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# THE ROLE OF MEGALIN IN THE TRANSPORT OF AMINOGLYCOSIDES ACROSS HUMAN PLACENTA

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## ACKNOWLEDGMENT

---

First, and foremost I would like to thank God for his unceasing blessings. I am thankful for the many gifts he has provided, like patience, good friends, excellent mentors and wisdom which all helped me to complete my dissertation.

My deep gratitude is to my advisor Dr. Mary Jayne Kenney for her academic support, guidance and patience. Her tolerance that helped me overcome many crisis situations and lead me to finish this dissertation successfully. Her mentorship and steady feedback helped me not only grow from the scientific aspect, but it also aided me to develop a personality of an assertive, confident independent thinker and problem solver. I am thankful for her the countless revisions for my presentations and papers including this dissertation.

I am grateful for the continuous guidance and support from my co-advisor Dr. Phillip Gerk. Through my PhD Journey, he was always there to provide genuine academic advice, technical assistance and continuous feedback. I am thankful for his encouragement to me to pay more attention to details, to improve my writing skills, and the corrections for my presentations and papers including this dissertation.

I am also indebted to the Department of Pharmacotherapy at VCU School of Pharmacy, especially those members of my doctoral committee for their input, valuable discussions and accessibility. In particular, I would like to thank Dr. Jürgen Ventiz, Dr. Patricia Slattum, and Dr. Douglas Sweet for their hard work, expertise and patience. Also, I would like to thank Dr. David Holdford, Dr. Donald Brophy and Dr. Susanna Wu-Pong for administrative assistance.

I would like to also thank Dr. Scott Walsh and Ms. Sonya Washington from the VCU school of Medicine for their valuable input in my project and assistance to get placental tissue samples for my project. I am as well grateful to Dr. Matthew Beckman for his step-by step guidance in my project, and his significant moral support.

I would like to thank the School of Pharmacy at my Home University, the University of Jordan, for financial support and providing the scholarship for my living expenses and tuition upon pursuing of the PhD degree. I also would like to acknowledge funding from the Thomas F. and Kate Miller Jeffress Trust and the VCU School of Pharmacy.

Many thanks are to my colleagues in the departments of Pharmacotherapy and Pharmaceutics as well as my friends in Jordan, and US for emotional support and academic guidance during the most difficult times, and also the Jordanian community in the US for its aid in accommodating life in a new country.

Finally and most importantly, I would like to sincerely express gratitude to my parents for whom I dedicate this dissertation to. Without them none of this could be possible. I would like to thank my father for his paramount emotional and financial support. For his guidance, and faith in my abilities to pursue this degree in spite of all cultural constraints. I would like to thank my mother for her unwavering love, patience, continuous encouragement, and emphasis on the importance of education.

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## LIST OF ABBREVIATIONS (ALPHABETIC ORDER)

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<sup>125</sup>I-TC-B<sub>12</sub>: Iodinated transcobalamin vitamin B<sub>12</sub> complex

AGs: Aminoglycosides

ANOVA: Analysis of variance

ARH: Autosomal recessive hypercholesterolemia protein

BBM: Brush border membrane

BCRP: Breast cancer related protein (BCRP)

BN16: Rat choriocarcinoma cells

BPE: Bovine pituitary extract

Cell lines 293: Human embryonic kidney cells

CHO: Chinese hamster ovary

ClC5: Chloride channel isoform 5

CME: Clathrin-dependent RME

CMF-HBSS: Calcium and Magnesium-Free Hank's buffered salt solution

CTBs: Cytotrophoblast cells

CUBAM: Cubilin-amnionless complex

Dab2: Disabled 2

DIDS: 4, 4'-diisothiocyanostilbene-2,2'-disulfonic acid

DMEM: Dulbecco's modified Eagle medium

DMSO: Dimethyl sulfoxide

DPBS: Dulbecco's phosphate buffered saline

DPM: Disintegrations per minute

dsRNA: Double-stranded RNA

EDTA: Ethylenediaminetetraacetic acid

FACS: Fluorescence activated cell sorting

FITC: Fluorescein isothiocyanate

FITC-BSA: Fluorescein-labeled bovine serum albumin

FR $\alpha$ : Folate receptor-alpha

GFP: Green-Fluorescent protein

GIPC: GAIP Interacting Protein, COOH Terminus; GAIP: G-alpha interacting protein.

HDL-C: High lipoprotein cholesterol

HEPG-2: Human hepatocellular carcinoma cells

hFcRn: Human neonatal Fc receptor

HK-2: Human kidney cells type 2

IAI: Intra-amniotic infection

IgG: Immunoglobulin G

IRB: Institutional Review Board

L2: Rat yolk sac cells

LCC-PK1: Lewis lung carcinoma porcine kidney cells

LDL-C: Low-density lipoprotein cholesterol

LRP: Low-density lipoprotein receptor-related protein

LSC: Liquid scintillation counter

MEF: Murine embryonic fibroblasts

ML: Mouse liver



MRP2: Multi-drug resistance related protein 2

NAG: *N*-acetyl- $\beta$ -D-glucosaminidase

NC: Nitrocellulose

NHE3: Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 3

NMHC IIA: Nonmuscle myosin heavy chain IIA

NPPB: 5-nitro-2-(3-phenylpropylamino) benzoic acid

NTC: Non template control samples

OK: Opossum kidney

PBS: Phosphate buffer Saline

p-gp: Multi-drug resistance P-glycoprotein

PTMs: Posttranslational modifications

PVDF: Polyvinylidene fluoride

PVT: Placental villous tissue

Q-PCR: Quantitative polymerase chain reaction

RAP: Receptor associated protein

rEGF: Human recombinant epidermal growth factor

RISC: RNA-interfering silencing complex

RK: Rat kidney

RME: Receptor-mediated endocytosis

RNAi: RNA interference

RT: Reverse transcriptase enzyme

SDS: Sodium dodecyl sulfate

shRNA: Short hairpin RNA

SITS: 4-Acetamido-4'-isothiocyano-2, 2'-stilbene disulphonic acid

STB: Syncytiotrophoblast

TEER: Transepithelial electrical resistance

u-PA: Urokinase--plasminogen activator

uPA-PAI: Urokinase-plasminogen activator-plasminogen activator Inhibitor .

VCU: Virginia Commonwealth University

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## ABSTRACT

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### **THE ROLE OF MEGALIN IN THE TRANSPORT OF AMINOGLYCOSIDES ACROSS HUMAN PLACENTA**

**By AmalAkour, BSc, PhD candidate**

**A dissertation submitted in partial fulfillment of the requirements for the Doctor of Philosophy of Pharmacotherapy at Virginia Commonwealth University.**

**Virginia Commonwealth University, 2012**

**Major advisor: Mary Jayne Kennedy, Pharm. D., Department of Pharmacotherapy and Outcomes Science**

**Co-advisor: Phillip Gerk, PharmD., Ph.D., Department of Pharmaceutics**

**Background:** Intra-amniotic infections (IAIs) are common complications of labor and delivery. If inadequately treated, these infections can lead to significant morbidity and mortality in the mother and the fetus. Intrapartum aminoglycoside (AG) administration is recommended for the management of IAIs. AGs are known to cross the placenta and achieve bactericidal concentrations in fetal serum. However, the highest and most persistent fetal levels are achieved in renal tissue. So, the fetus may be vulnerable to the nephrotoxic effects of AGs. Megalin, a 600 kDa endocytic receptor, is responsible for the uptake of AGs into renal proximal tubular epithelial cells. This receptor is also expressed in human term placenta and it is reasonable to speculate that it is similarly involved in the placental transport of AGs. However, the mechanisms responsible for placental AG uptake and transport have not yet been characterized.

**Objective:** To evaluate the role of megalin in the transport of AGs across human placenta.

**Specific aims:** (1) To assess and *compare* megalin expression in term and preterm placental villous tissue, and (2) assess the functional activity of megalin in *in vitro* placental models.

**Methods:** (1) Following IRB approval, placental tissue samples were collected from pregnant women undergoing term or preterm deliveries. Placental villous tissues were used to quantify megalin expression by western blotting and q-PCR (2) The human choriocarcinoma cell line (BeWo cells) were grown on Transwell plates, and then megalin expression and function were assessed.

**Results:** Megalin protein and mRNA expression were confirmed in samples of human placental villous tissues. Megalin mRNA expression declined steeply with gestational age till week 31 of gestation then it plateaued thereafter. Also, the expression in the early preterm (n=2) was six fold higher than that of both late preterm (n=3) and term placenta (n=10) ( $p < 0.05$ ). The uptake of  $^3\text{H}$ -gentamicin by the BeWo cells was time-dependent, saturable ( $V_{\text{max}} = 42.9 \pm 4.9$  nmol/mg protein/min;  $K_m = 2.93 \pm 0.68$  mM) and partially inhibited by megalin inhibitors.

**Conclusion:** Megalin is expressed in human placental villous tissues as well as the BeWo cells. When grown on Transwell® plates, the BeWo cells appear to be the most appropriate model to study the *in vitro* transport of AGs across the apical membrane. Time, temperature and concentration dependence of gentamicin uptake in the BeWo cells indicate protein-mediated transport. The inhibition data are consistent with megalin-mediated endocytosis of AGs.

## CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

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### I.THE USE OF AMINOGLYCOSIDES FOR THE MANAGEMENT OF INTRA-AMNIOTIC INFECTIONS

---

Intra-amniotic infections (IAIs) are common complications of labor and delivery occurring in up to 10% of term and 25% of pre-term pregnancies.<sup>1,2</sup> If inadequately treated, these infections can lead to serious complications for both the mother and/or fetus. Studies have shown that 5-10% of mothers developed bacteremia, while pneumonia, respiratory distress, and early-onset neonatal sepsis affect about 20%-40% of fetuses.<sup>2,3,4</sup> The rate of mortality for untreated infections is 10% and 25% for term and preterm fetuses, respectively. Aminoglycosides (AGs) in combination with penicillins cover the most common microorganisms encountered in IAIs (*E.coli* and Group B *Streptococcus*).<sup>5</sup> A Cochrane review<sup>4</sup> showed that the maternal administration of these antibiotics during labor or delivery (intrapartum) is at least as effective as postnatal administration in reducing maternal and fetal adverse outcomes. Due to their favorable pharmacokinetic profile, AGs are good candidates for intra-partum delivery, because they can readily cross the placenta into the fetal circulation and amniotic fluid, achieving fetal serum levels that are sufficient to exert a rapid bactericidal effect.<sup>6,7,8</sup>

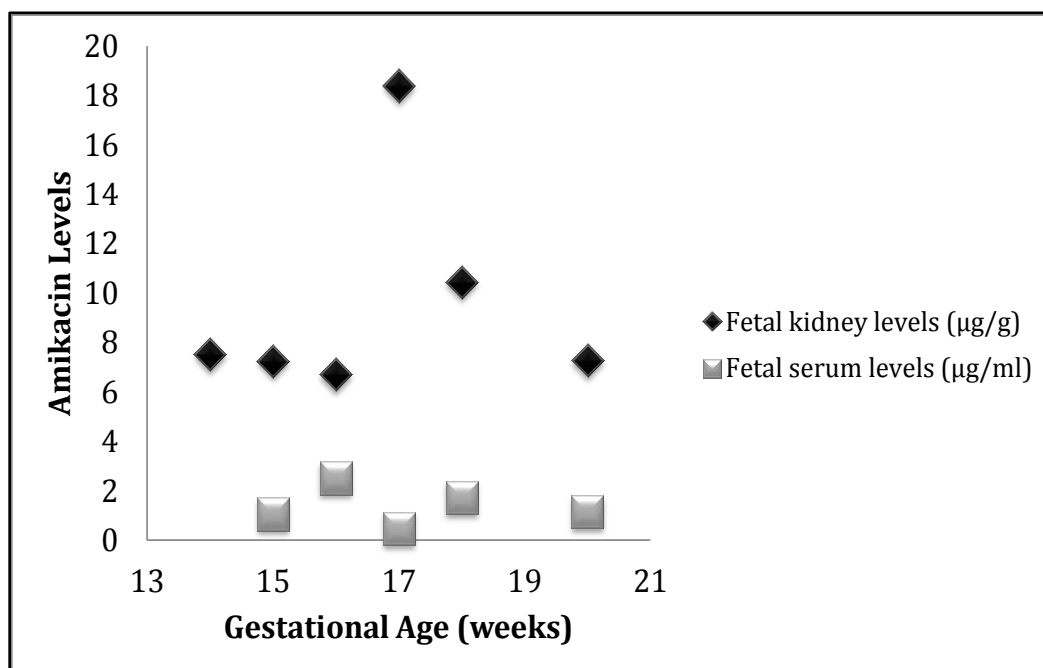
A review paper by Nahum *et al.*<sup>6</sup> stated that the administration of 40 and 80 mg of intramuscular gentamicin during labor resulted in peak cord serum levels that were



