PALMITIC ACID IMPEDES EXTRAVILLOUS TROPHOBLAST ACTIVITY BY INCREASING MRP1 EXPRESSION AND FUNCTION

Yunali V. Ashar

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PALMITIC ACID IMPEDES EXTRAVILLOUS TROPHOBLAST ACTIVITY
BY INCREASING MRP1 EXPRESSION AND FUNCTION

A dissertation submitted in partial fulfillment
of the requirements for the degree of

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New York

by

Yunali V. Ashar

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Yunali V. Ashar                        Sandra E. Reznik
ABSTRACT

PALMITIC ACID IMPEDES EXTRAVILLOUS TROPHOBLAST ACTIVITY BY INCREASING MRP1 EXPRESSION AND FUNCTION

Yunali V. Ashar

Normal function of placental extravillous trophoblasts (EVT), which are responsible for uteroplacental vascular remodeling, is critical for adequate delivery of oxygen and nutrients to the developing fetus and normal fetal programming. Proliferation and invasion of spiral arteries by EVT depends upon adequate levels of folate. Multidrug resistance-associated protein 1 (MRP1), which is an efflux transporter, is known to remove folate from these cells. We hypothesized that palmitic acid (PA) increases MRP1-mediated folate removal from EVT, thereby interfering with EVT’s role in early placental vascular remodeling. HTR8/SVneo and Swan-71 cells, first trimester human EVTs, were grown in the absence or presence of 0.5 mM and 0.7 mM PA for 72 h. PA increased ABCC1 gene expression and MRP1 protein expression in both cell lines. The rate of folate efflux from the cells into the media increased with a decrease in migration and invasion functions of the cultured cells. Treatment with N-acetyl cysteine (NAC) rescued the PA mediated upregulation of MRP1 and restored the invasion and migration of EVTs. Finally, in an MRP1 knockout subline of Swan-71 cells, there was a significant increase in the invasion and migration functions. The novel finding in this study that PA increases MRP1-mediated folate efflux provides a missing link explaining how PA compromises the in-utero environment.
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<tr>
<td>ABC</td>
<td>Adenosine triphosphate (ATP) binding cassette</td>
</tr>
<tr>
<td>MRP1</td>
<td>Multidrug resistance-associated protein 1</td>
</tr>
<tr>
<td>PgP</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>MRP7</td>
<td>Multidrug resistance-associated protein 7</td>
</tr>
<tr>
<td>EVTs</td>
<td>Extravillous trophoblasts</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>SP</td>
<td>Sodium palmitate</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>SL</td>
<td>Sodium linoleate</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin streptomycin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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CHAPTER 1
INTRODUCTION

1.1 Placenta

Placenta is an organ formed in the uterus during pregnancy and forms a barrier between maternal and fetal cavities[1]. It helps prevent the transfer of xenobiotics to the fetus and is involved in the exchange of gases and nutrients from the mother to the fetus and waste products from the fetus to the maternal circulation[2–6]. In addition, it is responsible for the production of various hormones and acts as an endocrine organ[1]. The development of the fetus and various pregnancy outcomes are dependent on the development of a healthy placenta[7].

The placenta consists of differentiated trophoblast cells which arise from the trophectoderm which is the outermost layer of blastocyst[1,7]. The human trophoblast cells are one of the most invasive cells as they invade up to the myometrium, but in the last trimester only one trophoblast layer forms an interface between the mother and the fetus[1,2]. The differentiation of the trophoblast cells takes place through two pathways – the villous pathway and the extravillous pathway[1]. The other cells originating from the trophectodermal layer are the cytotrophoblasts[1,7]. These cells are highly proliferative and undifferentiated cells [1]. These cells give rise to villi, which transform into primary and tertiary villi through the course of pregnancy[2,7]. Division, differentiation and fusion of cytotrophoblasts give rise to multinucleated syncytiotrophoblasts[7]. The formation of syncytiotrophoblasts is a characteristic of the villous pathway. These cells are responsible for the exchange of gases, nutrients and waste across the feto-maternal interface[1,2,7]. Syncytiotrophoblasts also allow nutrient uptake by the fetus since they
are in close contact with the placental vasculature[7]. The other function of these cells is to produce pregnancy-related hormones[1]. In the extravillous pathway, differentiated extravillous trophoblasts are formed from the proliferative cells which arise from basal plate villi from the placenta[1,7]. These cells have two subsets – interstitial trophoblasts and endovascular trophoblasts. The former invades deep into the myometrium which produces an attachment between the fetus and the mother. The latter increases the blood flow to the placenta by penetrating the uterine vasculature and replacing the maternal endothelial cells[1]. During the first trimester, blocking of the maternal arterioles is carried out by invasive endovascular cytotrophoblasts, which stops the premature onset of blood flow into the intervillous space. If this process is unsuccessful, there can be an increase in the oxygen levels leading to oxidative stress and disruption of placental villi and this may lead to pregnancy complications[7]. Environmental factors such as oxidative stress, hormones and growth factors regulate trophoblast differentiation [1]. Any disturbance in trophoblast differentiation especially in the migration of extravillous trophoblasts into the uterus, may lead to hypertension and proteinuria – a condition known as preeclampsia[1]. Thus, successful trophoblast differentiation and extravillous trophoblast invasion are required to reduce the risk of diseases such as intrauterine growth restriction and preeclampsia[7]. In recent years, studies have shown a role of microRNAs and trophoblast regulation. Abnormal levels of microRNA in the preeclamptic placenta hints at the connection between microRNA levels in the placenta and preeclampsia. The role of microRNAs in the pathogenesis of preeclampsia is unclear[1].
Figure [1]: Graphical representation of fetal-maternal interface

Adapted and modified from: Juarez et al., 2017.
Figure [2]: The development of the placenta. Placenta is developed from trophoectoderm, the outermost layer of blastocyst. The first layer of placental cells that are formed are cytotrophoblasts. Cytotrophoblasts are further differentiated into syncytiotrophoblasts and extravillous trophoblasts by villous and extravillous pathways respectively.
1.2 ATP-binding cassette transporters (ABC transporters)

ATP-binding cassette transporters, also known as ABC transporters, are the proteins that are present on the plasma membrane of the cells[8]. These transporters utilize the energy released by hydrolysis of ATP for the transport of the substrates in and out of the cell across the cell membrane[9–11]. This family of transporters is the largest and ubiquitous superfamily[11]. In humans, 49 ABC transporters have been identified, which are divided into 7 subfamilies and designated as ABC- A through G[8,10–12]. Structurally, the ABC transporters consists of two ATP binding cassettes also known as nucleotide binding domains (NBD) and two transmembrane binding domains (TBD). The NBD further comprises the highly conserved motifs Walker A and Walker B. In addition, NBD also contains ABC signature motifs and H and Q loops[9,11]. Binding of the two NBDs leads to the hydrolysis of ATP which provides the energy for transport and the TBD function as substrate identifiers and helps in their translocation across the cell membrane. The majority of these proteins are full transporters, while in some, the two subunits might bind as heterodimers or homodimers. Such proteins are known as half transporters[11]. Many of these proteins are responsible for the transport of substrates which include amino acids, iron, bile, nucleosides, cholesterol and its derivatives, vitamins, peptides, sugar, antibiotics and other hydrophobic drugs[9,11]. Their main function is to efflux cytotoxic compounds form the cell[13]. They are found in the gut, liver, kidney, placenta, eye, blood brain barrier, etc., where they decrease the bioavailability of certain drugs due to their efflux characteristics[13]. Their dysfunction may lead to various genetic diseases and their ability to pump out substrates from the cells leads to resistance to antibiotics, chemotherapeutic drugs, anti-epileptic medications,
herbicides and the phenomenon known as multidrug resistance (MDR)[8–12,14,15]. Many of the ABC proteins are responsible for the induction of MDR due to their efflux pumps[14]. Apart from efflux, other novel roles of ABC transporters have been identified which include developing a defense mechanism against tumor regulatory pathways and inhibition of apoptosis and improving the efficacy of drug sequestration by modifying the location of the ABC transporters in the intracellular and extracellular compartments[14]. ABCB1/PgP, ABCG2/BCRP/MXR, ABCC1/MRP1, ABCC10/MRP7ABCA1, ABCG1 and MRP2 are the most extensively studied ABC transporters[9].

1.2.1 ABCB1/ Pgp

Belongs to the ABCB family which is also known as the “MDR family of ABC transporters[11]” and was the first identified ABC transporter[9]. This protein is present in the brain, kidney, intestine and placenta[12]. It is encoded by ABCB1 gene which is located on chromosome 7p21. Pgp has a molecular weight of 160 kDa[9]. It is encoded by a single polypeptide chain which consists of two TMDs and two NBDs[14]. Highly expressed in the apical membrane of the cell, it is involved in pharmacokinetics [8] and is also responsible for protection against toxicants and xenobiotics. ABCB1 is responsible for the efflux of chemotherapeutic drugs such as paclitaxel, vincristine and doxorubicin[9,12]. In addition, certain epipodophyllotoxins and antibiotics are the substrates of Pgp[9]. The majority of anti-epileptic drugs that are prescribed are also substrates of Pgp[14]. It was the first transporter identified to have a feto-protective role[16].
1.2.2 ABCG2/BCRP/MXR

This was the first identified half transporter since it mediates multidrug resistance with one NBD and one TMD. It has a molecular weight of 72 kDa[9]. Encoded by the ABCG2 gene, this transporter consists of 655 amino acids and is located on chromosome 4q22[14]. It is mostly found in the plasma membrane[9]. Its expression is highest in the placental syncytiotrophoblasts, brain, liver kidney, colon and apical surface of the small intestine[8,9,12]. BCRP has a wide range of substrates such as organic anionic conjugates, TKIs, sulfate conjugates, anthracyclines and methotrexates[8,9]. Some of the substrates of ABCG2 overlap with the ABCB1 substrates which leads to a synergistic effect of ABCB1 and ABCG2 on the brain-to-plasma ratio of common substrates[14]. It was found that this synergistic effect was not due to the interaction between these two transporters, but due to their net efflux at the blood brain barrier[15]. ABCG2 is known to induce MDR in gastric, colon, small intestine and breast cancers and melanoma[14].

