based on their contraindication during pregnancy

2.2.3. Preeclampsia

Statin therapy for treating preeclampsia is currently being explored. Preeclampsia is a pregnancy specific condition defined by hypertension and proteinuria on or after the 20th week of gestation. It is the most common medical pregnancy complication, affecting 2-8% of pregnancies. Preeclampsia/eclampsia incidence is increasing and it is responsible for 10-15% of worldwide incidences of maternal mortality (Duley, L., 2009). Placental overproduction of anti-angiogenic factors have been implicated in preeclampsia. As well, elevated levels of sFlt-1 (soluble fms-like tyrosine kinase-1), endothelin-1 (sEng) and ET-1 (endothelin-1) (Aggarwal, PK. *et al.,* 2012) and inflammatory cytokines including TNF and interleukins (Eiland, E. *et al.,* 2012) contribute to endothelial damage in preeclampsia (Steinberg, G. *et al.,* 2009).

Current treatment for preeclampsia targets its symptoms; antihypertensive agents lower elevated blood pressure and magnesium sulfate prevents seizures (Duley, L., 2009). Aspirin has been used to prevent and treat preeclampsia. Some studies have shown positive results from aspirin treatment. However, large multicentered trials have failed to demonstrate its benefit (Eiland, E. *et al.,* 2012). Other possible therapies include calcium or antioxidant supplementation, but there is little evidence to support these methods of treatment (Eiland, E. *et al.,* 2012).

Statins have been suggested as treatment and possible prevention of preeclampsia. In animal models of preeclampsia, pravastatin decreased sFlt-1; increased placental growth factors and blood flow; amended IUGR; and improved hypertension and

proteinuria (Kumasawa, K. *et al.,* 2011, Singh. J. *et al.,* 2011). Currently, a double blind placebo controlled randomized study is underway in the U.K. investigating pravastatin use to improve preeclampsia symptoms (Current Controls Trials Ltd., 2012).

2.2.4. Recurrent Pregnancy Loss

RPL is defined as having experienced 2 or more consecutive miscarriages (American Society for Reproductive Medicine., 2008). While traditionally defined as 3 or more consecutive miscarriages, the definition had been recently changed to allow sufferers to undergo evaluation for their losses (Allison, JL. and Schust, DJ., 2009). Miscarriage is not a rare pregnancy complication; it affects approximately 25% of recognized pregnancies. However, 2 consecutive miscarriages affects only around 5% of couples trying to conceive, and 3 or more consecutive miscarriages will affect only about 1 % (American Society for Reproductive Medicine., 2008). With recurrent pregnancy loss, the likelihood of a subsequent loss is influenced by the number of previous losses. Therefore, after 3 consecutive losses the risk of miscarriage in the next pregnancy is 29%; after 6 losses the risk reaches 53% (American College of Gynecology., 2000). Etiologies for RPL and their frequencies are: uterine abnormalities such as bicornate, septate, or didelphic uteri (10-15%); genetic or chromosomal abnormalities in one or both parents (2-4%); antiphospholipid syndrome (15%); and embryonic aneuploidy (48%) (Jaslow, CR. *et al.,* 2012, American College of Gynecology., 2000). Additional causes include thrombophilias, metabolic abnormalities and endocrine irregularities including thyroid dysfunction and low progesterone levels (American Society for Reproductive Medicine., 2008, American College of Gynecology., 2000). However, for approximately 50% of couples undergoing evaluation, no definitive etiology can be determined.

Aneuploidy of the embryos could account for many of these losses, but testing of aneuploidy of products of conception is costly and complicated to undertake.

In an attempt to determine causes of unexplained RPL, one popular theory is that an immune response to the embryo and/or fetus results in attack of the products of conception and, ultimately, miscarriage (American College of Gynecology*.,* 2000). In this model of miscarriage, the theory suggests that an inflammatory response is triggered as a result of unknown invading tissue. During this response, an upregulation of cytokines respond to the foreign response, preventing necessary implantation (Kwak-Kim, J. *et al.,* 2010). Based on the suggested mechanism of pregnancy loss, researchers seek treatment that interferes with the inflammatory process.

It is reassuring to RPL sufferers that for about 60-70% of cases, subsequent pregnancies are successful (American Society for Reproductive Medicine*.,* 2008). Treatment in RPL cases depends on the results of the evaluation. For a patient with a uterine abnormality, corrective surgery may be necessary (American College of Gynecology*.,* 2000). Endocrine abnormalities, such as hyperthyroidism, can be corrected with medication, and hormonal abnormalities, such as luteal phase defects, may be supported by progesterone supplementation (American College of Gynecology*.,* 2000). In cases where age related factors are suspected, patients may undergo pre-genetic diagnostic screening of the embryos to test for aneuploidy (Go, KJ. *et al.,* 2009). In cases where genetic screening reveals a thrombophilia, anticoagulation therapy may be warranted (Gris, JC. *et al.,* 2004).

Despite potential therapies for determined causes of pregnancy loss, unexplained cases of RPL are not associated with a specific treatment. Treatment for unexplained

RPL varies among clinics. Therapy may include: progesterone suppositories to support the luteal phase (Ozlü T *et al.,* 2012); low dose aspirin to increase blood flow and vascular proliferation (Kaandorp, SP. *et al.,* 2010); intravenous immunoglobulin (IVIg) therapy to regulate immune response (Carbone, MM. *et al.,* 2012); and even low molecular weight heparins, such as dalteparin or enoxaparin, to limit blood coagulation (Kaandorp, SP. *et al.,* 2010). These therapies are common, but empirical evidence supporting their benefits is conflicting. A meta-analysis examining progesterone supplementation did not show an increase in live birth rates (Goldstein, P. *et al.,* 1989). Low dose aspirin, or low dose aspirin combined with anti-coagulants or prednisone did not show any difference in live birth rates compared to a placebo group (Kaandorp, SP. *et al.,* 2010, Clark, P. *et al.,* 2010, Laskin, CA. *et al.,* 1997). IVIg therapy has not been shown to be superior to placebo for treating RPL (Stephenson, MD. *et al.,* 1998, Christiansen, OB. *et al.,* 1995). There is conflicting evidence of the effectiveness of therapy coupled with the fact that therapy during pregnancy may not be compatible for all. Aspirin use during pregnancy is associated with an increased risk of gastroschisis (Kozer, E. *et al.,* 2002), as well as increased bruising (Bay, MJ. *et al.,* 2011). Low molecular weight heparins, administered as injections, cause discomfort to patients. Based on conflicting evidence of effectiveness and potential harms of use, additional therapies are being investigated (Tang, AW. and Quenby, S., 2010).

2.2.4.1. An Animal Model of RPL

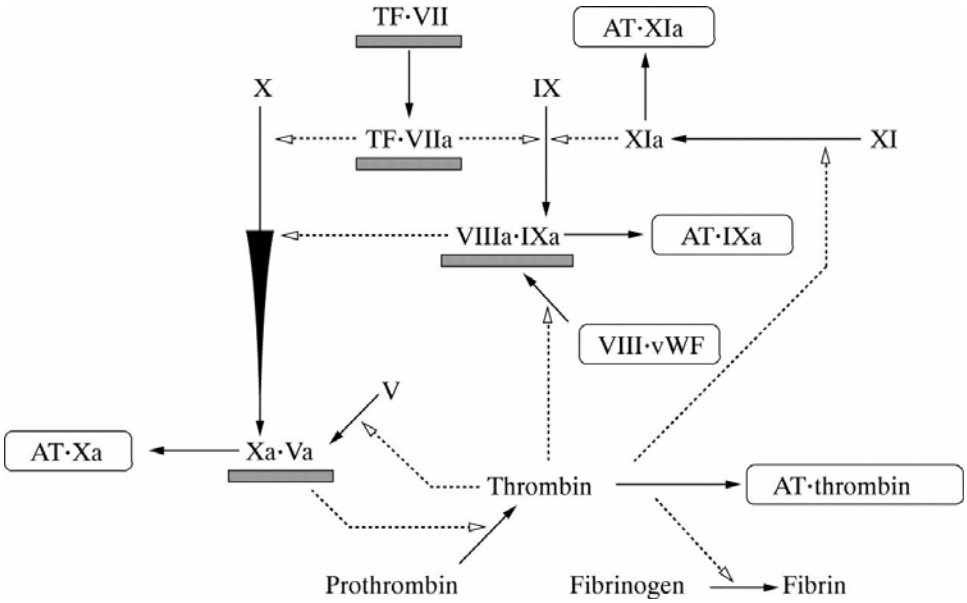
DBA/2- mated CBA/J mice exhibit immune mediated miscarriage. Since the detection of their increased resorption, they have been used as a model of RPL. Their increased resorption is associated with increased production of inflammatory cytokines.

(Clark, DA. *et al.,* 2004). Thus, through the animal model a better understanding into mechanism of immune mediated pregnancy loss as well as insight into appropriate treatment options is investigated. In the animal model, increased levels of TNF alpha, IFN-gamma, and IL-2 are reached (Tangri, S. *et al.,* 1994).

2.2.4.2. Tissue Factor and Potential Therapy

Tissue factor (TF) is a transmembrane glycoprotein responsible for the initiation of in vivo coagulation. TF, not normally exposed to circulating blood, comes in contact with Factor VII (FVII) when injury occurs. Once bound, the complex activates FVII which then becomes activated (FVIIa). The coagulation cascade continues with FVIIa binding Factor X (FX) to activate it to become FXa. The pathway leads to the conversion of thrombinogen to thrombin, fibrinogen to fibrin, and clot formation. This pathway is regulated by the Tissue Factor Pathway Inhibitor (TFPI) protein which complexes with FVIIa and Xa, preventing their binding to TF. (Petrillo, G. *et al.,* 2010). The TF coagulation cascade is illustrated in Figure 2.