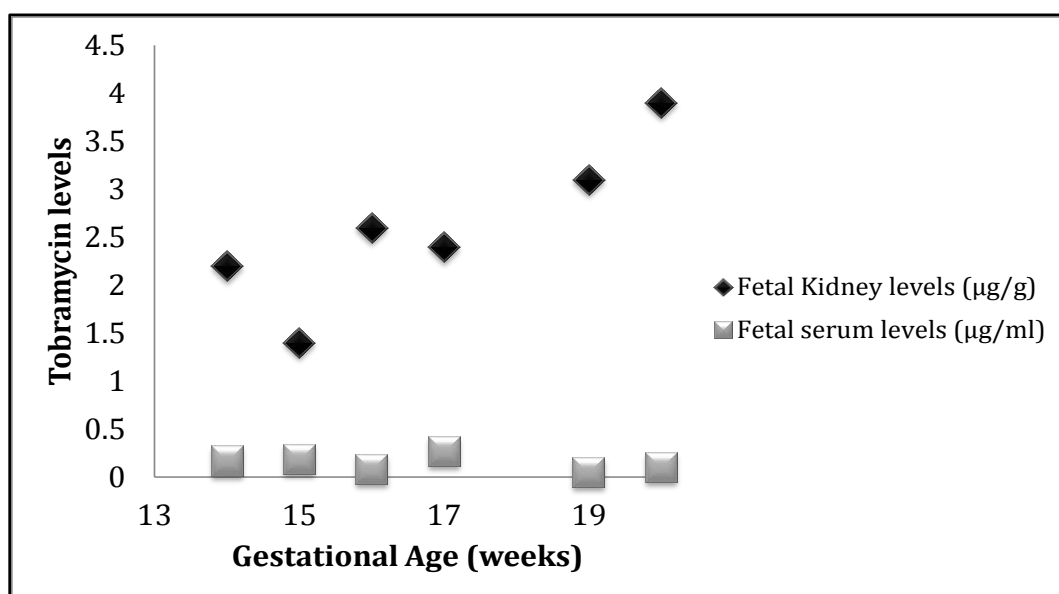
34%<sup>7</sup> and 42%<sup>8</sup> of associated maternal blood concentrations within 1-2 hours of administration; respectively. In the first study,<sup>7</sup> the peak maternal serum levels was  $3.65 \pm 0.5$   $\mu\text{g/ml}$  achieved 30 minutes after administration, while the peak cord levels of  $1.25 \mu\text{g/ml} \pm 0.15$  were achieved within 60-120 minutes. In the latter,<sup>8</sup> the maternal peak level was 2  $\mu\text{g/ml}$  while the cord levels were  $\sim 0.8$   $\mu\text{g/ml}$  and reached 90 minutes from administration. When gentamicin administered as an intravascular infusion (80 mg loading dose then infusion at a rate of 18.5 mg/hour), cord serum levels equal to 1.1  $\mu\text{g/ml}$  were achieved within the first two hours, representing about 30% of the associated maternal serum level.<sup>9</sup> Another study compared the level of gentamicin (dose was unreported) in maternal blood, cord blood, and placental membranes.<sup>10</sup> Interestingly, the gentamicin level was about 4 fold higher in **placental** membranes than that in cord blood (Maternal blood levels =  $3.5 \mu\text{g/ml} \pm 0.95$ ; cord blood levels =  $2.2 \mu\text{g/ml} \pm 0.95$ ; and placental levels =  $13.9 \mu\text{g/ml} \pm 10.0$ ). In two studies with a similar design, amikacin and tobramycin maternal-fetal pharmacokinetic properties were evaluated. After the administration of a single dose of intramuscular amikacin (7.5 mg/kg) to 30 pregnant females undergoing elective 1<sup>st</sup> and 2<sup>nd</sup> trimester abortion (6-20 weeks of gestation), two thirds of the placental tissue samples had amikacin levels of more than or equal to 8  $\mu\text{g/g}$  during the 20 hour interval between drug injection and delivery time while fetal serum levels were less than 4  $\mu\text{g/ml}$ .<sup>11</sup> In the other study which involves intramuscular tobramycin (2 mg/kg) to 35 pregnant females, the mean placental concentrations of tobramycin was 1.4  $\mu\text{g/g}$ ; which remain detectable until 34 hours. (The lowest detectable tobramycin concentrations were as the following: 0.06  $\mu\text{g/ml}$  in fetal serum; 0.1  $\mu\text{g/ml}$  in amniotic fluid; and 0.4  $\mu\text{g/g}$  in placenta). Tobramycin

did not appear in fetal serum until after 2.5 hours of maternal injection and all concentrations there were less than 0.6 µg/ml.<sup>12</sup>

Unfortunately, the clinical utility of AGs is limited by their tendency to accumulate in fetal renal tissue after maternal administration and achieve high and persistent tissue levels, even after administration of a single-dose. In the latter two maternal-fetal pharmacokinetic studies,<sup>11, 12</sup> the levels of both amikacin and tobramycin were highest (Figures 1-1 and 1-2) and most persistent in fetal renal tissue regardless of the gestational age. In the first study, the highest peak concentrations of amikacin attained following maternal administration were in the fetal kidney tissue and were 6-fold higher than in fetal serum and equal to that of maternal serum (Figure 1-1).<sup>11</sup> Concentrations up to 7.5 µg/g of tobramycin were also observed in fetal renal tissue for up to 34 hours after maternal administration (Figure 1-2).<sup>10</sup> Since there is a correlation between the rate and the extent of AGs accumulation in renal tissue and susceptibility to nephrotoxicity,<sup>12</sup> the fetus may be particularly vulnerable to nephrotoxic effects of AGs. Moreover, persistence of AGs in renal tissues after birth may increase the susceptibility of the newborn to injury during early postnatal period when AGs are routinely administered to prevent or treat infections acquired *in utero*.



**FIGURE 1-1:** A GRAPH ILLUSTRATING MEAN AMIKACIN LEVELS MEASURED IN FETAL SERUM AND KIDNEY AFTER THE ADMINISTRATION OF AMIKACIN (7.5 mg/kg) AT DIFFERENT GESTATIONAL AGES. Data in the diagram are depicted from table 1 in Bernard *et al.*, 1977.



**FIGURE 1-2:** A GRAPH ILLUSTRATING MEAN TOBRAMYCIN LEVELS MEASURED IN FETAL SERUM AND KIDNEY AFTER THE ADMINISTRATION OF TOBRAMYCIN (2 mg/kg) AT DIFFERENT GESTATIONAL AGES. Data in the graph are depicted table 1 in Bernard *et al.*, 1977.

Injury to the newborn kidney may have important structural and/or functional consequences. Animal studies showed that the final number of nephrons in pups born to pregnant rats exposed to gentamicin (75 mg/kg/day) from day 10 of gestation to term was reduced by 20% and pups' kidneys showed focal tubular lesions and progression to glomerular sclerotic lesions. Proteinuria was also seen in 3-month offspring.<sup>13</sup> In a study with similar design, delayed renal maturation with an alteration of the glomerular basement membrane was observed. Structural changes and tubular dysfunction have also been observed in newborn pups exposed prenatally to gentamicin.<sup>14</sup> *In utero* exposure to tobramycin (30-60 mg/kg/day) during early stages of nephrogenesis led to disruption of the maturation of the proximal tubules.<sup>15</sup> It is worth mentioning that care is needed when extrapolating data from animal studies to humans, since there are major interspecies differences in renal sensitivity to drugs. In humans, there is a single case report of a 4-year old child with renal dysplasia; his mother had received 300 mg of intravenous gentamicin for 10 days during the 7<sup>th</sup> week of pregnancy. However, this study described one case and there is a 4-year time period from time of AG exposure to the time of the nephrotoxicity. A recent study by Locksmith et al<sup>16</sup> showed that a high single dose of gentamicin given to mothers with chorioamnionitis imposes no additional risk of renal injury in neonates when compared to conventional dosing. Nevertheless, the later study was underpowered and allowed for only 2 days follow-up of neonates after birth, a time that is not long enough to confirm nephrotoxicity. Most studies have investigated the effect of early *in utero* exposure to AGs in animals and data about the effect of late gestational exposure to AGs on human fetal kidney are insufficient. Yet, the effect of AG exposure during late gestation or the intrapartum can be estimated from neonatal studies which have demonstrated evidence of

structural and persistent functional damage of neonatal kidney after AGs treatment.<sup>17</sup> In fact, approximately 50% of cases of drug-induced hospital-acquired renal failure in neonates are related to the use of AGs.<sup>17</sup> Tubular damage can occur in more than 50% of neonates and glomerular damage in fewer than 10%, despite adequate therapeutic dose monitoring.<sup>17</sup> These structural and functional consequences may be particularly important in *preterm infants* who have immature kidneys which continue to develop afterbirth. Given their high nephrotoxic potential, targeted strategies to minimize renal AG accumulation are therefore needed. In order for these strategies to be developed, the molecular mechanisms involved in the **placental** transport of AGs must be characterized. The next section of this chapter will provide an overview of the structure of human placenta and its relation to the transport function in general. Then, the transport of aminoglycosides will be discussed in detail.

## II. HUMAN PLACENTA: STRUCTURE AND ITS RELATION TO THE TRANSPORT FUNCTION

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Human placenta is the main link between the mother and fetus and provides the appropriate environment for fetal development and maturation.<sup>18,19</sup> To achieve this function, the placenta mediates the exchange of oxygen and carbon dioxide between mother and fetus, the transfer of essential nutrients such as amino acids, fatty acids, glucose, vitamins and minerals, and the disposal of the metabolic fetal waste. The transfer function of the placenta is facilitated by and related to its unique structure. The placenta is a discoid organ that is fetal in origin and composed of both fetal and maternal tissue.<sup>20</sup> The maternal portion of placenta is formed by decidua basalis which lines the pregnant uterus

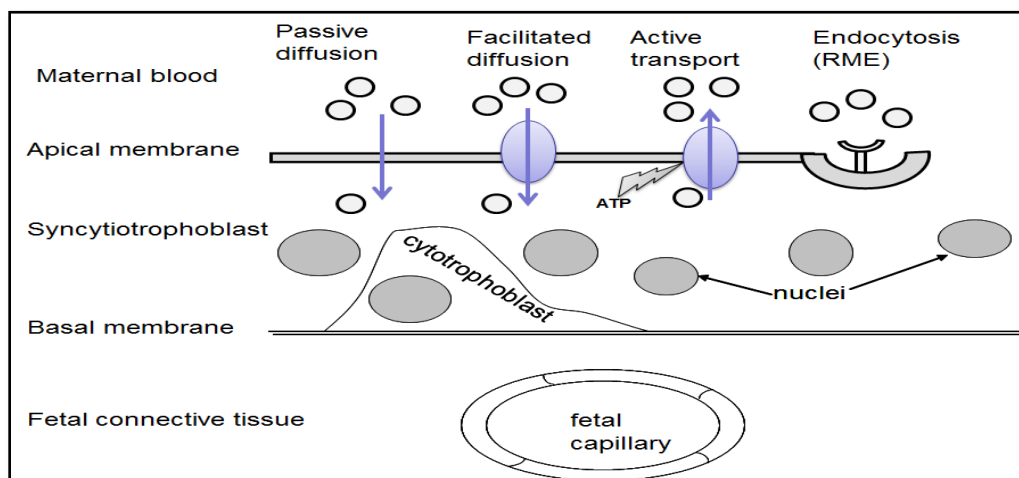
and covers the fetal villous tissue. The decidua basalis forms the deciduas septa which divides the placenta into several cotyledons, each representing an independent functional vascular unit of placenta.<sup>20</sup> Placental cotyledons contain villous trees, in which maternal and fetal circulations are separated by a placental barrier. This barrier consists of the trophoblast epithelium, inside of which are fetal capillaries. Trophoblasts include villous stroma, the cytotrophoblasts which fuse together to form the multi-nucleated syncytiotrophoblast.<sup>2</sup> The plasma membrane of syncytiotrophoblasts is polarized, consisting of the brush-border membrane, which is in direct contact with maternal blood, and the basal membrane that faces the fetal circulation (Figure 1-3). These two membranes exhibit distinctive protein composition having various enzymes, hormone receptors and transporters differentially localized between brush-border enzymes and the basal membrane.<sup>18</sup>

There are many drugs in therapeutic use that are intended to treat the mother and/or fetus. It is therefore important to understand the mechanisms involved in the transport of drugs across the placenta so that possible toxicity for the fetus can be avoided (in the case of maternally-directed treatment) and drug efficacy can be maximized (in the case of fetal pharmacotherapy). In addition, several essential nutrients need to cross the placenta and reach the fetal circulation to promote fetal growth and development. Depending on the substance properties, transport of drugs and endogenous substances across the placenta can be mediated by various mechanisms (Figure 1-3). While passive simple diffusion is believed to be the most common transport mechanism,<sup>18, 20</sup> the role of other transporter/receptor-mediated transport has become increasingly evident. Examples

include\_ but are not limited to: facilitated diffusion, active transport,<sup>18, 20</sup> and receptor-mediated endocytosis.<sup>21, 22, 23</sup>

AGs are assumed to undergo placental uptake by both passive diffusion and receptor-mediated transport processes. These assumptions are based on the information from the previous maternal fetal pharmacokinetic studies (section I) which suggest that there may be differences in the rate and/or extent of AG transfer between the 3 physiologic compartments (maternal blood to placenta > placenta to fetal blood) and hence that both receptor-mediated (placental uptake at the maternal side) and passive diffusion (membrane transfer at the fetal side) mechanisms may be involved. Given that these assessments were made under non steady-state conditions and most likely prior to attainment of equilibrium between the maternal and fetal compartments, these data cannot be used to draw definitive conclusions regarding the mechanistic basis of AG placental transport. However, they do provide some preliminary evidence that AG transfer may be a two-stage process involving multiple transport mechanisms. In renal proximal tubule cells for example, many mechanisms have been proposed to be involved in the cellular AG uptake: receptor-mediated endocytosis, penetration through cationic transporters and mast-cell derived granule-mediated internalization.<sup>24</sup> Similar mechanisms could be taking place in placenta where receptor-mediated endocytosis could contribute to the placental uptake of AG at the maternal side in combination with other pathway/s such as organic cationic transporters-mediated transport or passive diffusion. Moreover, another related mechanism/s should be involved in the intracellular trafficking of AGs to the basolateral side of the syncytiotrophoblasts in order for the drug to reach the fetal circulation. Section

III focuses on the receptor-mediated endocytosis as a mechanism of substrate uptake by human placenta.