1.2.3 MRPs (ABCC1-ABCC10)

They are another set of proteins which have been extensively studied[14]. ABCC1 and ABCC10 are both localized to the basolateral membrane of the epithelial membrane. They have molecular weights of 180kDa and 162kDa, respectively[9]. ABCC1/MRP1 was the first transporter identified in this family[10]. MRP1 prevents drug retention and promotes efflux out of cells and is mostly found in various physiological barriers such as the blood brain barrier and placenta. It is also one of the major transporters has been linked to drug resistance[10]. MRP7 also leads to multidrug resistance, most importantly, paclitaxel resistance[15]. Unlike MRP7, MRP1 does not confer resistance to taxanes[9]. MRP7 is found in the colon, testes, skin and pancreas[15].
Figure [3]: The structure of MRP1. MRP1 has two hydrophobic transmembrane domains containing six α-helices each having a nucleotide in the end. MRP1 has a central core, and a third transmembrane domain, which has five α-helices and an extracellular N-terminus.

Adapted and modified from Khamanehfar et.al., 2015
1.3 Localization of ABC transporters in the placenta

Use of medications during pregnancy poses a special concern because certain drugs can cross the placental barrier and cause harmful effects on the fetus[5]. Compounds that enter the maternal circulation passes through the apical membrane (in contact with the maternal blood) and basolateral membrane (in contact with the fetal circulation) of the syncyiotrophoblasts. The ABC transporters, which function as efflux proteins, are present on both these membranes[3]. These transporters prevent the passage of toxicants and xenobiotics into the fetal compartment thereby limiting fetal exposure to such compounds[2–6]. The transporters present in the apical membrane of the syncyiotrophoblasts are positioned to efflux the substrates towards the maternal circulation and the transporters present in the basolateral membrane efflux the substrates towards the fetal circulation[3,6]. Thus, the localization of these transporters in the syncytial membrane of the placenta determines the direction of efflux of the substrates [6,17]. The transporters present in the apical membrane of the syncyiotrophoblasts include Pgp, BCRP, MRP2, MRP3 and ABCA1, whereas MRP1 and 5, ABCG1 and MDR3 are present in the basolateral membrane[17]. Although MRP1 is highly expressed in the basolateral membrane, some findings suggest that MRP1 is also localized in the apical membrane, which implies bidirectional efflux of its substrates[2,3]. The expression of ABC transporters varies depending on gestational age and pregnancy conditions[2–6,16]. Apart from their presence in villous trophoblasts, the presence of ABC transporters in the extravillous trophoblasts (EVTs) has also been reported[18–20]. There is evidence of ABCA1, ABCA5 and MRP1 expression in EVT's. Although there are differences in
the functions and characteristics of villous and extravillous trophoblasts, the ABC transporters carry out their efflux functions in both types trophoblasts[18,20].

**Figure [4]: Localization of ABC transporters in the syncytiotrophoblast layer in the placenta.** There are various ABC transporters in the basolateral and apical membrane. The transporters present in the apical membrane efflux substrates towards the maternal compartment and the transporters present in the basolateral membrane efflux substrates towards the fetal compartment.

Adapted and modified from Bloise et al., 2016.
1.4 Modulators of ABC transporters affecting maternal/ fetal/ neonatal outcomes

Even though drug options during pregnancy are limited, majority of pregnant women take medications at some point during gestation[3,4]. Use of medications for certain chronic diseases such as asthma, diabetes, epilepsy, depression, schizophrenia and cancer is inevitable[3,4,21]. These medications could be the reason for fetal and neonatal abnormalities and pregnancy complications. Despite the placenta’s role in protection, its ‘leakiness’ is blamed for these outcomes[5].

The ABC transporters present on the basolateral membrane of the syncytiotrophoblasts transport drugs and xenobiotics from the maternal circulation to the fetal circulation[3]. These transporters are partly responsible for fetal drug exposure which could lead to teratogenesis and other abnormalities[21]. The characteristics of a drug, its interaction with the transporter, and the localization of the transporter determine the direction and extent of efflux[22]. While some drugs are substrates of these transporters, there are other drugs which can inhibit these transporters, modulating their function and expression. In addition to drugs, there are several other natural and synthetic modulators of ABC transporters, which include diet, oxidative stress, mutations and polymorphisms, maternal diseases and environmental toxicants. Furthermore, gestational age is a major factor determining the expression of ABC transporters in the placenta. There have been many reports suggesting an increase in the number of gestational complications caused by the modulation of ABC transporters. In addition, there are reports suggesting that partial or complete blocking of these transporters affects their protective activity.
1.4.1 Natural modulators of ABC transporters in the placenta

Gestational age, gestational diseases, infections, inflammations, oxidative stress, maternal stress and polymorphisms are some of the natural modulators of ABC transporters. These factors can affect one or more transporters and modulate their activities, leading to consequences which could prove lethal for the mother and the fetus, causing pregnancy complications, fetal deformities and preterm birth. Placental integrity greatly depends on maternal health and can be disturbed if these conditions are present[23].

There are various factors maintaining homeostasis during pregnancy, including cytokines and chemokines[24]. Inflammatory cytokines such as TNF-α, IL-6 and IL-1β, released in response to activation of the immune system, are involved in many pathological functions in pregnancy[24,25]. In some pregnancies, these cytokines and chemokines mediate strong inflammatory responses leading to pregnancy complications such as preterm birth, pre-eclampsia and fetal neural defects[24–28]. Evseenko et.al. demonstrated that TNF-α and IL-1β downregulated the expression of PgP and BCRP in the placenta, whereas, the expression MRP1 was up-regulated, compromising the maternal-fetal barrier and exposing the fetus to xenobiotics[28]. In conformity with these results, Petrovic et.al. showed downregulation of the key ABC efflux transporters on exposure to polyinosinic/polycytidylic acid (poly(I:C)) in pregnant rats. It was found that poly(I:C) induced TNF-α, IL-6 and IL-1β, which was believed to cause down-regulation of the ABC transporters[29]. TNF-α and IL-1β have little to no effect on MDR3 protein expression[28]. Increased levels of IL-8 are found in the cord blood and amniotic fluid of patients with intrauterine infection[4] as well as in chorioamnionitis[30]. The increase in
the IL-8 mRNA is associated with the mRNA levels of PgP and mRNA levels of BCRP[4].

The hormone estradiol is important during pregnancy because of its involvement in trophoblast differentiation, development of fetal adrenal glands, progesterone production and in maintaining pregnancy[31]. The levels of estradiol are increased throughout pregnancy[31]. Another major hormone is progesterone, which prepares the endometrium for implantation and is a key regulator of embryogenesis[31,32]. The hormone estradiol caused an increase in the expression of BCRP, Pgp and MDR3[28]. It is suggested that since estradiol plays a role in growth and differentiation of the placenta, the up-regulation of these transporters is required since they act to maintain the function of syncytiotrophoblasts and represent the mature syncytiotrophoblast phenotype[28]. In contrast, according to the study carried out by Wang et.al., estradiol decreases BCRP protein and mRNA thereby causing a decrease in its expression in BeWo cells. The down regulation in BCRP expression was believed to be mediated by estrogen receptor (ER)[33]. Estradiol has also been shown to reverse ABCG2 mediated multidrug resistance[34]. In a study conducted by Coles et.al., estradiol caused an induction of Pgp in JAR cells and also in NCI-ADR-RES cells overexpressing Pgp[35]. In another study conducted by Wang et.al., estradiol induced BCRP expression[36].

There is an increase in the concentration of progesterone throughout gestation and it is the highest at term[2,37]. Progesterone is another hormone which is important to maintain pregnancy[32,33,38]. It is well known that the placenta is responsible for producing various hormones during pregnancy, including progesterone in high concentrations[37]. Various studies have been done on cell and animal models to
demonstrate the effect of progesterone on ABC transporters. Progesterone is an required for the regulation of ABC transporters, most importantly PgP in the placenta as seen in in vivo and in vitro models[39–41]. According to the studies carried out by Coles et.al., progesterone causes an increase in the concentration of Pgp transporter in NCI-ADR-RES, cells overexpressing Pgp[35]. There have been contradicting studies showing a decrease in the expression of PgP in the presence of progesterone. According to Wang et. al, progesterone induces BCPR expression in the BeWo cell line and progesterone along with estradiol show a synergistic effect on the expression of BCRP[36]. Another study shows that progesterone upregulates the expression of PgP and BCRP in choriocarcinoma cell lines[28]. Progesterone also caused an increase in the expression of MRP1[28]. More studies are required to understand the effects of progesterone on the ABC transporters in the placenta.

The development of the first trimester placenta takes place in hypoxic conditions with PgP and BCRP being highly expressed[42–44]. Severe placental damage can occur if hypoxia persists in the second half of the pregnancy with abnormal remodeling of the spiral arteries in the second and third trimester causing pregnancy complications[44]. There is an increase in reactive oxygen species (ROS) markers and anti-oxidants in the third trimester, suggesting that pregnancy is associated with high oxidative stress[45]. The changes in oxygen levels in placental cells is sensed by hypoxia inducible factors (HIF) and these factors are found in the promoter region for the ABCA1 transporter[46,47]. Methyl mercury causes oxidative stress in the HTR-8/SVneo placental trophoblast cells[48,49]. In vitro and ex vivo studies have shown that hypoxia causes an increased expression of ABCA1[50]. Methyl mercury is the substrate of MRP1, which
mediates its efflux out of cells[49]. According to studies by Granitzer et. al., methyl mercury causes a dose dependent upregulation of MRP1 in HTR-8/SVneo cells, which explains the protective effect of MRP1 against methyl mercury induced oxidative stress in placental cells[49]. There is evidence of increased oxidative stress in patients with intrauterine growth restriction (IUGR)[51,52]. There is a decreased expression of BCRP in human placentas in cases of IUGR[53,54]. Moreover, when hypoxia was induced in BeWo choriocarcinoma cells, BCRP was downregulated in response to HIF-1α signaling activation[55]. In contrast, Pgp was upregulated in cytotrophoblasts obtained from first trimester human placentas exposed to hypoxia[56,57]. Severe oxidative stress was found in the placenta during fetal heart dysfunction causing a decrease in levels of PgP. This oxidative stress was reversed by the administration of vitamin C, which restored the protective function of the placenta[58].