Figure 2: The TF coagulation cascade



From: Laffan, M. Pulmonary Embolism. Thorax 1998;53:698-702 Copyright 1998, printed with permissions from BMJ Publishing Group Ltd.

TF has been shown to mediate inflammation in addition to initiating the coagulation cascade. The TF-FVIIa complex activates PARs leading to the production of inflammatory cytokines (Holroyd, EW. and Simari, RD., 2010). As well, activated factors in the coagulation cascade act as inflammatory mediators themselves, through protease activated receptor (PAR) activation. (Chu, AJ., 2011).

Elevated levels of TF could lead to thrombosis in pregnancy, which could lead to placental failure, growth restriction and eventual pregnancy loss. In the animal model of RPL, TF was shown to induce thrombi formation leading to decreased placental blood flow and subsequent decreased angiogenesis. In this model, TF was also found to mediate the release of a sFlt-1, a receptor for VEGF-1. VEGF-1 is necessary for trophoblast development and is gathered by the soluble receptor sFlt-1, restricting its

potential proliferation. Using pravastatin, TF was down regulated in this model, sFlt-1 levels were decreased, VEGF-1 levels restored and pregnancies were saved. (Redecha, P. *et al.,* 2009)

In addition to converting mevalonate into cholesterol intermediates produced by the mevalonic acid pathway, isoprenoids, are essential for the post translational modification of G proteins involved in cell signaling. (Buhaescu, I. and Izzedine, H *.,* 2007) An example of such G proteins are PARs, protease activated receptors. PARs play a role in inflammatory and coagulation response from tissue injury. As a responder to tissue injury, PARs have been shown to induce TF up regulation. By decreasing PAR prenylation, statins are able to decrease TF upregulation (Banfi, C. *et al.,* 2011).

2.3. Placental Transfer

2.3.1. Passive diffusion across the placenta

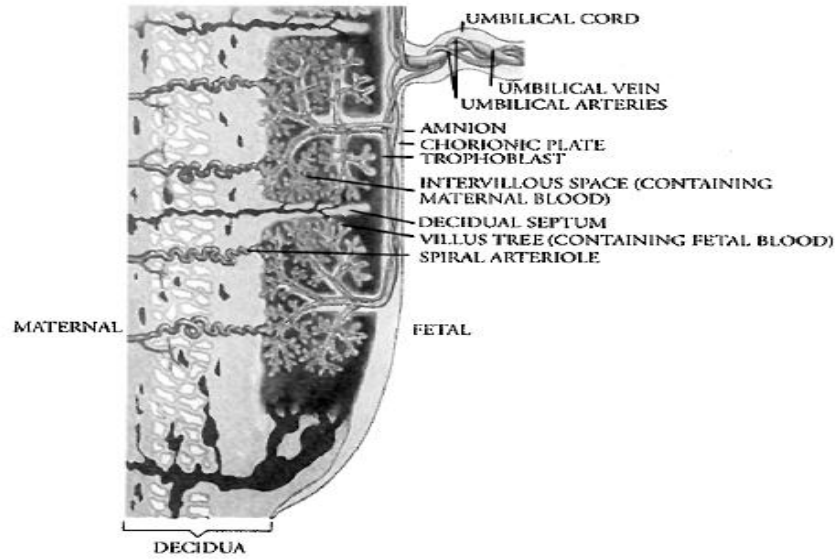
Placental structure differs according to species making it difficult to predict transfer and function across different species. The human placenta is haemochorial, where maternal blood is in direct contact with the trophoblast. This placenta is composed of maternal and fetal tissue divided into 20-40 functional units called cotyledons. In the cotyledon, pictured in Figure 3, maternal and fetal circulations are separated by a barrier consisting of fetal endothelium and the trophoblast which is composed of the villus stroma, cytotrophoblast and syncytiotrophoblast.

The syncytiotrophoblast borders the fetal compartment with the basal membrane and the maternal compartment with the syncytial microvillous apical membrane. The syncytial microvillous membrane contains maternal blood while the basal membrane contains fetal blood flow. These two membranes are responsible for the permeability or

impermeability of a molecule across the placenta. Molecules from maternal blood need to cross this placenta barrier to enter or exit the villus tree, containing fetal blood (van der Aa, EM. *et al.,* 1998). In the first trimester this barrier is at its thickest, around 20-30 um. As a pregnancy progresses this barrier thins and reaches 2-5 um at term, potentially resulting in maximum transfer (Włoch, S. Pałasz, A. Kamiński M., 2009).

The membranes responsible for permeability of the placenta are lipid membranes. Therefore, lipid substances readily diffuse across the membrane when they are less than 500 Da, and non-ionized. Transfer is dependent on the flow rate of maternal and fetal blood. In contrast, polar, hydrophilic compounds cross slowly, and transfer is limited by the permeability across the membrane. (van der Aa, EM. *et al.,* 1998)

Figure 3: A schematic representation of the human placenta.



From: van der Aa, EM. Peereboom-Stegeman, JH. Noordhoek, J. Gribnau, FW. Russel, FG. Mechanisms of drug transfer across the human placenta. Pharm World Sci. 1998 Aug;20(4):139-48. Copyright 1998, with permissions from Springer

The human placenta acts as the site of contact between the mother and the fetus. It is the site of transfer from maternal to fetal compartments, as well as fetal to maternal transfer. Essential to fetal development, nutrients and oxygen are transferred to the fetus while waste is transferred from the fetus to the mother. The placenta is permeable to most lipophilic molecules smaller than 500 Da where diffusion occurs passively. In addition to transfer, the placenta acts as the site of production of essential pregnancy hormones. Transfer across the placenta may also be modified by metabolizing enzymes contained in the placenta (Syme, MR. *et al.,* 2004).

While transfer across the placenta can occur via different mechanisms, the predominant mechanism of transport is passive diffusion. Ease of transfer depends on the properties of the molecule including molecular weight, pKa, lipid solubility, and protein binding. Generally, lipophilic molecules less than 500 Da are readily transferred across the placenta. Since only free molecules cross the placenta those highly protein bound are less likely to diffuse freely (Myllynen, P. *et al.,* 2007). Transfer by diffusion is dependent on blood flow rate and membrane thickness (Rubinchik-Stern, M. and Eyal, S., 2012).

2.3.2. Placental transporters

Passive diffusion occurs across a concentration gradient and no energy is required. However, not all molecules readily diffuse across the lipid membranes. There are molecules that require active transport to reach fetal circulation. Active transporters include ABC binding cassette transporters which efflux substrates against their concentration gradient. This family of transports include: P-glycoprotein (P-gp, MDR-

1/ABCB1), MDR3/ABCB4, BCRP/ABCG2 (breast cancer resistance protein), and MRPs/ABCC 1-6, 10-11 (multidrug resistance proteins).

P-gp is a large integral membrane protein located at the apical membrane of the syncytiotrophoblast where its peak activity occurs in the first trimester. It acts to eliminate hydrophobic, weak alkaline molecules from fetal circulation. It is essential in protecting the fetus from toxic metabolites and xenobiotics. While P-gp activity peaks in the first trimester, MDR3/ABCB4 activity peaks in the third trimesters. Transport of this basolateral protein is not well identified but is known to include phospholidylothoine, digoxin and paclitaxel.

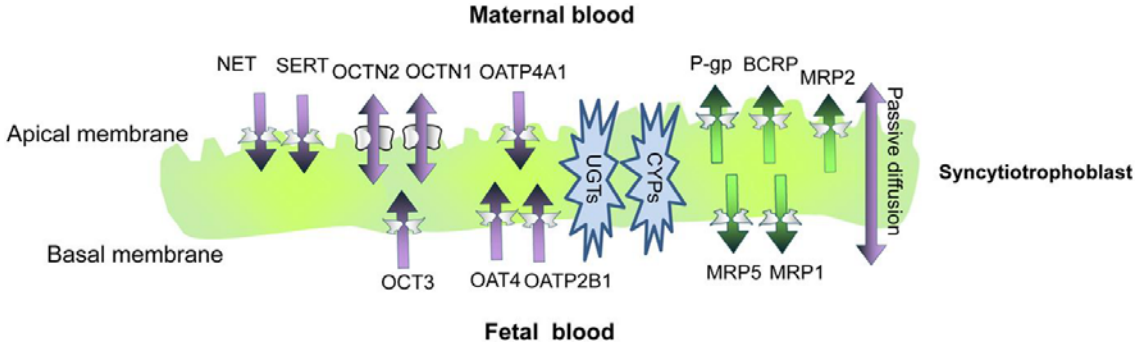
MRPs exhibit greater substrate specificity compared to P-gp. Substrates include: ampiphilic, lipophilic molecules and sulphate or glucoronic acid conjugates. The MRPs are expressed in various positions in the placenta and their expression differs by time in gestation. For example, MRP1 and MRP2 expression increased with gestational age. However, MRP1 is located in the endothelium of the capillary villi as well as the base of the syncytiotrophoblast. In contrast, MRP2 is only located in the microvilli of the syncytiotrophoblast. MRP3 exhibits stable expression throughout gestation and is located in the capillaries of the villi as well as the syncytiotrophoblast.

BCRP/ABCG2 proteins are located in the maternal facing syncytiotrophoblast as well as fetal facing placental capillaries.

The organic anion transporters (OAT) family of proteins includes OAT1, OAT3 and OAT4. They are located in the basal syncytiotrophoblast membrane and efflux from fetal circulation. In contrast to proteins that efflux substrates from the fetal to maternal circulation, there are proteins which actively transport substrates from the maternal to

fetal circulation. Polypeptide organic anion transporters (OATPs), including OATPA, OATPB, OATP8, OATPD and OATPE, transport substrates to the fetus (Włoch S, *et al.,* 2009) or mother, depending on their location. Additional influx transporters include: Organic Cation transporters (OCTN1, OCTN2) which are expressed at the maternally facing syncytiotrophoblast, as well as serotonin transporters (SERT) and norepinephrine transporters (NET) whose substrates include amphetamines. (Rubinchik-Stern, M. and Eyal, S., 2012). Transport direction is summarized in Figure 4.