**FIGURE 1-3: SCHEMATIC REPRESENTATION OF MATERNAL-FETAL INTERFACE AND THE VARIOUS TRANSPORT MECHANISMS (RME: receptor-mediated endocytosis)** (Adapted from Moe et al. 1995).

### III. RECEPTOR-MEDIATED ENDOCYTOSIS AS A ROUTE FOR SUBSTANCE UPTAKE BY THE PLACENTA

Endocytosis is a cellular absorption mechanism that serves as a major route for nutrient and macromolecule entry intrinsic to prokaryotic and eukaryotic cells to promote growth, maintenance, and function.<sup>23</sup> The rate of endocytosis-mediated transfer is influenced by membrane fluidity, mobility of the vesicle in the cytosol and, in the case of receptor-mediated endocytosis, the rate of receptor turnover.<sup>25</sup> There are three different subtypes of endocytosis. Fluid-phase endocytosis (pinocytosis) involves the entrapment of a solute present in the extracellular fluid in a plasma membrane invagination. The second type which is phagocytosis involves the engulfment and destruction of extracellular



material and is associated with innate and adaptive immunity in mammals. The third type of endocytosis is receptor-mediated endocytosis (RME). RME involves the selective internalization of specific extracellular ligands such as nutrients, hormones, antigens and other macromolecules into cytoplasmic vesicles through their interaction with a specific receptor. The ligand-receptor complex is delivered to the early endosome where the pH drops to allow complex dissociation. While the receptor can be recycled back to the membrane via the recycling endosome, the ligand can be further delivered to the late endosome where it is degraded, or can undergo intracellular trafficking to the other side of the cells where it can be released via exocytosis.<sup>26</sup> Exocytosis includes the fusion of the endosomal vesicle to the membrane followed by the release of the vesicles' contents. The process of endocytosis, intracellular trafficking and ligand transport across the opposite membrane of a polarized cell (transcytosis) is beyond the scope of this dissertation. RME frequently depends on clathrin as an accessory coat protein. Clathrin-dependent RME (CME) is known to regulate the absorption and trafficking of a variety of ligands, including transferrin, low-density lipoprotein, and tumor growth factor  $\beta$ .<sup>23</sup>

A wide variety of endogenous and exogenous substances have been shown to cross the placenta by RME. A summary of substrates that have been shown to undergo receptor-mediated endocytosis in different models of human placenta are presented below in Table 1-1.

Many essential nutrients including vitamins and minerals are transferred across the placenta by some type of endocytosis. The uptake of <sup>125</sup>I-Low-density Lipoprotein Cholesterol (<sup>125</sup>I-LDL-C) was shown to undergo RME in primary culture of human trophoblasts.<sup>27</sup> The initial stages of iron transfer to the fetus involve the internalization of

maternal iron-saturated transferrin bound to membrane receptors by RME, which can be inhibited by chloroquine.<sup>28</sup>

**TABLE 1-1: SUBSTANCES TRANSPORTED VIA RME ACROSS HUMAN PLACENTA.**

<b>Model</b>	<b>Substance</b>	<b>Reference</b>
<b>Primary culture of human term trophoblasts</b>	LDL-C <sup>a</sup>	27
<b>Primary culture of human term trophoblasts</b>	Carboxy-fluorescein liposomes	29
<b>Primary culture of human term trophoblasts</b>	Insulin	30
<b>Placenta explants</b>	Bovine Serum Albumin	31
<b>Isolated perfused cotyledon of human term placenta</b>	Iron	28
<b>Isolated perfused cotyledon of human term placenta</b>	Cobalt	28
<b>Human trophoblast placenta BeWo cells</b>	Riboflavin	23
<b>Human trophoblast placenta BeWo cells</b>	Folic acid	32
<b>Human trophoblast placenta BeWo cells</b>	Immunoglobulin G	33
<b>Human trophoblast JAR cells</b>	u-PA <sup>b</sup> and u-PAI <sup>c</sup> complex	34

<sup>a</sup>LDL-C: low-density lipoprotein cholesterol; <sup>b</sup>u-PA: Urokinase Plasminogen Activator, <sup>c</sup>u-PAI: Urokinase Plasminogen Activator Inhibitor.

Similarly, the transferrin-cobalt complex can be internalized by RME and can therefore be incorporated by the iron acquisition pathway.<sup>28</sup> Caveolae coat protein (Caveolin 1) was detected in human placental trophoblast (BeWo) cells co-localized with rhodamine-labeled

vitamin B<sub>2</sub>.<sup>23</sup> Thus, caveolae-mediated endocytosis is a putative mechanism for the uptake of riboflavin (Vitamin B<sub>2</sub>).<sup>35, 36</sup> In BeWo human trophoblast cells, the uptake of <sup>3</sup>H-folic acid was significantly although partially inhibited by unlabeled folic acid, the anion transport inhibitor 4-acetamido-4'-isothiocyano-2,2'-stilbene disulphonic acid (SITS) and the endocytosis inhibitor monensin at pH of 7.5. These uptake characteristics indicate that receptor-mediated endocytosis is involved in the transport of folic acid at the indicated pH in these cells via FR $\alpha$  (folate receptor alpha).<sup>32</sup> The iodinated transcobalamin complex B<sub>12</sub> (<sup>125</sup>I-TC-B<sub>12</sub>) was also efficiently endocytosed in rat yolk sac carcinoma cells. Therefore, RME is assumed to play an important role in the fetal supply of vitamin B<sub>12</sub>.<sup>37</sup>

Proteins and enzymes are also known to undergo transcytosis across the placenta. Endocytosed proteins undergo some degree of degradation or recycling. Only a few, such as immunoglobulins and albumin, are protected and hence make it to the fetal circulation.<sup>22, 31, 33</sup> Ellinger I. *et al.*<sup>33</sup> studied the transport of human immunoglobulin G (IgG) across BeWo choriocarcinoma cells. The cells demonstrated the expression of human neonatal Fc receptor (hFcRn) mRNA and the IgG-binding protein. Low pH (pH 6.0)-dependent IgG binding was confined to the apical but not to the basolateral plasma membrane of polarized grown cells, suggesting apical plasma membrane localization of hFcRn. They showed that IgGs undergo initial internalization by pinocytosis into mildly acidic early endosome that is followed by receptor-mediated transcytosis. The uptake of <sup>125</sup>I- or FITC- labeled BSA (bovine serum albumin) has also been studied in placenta explants. It was postulated that the entry of protein mainly involves the clathrin-dependent endocytic system and to a lesser extent megalin-mediated endocytosis.<sup>31</sup> Moreover, urokinase-plasminogen activator (u-PA) and to a greater extent the urokinase-plasminogen activator-plasminogen activator

inhibitor (uPA-PAI) complex were found to be taken up by endocytosis in human choriocarcinoma JAR cell-line.<sup>38</sup> In addition, the human insulin receptor is found to be predominantly localized in the apical plasma membrane of syncytiotrophoblasts of first-trimester placenta, but is mainly expressed in the fetal endothelium in a term placenta.<sup>30</sup> Some early biochemical studies using primary cell culture of human term placenta suggested receptor-mediated internalization of insulin.<sup>39</sup> However, later studies failed to localize the insulin receptor in coated pits or vesicles by immunohistochemistry.<sup>30</sup>

There are also many drugs used during pregnancy that cross the placenta and reach the fetal circulation. However, little is known about drugs that are transported across placenta via a mechanism involving endocytosis. Liposomes composed of equimolar concentrations of lecithin and cholesterol and containing carboxy-fluorescein were found to undergo endocytosis when their uptake was studied in a culture of human term trophoblasts.<sup>29,40,41</sup> Enzyme replacement therapy exploits the process of IgG transcytosis across the placenta to deliver enzymes to fetuses with lysosomal storage diseases. The maternal IgG is known to be transported across the placenta via neonatal Fc receptor which recognizes the Fc domain of IgG. Therefore, the absent enzyme can be carried to affected fetuses by combining it with the Fc-tag, which provides a way to treat these fetuses prenatally. To date, however, this method has only been used in pregnant mice with mucopolysaccharidosis type VII to deliver Fc-tagged B-Glucuronidase and corresponding human studies have not been performed.<sup>42</sup>

In the last few years, megalin, a 600-kDa protein has been identified as a major endocytic receptor in placenta and the functional activity of megalin in placenta has been sufficiently studied. The next section will concentrate on megalin, its structure, substrate

specificity, the molecular mechanism of megalin-mediated endocytosis, and what is available in the literature about its role in placental uptake of substances, namely, our drugs of interest aminoglycosides.

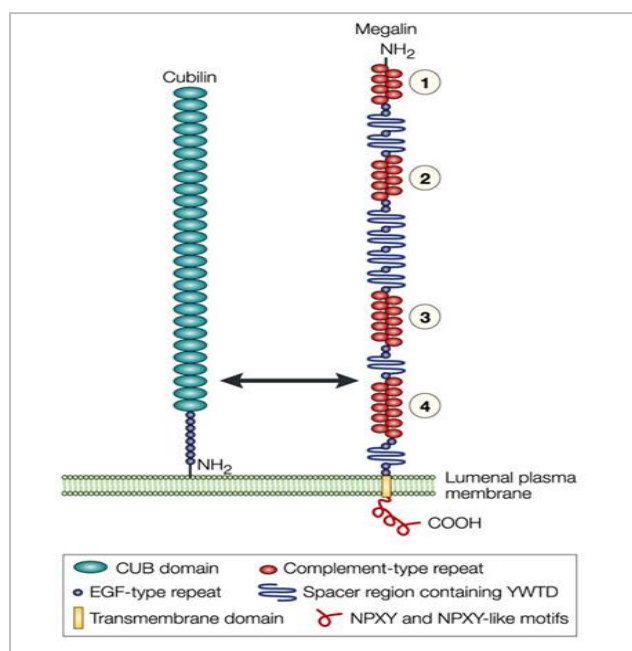
## IV. MEGALIN:

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### STRUCTURE AND DISTRIBUTION

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As previously mentioned, the endocytic receptor megalin has been shown to be involved in the receptor-mediated endocytosis of various substrates and in multiple tissues. Megalin (also called gp 330) is a multi-ligand, ~ 600 kDa, glycoprotein that belongs to the low-density lipoprotein receptor family (Figure 1-4).<sup>43</sup> This receptor has a large extracellular domain consisting of 4398 amino acids, which enables it to bind *a wide variety of substrates*. Examples of clinically important megalin substrates and inhibitors are presented in Table 1-2. The extracellular domain is made up of three types of repeats that are common to the LDL receptor family (Figure 1-4).<sup>43</sup> First, it contains 36 cysteine-rich complement-type repeats organized in four clusters which constitute the ligand-binding region. These repeats have homology to sequences with the complement component. Complement-type repeats are separated by the second type of repeats which are 16 cysteine-rich growth factor repeats followed by epidermal growth factor-like repeat which contains YWTD motifs. The latter are involved in the pH-dependent release of ligands. A single transmembrane domain is attached to this extracellular domain. The intracellular domain is followed by a short carboxyl cytoplasmic tail.<sup>44,45</sup>



**FIGURE 1-4: STRUCTURE OF MEGALIN AND CUBILIN.**

(EGF: Epidermal Growth Factor). Numbers from 1 to 4 represent the four cysteine-rich clusters of low-density lipoprotein-receptor type a repeats which constitute the ligand-binding regions. (Figure was reused with permission from Christensen E. and Brin H., 2002).