Pregnancy is a condition which can be complicated with other health conditions such as diabetes, infections, hypertension, depression, epilepsy, etc[59]. Maternal health may directly or indirectly affect the development of the fetus and the health of the child in later stages of life[59,60]. Gestational diabetes mellitus (GDM) usually develops in the second or third trimester and is one of the most common pregnancy complications. This condition is poorly managed in pregnant women, causing maternal and fetal morbidity and mortality[61,62]. GDM leads to excessive production of ROS, inducing oxidative stress[63]. Diabetes during pregnancy can significantly increase blood lipid levels[62]. Furthermore, placental enlargement has been associated with diabetic pregnancies[64,65]. In human fetoplacental endothelial cells derived from GDM subjects, ABCA1 and ABCG1 transporters were upregulated, mediating increased cholesterol
efflux[66]. This could be due to an increase in ROS causing liver X receptor activation[66,67]. Anger et.al. examined the effect of GDM on placental ABC transporters and found an increase in the mRNA levels of PgP, BCRP and MRP2. However, in insulin treated groups, the mRNA levels of these transporters were similar to the mRNA of the transporters in normal placenta[68]. No change in the localization was found upon comparing the placentas from diabetic pregnancies to normal pregnancies. However, decreased expression of PgP, MDR3 and MRP1 were observed in diabetic placentas, with basolateral transporters (MRP1 and MDR3) being more sensitive to GDM, altering the pharmacokinetics of the substrates[69].

Psychological conditions such as depression, anxiety and stress during pregnancy enhances the risk of adverse maternal and outcomes.[70–72]. Statistics show that around 3.3% of pregnant women have posttraumatic stress disorder[73]. Adrenal cortex produces glucocorticoids in response to stress and glucocorticoid signaling is crucial for adapting to stress[74–76]. ABC transporters have a role in the efflux of glucocorticoids towards the maternal compartment[77,78]. In animal models, glucocorticoid surge increased the expression of MRP1, whereas the expression of BCRP was decreased[79,80]. Sound stress resulted in increased levels of maternal cortisone in mice, with elevated cortisone levels in female fetuses, while the cortisone levels in male fetuses remained unaffected[81]. This was due to an increase in the levels of ABCA1a and MRP1 in the placentas associated with male offspring, which caused increased efflux of cortisone. There was no change in ABC transporter expression placentas associated with female offspring[81]. Synthetic glucocorticoids such as dexamethasone regulate the expression of PgP and MRPs in the placenta but decrease MRP1 mRNA levels[79,80,82]. Decrease
in BCRP protein and mRNA expression was further confirmed by Petropoulos et.al., but they found an increase in the expression PgP in mouse placenta upon treatment with synthetic glucocorticoids[77].

**1.4.2 Synthetic modulators of ABC transporters in the placenta**

In conditions where levels of ABC transporters are increased, such as in cancer, the co-administration of synthetic modulators of ABC transporters with chemotherapeutic drugs increases the efficacy of the chemotherapeutic drugs[83]. However, in the placenta, synthetic modulation of ABC transporters could either be beneficial or have detrimental effects on the mother and the fetus based on the transporter that is modified and the direction of the efflux of the transporter. ABC transporters are modulated upon exposure to environmental pollutants as well as toxic compounds such as pesticides and unwanted metals[87,88].

Mercury exposure during pregnancy can have unfavorable effects on fetal development, most importantly neurodevelopment[89]. An ex vivo study carried out on human placental cells suggests that mercury is a substrate of MRP1 and is transported out of the cells as a glutathione conjugate[90,91]. Maternal mercury exposure can lead to transfer of mercury towards the fetal compartment[90]. This study was supported by Granitzer et.al., who showed that inhibition of the MRP1 transporter increased intracellular concentrations of mercury, thereby increasing GSH, and increased oxidative stress and placental cell death[92]. Thus, mercury is a highly toxic metal transported by the MRP1 transporter which accumulates in the fetal compartment. [49,90,91]. However, Bridges et.al. confirmed the role of MRP2 in the transport of mercuric ions from the maternal compartment to the fetal compartment[93]. In rats, increased levels of
methylmercury in the fetal compartment leads to increased accumulation of this toxic compound in the fetal kidney, making it more susceptible to injury, affecting nephrogenesis and renal function[94]. Liu et.al. observed that cadmium downregulated the expression of ABCG2 and ABCB4 transporters, which led to fetal cadmium toxicity[87]. However, in the BeWo cell line, cadmium showed no effect on the expression of ABCG2 transporter, but its function was inhibited[95].

Organochlorine pesticides are the most effective pesticides because they are phosphoric acid esters and they directly inhibit the activity of acetylcholinesterase[96]. A recent study shows the role of ABC transporters in the transplacental transport of organochlorine pesticides. ABCB1, ABCG2, MRP1 and MRP2 are involved in the transport of these pesticides[88]. Although this pesticide is a substrate of the transporters present in both the apical and the basolateral membrane of the syncytiotrophoblast layer, the direction of transport of this pesticide is more towards the fetus[97]. This suggests that efflux by the transporters in the basolateral membrane is greater than efflux in the basolateral membrane. This difference in the transporter activity could be due to the difference in the binding affinities of the transporters for these pesticides[98]. DDT, which is a type of organochlorine pesticide, passes through the placenta and enters the fetal circulation. Chlorpyrifos, which is another organochlorine pesticide, is highly toxic to the JEG-3 choriocarcinoma cell line, even at minute concentrations[99]. In rats, developmental defects, IUGR and neurotoxicity were induced on prenatal exposure to chlorpyrifos[100]. In addition, chlorpyrifos altered the expression of ABCG2 in JEG-3 cells[101]. At concentrations found in food, pesticides were found to be the substrates of ABCG2 in rabbit placenta, thereby limiting the exposure of pesticides to the fetus[102].
This indicates that pesticide will enter and be limited to the maternal circulation [97,102]. Insect cells conferred resistance to pesticides due to upregulation of ABC transporters[103]. It is evident from these studies that pesticides can have detrimental effects on the fetus as well as the mother[97,98,101].

Pesticides can cross the transplacental layer and move towards the fetal compartment. The concentration of the pesticides reaching the fetus can be assessed by measuring the concentration in maternal blood as well as in the umbilical cord[104].

1.5 Effects of high fat diet in the placenta

There has been a significant decline in the maternal and fetal mortality rates in the past two decades; however, there is an increase in obesity and high fat diet related complications in the mother and fetus[105]. A balanced maternal diet is required for proper development of the fetus[106]. Maternal diet is known to impact maternal and fetal health[107]. Diet rich in fruits, vegetables, whole grains and selected fish is necessary for the best maternal and fetal outcomes[108]. Therefore, there are constant efforts being made to optimize the diet preconception to improve neonatal and perinatal outcomes[109]. Fetal growth determines development and future health adult life[110,111]. Most conditions that occur during adulthood originate in fetal life[112]. The mechanism of this early programming can include various epigenetic changes, modified by diet, and can impact more than one generation[112]. There are various studies suggesting that the fetus adapts to poor diet and this adaptation can lead to changes in genetic, physiologic and metabolic programming[112,113]. In addition to the fetus, high fat diet also impacts the mother[114]. High fat diet and obesity in women increases the risk of gestational diabetes mellitus, preeclampsia and abnormal
placentation[115,116]. Studies have shown that high fat diet is linked to decreased cardiac function and altered cardiac structure caused by high fat metabolic programing[117,118]. Interestingly, a recent study showed that the cardiovascular function programmed by high fat diet was reversed by maternal conjugated linoleic acid intake[119]. It has been observed that saturated and unsaturated fatty acids have contrasting effects on oxidative stress in the placenta[107]. Saturated fatty acids such as PA induced oxidative stress in the placenta, which further led to arterial hypertension, inflammation, and cardiovascular diseases in offspring[107,120,121]. Unsaturated fatty acids such as linoleic acid have a protective effect against oxidative stress and help improve trophoblast stem cell differentiation and function[107,122]. Interestingly, a recent study showed that conjugated linoleic supplement reversed the cardiovascular dysfunction in male offspring caused by high fat diet metabolic programing[119]. Moreover, oleic acid, which is a monounsaturated fatty acid, showed an increase in the invasion and migration of the extravillous trophoblasts, indicating an important role of unsaturated fatty acids in the function of EVTs[122]. In addition, unsaturated fatty acids showed a protective effect against saturated fatty acid induced autophagy[123]. Therefore, avoiding a high fat diet rich in saturated fatty acids and preconception and prenatal intake of a balanced diet having appropriate amounts of macro and micronutrients is essential for a healthy pregnancy[124–126].
Figure [5]: The effects of high fat diet in pregnancy. High fat diet causes an adverse intrauterine environment which can lead to pregnancy complications and may alter fetal programming.

1.6 Saturated fatty acid: PA

Fat is one of the most important macronutrients in the human diet[127], comprising 20-35% of total calories in a healthful diet[128]. The different types of fatty acids are saturated fatty acids such as palmitic and stearic acid, monounsaturated fatty acids such as linoleic acid, and polyunsaturated fatty which include oleic acid[129,130]. Intake of saturated fatty acid should be less than 10% of total fatty acid consumption[131]. Saturated fatty acids have a deleterious effect when consumed in higher concentrations[132–134]. Saturated fatty acid are linked to obesity, inflammation, insulin resistance and cardiovascular diseases[135,136].

One of the most consumed saturated fatty acids is PA[127,137]. It is the most abundant fatty acid available in nature and can be found in mammalian cells, plants,
fungi, bacteria and algae\[138\]. PA is a major component of palm oil\[139,140\]. With increasing availability and consumption of palm oil, the consumption of PA is also on the rise\[139\]. PA is also synthesized endogenously\[141\]. PA is synthesized in the cytosol by de novo fatty acid synthesis and starts with citrate conversion to acetyl-CoA\[142,143\]. PA is an important component of the cell membrane and functions as a substrate in lipid synthesis, fatty acid oxidation and transport, and carries out protein palmitoylation of signaling molecules\[142,144\]. Endogenous PA, which is synthesized by the fetus, is important in increasing the fatty tissue percentage of the fetus to 13-15%\[138\]. In addition to palm oil, PA can also be found in milk and milk products, red meat, cocoa butter, olive oil, chocolates, butter\[138,140\].