Figure 4: Placental Transporters



From: Rubinchik-Stern M and Eyal S (2012) Drug interactions at the human placenta: what is the evidence? Front. Pharmacol. 3:126. Copyright 2012, with permissions from Frontiers in Pharmacology

2.3.3. Models for Studying Placental Transfer

The placenta plays an essential role in fetal drug exposure. However, for many reasons the study of fetal drug exposure is challenging: 1) it is unethical to conduct controlled drug trials with pregnant women; 2) interspecies placental variability makes it difficult to extrapolate human fetal exposure from animal models; 3) available cell lines similar to placental tissue are difficult to grow and results from experiments with cell

lines may not reflect an in-vivo environment; 4) cord blood collection from neonates exposed during gestation requires the consumption of the drug during pregnancy which is difficult to obtain in medications that do not have a positive safety profile (Myren, M. *et al.,* 2007). In light of these challenges, an ex-vivo perfusion model has been developed. This model uses available placental samples and re-creates an in-vivo environment. The placenta is the only organ that can be ethically studied outside of the human body (Myren, M. *et al.,* 2007). Once it is no longer needed (i.e. after delivery) it is essentially biomedical waste. However, it still functions as it did in-utero for hours outside the womb (Di Santo, S. *et al.,* 2003). This model isolates one fetal vein/artery pair from a single cotyledon to determine transfer. Mimicking pressures, gases, and nutrients of the placental environment during pregnancy, this model is able to accurately reflect placental function during pregnancy and has proven itself to be an ideal model for studying placenta transfer and fetal exposure (Hutson, JR., 2011)

Chapter 3. Patients and Methods

3.1. Comparison of Tissue Factor Levels in Healthy Pregnant Women, Women with Recurrent Pregnancy Loss (RPL), and Lupus Anticoagulant Positive (LAC+) Women

3.1.1. Study recruitment

Patients were recruited through the LifeQuest Centre for Reproductive Medicine. The T.E.R.M. (Treatment and Evaluation of Recurrent Miscarriage) programme at LifeQuest investigates unexplained causes of 2 or more consecutive losses. Patients were recruited when planning a pregnancy after 2 or more losses, or when pregnant having previously experienced 2 or more consecutive losses. Patients were counseled regarding the rationale, participation details and risks involved. They were given the consent form (appendix A) and were allowed time to read, understand and ask questions regarding the study, in accordance with an internal ethics board at LifeQuest. Once the consent form was signed, they were given the requisition for sample collection.

Control and LAC+ sera were obtained from previously collected samples where participants had consented for samples to be used for research purposes.

3.1.2. Inclusion and exclusion criteria

Patients were eligible after having experienced 2 or more consecutive unexplained miscarriages. Exclusion criteria for the study would be any findings of explanation for their losses, including uterine abnormalities, hormonal abnormalities, chromosomal abnormalities and presence of antiphospholipid antibodies. As well, to avoid age related factors of pregnancy loss, patients over the age of 38 were excluded from the study.

3.1.3. Laboratory methods

To determine levels of tissue factor, serum was collected from patients in 5 mL BD Vacutainer ® tubes (Becton, Dickinson and Co., Franklin Lakes, New Jersey) with a gel for separation. Once collected, samples were centrifuged at 4 degrees Celsius for 15 minutes at 2500 rpm. Then, the samples were aliquotted into cuvettes (Diagnostica Stago, France). Once samples had been collected and aliquotted, they were stored at 80 degrees Celsius until tested as a batch. When ready to test, samples were thawed over night at 4 degrees Celsius and brought down to room temperature on the lab bench.

Tissue factor levels were analyzed using the *Abnova* Tissue Factor ELISA Kit (Cat # KA0506, *Abnova,* Walnut, CA, USA.). The assay utilizes a quantitative sandwich enzyme immunoassay. The 96 well microplate had been coated with a TF specific polyclonal antibody. The samples were sandwiched between the fixed TF specific antibody and a biotinylated polyclonal antibody specific to TF. Then, a streptavidin-peroxidase conjugate was added and colour proportional to the TF concentration appears with the addition of a peroxidase substrate. Finally, the intensity of the colour was measured at 450nm using an ELISA ELx800 Universal Microplate Reader (Bio-Tek Instruments Inc. Vermont, USA) and the concentrations of samples were calculated compared to TF standards. The limit of detection for the assay was 20pg/ml (*Abnova* Tissue Factor ELISA Kit insert).

3.1.4. Statistical analysis

Data were expressed as mean plus standard error of the mean (SEM). Comparison of values was determined using a Student's t-test for unpaired data or Mann Whitney U test where appropriate. Significance was determined when $p<0.05$.

3.2. Follow-up of Statin Exposed Pregnancies from the Motherisk Counseling Line

3.2.1. Use of previously published data

The current study used previously published data (Taguchi, N. *et al.*, 2008) collected by the Motherisk group. Similar to the current study, the previous study investigated the outcomes of pregnancies following exposure to statins. Pregnancies were from 1998- 2005. Information was collected by telephone interviews to determine birth defect risk following statin exposure.

For the current study, we aimed to gain more information on teratogenic risks of statins. The primary outcome was the occurrence of a major birth defect identified from birth. Secondary outcomes included IUGR, therapeutic abortions, spontaneous abortions, neonatal death, and stillbirths. We collected data on pregnancies occurring from 2006-2010. We included the previously published data in order to show the effects on a larger group of exposed pregnancies.

3.2.2. Patient recruitment

Patients were recruited when they called the Motherisk counseling line to inquire about the safety of statin use during pregnancies. Prospective pregnancy and maternal health information was collected at the time of the initial call. Retrospectively, pregnancy outcomes were collected once adequate time had passed for the pregnancy to proceed and potentially result in a live birth (Appendix B).

3.3. Placental Perfusion of Pravastatin

3.3.1. Drug preparation

The desired concentration of pravastatin was based on the clinically relevant peak serum concentration of a 40 mg daily dose, 50 ng/ml (Lilja, JJ *et al.,* 1999). Based on this

concentration of pravastatin (Sigma-Alrich Corporation, St. Louis, Missouri) a stock sample of 100ug/mL was prepared in methanol and stored at -20 degrees Celsius. When adding pravastatin to the experiment, 125 uL of stock solution was added to 250 mL of maternal perfusate, based on the calculation:

C1V1= C2V2 where C1= 100ug/mL, C2= 50ng/mL, V2= 250mL and V1 is the unknown variable.

3.3.2. Placental Procurement

Patients were recruited at Toronto's St Michael's Hospital while undergoing a scheduled caesarean section. Patients were approached for the study once deemed eligible (placenta not required to be sent to pathology, no communicable diseases). The study was previously approved by the hospital's research ethics board and recruited participants signed a consent form (appendix C) prior to delivery.

3.3.3. Perfusion Method

The perfusion method has been previously described by our laboratory (Hutson. JR. *et al.,* 2011, Pollex, EK. *et al.,* 2010). For our experiment, following delivery, the placenta was placed in a plastic container filled with heparanized phosphate buffered saline to prevent coagulation during transportation. The placenta was transported to an onsite perfusion laboratory. An artery-vein pair on the fetal side was isolated and cannulated and flow was established with 150ml of fetal perfusate solution (10.9g/L M199 tissue culture medium-Sigma Aldrich, St. Louis, MO, dextran 30.0 g/L, heparin 2000 IU, Kanamycin 100 mg/L, and 30 mM NaHCO3 to reach pH 7,4). Once excess tissue was discarded, the isolated lobule was placed in the PBS filled perfusion chamber, fetal side down, and clamped into place. This was done assuring continuous flow and

appropriate pressure (40-60 mm hg). Once clamped into place, flow was observed for leakage.

The experiment was stopped if volume leakage exceeded 4mL/hour. Then, the maternal flow was established with blunt tipped needles inserted under maternal tissue at the location of the fetal pair, and outflow collected from the surface of the maternal tissue. Blood was completely cleared with a round boiling flask containing 250 mL of maternal perfusate (10.9g/L M199 tissue culture medium-Sigma Aldrich, St. Louis, MO, dextran 7.5 g/L, glucose 2.77 mM, heparin 2000 IU, Kanamycin 100 mg/L, 1mM antipyrine and 25 mM NaHCO3 to reach pH 7.35).

Once cleared of fetal and maternal blood, the circuits were closed, perfusate solution was refreshed and the control hour started. The control hour ensured placental viability by determining markers of viability including $P0^2$, PCO^2, pH, glucose (mg/dL), hCG, and antipyrine transfer. $P0^2$, PCO^2, pH and glucose levels were determined by sampling every 15 minutes from the fetal artery (FA), fetal vein (FV), maternal artery (MA), maternal vein (MV) and using the Radiometer ABL 725 Blood Gas Analyzer (Copenhagen, Denmark). Oxygen content, delivery, transfer and consumption were calculated using the formulas in Table 1.

HCG levels and antipyrine diffusion were determined after the perfusion experiment was completed. HCG was collected from maternal and fetal sampling and levels were determined using ELISA (Alpha Diagnostic International, San Antonio, TX) with a standard curve of known concentrations. The sample concentrations and standards were determined with the Bio Tek Synergy HT microplate reader (Bio Tek Instruments,

Winooski, VT) read at 450 nm and unknown levels calculated using the standard curve and the equation : concentration = (absorbance – y-intercept)/slope.