Megalin co-localized with a 400 kDa glycoprotein, cubilin, is expressed on the apical side of many epithelia including the small intestine, strial marginal cells of inner ear cochlea, renal proximal tubule, visceral yolk sac, the placental cytotrophoblasts<sup>43</sup> and syncytiotrophoblasts.<sup>46</sup> On the other hand, megalin was found to be expressed without cubilin in choroid plexus, ependymal cells, epididymis, oviduct, thyroid cells, type II pneumocytes, the parathyroid hormone secreting cells of the parathyroid gland, the endometrium, the ciliary epithelium of the eye, and embryonic tissues such as the trophoectodermic cells.<sup>43</sup>

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## MOLECULAR MECHANISMS OF TRANSPORT

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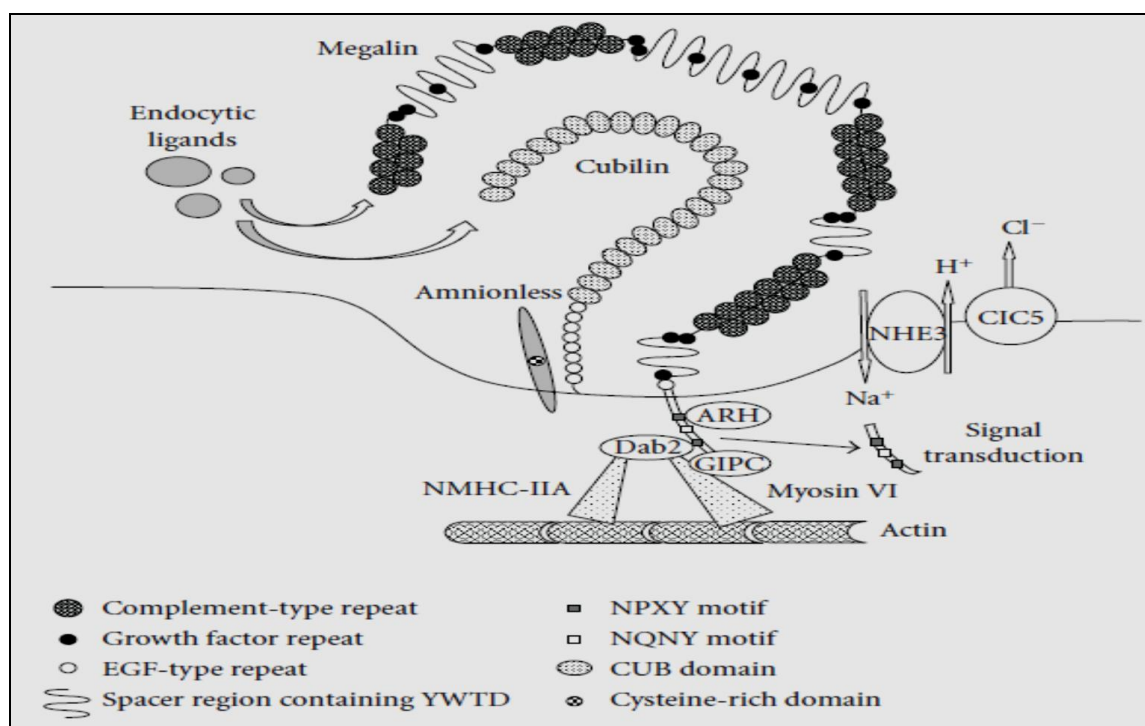
Most megalin substrates are cationic, thereby promoting their electrostatic interaction with the negatively charged binding sites of megalin. For some substrates, such as the AG antibiotic gentamicin, the first point of their attachment is the membrane acidic phospholipids.<sup>44, 47</sup> Other substrates such as albumin and transferrin, bind to cubilin first

and are then transferred to megalin for subsequent internalization.<sup>44, 47</sup> Thereafter, substrates are internalized into endosomes, and then to lysosomes through the process of endosome-lysosome fusion where substrates will be exposed to acidic conditions which are required for the receptor-ligand dissociation. Some receptors are recycled back to the plasma membrane to be re-used and the ligand is available for the use by the cell or for subsequent transfer to the other side (basolateral) of the epithelial cells.<sup>48</sup>

On the apical membrane of *renal tissues*, various molecules are involved in the process of receptor-mediated endocytosis (Figure 1-5). Megalin, playing a central role in the process, cooperates with other membrane proteins such as the cubilin-amnionless complex (CUBAM), Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 3 (NHE3), and the chloride channel isoform 5 (ClC5). Megalin and CUBAM directly bind a variety of ligands, whereas NHE3 and ClC5 are involved in endosomal acidification. Megalin also interacts with intracellular adaptor proteins such as autosomal recessive hypercholesterolemia protein (ARH), Disabled 2 (Dab2), and GAIP Interacting Protein (GIPC). Dab2 binds to motor proteins, myosin VI, and nonmuscle myosin heavy chain IIA (NMHC IIA), which may mediate endocytic trafficking of the molecular complexes through actin filaments.<sup>45</sup>

In addition, megalin is thought to be involved in signal transduction where ligand binding to megalin promotes, through protein kinase C, fragmentation of the intracellular COOH terminus of megalin. The resulting fragment is released into the cytosol then is translocated to the nucleus where it regulates gene transcription.<sup>49</sup> A recent study by Zheng B. *et al.*<sup>50</sup> illustrated the role of a group of proteins (PGS-PX1/nexin 13 or SNX13) in the regulation of endocytic trafficking in mouse visceral yolk sac endodermal cells. This study showed that in wild-type murine visceral yolk sac endoderm cells, megalin is mainly

seen immediately beneath the apical plasma membrane where it co-distributes with the coat proteins ARH and clathrin. In *SNX13* knockout mice, both megalin and ARH have a much broader distribution near the apical side of cytoplasm, suggesting redistribution of megalin and ARH which escorts megalin to and through the apical tubular endosomal system (Figure 1-5).<sup>45</sup>



**FIGURE 1-5:** MEGALIN AND ITS ASSOCIATED MOLECULES INVOLVED IN RECEPTOR-MEDIATED ENDOCYTOSIS IN PROXIMAL TUBULAR CELLS (Reused with permission from Saito et al. 2010).



**TABLE 1-2: LIST OF CLINICALLY IMPORTANT MEGALIN SUBSTRATES AND INHIBITORS****Substrates**

<b>Substrate</b>	<b>Reference</b>
transcobalamin-vitamin B <sub>12</sub> complex	37
vitamin D binding protein	46
retinol binding protein	51, 52
folate binding protein	53
liver-type fatty acid-binding protein	54
sex hormone binding globulin	55
parathyroid hormone	56
Ca <sup>+2</sup>	57
u-PA-PAI-I <sup>a</sup>	58
advanced glycation end products	59
alpha1-microglobulin	60
beta 2-microglobulin	51
immunoglobulin light chain	61
albumin	51, 62
transthyretin	63
insulin	64
prolactin	64
epidermal growth factor	64
myoglobin	65
hemoglobin	66
thyroglobulin	67, 68
trichosanthin	69
angiotensin II	70

bone morphogenic protein-1	71
coagulation factor III	72
lysozyme	51
cubilin	47, 73
gentamicin	63

### **Inhibitors**

Inhibitor	Inhibition mechanism(s)	Reference
RAP	Not fully elucidated With $\alpha 2$ -microglobulin, RAP is thought to down-regulate the receptor binding activity in the endoplasmic reticulum/Golgi compartments	74
EDTA <sup>c</sup>	Calcium ion chelation	75, 76
aprotinin	Competes with u-PAI at the complement type repeats	76
gentamicin	Competes with u-PAI at the complement type repeats	76
amikacin	Competes with u-PAI at the complement type repeats	76
polymyxin B	Competes with u-PAI at the complement type repeats	76
statins	Blocking the post-translational modification of GTP-binding proteins that are necessary for the correct function of megalin	77
cytochrome C	Competitive inhibitor for albumin	64
cytochrome C	Competitive inhibitor with gentamicin	78
lysosyme	Competitive inhibitor	64
lactoferrin	Inhibitor of the cholesterol synthesis induced by megalin but mechanism is unclear	79
cadmium	Reduced expression of megalin and CIC-5	80

<sup>a</sup>u-PAI-PAI: *Urokinase*-plasminogen activator-plasminogen activator inhibitor; <sup>b</sup>RAP: Receptor associated protein; <sup>c</sup>EDTA: ethylenediaminetetraacetic acid.

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## MEGALIN EXPRESSION IN HUMAN PLACENTA AND PLACENTAL MODELS

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Megalin has been purified from human placenta by affinity chromatography<sup>76, 81</sup> and immunoprecipitated by anti-megalin antibodies.<sup>82</sup> In addition, a receptor with a molecular weight of more than 200 kDa has been purified from human term placenta and identified by autoradiograph as the receptor-transcobolamin-vitamin B12 complex of the soluble fraction of placental membrane.<sup>81</sup> Although at that time megalin had not yet been identified, the addition of 10 mM of EDTA, a known megalin inhibitor, induced dissociation in the receptor-ligand complex, and the binding of TC-B<sub>12</sub> to megalin was blocked. Later studies have confirmed that megalin is involved in the trans-epithelial transport of TC-B<sub>12</sub>.<sup>37</sup> It is therefore expected that the receptor is most likely to be megalin.

The aforementioned studies only point to the expression of megalin in placenta as a whole tissue but they provide no information regarding the specific localization of the receptor at the cellular level. Most of the evidence regarding megalin expression in cytotrophoblasts, is available from immunohistochemical studies of human placental tissue obtained at term. The immunohistologic localization of megalin in human placental tissue displayed an intense staining by E11 (specific anti-megalin antibody), which was confined only to the cytotrophoblasts. Although some intracellular staining was present, the most intense staining was seen on the surface.<sup>82</sup> Moreover, immunohistochemical studies of placental explants<sup>31</sup> indicated that megalin was located selectively at the apical membrane of the syncytiotrophoblast, but not in the cytoplasm or the vascular endothelium. Larsson and coworkers<sup>83</sup> showed the localization of megalin in human placental cytotrophoblasts by immunohistochemistry.<sup>83</sup> In situ hybridization experiments showed that the antisense probes (pCAS-2) signaled the expression of human megalin mRNA in the

cytotrophoblasts.<sup>82</sup> In all studies, the expression of megalin was only qualitatively identified for example, by western blotting, but never quantitatively. Only one study detected megalin in human term placenta but no quantification was performed.<sup>38</sup> In addition, megalin expression in the placenta of earlier gestational ages, i.e. *preterm placenta* has never been assessed.

In human choriocarcinoma BeWo cells, there is no available evidence in the literature about megalin expression. However, our laboratory has demonstrated expression of LRP2 (megalin) mRNA in BeWo cells.<sup>84</sup> Furthermore, immunocytochemistry and immunofluorescence of the JEG-3 human choriocarcinoma cell line showed that megalin was expressed in the intracellular space and on the cell surface. Immunoprecipitation of JEG-3 proteins with E11 and G11 antibodies (produced by immunization of mice with human parathyroid cells) resulted in detection of a protein band at about 515 kDa. The identity of this protein was not revealed but it showed  $\text{Ca}^{+2}$  sensor activities typical of megalin.<sup>85</sup> Northern blot analysis of JEG-3 cells lysate showed that the proportion of cells expressing megalin mRNA increased as a function of the time that cells spend in culture and that the expression was up-regulated with vitamin D and retinoic acid in these cells.<sup>86</sup> One aim of this dissertation is to quantify megalin expression in human preterm and term placenta or cytotrophoblasts in addition to verify/quantify megalin expression in the other placental models such as BeWo cells.

## V. MEGALIN AND TRANSPLACENTAL AMINOGLYCOSIDE TRANSPORT – EXTRAPOLATION FROM THE KIDNEY

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Megalin is extensively expressed in proximal tubular cells of the kidney, the physiologic site of AG-induced renal injury.<sup>23</sup> Megalin is believed to play a vital role in the renal reabsorption of many polybasic drugs including the aminoglycosides (AGs).<sup>45</sup> The direct *in vivo* evidence supporting the role of renal megalin in gentamicin uptake mostly comes from pharmacokinetic studies that were done before megalin was fully characterized.<sup>87,88,89,90</sup> However, there is ample evidence from which we can indirectly conclude that megalin is involved in renal uptake of gentamicin. This indirect evidence can be based on gentamicin's ability to inhibit the renal accumulation<sup>91</sup> or to increase urinary excretion of other megalin substrates in experimental animals.<sup>92</sup> This was also confirmed by assessing the effect of megalin genetic ablation, or megalin blockade on gentamicin renal accumulation or excretion.<sup>93,94</sup> Urokinase-plasminogen activator (u-PA) and to a greater extent urokinase-plasminogen activator-plasminogen activator Inhibitor (uPA-PAI) complex were found to be taken up by endocytosis in human choriocarcinoma JAR cell-line. Being a competitive inhibitor of AG transport in kidney models, u-PA transport in JAR cells suggests a role of megalin in the transplacental transport of AGs. The role of megalin in the placental transport of AGs has not been directly investigated to date. However, maternal-fetal pharmacokinetic studies (section I) with gentamicin do provide some insight into AG transplacental transport. In renal proximal tubular cells, many mechanisms along with megalin-mediated endocytosis have been proposed to contribute to the cellular uptake of AGs.<sup>24</sup> Cubilin, purified from inner ear, was found to bind 6 AGs with affinities that are comparable to those of megalin. This finding suggests the role of cubilin as a potential

receptor/transporter for AGs.<sup>73</sup> However, the role of cubilin in AG transport has never been studied in any model. Similar mechanisms could be taking place in placenta where receptor-mediated endocytosis could contribute to the placental uptake of AG at the maternal side in combination with at least another transport mechanism.