Although PA is an important component of the cell membrane, it has deleterious effects on human health and is known to cause lipotoxicity\[145–147\]. Surprisingly, concentrations of PA are the highest compared to all other fatty acids in obese individuals\[136\]. PA is also known to increase low density lipoprotein levels (LDL) more than any other known fatty acids\[148,149\]. Interestingly, this acid may comprise more than half of the saturated fat intake in the United States\[144\]. Unlike unsaturated fatty acids and stearic acid, PA does not reduce high density lipoprotein and is associated with cardiovascular diseases\[127,133,148\]. Moreover, PA has been shown to increase ROS thereby stimulating pre-inflammatory mechanisms\[127\]. There is abundant evidence of PA’s role in inducing oxidative stress and inflammation in the placenta.\[150–153\]. Some level of oxidative stress is inevitable during pregnancy; therefore, it is very important that levels of oxidant and antioxidant production are balanced during the different periods of gestation\[154\]. Additional oxidative stress caused by excessive intake
of dietary PA should be avoided[155]. Some unsaturated fatty acids such as oleic acid and linoleic acids are able to ‘rescue’ PA induced inflammation[156,157]. In addition, there are other drugs as well as herbal products and supplements such as manganese, copper and zinc which have been shown to ameliorate inflammation caused by PA[158–161]. However, there is no evidence of the safety of these products during pregnancy and there are also very few antioxidants in clinical trials for use during pregnancy[161]. Antioxidants are potent molecules; therefore, it is highly recommended to consume a diet high in antioxidants before taking antioxidative drugs to overcome the ROS caused during pregnancy[155].

![PA structure](image)

**Figure [6]: PA structure**

Adapted from Pubchem

### 1.7 Folic acid in pregnancy

In 1931, British hematologist Dr. Lucy Willis discovered that an intrinsic factor could be used for the treatment of tropical macrocytic anemia[162]. It was not until 1942 that this intrinsic factor was isolated from spinach and named ‘folic acid’ (folium in Latin means plant)[162]. In the first half of the twentieth century, very few women in America had any contact with a medical doctor during pregnancy and labor[163]. In the second
half of the twentieth century, visits to the medical practitioner during pregnancy, hospitalization during childbirth, legislation for abortion and technologies such as IVF had extended throughout the United States[163].

Even though studies on folic acid deficiency on maternal health and fetal development started in the early 1950s, it was not until 1995 that the policies for taking folic acid or vitamin B during the first trimester of pregnancy were designed[164,165]. Since then, 400 mcg per day supplementation of folic has been recommended prenatally and during pregnancy[165]. Folic acid supplementation during the second and the third trimesters is also found to be beneficial for the cognitive development of the fetus[166].

During pregnancy, 20 ng/ml of folic acid is considered a normal physiological concentration, 2000 ng/ml is a supra-physiological concentration[167]. While folate deficiency during pregnancy has unwanted consequences in the fetus, excessive folate intake and concentrations in the body can also lead to undesirable effects on maternal and fetal health[168,169].

Although folic acid and folate are used interchangeably, there is a difference between these terms[170]. Folate is a term generically used to describe the various forms of vitamin B9: folic acid, tetrahydrofolate (THF), 5, 10-methylenetetrahydrofolate (5, 10-MTHF), dihydrofolate (DHF), and 5-methyltetrahydrofolate (5-MTHF)[171]. Folate cannot be synthesized in the human body and hence, folate needs to be acquired through diet or supplements to maintain normal levels[170]. Folic acid, which is a type of folate, is not active in the body and is metabolized in the liver into its active form 5-methyltetrahydrofolate[171]. This active form acts as a methyl donor in metabolic reactions such as homocysteine conversion to methionine, synthesis of various amino
acids and DNA precursor molecules[171]. Thus, folic acid is important for growth and development and very important for fetal development and maternal health during pregnancy[171]. Folic acid is a synthesized form of folate which can be taken in food or as supplements and has a higher bioavailability than naturally occurring folate[172]. While synthetic 5-methyltetrahydrofolate can also be taken as a supplement, naturally occurring 5-methyltetrahydrofolate is more advantageous[170]. The absorption of natural 5-methyltetrahydrofolate is not changed even when the pH of the gastrointestinal tract is altered[170]. In addition, the bioavailability of naturally occurring 5-methyltetrahydrofolate is not affected by metabolic defects[171].

1.8 Effects of folic acid imbalance during pregnancy

Folic acid has been clearly shown to reduce the risk of neural tube defects in the fetus[173–176]. Neural tube defects occur in 2 out of 1000 pregnancies[177]. They can cause malformations of the organs of the central nervous system, cranium, and spine[178]. Neural tube defects are a major cause of newborn mortality[178]. If 400 mcg of folic acid are taken daily, it takes about 20 weeks for folate blood levels to reach between 1000 and 1350 nmol/l, the concentration required to prevent neural tube defects[174]. Therefore, folic acid supplements should be started a few months before conception[174]. Folic acid is also very important for growth, especially in the embryonic and fetal stages[171]. Folic acid deficiency has also been linked to oral clefts, congenital heart defects, and limb defects[178–180]. Congenital heart defects are a leading cause of mortality in newborns, affecting 1 in every 250 births[178].

Recently, a mutation in the 5,10 methylenetetrahydrofolate reductase gene has been identified, which leads to reduced enzyme activity[181] and decreases re-
methylation of homocysteine to methionine[182]. The mutation in this enzyme results in a condition known as hyperhomocysteinaemia which causes levels of homocysteine to rise[182]. This can further increase the risk of congenital heart disease and neural tube defects in the fetus[183]. Folic acid increases the activity of mutant 5,10 methylenetetrahydrofolate reductase causing homocysteine levels to decrease[184], which is one of several mechanisms whereby folic acid prevents congenital cardiac anomalies and neural tube defects[185]. Recently a study found that folic acid antagonists increased the risk of neural tube defects, confirming the role of folic acid in the prevention of this anomaly[186].

In addition to preventing neural tube defects, folic acid is also required for cell proliferation and regulation of gene expression for differentiation of the placenta and fetal tissues[187]. Studies have found a link between preeclampsia and low folate levels[188]. Moreover, invasion and migration capacities of placental trophoblasts are altered in conditions of low folic acid[188,189]. Maternal intake of folic acid is associated with normal cognitive function and increases neurogenesis and synaptogenesis in offspring[190]. While studies have shown that folic acid is required in the first trimester of pregnancy and beneficial in the second trimester, the benefits of using folic acid longterm and throughout pregnancy is yet to be investigated[191].

Even though folate and folic acid are very important during pregnancy for proper development of the placenta and the fetus, excess intake of folic acid is also not advised[192]. Excess folic acid has shown to alter brain and gene expression in female offspring[193]. Another study also showed a change in gene expression and behavior in male offspring[194]. Excessive folic acid also leads to ectopic pregnancy. Methotrexate,
a folic acid antagonist, is used for the treatment of ectopic pregnancies in early unruptured stages[195–197]. Therefore, the daily supplementation of folic acid should not exceed 400 mcg and folate blood levels should fall between 1000 and 1350 nmol/l[178,184].

1.9 Extravillous trophoblasts and spiral artery remodeling

Trophoblast stem cells developed from the blastocyst are the building blocks for placental development[198]. Problems in placental development can affect fetal development and can lead to undesirable pregnancy outcomes such as preeclampsia, gestational diabetes mellitus, and intrauterine growth restriction[199]. Differentiation of cytотrophoblasts into extravillous trophoblasts by the extravillous pathway is a very important step in facilitating the flow of blood to and from fetus[200,201]. By two weeks after fertilization, two types of extravillous trophoblasts can be detected in the maternal compartment: interstitial cytotrophoblasts, which colonize the maternal stroma and endovascular cytotrophoblasts, which invade into the uterus and modify its vessels[202,203]. One of the most critical steps in placental development is stepwise modification of these vessels[204].

The uterine vessel that the EVTs invade is known as the spiral artery. The function of the spiral artery is to temporarily supply blood to the uterus during menstruation. Under normal conditions in the absence of pregnancy, these arteries are high-resistance low-flow arteries. During pregnancy they have the important role of supplying blood to the fetus and are transformed to low-resistance high-flow arteries. This transformation from high-resistance low-flow vessels to low-resistance high-flow vessels is known as spiral artery remodeling. Remodeling of the spiral artery is very
important for a successful pregnancy. Failure of the spiral arteries to remodel can lead to undesirable maternal and fetal consequences.

EVTs play a very important role in spiral artery remodeling. The EVT cells are highly proliferative and invasive. They tear apart the syncytiotrophoblast layer, migrate towards and invade the spiral artery to orchestrate spiral artery remodeling. The lumen of the spiral arteries has endothelial cells on the inside and is layered by vascular smooth muscle cells on the outside. During the first few weeks of gestation, the oxygen pressure is higher in the endometrium than the placenta[205]. This difference in oxygen pressure initiates extravillous trophoblast invasion which is further facilitated by activation of maternal natural killer cells[199]. However, very little is known about the factors affecting trophoblast invasion and migration during the first trimester of pregnancy and studies are being carried out to study the mechanisms of trophoblast invasion[206,207]. After the extravillous trophoblasts migrate towards the spiral artery, they disrupt the vascular smooth muscle cells, which are present on the outside of the lumen[199]. This disruption makes it easier for the trophoblast cells to replace the endothelial cells lining the inner wall of the lumen[207]. This process occurs throughout the spiral artery and is known as spiral artery remodeling[199,207–209]. Remodeling of the spiral artery transforms it into a high-flow low-resistance vessel, which ensures a sufficient blood supply to the placenta[210]. Remodeling results in an almost 10-fold increase in the diameter of the spiral artery and a 4-fold increase in blood flow through the artery[211]. Most of the disrupted vascular smooth muscle cells and the replaced endothelial cells undergo apoptosis[211]. Remodeling of the spiral artery is required for uninterrupted flow of blood from the mother to the fetus[212]. Failure to remodel has been linked to
early onset of preeclampsia[207,209,212–214]. In addition, improper remodeling of spiral arteries has also been linked to other major sequelae such as intrauterine growth restriction, placenta abruption, spontaneous rupture of membranes, and preterm birth[209].