Antipyrine levels were used as a marker for passive diffusion across the placenta, since antipyrine is known to readily transfer across the placenta by passive diffusion (Schroder *et al.,* 1985). To determine antipyrine concentration, standards and samples were analyzed with the UV – visible spectrophotometer W-160A (Shimadzu, Tokyo) at 350 nm. Unknown sample concentrations were determined using the standard curve and the equation: concentration = (absorbance – y-intercept)/slope.

Following the control hour, fetal and maternal perfusate levels were collected, and the experiment phase consisting of 3 hours was initiated. Maternal and fetal perfusates were refilled, including pravastatin solution in the maternal reservoir, added as previously described. Viability markers continued to be collected and detected with the ABL Gas Analyzer at 0, 10, 20, 30, 60, 90, 120, and 150 minutes. Samples to be analyzed for pravastatin concentration at 0, 10, 20, 30, 60, 90, 120, 150, and 180 minutes and samples to determine hCG and antipyrine transfer were collected at 0, 30, 60, 90, 120, 150 and 180 minutes. Samples were stored at -20 degrees Celsius until sample analysis.

Table 2: Oxygen Content, Delivery, Transfer and Consumption

values	calculations	variables
Oxygen content	$0.939/(BP-47) \times pO^2$	0.939= solubility of oxygen (umol/02/ml fluid 37 degrees Celsius, 1 atm pressure BP= barometric pressure mmHg 47- saturated vapour pressure of water at 37 degrees $P0^2 = p0^2$ of sample
Oxygen delivery	MA x Qm/WT	MA= 0^2 concentration of maternal artery perfusate sample (umol O^2/ml perfusate) Qm –flow rate maternal flow (mL/min) WT weight of lobule (g)
Oxygen transfer	Qf x (FV-FA)/WT	Qf= flow rate fetal flow (mL/min) FV= O^2 fetal vein (umol O^2/ml perfusate)

		FA= 0^2 fetal artery (umol O^2/ml perfusate) WT weight of lobule (g)
Oxygen consumption	O^2 lost from maternal – O^2 transferred to fetal	O^2 lost = 0^2 maternal delivery calculated above (umol/min/g) 02 transferred- o2 transfer calculated above (umol/min/g)

3.3.4. Sample Detection

Pravastatin and d3-pravastatin were obtained from Toronto Research Chemicals (North York, Canada). A standard curve was prepared in blank maternal perfusate. Aliquots (100 µl) of standards and samples were precipitated in 300 µl of acetonitrile containing d3-pravastatin, and centrifuged for 20 minutes at 19 000 rpm at 4 °C. The supernatant was diluted 1:2 in 0.05% formic acid. The diluted samples were injected (50 µl) by a TLX2 high-performance liquid chromatography system (Thermo Scientific, Pittsburgh, Pennsylvania) onto a Hypersil GOLD column (50 x 3 mm particle size; Thermo Scientific), attached to a TSQ Vantage triple-quadruple mass spectrometer with a HESI II probe (Thermo Scientific). The mobile phases were 0.05% formic acid and acetonitrile containing 0.05% formic acid. Pravastatin was then detected in negative

mode, using the transitions m/z 423.4 to 321.4 for pravastatin, and m/z 426.4 to 321.4 for d3-pravastatin. The range of pravastatin quantification was 1ng/mL to 100 ng/mL.

Chapter 4. Results

4.1. Comparison of Tissue Factor Levels in Healthy Pregnant Women, Women with Recurrent Pregnancy Loss (RPL), and Lupus Anticoagulant Positive (LAC+) Women.

A total of 26 patients were included in the study. 6 patients were LAC +, 9 controls with healthy pregnancy histories, and 11 patients with RPL. There was a trend in TF levels (Figure 5). The mean TF levels (with SEM) for the LAC+ group were: 334.3 ± 111.9 pg/ml non-pregnant and 293.0 ± 69.9 pg/ml during pregnancy. For the control group the mean TF levels were: 116.6 ± 29.6 pg/ml non-pregnant and 107.8 ± 69.9 pg/ml during pregnancy. For the RPL patients the average TF levels were: 147.2 ± 34.9 pg/ml non-pregnant and 235.1 ± 75.6 pg/ml during pregnancy. There were no significant differences between the RPL and controls or between the RPL and LAC+ women.

In contrast, LAC+ women had significantly higher TF levels compared to controls, both before and during pregnancy ($p<0.05$).

Table 3: Comparison of TF levels among LAC+ and RPL

	LAC+ n=6	RPL n=11	P value
Non-pregnant	334.3 ± 111.9	147.2 ± 34.9	0.06
Pregnant	293.0 ± 69.9	235.1 ± 75.6	0.62

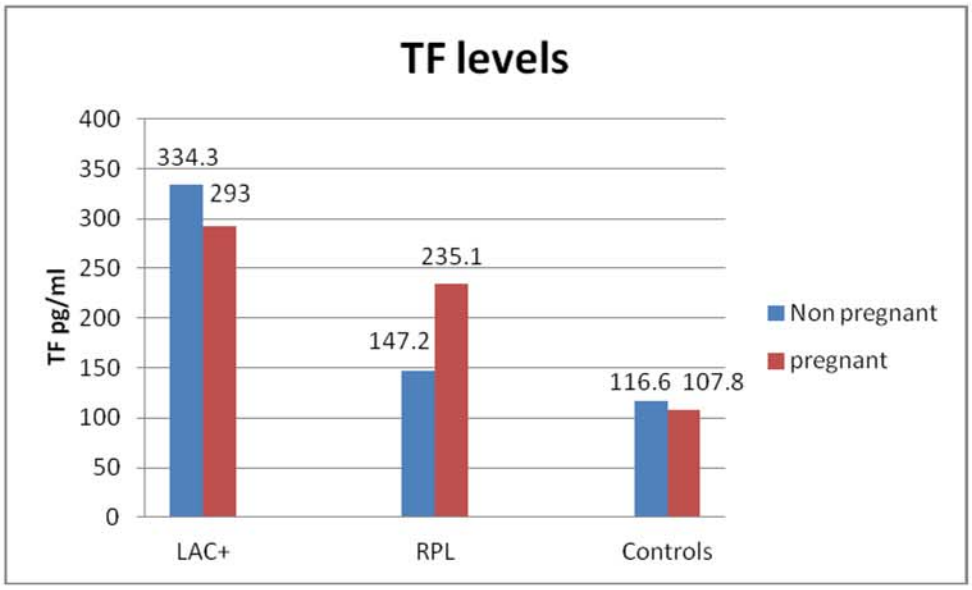
Table 4: Comparison of TF levels among RPL and Controls

	RPL n=11	Controls n=9	P value
Non-pregnant	147.2 ± 34.9	116.6 ± 29.6	0.52
Pregnant	235.1 ± 75.6	107.8 ± 69.9	0.28

Table 5: Comparison of TF levels among LAC+ and Controls

	LAC+ n=6	Controls n=9	P value
Non-pregnant	334.3 ± 111.9	116.6 ± 29.6	0.042
Pregnant	293.0 ± 69.9	107.5 ± 69.9	0.032

Figure 5: Comparison of TF Levels among Subgroups



4.2. Follow-up of Pregnancies Exposed to Statins

Data were collected on the total number of inquiries regarding the safety of statins from 2006-2010. The callers included: pregnant women exposed to statins, women exposed to statins planning a pregnancy, and inquiries made by health professionals regarding the safety of statins during pregnancy. The number of total inquiries per year are illustrated in Figure 6. Inquiries into the safety of statins during pregnancy were consistent over time, with rosuvastatin inquiries rising over time. There were no inquiries regarding the safety of lovastatin or fluvastatin over this five year time period.

Regarding pregnant patients exposed to statins, there were 123 callers from 1998-2012. Outcomes were collected for 83 exposed pregnancies, among which 64 outcomes were collected in the previously published study (Taguchi, N. *et al.,* 2008). Maternal characteristics are listed in Table 6. The exposed groups were matched to a control group not exposed to known teratogens. The groups were matched according to the week of initial consult and according to age. Known characteristics are listed in Table 6, significance was determined with Student's t-test and z-test, where appropriate. Maternal weight and diabetes were significantly more prevalent in the exposed group compared to controls. There were no significant differences in pregnancy outcomes between groups, *p* values were determined with a z-test. Pregnancy outcomes are listed in Table 8. Minor anomalies in the exposed group included one case of ankylglossia and one case of a cervical soft tissue mass, which was surgically removed.