## VI. ONTOGENY OF MEGALIN EXPRESSION AND FUNCTION IN PLACENTAL TISSUE

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As previously mentioned (Section V.3), the effect of gestational age on the mRNA or protein expression of megalin in human placenta has never been studied. Placental megalin-mediated endocytosis is assumed to have a vital physiologic role in the internalization of various nutrients, vitamins and minerals, which is necessary for embryonic development and normal growth. Megalin is thought to be involved in the receptor-mediated endocytosis of high density lipoprotein cholesterol (HDL-C) and the vitamin B<sub>12</sub>-transcobalamin complex across rat yolk sac.<sup>28,29</sup> In addition, it contributes to the placental uptake of albumin across human placental explants.<sup>30</sup>

Considering the function of placenta as a whole organ along with the megalin role per se, makes it reasonable to expect that megalin expression and function will change by gestational age. The rate of fetal growth and organ development will vary during gestation. The extent to which fetuses need and utilize nutrients, therefore, differs according to their gestational age. Human placenta itself undergoes structural changes to accommodate these continuously changing fetal requirements. For example, as pregnancy proceeds, the STB becomes the predominant layer, whereas the CTB becomes discontinuous. Consequently, we can assume that the expression and function of placental proteins including megalin will be

changed *as a function of gestational age* to harmonize with the concomitant changes in placental barrier function. The direction of the change can be predicted from the physiologic-relevance of gestational age-dependent expression of placental megalin in terms of placental function and fetal development. It is *hypothesized* that megalin will have higher extent of expression and functional activity in preterm placentas as compared to term placentas. This assumption is based on the fact that preterm fetuses are actively developing and thus have higher nutritional requirements. However, we cannot draw any definite conclusions at this time.

*One of the specific aims of this dissertation is to compare megalin expression in term placenta and preterm placentas.* This knowledge is crucial because it will have significant implications for our understanding of the effect of developmental maturation on the body's ability to handle AGs. Renal megalin mediates the renal uptake and the accumulation of aminoglycosides which is directly related to the increased risk of AGs-induced nephrotoxicity.<sup>76</sup> Although megalin was also found to be expressed in term placenta,<sup>81</sup> the involvement of megalin in the placental uptake of aminoglycosides has not been studied yet. If we assume that placental megalin contributes to the maternal-fetal transfer of aminoglycosides, the hypothesized greater levels of megalin expression and function in preterm fetuses will result in a higher rate and/or extent of AG transport across human placenta, meaning that more of the antibiotic will reach fetal serum and thus be available at the site of AG-induced toxicity (fetal renal tissue) ultimately exposing the fetuses to higher risk of AG-induced nephrotoxicity at earlier gestational age. However, the ontogeny of megalin expression in fetal kidney and the presence of other risk factors for fetal renal injury will dictate the overall risk of nephrotoxicity. Preterm neonates with immature

developing kidney are expected to be more prone to AG-induced nephrotoxicity. Greater attention, therefore, should be paid to the level of expression and function of megalin in preterm placentas and renal tissues. These antibiotics were found to induce nephrotoxicity in fetal rats and hence the pregnancy risk category of AGs is C. Information about age-dependent changes of megalin function and expression in placenta and kidney can lead to implementing different therapeutic approaches to treat pregnancy-related infections according to gestational age.

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## VII. CONCLUSION

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Intra-amniotic infections have serious consequences for both the mother and the developing fetus. Aminoglycosides are part of a standard therapeutic regimen used in the treatment of these infections. AGs can readily cross the placenta and achieve bactericidal levels in the fetal serum. Unfortunately, the clinical utility of AGs is limited due their ability to accumulate in fetal kidney and cause a concentration-dependent nephrotoxicity. Therefore, the placental uptake of these antibiotics merits further investigation.

Human placenta represents a vital transport organ between the mother and the developing fetus. Many transport mechanisms play a role in nutrient delivery, drug transport, and gas exchange across the placenta. These mechanisms include passive and facilitated diffusion, active transport and endocytosis. Megalin, a 600 kDa, is an endocytic receptor protein that is expressed in the apical epithelia of several tissues. Megalin is extensively expressed in renal proximal tubules and plays a role in the receptor-mediated endocytosis of aminoglycosides. Being expressed in placenta, megalin may be similarly



involved in placental transport of AGs. However, the role of megalin in the placental transport of these antibiotics is unknown.

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## IX. SIGNIFICANCE

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Pharmacologic blockade of the megalin receptor has been shown to limit renal accumulation of AGs and prevent nephrotoxicity in animal models. This strategy may be useful in preventing renal drug accumulation in fetal kidney during intrapartum AG administration but only if placental transport remains unaltered by megalin receptor blockade. Therefore, understanding the mechanisms responsible for the placental transport of AGs will help us develop strategies to limit AG accumulation in fetal renal tissues without comprising placental transfer, thus potentially protecting the kidney from injury occurring as a result of exposure *in utero*.

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## X. HYPOTHESES AND SPECIFIC AIMS

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The *overall objective* of this dissertation project is to characterize the molecular mechanisms responsible for the placental transport of AGs. We hypothesize that (1) the expression of the megalin receptor differs in human term and preterm placentas; (2) placental uptake and transfer of AGs occurs via megalin-mediated endocytosis. These hypotheses are to be addressed through the following **specific aims**:

1. Characterize and compare megalin expression in human term and preterm placenta.
2. Assess the functional activity of megalin in *in vitro* human placental models.

## CHAPTER 2 : EXPRESSION OF MEGALIN IN HUMAN PLACENTAL TISSUE

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### I. PRELIMINARY PROTEIN EXPRESSION STUDIES

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#### INTRODUCTION

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The first specific aim of this dissertation is to characterize and compare megalin expression in human term and preterm placental tissues. As described in chapter 1, previous data showed that the megalin protein is expressed in various placental models. Megalin protein is qualitatively detected by immunohistochemistry and ligand immunoblotting in human placental cytotrophoblasts, syncytiotrophoblasts as well human placental tissues. In this project, we first attempted to detect the protein by Western blotting.

Western blotting (immunoblotting) is an analytical method used to detect specific proteins in a homogenate of tissues or cell extracts. It depends on separation of proteins by gel electrophoresis according to their size. The separated proteins are then transferred electrically to a membrane (usually PVDF or nitrocellulose). Finally, the target protein will be detected by appropriately matched primary antibodies. Once detected, the target protein will be visualized on a blotting membrane by chromogenic, chemiluminescence, radioactive or fluorescence imaging system. In this project, we used fluorescent secondary

antibodies which bind to the primary antibody, and visualize the fluorescent signal with Odyssey® Infrared Imaging System. This system is capable of yielding a linear range of quantification of 500-fold or greater.<sup>95</sup>

The western blotting is characterized by overall simplicity when compared to other immunological techniques. It also provides information that cannot be obtained by these techniques. Since proteins are separated by size then detected by a “specific” antibody, the western blotting provides means not only to confirm the identity of a target protein, but also to quantify the protein relative to a normalizing control (e.g.  $\beta$ -actin and tubulin). In addition, even if data do not match the expectations, this urges a deeper look into possible explanations. For example, when bands are seen at smaller size than expected, it could indicate protein degradation. If, however, bands observed at higher molecular weight, this can indicate glycosylation or multimer formation. Successful western blotting depends on the specificity of interaction between the antibody and the antigenic region of the protein (epitope). For the experiments in this section, we have tried to polyclonal antibodies from Santa Cruz®. One is a rabbit antibody raised against the C-terminus of human megalin (H-245), and the other is an affinity purified goat antibody that is raised against a peptide mapping near the C-terminus of megalin of human origin. The use of polyclonal antibody will enable us to identify the entire target proteins via binding to multiple sites which lead to protein detection with higher degree of sensitivity, although specificity may be somewhat compromised.

A literature review was performed to identify various Western blotting experimental conditions that have been used to successfully detect the megalin protein in

various tissue types. A stepwise approach of method optimization was then initiated. Table 2-1 describes the experimental conditions previously reported in the literature. The first specific aim of my dissertation project is to characterize and compare megalin expression in human term and preterm placental tissues. As described in chapter 1, previous data showed that the megalin protein is expressed in various placental models. Megalin protein is qualitatively detected by immunohistochemistry and ligand immunoblotting in human placental cytotrophoblasts, syncytiotrophoblasts as well human placental tissues. In our project, we first attempted to detect the protein by western blotting.

**TABLE 2-1: SUMMARY OF WESTERN BLOTTING EXPERIMENTAL CONDITIONS USED TO DETECT MEGALIN**

<b>Tissue</b>	<b>Conditions</b>	<b>Loaded protein</b>	<b>Gel%</b>	<b>Membrane</b>	<b>1°Antibody (Dilution)</b>	<b>Observed MW (kDa)</b>	<b>Ref</b>
<b>Rat renal cortex</b>	?	?	6	PVDF	Anti-rat megalin rabbit anti-serum (1:5000)	unspecified	<sup>96</sup>
<b>Megalin purified from rat kidney BBM</b>	Non-reducing	10 µg	4-16	NC	Anti-rat megalin rabbit antiserum (1 :4, 000)	>218	<sup>66</sup>
<b>Rat kidney BBM</b>	Reducing	?	6	PVDF	Anti-rat megalin rabbit antiserum (1 :4000)	unspecified	<sup>97</sup>
<b>Rat kidney (cortex, medulla and papilla), lung, brain and liver</b>	Reducing and non-reducing	20 µg	6	PVDF	Anti-rat megalin rabbit antiserum (1:5,000)	>212	<sup>92</sup>
<b>Kidney membrane proteins</b>	?	50 µg	?	NC	Polyclonal rabbit IgG 612 (5 µg/ml) directed against purified rat megalin	>200	<sup>98</sup>
<b>HK-2</b>	Reducing	20 µg	4-15	PVDF/NC	Polyclonal goat (1:1,000)	unspecified	<sup>99</sup>
<b>OKcells membranes</b>	Non-reducing		4-16	NC	Sheep anti-rat megalin	600	<sup>100</sup>
<b>Membranous inner ear tissue</b>	?	?	4-16	PVDF	Polyclonal sheep anti-human megalin (1:20,000)	>200	<sup>73</sup>

<b>Mouse embryonal teratocarcinoma F9 cells</b>	?	20 µg	4-12	PVDF	?	>200	101
<b>Mice renal membrane extract</b>	?	1 µg	?	?	?	unspecified	93
<b>Urine lyophilizates</b>	Non-reducing	?	5%	PVDF	Sheep anti-rat megalin (1:20,000)	600	102
<b>L2 cells, BN16, cell lines 293, LLC-PK-1, and MEF (purified protein)</b>	Non-reducing	20 µg	4-15	?	Goat anti-megalin	>250	103
<b>Lysates of mouse spinal cord and brain, megalin-enriched kidney fraction or megalin-free liver fraction, and lysates from LLC-PK1 cells</b>	?	?	4, 12	NC	Anti-megalin MM6 antiserum	>250	104
<b>Isolated BBM of Caco-2 cells</b>	Non-reducing	75-100 µg	5	PVDF	Antiserum to rat renal megalin (1 : 5000)	>200	105
<b>Human breast cancer cells (T-47D)</b>	Reducing	60 µg	?	NC	Polyclonal rabbit anti-megalin antibody	>220	106
<b>Endometrial stromal cells, CHO cells, mouse F9 carcinoma, human myocardium, and 3<sup>rd</sup> trimester placenta</b>	?	250 µg	?	NC	Polyclonal rabbit anti-megalin antibody	>200	38

BBM: brush border membrane, BN16: rat choriocarcinoma cells, Cell lines 293: human embryonic kidney cells, CHO: Chinese hamster ovary, HK-2: human kidney cells type 2, L2: rat yolk sac cells, LLC-PK1: Lewis lung carcinoma porcine kidney cells, MEF: murine embryonic fibroblasts, NC: nitrocellulose, OK: opossum kidney, PVDF: polyvinylidene fluoride, MW: molecular weight. The question mark (?) in any column indicates that the corresponding parameter was not reported.

There are several limitations to these previously reported studies that must be considered. While all of these studies showed blots with bands that claimed to be megalin, most of these blots lacked a molecular weight marker.<sup>96, 66, 92, 99, 93</sup> The absence of the marker makes it difficult to identify the apparent molecular weight of the band of interest, that would have otherwise been done by comparing the relative migration of the megalin band to one with a predefined molecular weight. Moreover, some papers showed megalin bands that were located at a molecular weight that is greater than 200 kDa with no further test of the band's identity. Also, the bands' intensity was not normalized to a control or reference protein (ex.  $\beta$ -actin).<sup>96, 107</sup> Given the limitations of the current literature, we sought to develop a western blotting protocol for megalin protein detection based on optimization of methods reported in previous literature using two different *in vitro models* of human placenta (placental tissue fragments and placental villous tissue).

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## METHODS

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Nine frozen term and preterm placental samples were kindly provided by Dr. Scott W. Walsh via Ms. Sonya Washington (School of Medicine/ Virginia Commonwealth University Medical Center, Richmond, VA). A homogenate of human trophoblast cells (the BeWo cells) was also available. Samples were thawed on ice and homogenized in 1:10 Phosphate Buffer Saline (PBS) containing a cOmplete® protease inhibitor cocktail tablet (one tablet per 25 ml of buffer) (Roche Applied Science, Indianapolis, IN). Polytron PT 10-35 homogenizer with a PTA 10 TS generator (Kinematica, Lucerne, Switzerland; speed setting 6.5) was used to homogenize samples for about 1 minute on ice. Protein concentrations were determined by a modification of Lowry's protein assay using bovine

serum albumin as a standard. Approximately 30-80 µg of total protein were loaded onto polyacrylamide gel (Novex<sup>®</sup>, Invitrogen, Carlsbad, CA) and were subsequently separated by electrophoresis at 120 V for about 45 minutes (Table 2-2 illustrates experimental conditions and type of samples). Following separation, the protein was transferred onto a nitrocellulose or PVDF membrane on ice for 3 hours at 70 V using the Bio-Rad<sup>®</sup> Mini Trans-Blot<sup>®</sup> electrophoretic transfer cell. Membranes were blocked overnight at 4° C using the Odyssey<sup>®</sup> Blocking Buffer (Li-Cor Biosciences, Lincoln, NE). Binding of the primary antibodies (rabbit or goat anti-human megalin, 1:200; Santa Cruz Biotechnology; mouse anti-human β-actin, 1:2000, Sigma Aldrich) and secondary antibodies (goat anti-mouse Alexa Fluor 680; goat anti-rabbit IR Dye 800; Li-Cor) were performed in the Odyssey<sup>®</sup> blocking buffer at room temperature for 1 hour in the dark. The resulting fluorescent complexes were detected and the band intensities visually compared to β-actin using the Odyssey Infrared Imaging System (Li-Cor). Rat kidney tissue was processed similarly and used as a positive control.