During the first few weeks of gestation, the embryo does not require maternal blood[209,215]. At this stage, the embryo obtains its nutrients through histotrophic nutrition, in which the local macromolecules are responsible for supplying and maintaining the embryo[216]. Another important function of extravillous trophoblasts is to block the spiral arteries by forming a trophoblast pug[215,217]. This prevents precocious flow of blood to the fetus[218]. Around 10 weeks of gestation, when the fetus requires maternal blood, the trophoblast plugs dissolve and the fetus switches to hemotrophic nutrition where the fetus gets its nutrients and oxygen from maternal blood[217]. Therefore, abnormal placentation causes intermittent perfusion, leading to undesirable maternal and fetal outcomes[218]. Interestingly, there is a significant decrease in the proliferative activity of the EVTs as the fetus switches from histotrophic to hemotrophic nutrition[219].
Figure [7]: Spiral artery remodeling. Extravillous trophoblasts (EVTs) migrate and invade towards the spiral artery and replace the endothelial cells by disrupting the vascular smooth muscle cells.
Figure [8]: Spiral arteries during absence of pregnancy, normal pregnancy, and abnormal pregnancy. EVTs are not present in absence of pregnancy. During normal pregnancy, EVTs invade the spiral artery for spiral artery remodeling. In abnormal pregnancy, the migration and invasion functions of EVTs is compromised which affects spiral artery remodeling.
Hypothesis statement:

High fat diet is known to cause various pregnancy complications which may lead to fetal programming. Saturated fatty acids have been shown to induce oxidative stress in placental cells, whereas unsaturated fatty acids have been shown to have a protective effect. ATP-binding cassette (ABC) transporters, which are efflux transporters and make up one of are the largest families of transmembrane proteins, are present in abundance in the placenta. However, ABC transporters in the placental extravillous trophoblast (EVTs) cells have not been extensively studied. We hypothesized that fatty acids would modulate ABC transporter expression and function in the placental extravillous trophoblast cell lines. Currently, there are no published reports regarding the effect of fatty acids on the ABC transporters of the extravillous trophoblasts.
CHAPTER 2
MATERIALS AND METHODS

2.1 Reagents

The HTR-8/ SVNeo cell line was provided by Dr. Charles Graham (Queens’s University, Ontario, Canada. The Swan-71 cell line was donated by Dr. Gil Mor (Yale University, Connecticut, USA). JEG-3 and BeWo cell lines were obtained from ATCC (Virginia, USA). DMEM, RPMI and fatty acid-free BSA were purchased from VWR International Inc. (Bridgeport, New Jersey, USA). EMEM and F-12K media were obtained from ATCC (Virginia, USA). Palmitic and linoleic acids were obtained from TCI Chemicals (Portland, Oregon, USA). FBS was purchased from Atlanta Biologicals (Georgia, USA). Monoclonal antibodies against ABCB1, ABCG2, ABCC1, ABCC7 and GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA). N-acetylcysteine, trypsin and Alexa Fluor 488 conjugated goat anti-rabbit IgG secondary antibody were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). [3H]-folic acid, diammonium salt (50µCi) was a product of Moravek Biochemicals, Inc (Brea, California, USA). Corning transwell with permeable polycarbonate membrane inserts was purchased form Thermo Fisher Scientific Inc. (Rockford, IL, USA). The plasmid for ABCC1 for CRISPR/Cas9 study was purchased from VectorBuilder. Mouse embryonic fibroblasts, 2-mercaptoethanol and sodium pyruvate were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Mitomycin C, heparin sodium salt and M2 medium were purchased from Sigma Aldrich (Missouri, USA). Recombinant Human FGF-4 was obtained from Peprotech (New Jersey, USA). EDTA, Tetrasodium Tetrahydrate Salt and Matrigel matrix were purchased from Millipore Sigma (Massachusetts, USA). Plasmid
for ABCC1 gene knockout was built and purchased from VectorBuilder (Illinois, USA). In addition, plasmid extraction kit was purchased from Thermofisher.

2.2 Cell lines and cell culture

Placental first-trimester extravillous trophoblast cell lines HTR-8/SVNeo and Swan-71 were used for in vitro studies. The HTR-8/SVNeo cell line was derived from first trimester human extravillous trophoblasts by transfection with the gene encoding for Simian virus 40 large T antigen for immortalization. RPMI supplemented with 10% FBS and 1% penicillin streptomycin (PS) was used to culture HTR-8/SVNeo cells. The Swan-71 line was established from first trimester human extravillous trophoblasts and immortalized by transfection with cells infected with human telomerase reverse transcriptase (hTERT). These cells were cultured in DMEM containing 10% FBS and 1% penicillin streptomycin (PS). JEG-3 and BeWo which are human choriocarcinoma cell lines were also used for in vitro studies. The JEG-3 cell line was cultured in EMEM with 10% FBS and 1% penicillin streptomycin (PS) and the BeWo cell line was grown in F-12K medium. All the cell lines were incubated at 37 °C and 5% CO₂. HTR-8/SVNeo and Swan-71 cell lines represent extravillous trophoblast cells of the placenta whereas JEG-3 and BeWo cell lines represent syncytiotrophoblasts. For CRISPR/Cas9 gene knockout, cells were incubated in Opti-MEM medium during transfection. The ABCC1 knockout subline of Swan-71 was cultured in DMEM with 10% FBS and was further supplemented with 0.6 mg/ml of G418.

2.3 Conjugation of palmitic and linoleic acids with BSA

Sodium palmitate and sodium linoleate are not soluble in polar solvents. Therefore, these compounds are insoluble in water or cell culture media. To increase their
solubility in cell culture media, sodium palmitate (SP) and sodium linoleate (SL) were conjugated with fatty acid-free BSA. 10% fatty-acid free BSA was prepared by dissolving fatty-acid free BSA in distilled water. Absolute ethanol (200 proof) was diluted to 50% with water. SP and SL were added to pre-warmed (55°C) diluted ethanol to achieve a final concentration of 200mM. This solution was further diluted to 50 mM using pre-warmed 10% fatty acid-free BSA and conjugated for 2 h at 40°C in a shaking water bath with manual shaking every 30 min. The final conjugate was sterile filtered using syringe filters with a pore size of 0.8 uM and stored at -20°C. The conjugated palmitic and linoleic acids are stable at -20°C for 2 months of freeze-thaw cycles. Before treatment, the conjugated drugs were thawed in a water bath until a clear and homogenous solution of the drugs without any turbidity was obtained.

2.4 Cell viability assay

MTT assay was carried out to determine the non-cytotoxic concentrations of palmitic and linoleic acids in the HTR-8/SVNeo, Swan-71, JEG-3 and BeWo cell lines. Cells were seeded in 96 well plates, 5000 cells per well, with a final volume of 180 uL per well, and incubated at 37°C in the presence of 5% CO₂ for 24 h. After 24h, the cells were checked for attachment under the microscope. Once attachment was confirmed, the cells were incubated with different concentrations of palmitic and linoleic acids ranging from 0 to 2.5 mM to make up the final volume of 200 uL per well at 37°C in the presence of 5% CO₂ for 72 h. After 72h, 20 uL of MTT (2 mg/ml) was added, and the cells were further incubated for 4 h. The plate was constantly checked under the microscope for formation of purple formazan crystals. Subsequently, the supernatant containing media and MTT was removed and 100 uL of DMSO was added to each well to dissolve the
formazan crystals. The plate was further left on a shaker until the formazan crystals dissolved. The absorbance was read at 570 nm in an Opsys microplate reader (Dynex Technologies, Chantilly, VA). The viability of untreated cells was set as 100%. IC50 was calculated with a concentration viability curve using a modified Bliss method. For each cell line, the concentrations at which palmitic and linoleic acids were associated with 80% cell viability were selected for further studies. The cell viability assay was performed in triplicate and each assay was run at least three times.

2.5 Preparation of cell lysates

To determine 1) the expression of ABC transporters (PgP, BCRP, MRP1, MRP7) in the placental cell lines, 2) the effect of palmitic and linoleic acids on the expression of these transporters, 3) the effect of PA on reduced folate receptor 1 (RFC1) and 4) the effect of NAC on PA treated cells, cells were treated with PA at different time points (0-72 h). After the cells showed 80% confluency, they were scraped and collected. RIPA lysis buffer was added to the cells, followed by vortexing for a few seconds. The resuspended cells were incubated for 30 min on ice. They were then centrifuged in a microcentrifuge at 12000 rpm for 20 min at 4°C. After 20 min, the cell debris was removed, and the cell lysates were stored at -80°C. Protein concentrations were determined using bicinchonic based protein assay (BCA assay)

2.6 Immunoblot analysis

After determining their protein content, equal concentrations (40 ug) of protein aliquots were loaded and resolved by SDS-PAGE and transferred to a PVDF membrane previously soaked in methanol for 10 min. The membrane was blocked for 2 h in 5% skim milk prepared in TBST (10 mM Tris HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween
20). The membrane then was left overnight at 4°C with primary monoclonal antibodies against ABCB1, ABCG2, ABCC1 and ABCC7, each having a dilution of 1:500, RFC1 at a 1:200 dilution and GAPDH at 1:1000. The membrane was then washed with TBST and incubated with horseradish peroxide (HRP)-conjugated secondary antibody (1:1000) in 5% skim milk on a shaker at room temperature for 2 h. Bands were detected using an ECL detection system (LI-COR biosciences, Nebraska) and then analyzed using ImageJ software (Maryland, USA). To determine the effects of palmitic and linoleic acids on the expression of specific proteins, cells were first incubated with or without palmitic or linoleic acid for 72 h. Lysates were collected and their protein concentrations determined using a BCA assay. Western blot was performed as described above.

2.7 RNA extraction and quantification

Total RNAs were isolated from HTR-8/SVNeo and Swan-71 cell lines after pre-treatment with PA at 0 and 72 h. Cells were lysed using TRIzol RNA extraction reagent (Life Technologies, Inc.). The RNA from the lysed cells was extracted by adding chloroform and centrifuging at 16000 g for 5 min at 4°C. Isopropanol was used to precipitate the RNA and the pellets were washed using 75% ethanol prepared in DEPC water. The pellets were air dried and further dissolved in DEPC water. The solution was incubated at 55°C for 20 min after which the cells were incubated overnight at 4°C. RNA concentrations were quantified at 260 nm with a spectrophotometer (Eppendorf Biospectrometer, Hauppauge, NY). RNA samples with an A260/A280 ratio between 1.6 and 2.0 were used for further analysis.
2.8 RT-qPCR Assay

Polymerase chain reaction (PCR) was carried out to determine if there was a change in the mRNA levels of the *ABCC1* gene in HTR/SVNeo and Swan-71 cells. Reverse transcription of total RNA into cDNA was carried out using SuperScriptII reverse transcriptase (Life Technologies, Inc.). The cDNAs were incubated overnight at 55°C. ABCC1 forward and reverse primers were ordered from Fisher Scientific company, LLC. Quantitative PCR was carried out using SYBR Select Master Mix (Life Technologies, Inc.) in an AriaMx (Agilent Technologies, Santa Clara, CA) real time qPCR system. The data were calculated according to the comparative ΔΔCT method and presented as relative fold-change compared to the control.

2.9 [3H]-Folic acid accumulation assay

The effect of PA on the accumulation of [3H]-folate was evaluated in HTR-8 and Swan-71 cells. Cells pre-treated with PA at different time points from 0 to 72 h were further incubated with MK-571 for 2 h. The cells were then washed with PBS and lysed using lysis buffer. The lysed cells were then added to 5 mL scintillation fluid in scintillation vials. Radioactivity was detected using a Packard TRI-CARB 1900CA liquid scintillation analyzer.