Figure 6: Inquiries per Year

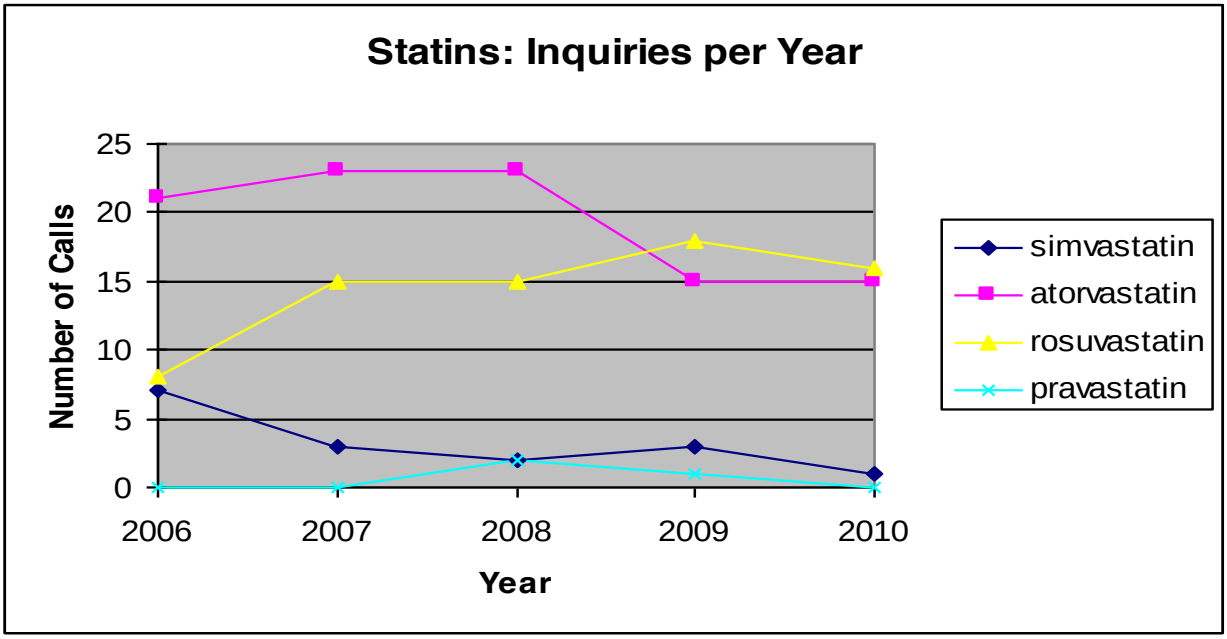


Table 6: Maternal Characteristics of Exposed and Control Groups ± sd

	Exposed Group (n=83)	Control group n=83	*P* value
Maternal age, yrs	34.0 ± 5.3	33.0 ± 4.0	0.17
Gravidity, n	2.7 ± 1.6	2.4 ± 1.0	0.14
Parity, n	1.4 ± 1.2	1.1 ± 0.8	0.06
Previous spontaneous abortion, n	0.8 ± 1.3	NA	
Gestational age at recruitment, wks	6.7 ± 3.6	7.1 ± 3.4	0.46
Maternal weight at recruitment, kg	75.6 ±19.3	66.4 ± 11.1	<0.001
Maternal diabetes, n (%)	14 (16.9)	3 (3.6)	0.01

Table 7: Maternal Exposure to Statins

Medication	N	Discontinuation of Medication (pregnancy weeks, mean ± sd)	Dose (mean ± sd)
Atorvastatin	54	6.1 ± 2.3	16.8 ± 10.9
Rosuvastatin	11	7.4 ± 2.0	10.0 ± 5.9
Pravastatin	8	8.2 ± 9.0	26.3 ± 15.0
Simvastatin	4	7.2 ± 4.4	15.0 ± 14.1

Table 8: Pregnancy Outcomes

	Exposed (n=83)	Controls (n=83)	*P* value
Livebirths, no. (%))	58 (69.9)	66 (79.5)	0.21
Spontaneous Abortions, n (%)	20 (24.1)	15 (18.1)	0.45
Therapeutic Abortions, n (%)	4 (4.8)	0 (0)	0.13
Stillbirths, n (%)	1 (1.2)	1 (1.2)	1.0
Birth defects, n (%)	2 (2.4)	2 (2.4)	1.0

4.3. Placental Perfusion of Pravastatin

Of 54 collected placentae, 2 were successfully perfused with pravastatin. The masses of the cotyledons were 20.4 grams and 10.82 grams. The measures of viability and function of the placentae were within normal ranges throughout the experiments and are listed in Table 9. The rate of disappearance of antipyrine from maternal perfusate was 0.030 ± 0.002 umol/g/min, and appearance into fetal perfusate was 0.24 ± 0.003 umol/g/min for the entire 180 minute experiment. Mean antipyrine transfer is illustrated in figure 3 as mean ± SEM.

After adding pravastatin (50 ng/ml) to maternal circulation, there was a rapid decrease in the concentration from the maternal compartment. However, pravastatin in the fetal circulation was not detected until 1 hour into the experiment, suggesting uptake by placental tissue. Transfer was determined for 180 minutes. After the 3 hour experiment, the mean fetal concentration of pravastatin was 4.4 ng/ml ± 0.8 and the mean fetal to maternal ratio of pravastatin was 0.17 ± 0.04.

Table 9: Viability Parameters throughout Perfusions (mean ± SEM)

Viability parameter	Pre-control	experiment
hCG production (mIU/g/min)	299.5 ± 175.5	98.7 ± 36.3
Oxygen (μmol O2/g/min)		
Transfer	1.0 ± 0.00	1.0 ±0.00
Delivery	0.495±0.13	0.48± 0.09
Consumption	0.23±0.06	0.22±0.04
Glucose Consumption (μmol/g/min)	0.38±0.006	0.145±0.01

Figure 7: Mean Antipyrine Transfer ± SEM

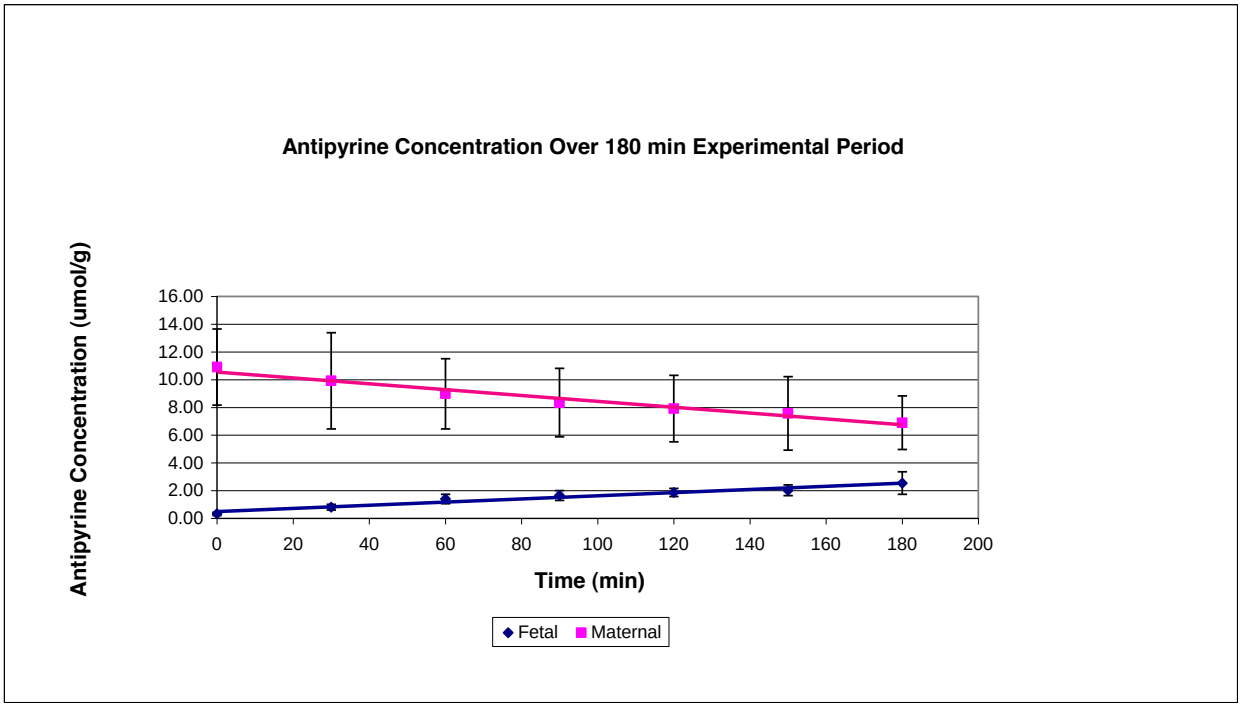


Figure 8: Pravastatin Concentrations in the Maternal and Fetal Circulations over 180 Minute Experiment