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## RESULTS

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Initial immunoblot analyses were performed on placental tissue samples (4 preterm and 5 term samples) and the human choriocarcinoma BeWo cells. Rat kidney tissue was used as a positive control. We could detect a specific 130kDa band in all samples. Representative western blots obtained using the various experimental conditions are presented in Figures 2-1 to 2-3. The experimental conditions used to generate each image are detailed in Table 2-2.

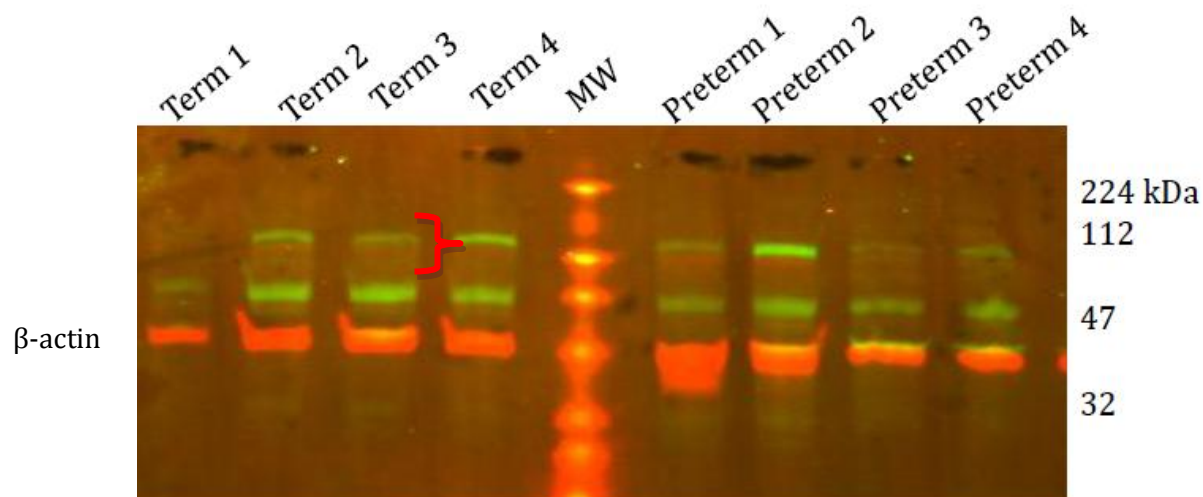


The molecular weight of megalin is ~600 kDa, so we would expect a band to appear at a molecular weight higher than 224 kDa. We were unable to detect the full-size 600 kDa megalin protein in human placental tissues using methods previously published in the literature. However, a distinct band appears at about 130 kDa in placental tissue samples, the BeWo cells and rat kidney. The specific 130 kDa band may represent a megalin fragment. To confirm our conclusion, the bands were sent for amino acid sequencing.

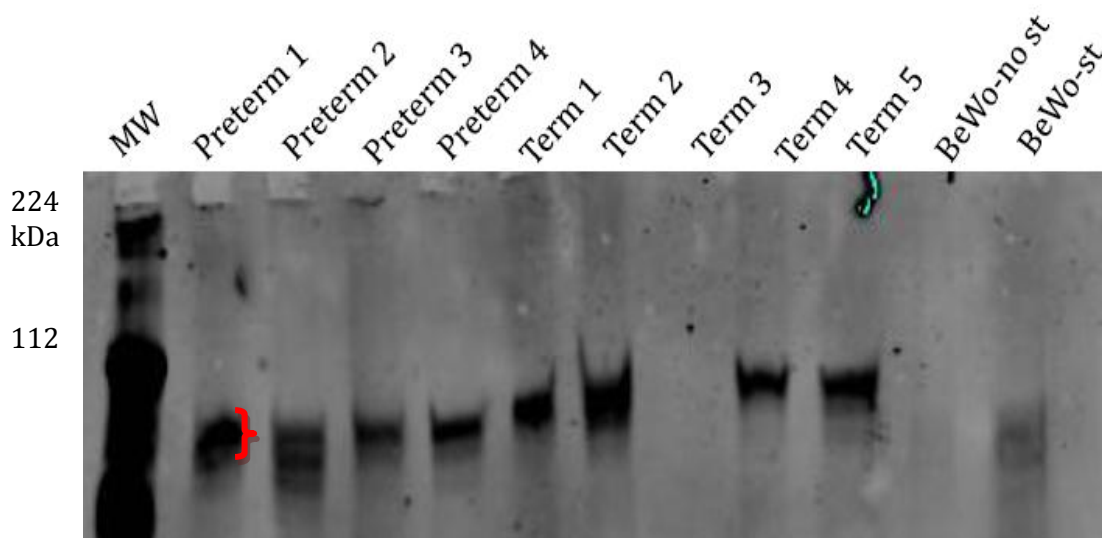
**TABLE 2-2:** EXPERIMENTAL CONDITIONS USED TO DETECT MEGALIN BY WESTERN BLOTTING IN PLACENTAL TISSUES CORRESPONDING TO DIFFERENT IMMUNOBLOTS (2.1, 2.2 AND 2.3).

Figure	Samples	Conditions	Amount protein	of % gel	Membrane	Primary antibody
2-1	4 term and 4 preterm	Reducing	40 µg	4-20%	PVDF <sup>a</sup>	Polyclonal rabbit
2-2	5 term and 4 preterm	Non-reducing	70 µg	8%	NC <sup>b</sup>	Polyclonal rabbit
2-3	5 term and 4 preterm	Reducing	50 µg	8%	NC	Polyclonal goat

(<sup>a</sup>PVDF: Polyvinylidene Fluoride; <sup>b</sup>NC: Nitrocellulose,).



**FIGURE 2-1: IMMUNOBLOT OF 4 TERM AND 4 PRETERM PLACENTAL TISSUES.**



**FIGURE 2-2: IMMUNOBLOT OF 4 PRETERM, 5 TERM PLACENTAL TISSUE, BEWO CELLS WITHOUT SURFACTANT AND BEWO CELLS WITH A SURFACTANT.**

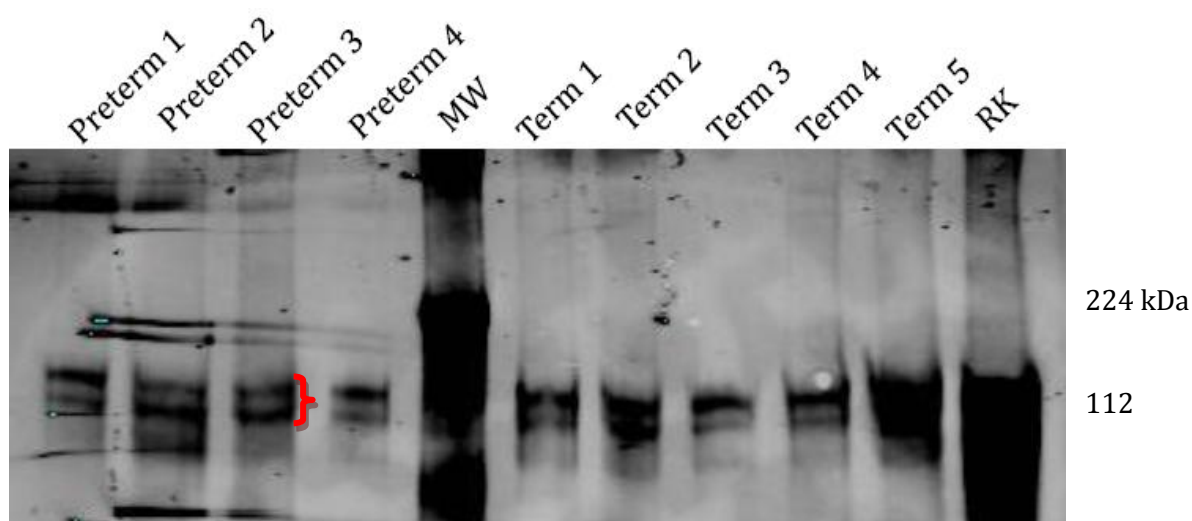


FIGURE 2-3: IMMUNOBLOT OF 4 PRETERM PLACENTAL TISSUES, 5 TERM AND RAT KIDNEY.

### **Silver staining and band identity**

In order to assess bands' identity, the bands of interest were excised from the gel and sent for amino acid sequencing. After protein loading and electrophoresis, polyacrylamide gels were silver-stained. The bands of interest were cut then sent to the Mass spectroscopy laboratory (Department of Chemistry, Virginia Commonwealth University). The silver staining protocol followed was provided by the mass spectroscopy facility due to its compatibility. Briefly, the gel was fixed for 12-18 hours with 50% Methanol, 12% acetic acid, and 0.05% formalin. After washing with 35% ethanol, it was sensitized with 100 mM sodium thiosulfate and potassium ferricyanide then stained with 0.2% silver nitrate. Blots were stored in 1% acetic acid until use. Amino acid sequencing and subsequent detection by mass spectroscopy of the 130-kDa band, revealed 14 different proteins (Table 2-3). Unfortunately, megalin was not among them. Table 3 lists the

identified proteins, the corresponding molecular weight, and the relative quantification of the protein in human placenta and rat kidney. Results from amino acid sequencing could either mean that the megalin protein existed in the sample but in low amounts that were beyond the lower limit of detection of the spectrometer, or that the band did not represent a megalin fragment.

Amino acid sequencing and subsequent detection by mass spectroscopy of the 130-kDa band, revealed 14 different proteins listed by the identified proteins, the corresponding molecular weight, and the relative quantification of the protein in human placenta and rat kidney (Table 2-3). The list did not include megalin (LRP2).

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## DISCUSSION AND CONCLUSION

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Using the western blotting methods previously described in the literature to detect megalin, we were unable to detect the full-size protein. However, we consistently detected a 130 kDa band in placental tissue samples, BeWo cells and the positive control (rat kidney). In addition, the same band was detected using two different primary antibodies directed toward the C-terminus of the protein; polyclonal rabbit and goat anti-megalin antibody.

**TABLE 2-3: MASS SPECTROSCOPY REPORT.**

<b>Identified Proteins</b>	<b>Molecular Weight</b>	<b>Human placenta</b>	<b>Rat Kidney</b>
Serum albumin	69 kDa	23	0
Catalase	60 kDa	9	0
Hemoglobin subunit beta	16 kDa	0	4
Uncharacterized protein	72 kDa	0	4
Acyl-coenzyme A synthetase ACSM2, mitochondrial	64 kDa	5	0
Hemoglobin subunit alpha	15 kDa	0	3
Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	62 kDa	3	0
Glutamate dehydrogenase 1, mitochondrial	61 kDa	2	0
Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	59 kDa	2	0
Transketolase	71 kDa	2	0
Pyridine nucleotide-disulfide oxidoreductase domain-containing protein 2	63 kDa	2	0
Complement C3 (Fragment)	186 kDa	2	0
Liver carboxylesterase 4	62 kDa	3	0
Glucose-6-phosphate isomerase	63 kDa	2	0

There are two possible interpretations of these results. The protein may have been present in the band but in such a low amount that mass spectroscopy could not detect it or, conversely, the band truly did not represent a megalin fragment. Knowing that detection limits of LC-MS is in the femtogram ranges,<sup>108</sup> one would expect that the first option is not likely. However, analytical studies of mass spectroscopy sensitivity showed that the availability of the minimum quantity of an analyte is not enough to obtain an interpretable

mass spectrum.<sup>109</sup> For example, in cases where samples are complex (e.g.: tissues, plasma), the mass spectrometer might not detect the protein/peptide of interest even if the protein of interest exists within the detection limits because the more abundant proteins could be masking the less abundant ones.<sup>109</sup>

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## II. METHOD OPTIMIZATION

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### INTRODUCTION

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Given that we couldn't detect the full-size megalin protein using the western blotting methods described in the literature, we re-examined our experimental conditions in a systematic manner in an attempt to detect the desired band. The following modifications were made to the experimental protocol:

- a) Using freshly collected samples instead of samples stored at -80°C for few years.
- b) Changing the type of the placental model analyzed. Placental pieces represent a more complex structure when compared to the villous tissue which could have introduced much interference during protein detection. The target protein could account of very low percentage relative to the total amount of protein in the preparation. So, in that case we need to load a large amount of total protein in order to have a detectable signal. On the other side, placental villous tissue fragments, prepared from placental pieces, represent the functional unit of placenta consisting of fetal capillaries, cytotrophoblasts and syncytiotrophoblasts. Megalin had been previously located in CTBs and STBs. Therefore, villous tissues provide a purer and

less heterogeneous model where receptor is more likely to be found while loading less amounts of protein.

- c) Using tissue protein extraction buffer instead of PBS for sample homogenization.

t-PER is a commercially available lysis buffer used for the extraction extracellular, or intra-membranous proteins.