2.10 [3H]-Folic acid efflux assay

To determine whether the increase in accumulation of [3H]-folate was due to a decrease in the efflux of [3H]-folate, an efflux assay was carried out. The reduction in efflux was determined by using PA pre-treated cells. The cells were further incubated with [3H]-folate for 6 h. Cell lysis was carried out using lysis buffer and the lysed cells
were added to 5 ml scintillation fluid in scintillation vials. Radioactivity was detected using a Packard TRI-CARB 1900CA liquid scintillation analyzer.

### 2.11 Immunofluorescence assay

An immunofluorescence assay was performed to determine the expression and localization of ABCC1 in HTR-8/SVNeo and Swan-71 cell lines. Cells were seeded (10 \times 10^4 cells per well) in a 24 well plate overnight. HTR-8/SVNeo and Swan-71 cells were then treated with 0.5 mM or 0.7 mM PA, respectively for 0, 24, 48 and 72 h. Formaldehyde (1 mL at 4%) was added to each well to fix the cells. The cells were incubated with 1 mL of ice-cold 0.25% Trition-X 100 solution for 15 min to increase permeability. Blocking was carried out with 6% BSA and the cells were further incubated with 1 mL monoclonal anti-P-Glycoprotein antibody per well. The plate was covered with aluminum foil at and stored at 4°C. Monoclonal anti-P-Glycoprotein antibody was diluted to 1:200 in 6% BSA. Subsequently, the cells were incubated with 1 mL Alexa flour 488-conjugated goat anti-rabbit IgG per well for 2 h at room temperature. A 1:1000 dilution of Alexa flour 488-conjugated goat anti-rabbit IgG was prepared in 6% BSA. The nuclei were counterstained by DAPI at room temperature for 15 min. Fluorescence staining was carried out in a dark room. Cells were washed thrice with PBS after each step. The fluorescent images were taken using EVOS FL Auto fluorescence microscope (Life Technologies Corporation, Maryland, USA).

### 2.12 Transwell migration assay

In order to determine the functional effects of upregulation of ABCC1 on extravillous trophoblasts, a migration assay was performed. HTR-8/SVNeo and Swan-71 cells were pre-treated with 0.5 mM and 0.7 mM PA, respectively, for 0, 24 and 48 hrs.
Cells in 500 uL serum-free media were added to each transwell (75,000 cells per well), whereas media with 10% FBS (1mL) was added to the lower chamber as a chemoattractant. After 24 h, the cells were fixed with 4% formaldehyde. Subsequently, the non-migrated cells were removed from the transwell with a cotton swab. Each transwell was further incubated with 0.5% crystal violet for 15 min. Transwells were washed thrice with PBS after each step. Brightfield images were taken using an EVOS FL Auto fluorescence microscope (Life Technologies Corporation, Maryland, USA). For quantification, the cells were trypsinized and counted using a hemocytometer.

### 2.13 Transwell invasion assay

To evaluate the effect of PA on the invasion of extravillous trophoblast cell lines, an invasion assay was carried out. HTR-8/SVNeo and Swan-71 cells were pre-treated with 0.5mM and 0.7 mM PA, respectively for 0, 24 and 48 hrs. 24 h before adding cells to the transwell; each transwell was coated with Matrigel diluted with serum free media (1:25) and incubated at 37°C and 5% for solidification. Subsequently, 75,000 cells were suspended in 500uL serum free media and added to the transwell coated with Matrigel. Media containing 10% FBS was added to the lower chamber. After 24 h, the transwell was washed with PBS and the cells were fixed with 4% formaldehyde for 15 mins. The non-invasive cells and Matrigel were gently scraped from the transwell using a cotton swab. The transwell was further washed with PBS and the cells were stained by incubating the transwell in 0.5% crystal violet. Brightfield images were taken using an EVOS FL Auto fluorescence microscope (Life Technologies Corporation, Maryland, USA). For quantification, the cells were trypsinized and counted using a hemocytometer.
Figure [9]: Invasion and migration assay. The cells were added to a transwell in a 24 well plate in media without FBS. Media with FBS was added to the bottom well. The cells were left overnight and the cells which diffused through the transwell were counted. For the invasion assay, Matrigel was added to the transwell before the addition of the cells.

2.14 NAC treatment to inhibit ROS

To confirm the mechanism for the upregulation of MRP1 by PA, cells were treated with 1mM NAC for 2 h and then treated with PA at different time points. Cell lysates were collected, and western blot was carried out to assess the expression of MRP1.

2.15 Determining cytotoxic concentrations of G418

To confirm the role of MRP1 in impeding the function of extravillous trophoblast cells, the ABCC1 gene was knocked out using the CRISPR/Cas9 method. An ABCC1 gene knockout subline of Swan-71 cells was constructed using a clustered regularly
interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 system. The vector consists of a U6 promoter that regulates the transcription of guide RNA (gRNA), a CBh promoter that regulates the expression of Cas9 nuclease, and a CMV promoter that regulates the transcription of the neo gene responsible for resistance to G418. The gRNA of the human \textit{ABCC1} targeting vector contains a specific 20 bp guide sequence of 5\textquotesingle-GTTGACAATCTCCCCGACCG-3\textquotesingle selected from exon 10 of the human \textit{ABCC1} gene and a scaffold sequence specific for Cas9 protein. Since the plasmid contained the neomycin resistance gene, the cytotoxic concentrations of G418 on HTR-8/SVNeo and Swan-71 cells could be determined. For determining the cytotoxic concentrations of G418 in both cell lines, 500 cells were first seeded in a 96 well plate. The plate was left in the incubator with 5\% CO\textsubscript{2} at 36\textdegree C. After 24 h, the cells were treated with concentrations of G418 ranging from 0 to 10 mg/ml. The plates were left in the incubator for 14 days and the cytotoxicity was evaluated for both cell lines. The cells were checked every 2 days and the medium was changed as required. The cytotoxicity of G418 in the cells was determined visually by observing the cells under the microscope or by MTT assay.

\textbf{2.16 Transfection of \textit{ABCC1} knockout gene into Swan-71 cells}

No cytotoxicity of G418 in HTR-8/SVNeo cells was observed at any of the concentrations tested. However, G418 was highly toxic in Swan-71 cells where 100\% cell death was observed at concentrations as low as 0.6 mg/ml. Therefore, Swan-71 cells were used for transfection. Firstly, 1 million cells were seeded in two 100 mm\textsuperscript{2} petri dishes using DMEM as a medium. One dish was used as a control and the other dish was used for transfection. The cells were allowed to grow and attach for 24 h in an incubator.
with 5% CO₂ at 36°C. After 24 h, transfection was carried out in one of the plates. Two master mixes were prepared. After a 10 min incubation, both master mixes were combined and added to the cells in 10 ml of OPTI-MEM medium. The cells were left in an incubator with 5% CO₂ at 36°C for 24-48 h until complete confluency was achieved. Once the cells were confluent, the media was aspirated, and the cells were washed thrice with PBS. Further, 10 ml of media containing 10% FBS and 0.6 mg/ml G418 were added to the transfected cells. With medium changes every third day, the transfected cells were incubated with this selection medium for 14 days. Then, single positive colonies were obtained by limited dilution. Measurement of protein expression using western blotting was conducted to verify the knockout of ABCC1. The ABCC1 knockout subline was then used for invasion and migration assays.

2.17 Statistical Analysis

All experiments were repeated at least three times. The statistical significance between two groups was determined with Student’s t-test, whereas comparisons of multiple groups was carried out by one-way ANOVA, followed by Bonferroni’s post-test using Microsoft Excel software. A probability value of *P < 0.05 was considered to be significant.
CHAPTER 3

RESULTS

3.1 Cytotoxicity of linoleic acid and PAs in JEG-3, BeWo, HTR-8 and Swan-71 cells

Before studying the role of the ABC transporters in the placenta, non-cytotoxic concentrations of palmitic and linoleic acids in the four placental cell lines were determined using an MTT assay. Non-cytotoxic concentrations at which cells are 80% cell viable were selected. As shown in figure 10A, there was 80% cell survival at 0.5 mM PA in JEG-3, BeWo and HTR-8/SVNeo cells. However, in Swan-71 cells, there was 80% cell survival at 0.7 mM. On treatment with linoleic acid, 80% cell survival was obtained at 1mM as observed in figure 10B. Therefore, 1mM linoleic acid was used in all the cell lines, 0.5 mM PA was used for JEG-3, BeWo and HTR-8/SVNeo cell lines and 0.7 mM PA was used for the Swan-71 cell line.
The viability of placental cells on treatment with PA
Figure [10]: Cytotoxicity of (A) PA and (B) linoleic acids in HTR-8/SVNeo, JEG-3, BeWo and Swan-71 cell lines. IC$_{20}$ values were obtained from the cytotoxicity assay. IC$_{20}$ ± SD: The drug concentration that inhibited cell survival buy 20% (mean ± SD). The results represent 3 independent experiments, performed in triplicate.