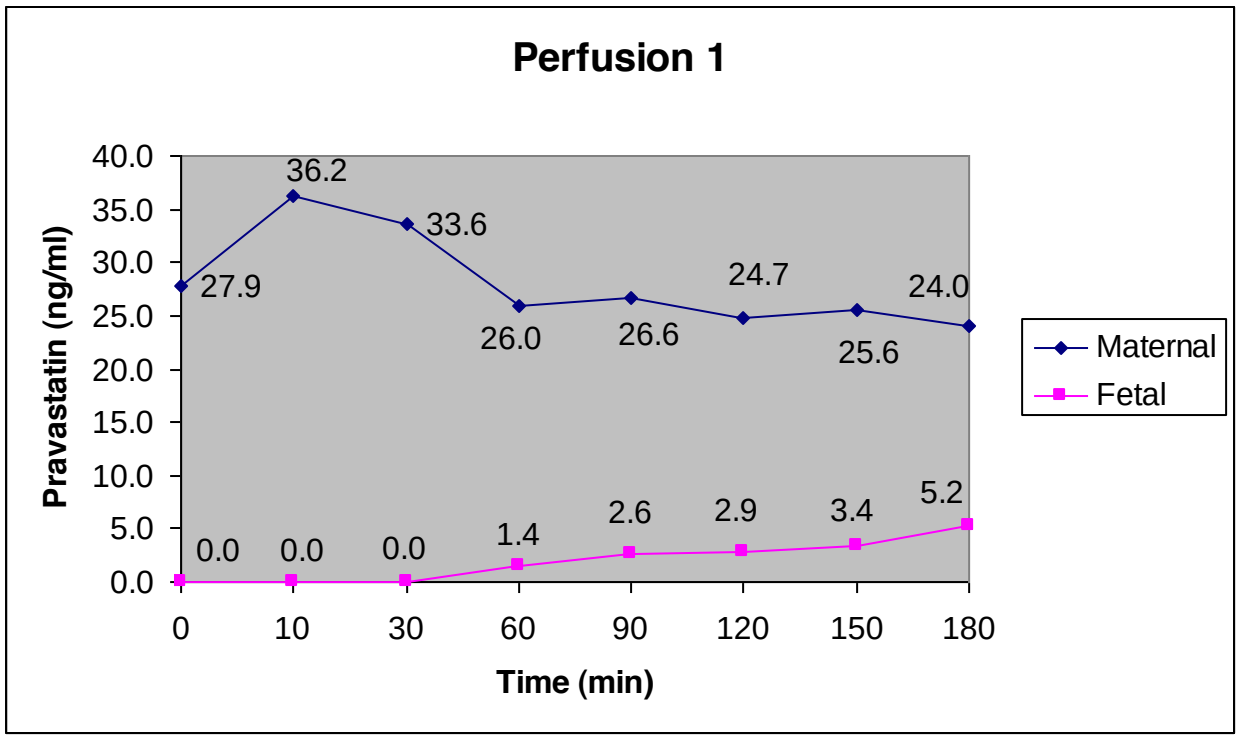
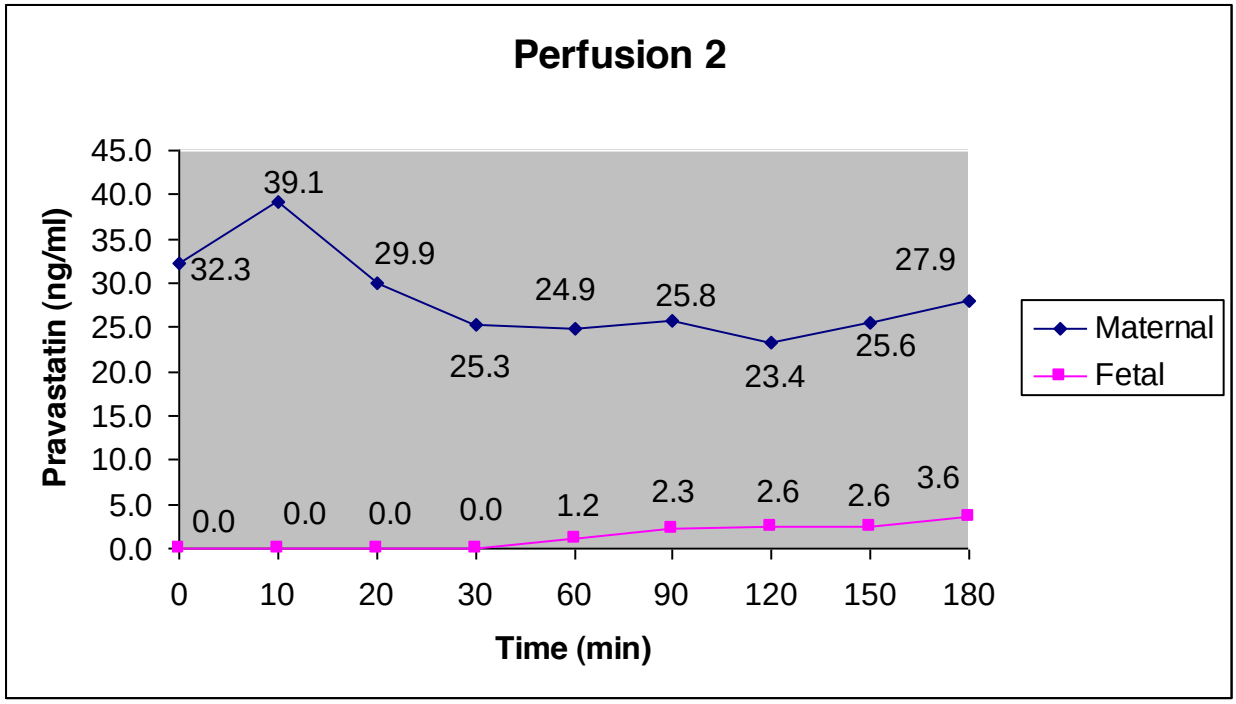


Figure 9: Pravastatin Concentrations in the Maternal and Fetal Circulations over 180 Minute Experiment



Chapter 5. Discussion

This thesis focuses on assessing the risks of statin exposure in pregnancy in the context of promising new reproductive indications for this class of drugs. This evaluation includes their potential role in alleviating recurrent pregnancy loss, their ability to cross the human placenta, and the "real life" evidence of them causing congenital malformations.

5.1. Tissue Factor Levels during Pregnancy

In our study, LAC+ women had significantly higher levels of circulating TF compared to controls, whether they were pregnant or not pregnant. LAC is the circulating antiphospholipid antibody most likely associated with adverse pregnancy outcomes (Lockshin, MD. *et al.,* 2012). The six women in our LAC+ cohort had adverse pregnancy outcomes including IUGR and pre-eclampsia, even though they had been treated with low molecular weight heparin and low dose aspirin. Their significantly elevated TF levels may warrant evaluation for treatment which targets this pathway.

In contrast, TF levels in the RPL cohort were not significantly elevated compared to controls, in both pregnant and non-pregnant states. However, TF levels in the LAC+ cohort were not significantly elevated compared to the RPL group, either. Rather, a trend was observed in TF levels of the RPL group that appeared to be elevated compared to controls, but not as elevated as the LAC+ group. Interestingly, even though expected success rates of a subsequent pregnancy of RPL women are expected to be 60-70%, (American Society for Reproductive Medicine*.,* 2008) in our small cohort of RPL patients, 100% of them had successful pregnancies. This may indicate that the current

therapy may be effective in treating RPL. However, a larger sample is required to determine accurate success rates of therapy.

5.1.1. Study Limitations

The sample size was small for each of our study groups, resulting in low power in the results. As well, since there were no miscarriages in our RPL group we could not determine TF levels associated with pregnancy loss. Future work with increased numbers will allow for evaluation of the association of tissue factor with unexplained recurrent pregnancy loss as well as adverse pregnancy outcomes in LAC+ women.

5.2. Statin Exposure during Pregnancy.

Pregnancy outcomes in pregnancies exposed to statins did not differ compared to unexposed controls with respect to rates of birth defects, miscarriages, neonatal deaths or stillbirths. The exposed group had a non-statistically significant increase in occurrences of therapeutic abortions. However, the therapeutic abortions in the exposed group were not due to detected fetal malformations. Rather, they were due to medical conditions that were interpreted by the clinicians as incompatible with pregnancy, or due to concerns regarding exposure to statins. Increased maternal weight, which may be associated with miscarriage and birth defects (Lashen, H. *et al.,* 2004, Watkins, ML. *et al.,* 2003), was significantly higher in the exposed group. Maternal diabetes was also significantly greater in the exposed group, and may also be associated with an increased rate of birth defects (Evers, IM. *et al.,* 2004); however, it was not known whether the disease was Type 1 or Type 2. The birth defects reported were minor and did not appear to suggest a specific pattern indicative of an error in cholesterol biosynthesis.

Thirteen years (1998-2010) of collected data from the Motherisk Program did not include inquiries regarding exposure to fluvastatin or lovastatin. This is interesting to note since the initial case reports published described 9 of 31 incidences of birth defects occurring after exposure to lovastatin (Edison, RJ. and Muenke, M. ., 2004). As well, cerivastatin was removed from the market in 2001 (Furberg, CD. and Pitt, B., 2001); this was another statin not identified in our cohort. However, it was one of the statins described in the 2004 published case reports as being associated with a birth defect. Additionally, rosuvastatin was released into the market in 2003 (Culhane, NS. *et al.,* 2005), and it was missing from the group of statins described by Edison and Muenke. However, in our collection of all statins, rosuvastatin inquiries consistently increased over time. The differences in statin exposure observed in our cohort compared to the 2004 published case reports, suggest a change in pattern of exposure; this further necessitates the need for re-evaluation of the statins to reflect those that women are actually exposed to.

5.2.1. Study Limitations

While 123 exposed women contacted Motherisk during their pregnancy, outcomes could only be determined for 83 pregnancies and recall bias can be associated with follow-up. After pregnancy, women may have difficulty remembering details of their pregnancy progression and medication cessation. However, since initial data were collected when women first called to inquire about drug safety, it was confirmed that they had been exposed to the drug at the time of their initial call.

5.3. Placental Transfer of Pravastatin

Previously, it had not been believed that pravastatin crossed the placenta in a substantial amount (Edison RJ. and Muenke, M., 2004, Aubuchon, M. *et al.,* 2011). This assumption was based on the hydrophilic property of pravastatin, limited transfer in animal testing (Edison and Muenke., 2004), as well as illustrations of the safety of pravastatin exposure during pregnancy reported in case reports and cohort studies (Edison and Muenke., 2004, Ofori *et al.,* 2007). In addition to its hydrophobic property limiting its transfer via passive diffusion, pravastatin is a substrate for OATP1A2, OATP1B1, OATP2B1 (Shirasaka, Y. *et al.,* 2010, Ieiri, I. *et al.,* 2009) which may transport pravastatin to the fetal circulation. In addition, pravastatin is a substrate for MRP2 (Ieiri, I. *et al.,* 2009, Hasegawa, Y. *et al.,* 2010) which may efflux the drug back into maternal circulation.

Our study showed that pravastatin is transferred across the placenta, albeit at low concentrations, and not measureable until after one hour. While pravastatin has the potential to cross the placenta, it may actually transfer to a lesser extent than our experiment showed. The closed system perfusion model we used maintained a constant concentration throughout the experiments. In reality, the half-life of pravastatin is just 2 hours. After 2 hours, our experiment showed that the concentration of pravastatin in fetal circulation was 2.75 ng/ml ± 0.15. It continued to increase to reach its maximum concentration after three hours in our experiment, 4.4 ng/ml ± 0.8. It is likely that this maximum concentration observed would have been much lower since maternal concentration of the drug decreases by half after 2 hours (Singhvi, SM. *et al.,* 1990). Protein binding may account for a decrease in drug transfer, but pravastatin's protein

binding is 50%; allowing transfer during the transition from free to bound drug. In the first hour of the perfusion, we observed a rapid decrease in maternal drug concentration without an increase in fetal concentration. This suggests that drug transfer occurred only after the placenta was fully saturated.