- d) Using a lower percent and gradient tris-glycine polyacrylamide gels (4-15%) to separate proteins.

- e) Loading a greater amount of total protein into the gel (100-150 µg).

Shift to the in-gel western methods developed by Li-Cor® where we omitted the transfer step. Large molecular weight proteins are poorly transferred to membranes. So, the reason we are not detecting a huge protein like megalin could be due to its retention on the gel. The in-gel western is similar to the western blotting in the initial steps of sample preparation and protein separation. However, it skips the transfer step so that the proteins are directly fixed on the gel after electrophoresis. Thereafter, the gel is probed with the primary and secondary antibodies and visualized under the Odyssey® Imager.

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## METHODS

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STUDY SUBJECTS AND DATA COLLECTION: Pregnant adult females (18 to 45 years) admitted to Virginia Commonwealth University (VCU) Medical Center for labor and delivery were enrolled. Women delivering at term ( $\geq 36$  weeks) and preterm ( $< 36$  weeks) were included. Subjects were excluded if the following criteria were met: (1) maternal history of diabetes, pre-eclampsia, hypertension or HIV infection; (2) maternal history of smoking or drug and/or alcohol abuse; and/or (3) documented or suspected placental

disorders. The research protocol and informed consent were reviewed and approved by the VCU Institutional Review Board (VCU IRB protocol number is 04212) prior to study initiation. For the collection of placentas from term deliveries, written informed consent and research approval were obtained from all subjects before sample and data collection. Maternal information including race and placental weight were collected. In addition, information in the subjects' medical records regarding the previous and current medical history were reviewed for inclusion/exclusion (but not recorded). Neonatal gestational age was also obtained. With respect to the preterm placental tissues samples, there were some difficulties obtaining the samples using the IRB protocol number 04212 which did not permit the collection of preterm placental tissues. In addition, the standard hospital protocol requires that all preterm placental tissues must be examined by the Department of Pathology before they can be available for research purposes. This meant that samples could stay for several hours in the refrigerator (i.e. at 4°C) before they are available for analysis. This might affect the quality of the sample with regard to megalin protein and/or mRNA expression stability. Moreover, preterm births are not as common as term births, so obtaining a sufficient number of preterm placental tissues was problematic especially since preterm births often occur on an unscheduled basis outside of normal working hours. The latter made the process of subject consenting prior to sample collection challenging. Due to these difficulties regarding the collection of preterm placentas, a second VCU IRB protocol which establishes a placental tissue registry and waives the requirement for informed consent was created and subsequently approved by the IRB (Appendix A). Upon approval of the new IRB protocol (14035), preterm placental samples were collected via Sonya Washington (Department of Gynecology and Obstetrics, School of Medicine, VCU).



MODEL PREPARATION AND WESTERN BLOTTING: Unless otherwise mentioned, placental tissues were collected within 60 minutes of delivery. Placental weight for the term samples was recorded. The tissues were visually inspected for presence of any gross abnormalities. Any tissues with visible infarcts, calcifications, hematomas or other abnormalities were excluded from analysis. Sample collection continued until a total of 15 “evaluable” placentas (n=10 term and n=5 preterm) were obtained. Pieces of placental tissue were snap-frozen in liquid nitrogen and stored at -80°C. Villous tissue fragments were prepared using methods previously described by Dr. Gerk’s laboratory.<sup>110</sup> Tissues were processed on ice and under aseptic conditions. The umbilical cord was cut gently to release any blood first then it was excised. Triangular wedges of tissue (approximately 100 grams) were cut starting at the point of cord insertion and extending out towards the periphery. The basal and chorionic plates were removed and the tissues were rinsed with ice-cold sterile saline and blotted with sterile gauze. While taking time into consideration, efforts were made to remove blood vessels and blood clots. Tissue wedges were then gently cut into small pieces, washed in antibiotic-supplemented Dulbecco’s phosphate buffered saline (DPBS), filtered through gauze and subsequently minced into smaller pieces. After several cycles of mincing and washing, 300-400 mg of the resulting villous tissue was frozen. Frozen placental villous tissue samples were homogenized in 1:10 tissue protein extraction buffer (t-PER; Thermo Scientific Pierce Inc.) which contained 1:100 Halt Protease inhibitor (Thermo Scientific, Rockford, IL) using a Polytron PT 10-35 homogenizer with a PTA 10 TS generator (Kinematica, Lucerne, Switzerland; speed setting 6.5) for 30 seconds to 1 minute on ice. Protein concentrations were determined in tissue supernatants using bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL) using bovine serum albumin

as a standard. Approximately 100-200 µg of membrane protein were loaded onto a 4-12% polyacrylamide BioRad<sup>®</sup> tris-glycine denaturing gels (Biorad, Hercules, CA) separated by electrophoresis at 125 V for about 1 hour and 30 minutes. Two molecular weight standards were used: The HiMark<sup>™</sup> Pre-stained standard (31-460 kDa)(Invitrogen, Carlsbad, CA) and the Bio-Rad<sup>®</sup> Precision plus Protein All Blue standard (10-225kDa). The separated proteins were fixed by 50% isopropyl alcohol and 12% acetic acid for 15 minutes at room temperature. After the fixation, the gel was washed with ultra-pure water. The binding of the primary antibodies (rabbit anti-human megalin, 1:200, Santa Cruz Biotechnology; mouse anti-human  $\beta$ -actin, 1:2000, Sigma Aldrich) and secondary antibodies (goat anti-mouse Alexa Fluor 680; goat anti-rabbit IR Dye 800; Li-Cor) were performed in 5% bovine serum albumin (BSA) at 4°C overnight and at room temperature for 1 hour in the dark; respectively. The resultant fluorescent complexes were detected and the band visualized using the Odyssey Infrared Imaging System (Li-Cor). Rat kidney tissue and mouse liver tissue, which were processed in a similar way, were used as a positive control and negative control; respectively. Mouse liver was kindly provided by Dr. M. Beckman (Department of Pharmacotherapy and Outcomes Science, VCU School of Pharmacy). Moreover, gels with the potential band were silver- stained and sent to the VCU Chemical and Proteomic Mass Spectrometry Core Facility (VCU Department of Chemistry) for amino acid sequencing as described before (see chapter 2 section I).

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## RESULTS

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### SAMPLE CHARACTERISTICS

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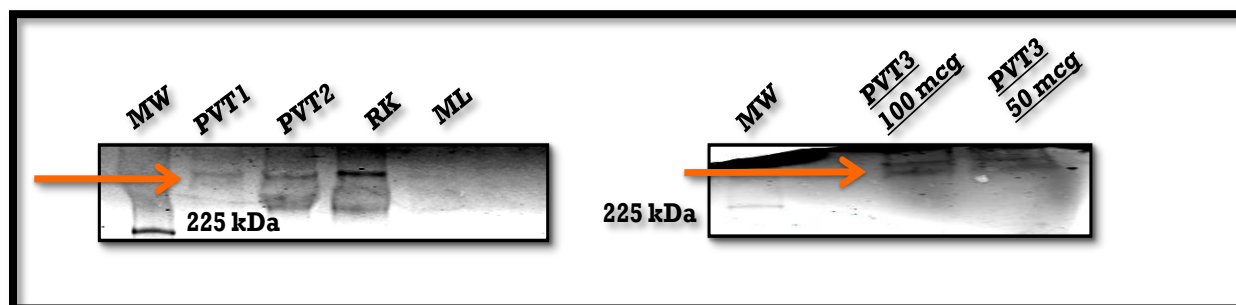
Ten term placentas were collected and processed, unless otherwise mentioned, as described in the methods section. Five preterm placental tissue samples were obtained through the Department of Gynecology and Obstetrics, School of Medicine, VCU). Table 2-4 describes the placental samples illustrating neonatal gestational age, number of subjects, weight of placenta (in grams) and maternal race when available. All samples were free of gross abnormalities. No visible infarcts, calcifications, hematomas or other abnormalities were noticed. Mothers were illness-free and had no history of smoking or alcoholism. Preterm placenta samples (< 35 weeks) were supplied as a ready cut placental villous tissue. The only clinical information available was gestational age. Nevertheless, the inclusion and exclusion criteria were the same for both term and preterm placental tissue samples.

**TABLE 2-4:** SUMMARY OF PLACENTAL SAMPLE CHARACTERISTICS. Samples are described by neonatal gestational age (weeks), weight of placenta (grams) and race. The shaded areas represent unavailable data.

Gestational age (weeks)	N	Weight of placenta in g Mean (SD)	Race	Time from delivery to sample collection
42	3	695.3 (58.58)	White: 1 African American:2	Fresh frozen
39	6	760.2 (87.57)	White:2 Hispanic:2 African American: 1 Middleeastern:1	Fresh frozen
38	1	716.2 (n/a)	African American	Fresh frozen
35	1			1 hour
34	1			5 hours
33	1			3 hours
31	1			7 hours
26	1			5 hours

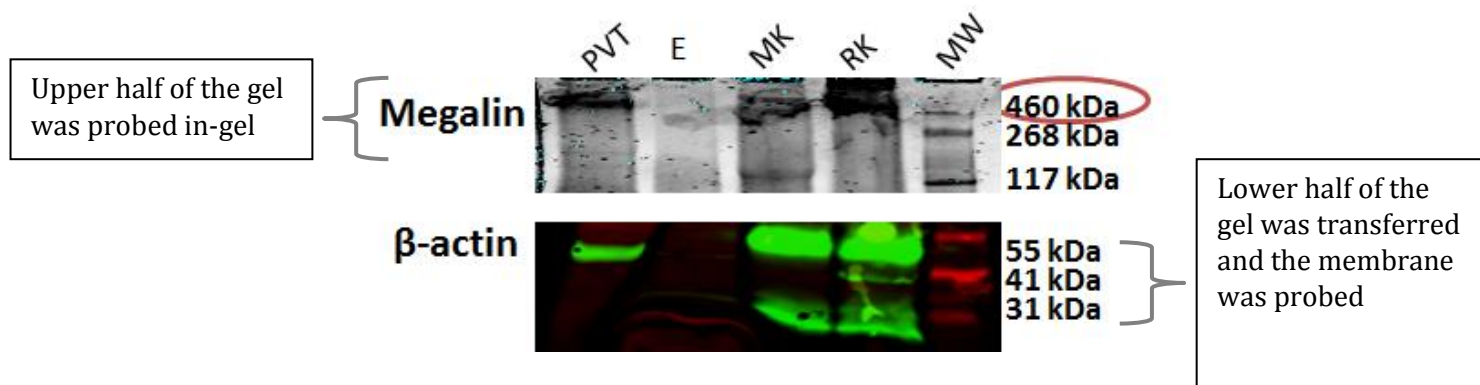
#### WESTERN BLOTTING

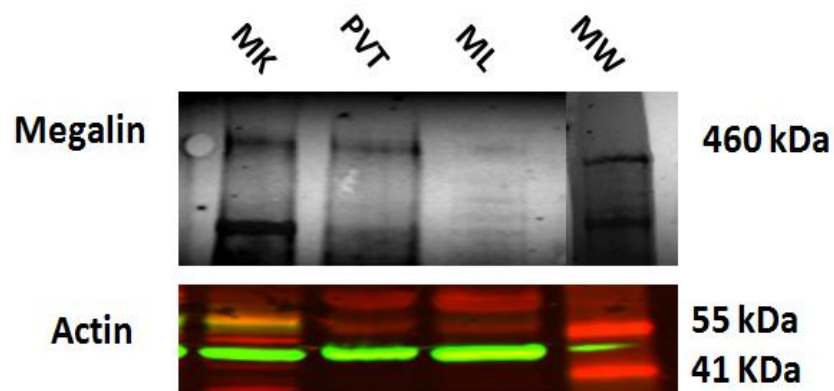
Immunoblots of three term placental villous tissues (gestational age is 42 and 39 weeks for placental villous tissues 1 and 2; respectively) and rat kidney show specific protein bands greater than 225 kDa, whereas the band was absent in the mouse liver (Figure 2-4: the blot on the left side). In addition, halving of the amount of total protein led to decreased intensity of the corresponding band in a third sample of placental villous tissue (gestational age is 39 weeks) (Figure 2-4: the right blot).



**FIGURE 2-4:** IMMUNOBLOTS OF PLACENTAL VILLOUS TISSUES 1, 2 AND 3 (PVT1, PVT2 AND PVT3), RAT KIDNEY (RK) AND MOUSE LIVER (ML).

In order to normalize our band intensities, the gel was probed with the primary anti- $\beta$ -actin antibody. Unfortunately, the double color detection of megalin and actin could not be achieved simultaneously on the gel. Therefore, we cut the gel and into two halves where megalin was detected by the in-gel method while actin was transferred and blotted according the conventional western blotting methods. In these latter experiments the HiMark™ ladder with an upper 460-kDa band was used. Figure 2-5 showed that a specific band appeared at a molecular weight higher than 460 kDa, and parallel band appeared in both mouse and rat kidney tissue samples where megalin is known to be expressed.





**FIGURE 2-5:** IMMUNOBLOTS PLACENTAL VILLOUS TISSUE (PVT), MOUSE KIDNEY (MK), RAT KIDNEY (RK) AND MOUSE LIVER (ML).E: Empty lane.