3.2 Expression of ABC transporters in the placental cell lines

To evaluate the modulation of ABC transporters by the two fatty acids, it was important to determine the expression levels of the ABC transporters in the four placental cell lines. Expression of the four most commonly studied ABC transporters, PgP, BCRP, MRP1 and MRP7, was evaluated using a western blotting. SW-620 and SW-620/AD300 were used as negative and positive controls, respectively, for the expression of PgP; H460 and H$60$/MX20 were used as negative and positive controls, respectively for
BCRP. KB-31 and Kb-CV60 were used as negative and positive controls for MRP1, and HEK293 and HEK293/MRP7 were used as a negative and positive controls for MRP7. As is shown in Figure 11A, there was no expression of PgP in any of the four cell lines. However, BCRP was present in the BeWo cell line as shown in Figure 11B. As is shown in Figure 11C, some expression of MRP7 was found in the HTR-8/SVNeo cells, but this expression was only visible upon overexposing the membrane. One of the major findings of this study was the presence of MRP1 in all the four cell lines as is shown in Figure 11D. Hence, the effect of palmitic and linoleic acids on the modulation of MRP1 was studied in all four cell lines. The modulation of BCRP by both fatty acids in the BeWo cell line was also studied.
**Figure [11]: The expressions of ABC transporters in placental cell lines.** The expression of (A) BCRP, (B) PgP, (C) MRP1 and (D) MRP7 were studied in the four placental cell lines. Negative and positive controls were used for each transporter and equal amounts of total cell lysates were used for each sample. Western blot was performed and grayscale ratios (E) were determined with ImageJ. *P < 0.05 versus the control.
3.3 MRP1 is modulated by treatment with PA in HTR-8/SVNeo and Swan-71 cells

The modulation of BCRP in BeWo cells and MRP1 in JEG-3, BeWo, HTR-8/SVNeo and Swan-71 cells by treatment with linoleic and PAs was evaluated by using western blotting. The cells were treated with the above-mentioned concentrations of linoleic and PAs and the expression of the ABC transporters was evaluated at different time points from 0-72 h. It was observed that on treatment with both palmitic and linoleic acids, there was no change in the expression of BCRP in the BeWo cell line. In addition, the expression of MRP1 was also not altered upon treatment with linoleic acid in all four cell lines as shown in Figures 12A and 12B. As is shown in Figure 12C, treatment with PA also did not alter the expression of MRP1 in JEG-3 and BeWo cells. However, as shown in Figure 12D, there was a time dependent increase in the expression of MRP1 in the HTR-8 and Swan-71 cell lines upon treatment with 0.5 mM and 1 mM PA, respectively. Based on these results, HTR-8/SVNeo cell, which represents first trimester extravillous trophoblast cells were selected for further studies.
C

**JEG-3**

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**BeWo**

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**Graphs**

![Graph 1](image1.png)

![Graph 2](image2.png)
Figure [12]: Effect of linoleic and PAs on MRP1 in placental cell lines. The effect of linoleic and PAs on the expression of MRP1 was tested after placental cells were treated with linoleic acid (1mM) or PA (0.5mM) from 0 to 72 h. (A) and (B) Effect of linoleic acid on MRP1 expression in placental cell lines. (C) and (D) Effect of PA on MRP1 expression in placental cell lines. Grayscale ratios were determined with ImageJ. *P < 0.05 versus the control.
3.4 *ABCC1* gene expression is increased in HTR-8/SVNeo and Swan-71 cells upon treatment with PA

To determine the effect of PA treatment on ABCC1 gene expression, qRT-PCR analysis was performed. As shown in Figures 13A and 13B, after 72 h of treatment with PA, there was a 2.5-fold upregulation in *ABCC1* gene expression in the HTR-8/SVNeo cells compared to the control. Similarly, in Swan-71 cells, treatment with PA caused an approximately three-fold increase in the expression of the *ABCC1* gene. These results indicate that in addition to the increase in MRP1 protein expression, the expression of the *ABCC1* gene is also increases upon treatment with PA.

![Graph showing mRNA expression of MRP1 after treatment with PA](image)
Figure [13]: Effect of PA on ABCC1 expression. TRizol was used to extract total RNA as per the manufacturer’s instructions. Reverse transcription was carried out and qRT-PCR was performed. (A) Relative ABCC1 mRNA expression in HTR8/SVNeo cells, (B) Relative ABCC1 mRNA expression in Swan-71 cells, treated with PA for 72 h. The change in gene expression is expressed as relative change. GAPDH was used as a reference gene. Each data point is represented by mean ±SD of at least three independent experiments. *P < 0.05 versus the control.

3.5 PA increases [3H]-folic acid efflux in HTR-8/SVNeo cells

The effect of PA on the function of MRP1 was studied using an efflux assay. As shown in Figure 14, there was a time dependent increase in the efflux activity of HTR-
8/SVNeo cells upon treatment with PA as compared to the control. Efflux activity was highest in cells treated with PA for 72 h.

Figure [14]: Effect of PA on the \([\text{H}]\)-folic acid efflux. Untreated HTR8/SVNeo cells were used as a control. Error bars indicate SD and the mean values are from three independent experiments. \(*P < 0.05\) compared to the control group.

3.6 MK-571 increases the intracellular accumulation of \([\text{H}]\)-folic acid in HTR-8/SVNeo cells

To demonstrate that the increased efflux of \([3H]\)-folic acid was because of the decrease in the accumulation of \([3H]\)-folic acid, an accumulation assay was performed. MK-571 was used as an MRP1 inhibitor. Figure 15 shows that there was a time-dependent increase in the intracellular accumulation of \([3H]\)-folic acid from 0 to 72 h in
HTR-8/SVNeo cells as compared to the control. The increase in the accumulation of [3H]-folic acid was the highest in cells treated with PA for 72 h.

**Figure [15]: Effect of MK-571 on the intracellular accumulation of [3H]-folic acid in PA treated cells.** Effect of MK-571 on the intracellular accumulation of [3H]-folic acid in PA treated HTR8/SVNeo cells. Mean values obtained from three independent experiments and error bars indicate SD. *P < 0.05 compared to the control group.

**3.7 PA does not alter the cellular localization of MRP1**

We further evaluated whether PA altered the cellular localization of MRP1 using an immunofluorescence assay. HTR-8/SVNeo and Swan-71 cells were treated with PA for 72 h. The MRP1 transporters are located on the cell membrane of both cell types.
Upon treatment with PA, there was no change in the intracellular distribution of MRP1 at 72 h compared to the control in both HTR-8/SVNeo and Swan-71 cell lines. However, an increase in the MRP1 expression was seen upon PA treatment at 72 h, which further confirms our western blot and qRT-PCR results.
**Figure [16]: Effect of PA on the subcellular localization of MRP1.** MRP1 protein expression in HTR8/SVNeo and Swan-71 cells after treatment with PA at 0 and 72 h. Nuclei were counterstained by DAPI. Images have been merged using Photoshop software.

### 3.8 PA decreases migration and invasion in HTR-8/SVNeo and Swan-71 cells

To ascertain whether PA decreases the invasion and migration capacity of extravillous trophoblast cells, invasion and migration assays were carried out using the Boyden chamber method. The cells that diffused through the transwell were considered to be the migrated or invasive cells. The cells were treated with PA at different time points. As shown in Figures 16A and 16B, there was decreased migration of both cell lines as length of exposure to PA increased. Migration was the lowest in cells treated with PA for 72 h.
For the invasion assay, cells were treated with PA at different time points and the cells that passed through the Matrigel and the transwell membrane were considered the invasive cells. Figure 16A and 16B demonstrated that as the time of exposure to PA increased, there was decreased invasion in both HTR-8/SVNeo and Swan-71 cells. Invasion was the lowest in cells treated with PA for 72 h.
**Figure [17]: Effect of PA on migration and invasion of EVTs.** The effect of PA treatment on the migration and invasion of (A) HTR8/SVNeo cells (B) Swan-71 cells. PA untreated cells were used as a control. Quantification was carried out by counting the migrated or invaded cells using a hemocytometer. Mean values were obtained from three independent experiments and error bars represent SD. *P < 0.05.
3.9 NAC rescues PA treated cells from MRP1 upregulation

To confirm that the upregulation of MRP1 resulted from increased oxidative stress, cells were treated with the anti-oxidant NAC. HTR-8/SVNeo and Swan-71 cells were pre-treated with 1 mM NAC for 2 h, and then were treated with PA. The expression of MRP1 was evaluated in both cell lines with and without NAC treatment using a western blot. As shown in Figure 17A, the expression of MRP1 in NAC and PA treated cells was much lower compared to cells treated with PA alone and was comparable to the expression of MRP1 in the control untreated cells. It was also observed that the expression of MRP1 in the cells treated with NAC alone was lower compared to the control untreated cells. These results indicate that the upregulation of MRP1 by PA is mediated by oxidative stress and is prevented upon treating the cells with 1 mM NAC.
Figure [18]: Effect of NAC on MRP1 in PA treated cells. The effect of PA on the expression of MRP1 in NAC (1mM) treated cells was tested in (A) HTR8/SVNeo and Swan-71 cell lines. Grayscale ratios (B) were determined with ImageJ. * P < 0.05 versus the control.
3.10 NAC increases the invasion and migration capacities of EVT

to further demonstrate the effect of NAC treatment on the function of EVT
migration and invasion experiments were carried out. We found that in the NAC and PA

treated group, there was an increase in migration in both HTR-8/SVNeo and Swan-71
cell lines, as shown in Figure 18. A similar result was observed in the invasion assay
where invasion capacity was retained in HTR-8/SVNeo and Swan-71 cell lines treated
with NAC. The invasion and migration in the NAC and PA treated cell lines was
comparable to the invasion and migration of the controls in both cell lines showing that in
addition to preventing upregulation of MRP1, NAC is also able to restore the migration
and invasion functions of EVT.
Figure [19]: Effect of NAC on migration and invasion in PA treated EVTs. The effect of NAC on the migration and invasion of PA treated (A) HTR8/SVNeo cells and (B) Swan-71 cells. PA untreated cells were used as a control. Quantification was carried out by counting the migrated or invaded cells using a hemocytometer. Mean values were obtained from three independent experiments and error bars represent SD. *P < 0.05.

3.11 MK-571 partially increases invasion and migration in HTR8/SVNeo and Swan-71 cell lines

To demonstrate that the decrease in migration and invasion functions of EVT cells is MRP1 mediated, cells were treated with MK-571, an MRP1 inhibitor. The cells were treated with MK-571 for 2 h and then treated with PA. As shown in Figure 20A, we
found that in cells treated with MK-571 and PA, there was an increase in the migration in both HTR-8/SVNeo and Swan-71 cell lines. In addition, there was also an increase in invasion cells treated with both MK-571 and PA as shown in Figure 20B. The increases in migration and invasion were not significant compared to cells treated with PA alone. The fact that the increases in invasion and migration did not reach statistical significance could be due to weak inhibition of MRP1 by MK-571.
Figure 20: Effect of MK-571 on migration and invasion of PA treated EVTs. The effect of MK-571 on the migration and invasion of PA treated (A) HTR8/SVNeo cells and (B) Swan-71 cells. PA untreated cells were used as a control. Quantification was carried out by counting the migrated or invaded cells using a hemocytometer. Mean values were obtained from three independent experiments and error bars represent SD. (*) indicates p < 0.05.
3.12 Knockout of *ABCC1* gene by CRISPR/Cas9 completely restores migration and invasion in Swan-71 cells

MK-571 was partially able to increase invasion and migration of the EVTs. To evaluate the role of MRP1 upregulation in the decrease in migration and invasion of EVTS further, we carried out a complete knockout of the *ABCC1* gene in Swan-71 cells using the CRISPR/Cas9 gene knockout method. As seen in Figure 21, the *ABCC1* gene has been completely knocked out of Swan-71 cells. We observed that upon complete knockout of the *ABCC1* gene in Swan-71 cells, there was a significant increase in migration after treatment with PA compared to control cells treated with PA, as shown in Figure 22A. Similar results were observed in the invasion assay, as shown in Figure 22B, where upon comparing the invasion of control Swan-71 cells treated with PA to Swan cells lacking the *ABCC1* gene treated with PA, there was a significant increase in invasion in the *ABCC1* gene knockout subline. These results indicate that MRP1 plays an important role in the invasion and migration of extravillous trophoblasts.
Figure [21]: MRP1 knockout in Swan-71 cells. MRP1 was knocked out in Swan-71 cells using CRISPR/Cas9. Quantification of the protein expression was done by gray scale values. Error bars are representative of mean ± SD from 3 independent experiments.
Figure 22: Migration and invasion in MRP1 KO cells treated with PA. The effect of PA treatment on MRP1 Ko cells on (A) migration and (B) invasion. PA untreated cells were used as a negative control and PA treated Swan-71 cells were used as a positive control. Quantification was carried out by counting the migrated or invaded cells using a hemocytometer. Mean values were obtained from three independent experiments and error bars represent SD. * P < 0.05.
CHAPTER 4

DISCUSSION

There has been a significant rise in the cases of pregnancy complications in the last decade[220].