5.3.1. Study Limitations

In our study, we were able to successfully perfuse 2 placentae with pravastatin; ideally, 4 successful perfusions are necessary to establish transfer. However, results from both perfusions were very similar illustrating transfer with a SEM of ± 0.8. This small margin of error is reassuring with respect to the accuracy of our results.

The clinically relevant dose of pravastatin, 40mg/day, has a Cmax of approximately 50ng/ml (Lilja, JJ. *et al.,* 1999). This was the targeted maternal dose for the perfusion. However, the maximum observed concentration was slightly lower, approximately 40 ng/ml. Furthermore, steady state was not reached in our 3 hour experiment; this resulted in a limited ability to extrapolate overall fetal exposure. Nevertheless, pravastatin transfer into fetal circulation appears low, and it is likely lower than observed in our experiment.

5.5. Should FDA Pregnancy Classification of Statins be Changed?

In summary, our results suggest that the risk of malformations from statin exposure during gestation may be significantly lower than initially presumed. The FDA classification system defines category X medications as those which "*studies in animals or humans have demonstrated fetal abnormalities or if there is positive evidence of fetal risk based on adverse reaction reports from investigational or marketing experience, or both, and the risk of the use of the drug in a pregnant woman clearly outweighs any*

possible benefit." (Federal Register. 2008) Based on this classification of drug safety during pregnancy, statins do not appear to fit into this contraindicated class. This system, while essential for proven teratogens such as thalidomide, may be detrimental when generalizations are made with regard to families of drugs that are classified without discrimination among newer formulations with different physiochemical properties. Animal studies, with the exception of lovastatin administered at maternally toxic doses (Lankas, GR. *et al.,* 2004), have not demonstrated increased risk of malformations. Small controlled studies - in humans - have also not demonstrated increased risk to the fetus (Ofori, B. *et al.,* 2007, Taguchi, N. *et al.,* 2008, McGrogan, A*.,* 2009, Colvin, L. *et al.,* 2010, Winterfeld, U. *et al.,* 2011).

While being fully cognizant of the importance of erring towards the side of caution with respect to the use of any medication during pregnancy, the lack of specificity in the classification system not only created an unnecessary burden of fear for women who are taking a drug that has no history of teratogenicity but is nonetheless labeled Category X, but also inadvertently obstructs appropriate evaluation of a drug's therapeutic potential. Additionally, women who require statin therapy for cardiovascular health are not provided with justified facts regarding cessation of statins during their childbearing years and are withheld from determining the most accurate risk: benefit assessment. As well, women who suffer from obstetrical complications are not provided with the option of pursuing therapy, albeit experimental, which may improve their outcomes.

Originally, it was believed that suspending statin therapy during gestation would not interfere with long term maternal health. However, it has been twenty-five years

since the first statin, lovastatin, was released into the market (Endo, A., 2010). Since then, obesity rates have increased dramatically (Flegal, KM. *et al.,* 2002) along with associated cardiovascular effects (Mokdad. AH. *et al.,* 2001, Rabkin SW. *et al.,* 1977, Messerli, FH., 1982, Manson, JE. *et al.,* 1995). Another risk factor is that women are delaying pregnancy (Heffner, LJ.,2004) and are therefore subject to higher LDL cholesterol levels associated with advancing age (Assmann, G. *et al.,* 1998.). The current recommendation for women on statin therapy is to stop statins at least 4 weeks before trying to conceive (Goldberg, AC. *et al.,* 2011). Taking into consideration the time needed to conceive, nine months of pregnancy, breastfeeding and planning a subsequent pregnancy - this may result in many years of avoiding cholesterol lowering therapy, potentially a woman's entire childbearing years. While short term interruption may not interfere with long term cholesterol lowering therapy, a longer than desired length of time without the benefits of statins has severe and detrimental effects on a woman's cardiovascular health (Taylor, F. *et al.,* 2011).

There is also emerging evidence that an in-utero hypercholesterolemic environment has detrimental long term health effects for offspring (Napoli, C. *et al.,* 2000, Palinski, W. *et al.,* 2001, Elahi, MM. *et al.,* 2008). New research suggests that the pleiotropic effects of statins may be beneficial to women experiencing obstetrical complications (Sathyapalan T. *et al.,* 2012, Banaszewska, B., 2011, Rashidi, B., 2011, Raja-Khan N., 2011, K, *et al.,* 2011, Singh J, *et al.,* 2011, Bruner-Tran, KL., 2009, Oktem, M. *et al.,* 2007, Redecha, P. *et al.,* 2009). However, due to their contraindication and negative reputation, investigation into how they may help pregnancy related conditions are hindered.

Currently, the FDA has classified all of the statins as contraindicated despite their varying chemical properties. Pravastatin is a hydrophobic compound whose rapid elimination and extensive hepatic specificity results in low systemic bioavailability and even lower transfer to fetal circulation. Pravastatin warrants reconsideration for cases of hypercholesterolemia during pregnancy that cannot be alternatively managed. It is also needed for exploration of novel therapy for pregnancy related conditions.

In summary, this thesis provides new insight into the risk:benefit assessment of statin exposure during gestation that should be considered by clinicians and regulators caring for women of reproductive age. More studies will be needed to increase the power of each of the observations I have made, but overall there is no doubt that the currently held vision of statins being major human teratogens is most probably incorrect and should be reframed to allow more accurate risk: benefit assessment.

Chapter 6. Conclusions & Future Directions

Statins are currently contraindicated during pregnancy. However, this thesis has challenged the contraindication. The benefit of treating hypercholesterolemia during pregnancy may outweigh putative risks of birth defects. As well, new indications may warrant reconsiderations for novel therapy.

Increased tissue factor levels have been reported in animal models of recurrent pregnancy loss. In our pilot study, we observed a trend in human RPL cases. Tissue factor levels appeared to be elevated compared to controls. However, this increase was not statistically significant. Women who were LAC+ who had adverse pregnancy outcomes had a statistically significant increase in tissue factor levels compared to controls. Our sample sizes were small. Further studies are necessary to elucidate the relationship between tissue factor, unexplained pregnancy loss and adverse pregnancy outcomes in LAC+ women. A larger study would be able to determine if pravastatin is indeed an appropriate treatment option for women with these pregnancy conditions.

Follow-up of women exposed to statins during pregnancy did not show increased occurrence of adverse pregnancy outcomes or birth defects. However, our cohort was small and continuation of follow-up is necessary to increase sample size as well as to show prevalence of statin exposure during pregnancy.

Placental perfusions of pravastatin showed that pravastatin transfer across the placenta is limited. In reality, this transfer is further limited by the short elimination half-life of pravastatin. After the 3 hours experiment, fetal: maternal concentration was 0.17 ± 0.04. Further studies are necessary to determine the role of the placenta in preventing

transfer. It would be interesting to determine placental concentration of pravastatin, to determine if the placenta itself accumulates the drug.

In conclusion, the current investigations did not determine an increased risk of birth defects in statin exposed pregnancies. Furthermore, they elucidated the need for re-evaluation of the current contraindication to benefit women with dangerous cardiovascular conditions. In light of our safety assessments, statins may be appropriate for obstetrical complications. However, further studies must be conducted to fully comprehend the potential of statin therapy for obstetrical complications.

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Appendix A: Consent Form for Tissue Factor in RPL Study

The T.E.R.M. Programme
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Toronto, ON, M5G 2K4
Tel 416-593-6433 Fax 416-599-8624

You are being asked to participate in a study as part of the T.E.R.M Programme. This study involves one blood sample during your pregnancy which will be taken when you test your beta-HCG levels at your first ultrasound. Two tubes of blood will be taken to test for tissue factor; a protein associated with inflammation and thought to be connected with recurrent pregnancy loss.
You will not receive any direct benefit from this study. However, information gained may influence our understanding of factors involved in maintaining a healthy pregnancy.
You may be asked to give a blood sample after your pregnancy is completed.

As a participant of the study, your personal information will remain confidential, and only researchers involved in the study will have access to your medical information.
If you choose not to participate your care will not be affected in any way.

If you have any questions, please contact me at (416)-593-6433 or jdavidovits@lifequestivf.com.

Judith Davidovits
Study Coordinator
T.E.R.M. Programme

Consent:

I voluntarily consent to take part in the study. I have received a copy of this consent.

_____	_____	_____
Patient's Name (Print)	Patient's Signature	Date

Appendix B: Motherisk Follow-Up Questionnaire

Pregnancy Follow Up
MOTHERISK PROGRAM

ID NUMBER _____
Date of Interview: _____
Interviewer: _____

A. GENERAL

Mother's FIRST NAME _____ Street Address _____
Mother's LAST NAME _____ City/Province _____
Telephone (H) _____ (W) _____ Postal Code _____

B. PREGNANCY OUTCOME

How did your pregnancy end? ❒ Live birth ❒ Miscarriage (<20 wks) ❒ Fetal Death (≥20wks) ❒ Elective abortion

If miscarriage, fetal death or elective abortion:
At how many weeks? _____
Were defects detected? ❒ No ❒ Yes
If Yes, describe _____
_____
_____
How? By ❒ ultrasound ❒ amniocentesis
done at _____ weeks

If live birth: ❒ boy ❒ girl
Child's FIRST NAME _____
Child's LAST NAME _____
Child's DATE OF BIRTH _____
Child's doctor _____
street address _____
city/province _____
telephone _____

C. DISEASES COMPLICATING PREGNANCY

			Details: *diagnosis* *onset* *medication/doses* *hospitalization?*
Amniotic fluid alterations	❒ No	❒ Yes	_____
Cancer	❒ No	❒ Yes	_____
Cardiovascular	❒ No	❒ Yes	_____
Central nervous system	❒ No	❒ Yes	_____
Dermatology	❒ No	❒ Yes	_____
Ears, eyes, nose, throat	❒ No	❒ Yes	_____
Endocrine	❒ No	❒ Yes	_____
Gastrointestinal	❒ No	❒ Yes	_____
Genito-urinary	❒ No	❒ Yes	_____
Hematology	❒ No	❒ Yes	_____
Infectious Disease	❒ No	❒ Yes	_____
IUGR/growth problems	❒ No	❒ Yes	_____
Musculo-skeletal	❒ No	❒ Yes	_____
Psychiatric	❒ No	❒ Yes	_____
Respiratory	❒ No	❒ Yes	_____
OTHER			_____

TELEPHONE LOG

<VM >Verbal Message <MM >Machine Message <Busy>
DATE/TIME NUMBER DIALED RESULT <OS >Out of Service <W >wrong number <NA >No Answer

_____
_____

D. EXPOSURES DURING PREGNANCY

- Did you use any **herbal** preparations?
- Did you use any **vitamins** (prenatal or other supplements)?
- Did you use anything for **allergies, anxiety, colds, constipation, depression, diarrhea, headache, heartburn, pain, weightloss**?

Over-the-Counter/Prescription medications and Radiation

Drug Name or Radiation Type	Start Date	Stop Date	Dose (mg,g,mL)	Frequency (od,qhs,bid,tid)
1		ongoing ❒		
2		ongoing ❒		
3		ongoing ❒		
4		ongoing ❒		

Indication for Medication	*Prescribing Physician*	*Details about medical condition*
1.		
2.		
3.		
4.		

Social Drugs/Others	Dose	Frequency	Started	Stopped
Ethanol wine [ ] ❒ no	_____ ounce [ ]	per day [ ] week [ ] weekend [ ] month [ ]		
liquor [ ] ❒ no	_____ glass [ ]			
beer [ ] ❒ no	_____ bottle [ ]			
Tobacco ❒ no	_____ cigarettes	per day [ ] week [ ] weekend [ ] month [ ]		
Cocaine ❒ no	_____	per day [ ] week [ ] weekend [ ] month [ ]		
Heroin ❒ no	_____	per day [ ] week [ ] weekend [ ] month [ ]		
Marijuana ❒ no	_____	per day [ ] week [ ] weekend [ ] month [ ]		
Heat jaccuzi [ ] ❒ no	_____	per day [ ] week [ ] weekend [ ] month [ ]		
electric blanket [ ] ❒ no	_____	per day [ ] week [ ] weekend [ ] month [ ]		
Radiation ❒ no	_____	per day [ ] week [ ] weekend [ ] month [ ]		
Exercise ❒ no	_____	per day [ ] week [ ] weekend [ ] month [ ]		

Occupation during pregnancy (what does she do?) _____

Started _____ Stopped _____ Reason for Stopping _____

EXPOSURES? chemical ❒ no ❒ yes: _____

computer ❒ no ❒ yes: _____

radiation ❒ no ❒ yes: _____

noise/vibration ❒ no ❒ yes: _____

E. TESTS DURING PREGNANCY

1. Triple screening ❒ no ❒ yes: at ____ weeks Reason _____
2. Amniocentesis ❒ no ❒ yes: at ____ weeks Reason _____
3. Glucose Tolerance Test ❒ no ❒ yes: at ____ weeks Reason _____
4. Ultrasound ❒ no ❒ yes: at ____ weeks Reason _____
   at ____ weeks Reason _____
   at ____ weeks Reason _____
5. Chorionic villus sampling ❒ no ❒ yes: at ____ weeks Reason _____
6. Other _____ ❒ no ❒ yes: at ____ weeks Reason _____

RESULTS: # ____: _____
____: _____
____: _____
____: _____
____: _____

F. DELIVERY INFORMATION

Maternal

Weight pre-pregnancy _____ lb _____ kg
gain _____ lb _____ kg
Total length of labour _____ hours
PROM? ❒no ❒ yes: _____ hours before onset of labour
(Premature Rupture of Membranes)

Method ❒ vaginal, vertex ❒ C/S emergency
❒ vaginal, breech ❒ C/S repeat
❒ C/S scheduled
Assistance: ❒ vacuum reason: _____
❒ forceps

Hemorrhage? ❒no ❒yes
Transfusion? ❒no ❒ yes
Pain relief? Anaesthetics ❒ no ❒ yes
epidural ❒ no ❒ yes
analgesic ❒ no ❒ yes
specify: _____

Neonatal

Hospital/City _____
Gestational age at birth _____ weeks _____ days
Birth weight _____ lb _____ oz (_____ grams)
1 oz = 28.4 g

Head Circumference _____ cm
Apgar scores 1 minute _____ 5 minute _____

A ppearance
P ulse
G rimace
A muscle activity
R espiration

Fetal Monitoring ❒no ❒ yes external [ ] internal [ ]
explain _____
Fetal distress ❒ no ❒ yes
explain _____
Meconium ❒ no ❒ yes

You originally called the Motherisk Program to find out whether your exposure increased your baby's risk for being born with a major birth defect. At that time, we explained to you that every pregnancy has a 3-5% baseline risk for malformations.

For our own documentation, which will help other women exposed to the same drug that you were exposed to, would you share with us whether your child was born with any birth defects?

DEFECTS ❒ no ❒ yes Refused to answer ❒

_____
_____
_____
_____

G. NEONATAL HEALTH

Health in hospital intensive care? ❒ no ❒ yes Home at: _____ days

_____

_____

_____

Breast feeding ❒ no ❒ yes stopped _____ months

Medication during lactation ? ❒ no ❒ yes (specify details in section D)

Name: _____

Infant side effects? ❒ no ❒ yes: _____

Formula feeding ❒ no ❒ yes started _____ months stopped _____ months

Solids ❒ not yet ❒ yes started _____ months type: _____

Problems with feeding? ❒ no ❒ yes explain: _____

Infant health problems since discharge from hospital?

Details: *diagnosis onset medication dose hospitalization?*

Cancer	❒ No	❒ Yes	_____
Cardiovascular	❒ No	❒ Yes	_____
Central nervous system	❒ No	❒ Yes	_____
Dermatology	❒ No	❒ Yes	_____
Ears, eyes, nose, throat	❒ No	❒ Yes	_____
Endocrine	❒ No	❒ Yes	_____
Gastrointestinal	❒ No	❒ Yes	_____
Genito-urinary	❒ No	❒ Yes	_____
Hematology	❒ No	❒ Yes	_____
Infectious Disease	❒ No	❒ Yes	_____
Musculo-skeletal	❒ No	❒ Yes	_____
Respiratory	❒ No	❒ Yes	_____

OTHER _____

H. MILESTONES

At what age did the infant first:		*Normal range*
Smile (recognition)	_____	[2 months]
Lift head on own	_____	[3 months]
Sit unaided	_____	[6-8 months]
Crawl	_____	[8-10 months]
Stand on own	_____	[8-10 months]
Speak first word	_____	[8-12 months]
Walk unaided	_____	[12-15 months]

Infant's age at followup _____ months

Infant's weight _____ kg (_____ lb _____ oz)

as of: ❒ follow up ❒ last MD visit

Infant's height/length _____ cm (_____ inches)

as of: ❒ follow up ❒ last MD visit

Last MD visit : _____ (date or baby's age)

Appendix C: Consent Form and Requisition for Placental Perfusion

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

Study Title: The Role of the Placenta in Fetal Toxicology

Investigators:

On Site Primary Investigator:
Dr Howard Berger, Perinatologist, St. Micheal's Hospital, Department of Obstetrics and Gynecology, Phone: (416) 867- 7460 Ext. 8408
(Available Monday - Friday 9 am – 4 pm)

Off-Site Primary Investigator:
Dr.Gideon Koren, The Hospital for Sick Children, Department of Clinical Pharmacology
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 question s 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.

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. They 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 fetus 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.

Description of Research:

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 to continue our study of the transport of medication across the placenta. These tissues will be studied immediately after birth and then disposed of in the usual fashion.

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, we will not collect it as part of this research study.

Potential Harms (Injury, Discomforts or Inconveniences):

Collection of these samples will not affect your labour or the delivery of your baby. The 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:

Your consent to collect your placenta will be of no direct benefit to you. The results from this study may improve our understanding of drug transfer in 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.

Protecting Your Health Information:

These consent forms and data collection forms will be held in the strictest confidence. To protect your anonymity, your name will not appear on any record. Information from this study will be kept in a locked filing cabinet in the locked laboratories at the Hospital for Sick Children for three years. Information from this study will also be kept on a password protected computer database in the research laboratories at the Hospital for Sick Children. Your name will not be used in any publication. None of the research results will be placed in your medical records.

Participation and Withdrawal:

Your participation in this study is voluntary. If you do not want to participate in this study, or wish to withdraw at any time, you are free to do so and this will in no way affect your present or future care.

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.

Publication of Results:

Once the study is complete the information will be summarized and submitted to a medical journal for publication. The outcome of this study may also 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 completely maintained.

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 such commercial products that will remain with the Hospital for Sick Children and their research partners.

Research Ethics Board Contact:

If you have any further questions about your rights as a research participant, you may contact Dr. Julie Spence, Chair, Research Ethics Board, 416-864-6060 ext 2557.

Futher 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

Leading with Innovation
Serving with Compassion

ST. MICHAEL'S HOSPITAL
A teaching hospital affiliated with the University of Toronto

PLACENTAL TRANSFER STUDY REQUISITION

Dear Nurse,
Name:……..
J Number: …… is participating in the Placental Transfer study.
Could you please:

1. Take care **not to damage the placenta or attached membranes** during and following delivery. Please attach a **clamp** near the base of the placenta.
2. **Immediately** following delivery, simply leave placenta in the steel basin and bring placenta to the adjacent soil room where the research student will be waiting with a collection container.

Thank you for your help!