Increase in the consumption of Western diets high in fat has significantly increased the incidence of preterm birth, ectopic pregnancy, miscarriages, and intrauterine growth restriction[220]. High fat diet can also put the fetus at a significantly higher risk for metabolic disorders later in life[221]. Thus, fetal programming may also be altered in obese and pregnant women consuming high fat diet[118,222,223]. One of the major conditions in women of reproductive age is obesity[224]. Non-communicable maternal diseases (NCDs) have been identified as a new focus area by The International Federation Gynecology[225]. Therefore, much work has recently been done for the identification and prevention of NCDs in both the mother and fetus[226–228]. One of the major risk factors for NCDs is maternal nutrition and diet[229,230].

Although various ABC transporters have been identified in the placenta, there are limited studies on their localization and their function in the maternal-fetal interface and developmental changes[231]. The alterations in the expression of these transporters during different stages of gestation or due to ABC transporter modulators can lead to changes in the transfer of substrates to and from the fetus[232–234]. However, there is limited evidence about the function and presence of ABC transporters in the extravillous trophoblasts[3,235].

There is evidence for the presence of PgP, BCRP and MRP1 in the JEG-3 and BeWo cell lines which represent the syncytiotrophoblast layer of the placenta. Consistent
with these studies, we found the BCRP and MRP1 expression in the BeWo cell line; however, we did not find the PgP expression in JEG-3 and BeWo cell lines. In addition, we did not find the BCRP expression in the JEG-3 cell line. Previous studies have shown the presence of PgP, BCRP, and MRP1 in the HTR-8/SVNeo cell line[92,236,237]. We found the MRP1 expression in HTR-8/SVNeo cells. In addition, we were also able to see minimal expression of MRP7; however, we did not find any expression of Pgp and BCRP in this cell line. There is no previously reported evidence of any ABC transporter in the Swan-71 cell line. Our western blot results showed the presence of the MRP1 transporter in the Swan-71 cell line. Conversely, PgP, BCRP and MRP7 were absent in this cell line. One of the most important findings of this study was the presence of MRP1 in all four placental cell lines.

MRP1 is localized in the basolateral membrane of the syncytiotrophoblasts[238]. There is also MRP1 expression in EVT's[236]. Various chemotherapeutic drugs, heavy metal conjugates, leukotrienes, glutathione and its conjugates, sulfate conjugates, and folates are substrates of MRP1[239]. Since MRP1 is present in the basolateral membrane, it effluxes substrates towards the fetus[240].

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**Table [1]: Substrates of MRP1.**

Adapted and modified from Jiye et.al., 2015.
The expression and function of MRP1 increases in the presence of oxidative stress[241,242]. As demonstrated in previous studies, PA induces oxidative stress in placental cells and linoleic acid has a protective effect. We tested for expression of MRP1 upon treatment with palmitic and linoleic acids and found that there was no change in the expression of MRP1 upon treatment with linoleic acid in any cell line. This result could be due to the protective effect of linoleic acid against oxidative stress. However, there was upregulation in MRP1 expression in HTR-8/SVNeo and Swan-71 cells upon treatment with PA. This finding could be because of induction of oxidative stress with PA treatment of placental cells. In addition to protein expression, our qRT-PCR results indicate that mRNA levels of ABCC1 gene also increase upon treatment with PA. Upregulation of the MRP1 protein and ABCC1 gene in the extravillous trophoblast cells indicates that the effect of PA on MRP1 could be specific to EVTs.

Folic acid is an important substrate of MRP1[243]. Under normal conditions, folic acid efflux by MRP1 is required to maintain homeostasis[244]. However, MRP1 upregulation may increase MRP1 function thereby causing more efflux of folic acid than required. We investigated the effect of PA on the function of MRP1. Interestingly, we observed that treatment with PA increased the efflux of $[^{3}\text{H}]$-folic acid from cells. Furthermore, blocking the function of MRP1, by MRP1 inhibitor MK-571 increased the cellular accumulation of $[^{3}\text{H}]$-folic acid. These results indicate that in addition to MRP1 protein and ABCC1 gene expression, the function of the MRP1 transporter is also increased on treatment with PA. We further studied the effect of PA on intracellular localization of MRP1. Our immunofluorescence results indicate that there is no change in the intracellular localization of MRP1 protein upon treatment with PA.
In the last two decades, there has been increasing evidence showing the effect of folic acid deficiency on birth defects[245]. Therefore, prescribing folic acid supplements before conception and during pregnancy has become important[246]. In addition, folic acid is also required for proliferation, invasion and migration of EVT[189,247]. Increased rates of efflux of folic acid due to upregulation of MRP1 by PA may cause decreased migration and invasion capacities of EVT. Our migration and invasion results show that treatment with PA decreased invasion and migration in both EVT cell lines. These results show that decrease in the invasion and migration of EVT could be due to increases in folic acid efflux mediated by MRP1 upregulation. High concentrations of folic acid have been shown to increase migration and invasion capacities in cells in which invasion and migration has been compromised[189].

Oxidative stress causes damage to various biological molecules and is a cellular process implicated in the etiology of many illnesses[248]. As mentioned earlier, PA induces oxidative stress and MRP1 expression and function is increased in the presence of oxidative stress. Glutathione (GSH) is the body’s master antioxidant[249]. GSH is a substrate of MRP1 and increases in MRP1 expression could lead to increases in GSH efflux rates. Decreased GSH in cells could further increase oxidative stress. To confirm the role of MRP1 in oxidative stress, we studied the effect of NAC on MRP1 expression and function in PA treated cells. NAC is a synthetic derivative of L-cysteine and a precursor of GSH[248]. NAC was used instead of GSH for this study because GSH is a tripeptide consisting of glutamate, cysteine and glycine, making it difficult to enter the cells[250,251]. As seen in our western blot results, NAC was able to decrease the expression of MRP1 in PA treated EVT cells, indicating that decreased oxidative stress
caused a decrease in MRP1 expression. Moreover, NAC was also able to restore invasion and migration in PA treated EVTs. These results indicate that NAC was able to ‘rescue’ MRP1 upregulation in EVTs and was able to restore the invasion and migration functions of EVTs. This explains the role of oxidative stress in PA induced upregulation of MRP1.

Figure 23: Mechanism of action of N-acetylcysteine and MRP1 upregulation by PA.
PA induces oxidative stress in the placenta, which increases the expression of the MRP1 protein, leading to increased GSH efflux. NAC is a GSH precursor and increases intracellular GSH concentration and reduces oxidative stress.
Diverse factors contributing to decreased invasion and migration in EVTs have been recently investigated[151,252]. We tested whether blocking MRP1 would restore invasion and migration capacities of EVTs. MRP1 inhibition by MK-571 only partially increased the invasion and migration of the EVTs. This partial effect could be due to weak inhibition of MRP1 by MK-571[253]. To further confirm the role of MRP1 in the decrease in invasion and migration of EVTs, we knocked out MRP1 in Swan-71 cell line. G418 was used for antibiotic selection since the plasmid contained a neomycin resistant gene. Therefore, it was necessary to achieve cytotoxic concentrations of G418 to isolate transfected colonies. MRP1 knockout was unsuccessful in HTR8/SVNeo cells since G418 was noncytotoxic up to 15 mg/ml concentration. However, in Swan-71, cytotoxic concentrations of G418 were achieved at 0.6 mg/ml. PA treated MRP1 knockout Swan-71 cells showed a significant increase in migration and invasion compared to PA treated control Swan-71 cells. This indicates that PA induced MRP1 protein upregulation is responsible for the decreased migration and invasion functions of MRP1, possibly due to increased folic acid efflux.

It is known that high fat diet causes pregnancy complications. However, the mechanisms involved in this effect are unclear. Various studies have shown that high fat diet compromises invasion and migration capacities of EVTs. ABC transporters are localized throughout the placenta and are very important to maintain homeostasis by carrying out efflux functions. ABC transporters of the syncytiotrophoblasts have been extensively studied. However, there are very few studies which elucidate the function of ABC transporters in the EVTs. EVTs are very important in the first trimester of pregnancy for spiral artery remodeling. Failures in spiral artery remodeling can cause
intermittent perfusion of blood, which may lead to undesirable maternal and fetal outcomes. MRP1 is highly expressed in EVTs and perform an important role in folate efflux. Therefore, it is very important to study the effect of PA on MRP1 mediated folate efflux.

MRP1 upregulation by PA caused a decrease in the migration and invasion of EVT cell lines, which was restored when MRP1 was knocked out in Swan-71 cells. This supports the role of MRP1 in the invasion and migration of EVTs. However, whether a similar effect is seen in primary EVTs or mouse giant cells requires further investigation. The novel finding in this study that PA increases MRP1-mediated folate efflux provides a missing link explaining how PA compromises the in-utero environment. Elucidation of pathways connecting maternal high fat diet consumption to fetal programming provides insights that can lead to future therapeutic approaches to safeguard the developmental origins of human health.
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VITA

Name: Yunali V. Ashar

Baccalaureate Degree: Bachelor of Pharmacy,
Dr. Bhanuben Nanavati College of Pharmacy, Mumbai, Major: Pharmacy

Date Graduated: June 2015