Mass Spectroscopy was also done to confirm the band's identity and the analysis identified high molecular weight protein (505-kDa protein which is low-density lipoprotein receptor-related protein 1; LRP1). Table 2-5 summarizes the group of the identified proteins with the corresponding molecular weight.

**TABLE 2-5: MASS SPECTROSCOPY REPORT.**

<b>Identified Proteins</b>	<b>Molecular Weight</b>
<b>Isoform 1 of Myosin-9</b>	227 kDa
<b>Isoform 1 of Clathrin heavy chain 1</b>	192 kDa
<b>Prolow-density lipoprotein receptor-related protein 1 (LRP1)</b>	505 kDa
<b>Solute carrier family 2, facilitated glucose transporter member 1</b>	54 kDa
<b>Isoform 1 of Collagen alpha-1(XIV) chain</b>	194 kDa
<b>Keratin, type I cytoskeletal 9</b>	62 kDa
<b>Actin, cytoplasmic 1</b>	42 kDa
<b>Keratin, type II cytoskeletal 1</b>	66 kDa
<b>Complement C3 (Fragment)</b>	187 kDa

## DISCUSSION AND CONCLUSION

Immunoblot analysis showed a band at a molecular weight higher than 225 kDa in 3 term placental villous tissue samples and in rat kidney (positive control). The corresponding band was very weak or absent in mouse liver (negative control). In addition, other blots that used a different molecular weight marker (MW up to 460 kDa), we could see a specific band in a fourth term placental villous tissue sample and in mouse and rat kidney tissues, the band was almost absent in liver. On the contrary, the mass spectroscopy analysis failed to detect megalin. Despite the mass spectroscopy results, that the obtained band most likely represents megalin for the following reasons:

- a. We used an antibody that was developed against the C-terminus of megalin, so it should bind to LRP2 (megalin) and not LRP1. So, it is possible that LRP2 is present

in the band, and that it was just too low for the MS to detect it. When the required protein probability level was decreased to 80%, the spectrometer could actually detect one potential hit for LRP2 (as reported by the mass spectrometry personnel). Dropping the probability level was not recommended, but it might indicate that the expected fragment representing LRP2 protein might be present at extremely low levels.

- b. LRP1 is most abundantly expressed in the liver. Liver tissue showed a very faint band, if any, on the Western blot. LRP2 is most abundantly expressed in kidney which showed the most intense band.
- c. The database used to identify proteins was SEQUEST®. SEQUEST is a proprietary tandem mass spectrometry data analysis program which identifies collections of tandem mass spectra to peptide sequences that have been generated from databases of protein sequences.<sup>111</sup> SEQUEST, like many engines, identifies each tandem mass spectrum individually. The software evaluates protein sequences from a database to compute the list of peptides that could result from each. The peptide's intact mass is known from the mass spectrum, and SEQUEST uses this information to determine the set of candidate peptides sequences that could meaningfully be compared to the spectrum by including only those which are near the mass of the observed peptide ion. For each candidate peptide, SEQUEST projects a theoretical tandem mass spectrum, and SEQUEST compares these theoretical spectra to the observed tandem mass spectrum by the use of cross correlation.<sup>111</sup> The candidate sequence with the best matching theoretical tandem mass spectrum is reported as the best identification for this spectrum. *While very successful in terms of sensitivity,*



*it is quite slow to process data and there **are concerns against specificity, especially if multiple posttranslational modifications(PTMs) are present.***<sup>112</sup> As a very large protein, it is expected that megalin should have several post-translational modifications. In fact, studies have shown that megalin carries a unique oligo/poly alpha2,8 deaminoneuraminic acid as PTM (glycosylation) in normal tissues and carcinoma cells.<sup>113</sup> Therefore, the reason mass spectroscopy could not find megalin is probably because the software was looking for fragments that did not exist (i.e., fragments of the unmodified protein).

- d. In both of the mass spectroscopy reports, (section I and II) a fragment of complement C3 protein was detected. Megalin structure contains 36 cysteine-rich complement-type repeats organized in four clusters which constitute the ligand-binding region. These repeats have homology to sequences with the complement component. It is reasonable to expect that the spectrometer is detecting this homologous fragment of megalin.

It is therefore plausible that the western blotting data support the detection of megalin protein expression in placental villous tissue. In any case, due to the controversy of the protein expression results, we could not solely rely on western blotting data to confirm megalin detection. Even if the band most probably represents megalin, we could not quantify the protein using western blotting. Megalin band is detected on the gel, while the  $\beta$ -actin is visualized on the PVDF membrane after the transfer. So, normalization of megalin band intensities to those of  $\beta$ -actin would be inaccurate. As a result, in order to make quantitative comparisons of megalin expression between term and preterm placental tissues, we decided to quantitate megalin mRNA.

### III. MEGALIN mRNA EXPRESSION

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#### INTRODUCTION

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Protein expression data showed the detection of a band on the immunoblots of placental villous tissue that is most likely megalin. However the data were inconsistent among different blots. In addition, normalization of megalin band intensities to  $\beta$ -actin was difficult to attain and finally, the mass spectroscopy results were inconclusive. Based on all of the above, q-PCR (quantitative polymerase chain reaction) methods were utilized to detect megalin mRNA expression and to study its relation to the gestational age, q-PCR is a method by which we can simultaneously amplify and quantify the **target mRNA** molecule. The key advantage of this technique is that it allows the detection of DNA products as the reaction progresses in a real time manner. In this project, we used a fluorescent reporter probe that only detects the cDNA which has the probe sequence, improving the specificity of detection. For quantification, we are comparing the mRNA expression of megalin to that of 18S ribosomal RNA.

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#### METHODS AND SAMPLE SIZE CALCULATIONS

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Samples of term and preterm placental villous tissue were collected according to the methods described in section II. Since there are currently no data regarding the differential expression of the placental megalin mRNA in preterm and term samples, the number of samples needed to see the effect of ontogeny on megalin mRNA expression was estimated from ontogenic data of mRNA expression of other transporters that are expressed on the apical (maternal) side of trophoblasts including: the multi-drug resistance P-glycoprotein (P-gp), multi-drug resistance related protein 2 (MRP2) and breast cancer related protein

(BCRP). Sample size calculation was performed using nQuery advisor 7.0® ( $\alpha=0.05$ ,  $\beta=0.8$ ). Using these estimates and effect size ( $\Delta$ ) (table 2-6), an average sample size of 6 subjects per group is required to detect significant difference in megalin mRNA expression.

**TABLE 2-6: SAMPLE SIZE CALCULATION**

<b>Transporter</b>	<b>P-gp</b>	<b>P-gp</b>	<b>MRP2</b>	<b>BCRP (ABCG2)</b>
<b><math>\alpha</math></b>	0.05	0.05	0.05	0.05
<b><math>\sigma</math></b>	1.50	0.50	0.44	0.7
<b><math>\beta</math></b>	0.80	0.80	0.80	0.8
<b><math>\Delta</math></b>	6 X (preterm> term)	1.025 (preterm>term)	0.75 (term> preterm)	0.87 (pre>term)
<b>Normalized to</b>	Expression in term	GAPDH	18S rRNA	18S rRNA
<b>n/group</b>	3	5	7	12
<b>Reference</b>	114	115	116	117

A.

#### RNA ISOLATION AND POLYMERASE CHAIN REACTION (q-PCR)

Total RNA was isolated from fresh frozen placental villous tissue (about 100 mg) using the Trizol® reagent (Invitrogen, Carlsbad, CA). Samples were homogenized in 1 ml of Trizol, and then 0.2 ml of chloroform was added to every 1 ml of Trizol®. After centrifugation of samples at 10,000 rpm for 15 minutes, the upper most aqueous layer was separated for total RNA separation. To form the RNA pellet, 0.5 ml of isopropyl alcohol was added to the aqueous layer and centrifuged at 10,000 rpm for 10 minutes. RNA pellets

were then washed with 70% ethanol. Total RNA (2.5 µg) was first digested by DNase I (Promega, Madison, WI) and the total concentration of mRNA was subsequently measured using the Nanodrop® 2000c (Thermo Scientific, Rockford, IL). Purity of the RNA preparation was checked by the 260/280 nm ratio. Samples with ratios between 1.7-2.1 were subjected to reverse transcription in a 20 µl reaction mixture that is composed of reverse transcriptase enzyme (RT), 25 mM MgCL<sub>2</sub>, 5X reaction buffer, PCR nucleotide mix and nuclease-free water added to oligo(dT)<sub>15</sub> and random primers (0.5 µg/reaction) (Goscript® Promega Reverse Transcription System; Madison, WI). RNA in the reverse transcription mixture was denatured at 70° C for 5 min, and then annealed at 25°C for 5 min. First strand synthesis occurred at 42°C for 60 min and finally RT was inactivated 70° C for 15 min. Taqman® Universal PCR master mix and 40X Taqman® human megalin probes rs\_2229263(probe1) and rs\_2225252 were added to the resultant cDNA (Applied biosystems, CA, USA). The probe with higher sensitivity was selected for quantification of gene expression. Megalin mRNA expression was normalized to that of 18S which acts as our reference gene; the latter was measured using Taqman® gene expression assay (Hs99999901\_s1). PCR reactions were performed using Bio-Rad C1000 Thermal Cycler® via the Bio-Rad C1000 Manager software®. Gene assays are validated by the company and PCR efficiency reported was ≥ 90%. The expression of mRNA was expressed as the normalized expression ratio as described by Livak *et al*<sup>118</sup> which assumes similar efficiencies of amplification for both the target and the reference gene. This method uses the number of cycle at which fluorescence signal is detected (Ct) to quantify gene expression. It normalizes the expression of target gene (megalin) to that of a reference gene

(18S) and also to that of a calibrator sample (arbitrary sample that is designated as a control), then the ratio is multiplied by 100% to show percentage:

$$\text{Normalized expression ratio} = 2^{-\Delta\Delta Ct}$$

Where  $\Delta\Delta Ct = \Delta Ct (\text{test sample}) - \Delta Ct (\text{calibrator sample})$

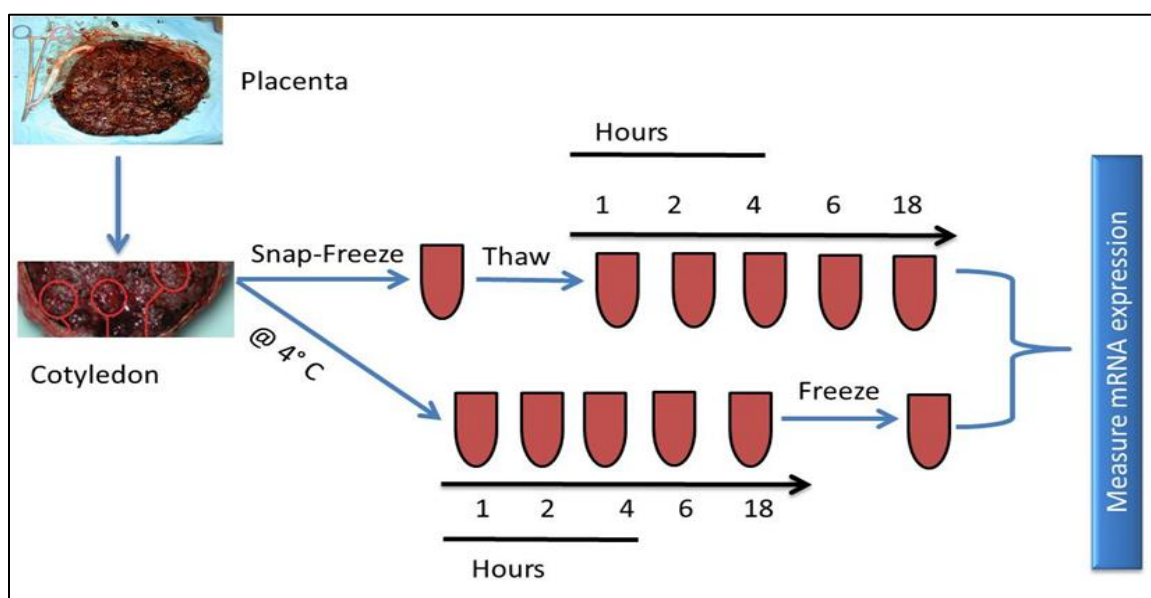
And  $\Delta Ct = Ct (\text{target gene}) - Ct (\text{reference gene})$

## B. STABILITY OF MEGALIN mRNA

**Rationale:** While the collection of term placental tissues was relatively prompt and was performed at an average of one sample/week, obtaining preterm tissue samples was challenging. Standard protocol at VCU Hospital requires that all preterm placentas are evaluated by the Department of Pathology prior to becoming available for research purposes. Because of the delay in sample processing, mRNA stability is therefore a concern. A sample which is freshly collected within a maximum of 60 minutes after delivery (term samples) will not have the same quality or levels of mRNA expression in a sample that has been setting in the refrigerator for 2-48 hours (preterm samples), as permitted by the IRB protocol. These differences may impact conclusions regarding differences in the expression of megalin as the megalin mRNA levels may decline with time in the preterm samples. Moreover, getting an approval for an IRB protocol which waives the requirement for a consent form required several months. Therefore, in the meantime we examined the stability of mRNA in human *term* placenta tissue samples:

In the first study, term tissues were collected from 6 subjects and divided into two 3-subject groups that were stored in two different ways (Figure 2-6 illustrates a schematic diagram of the process), then Megalin mRNA levels were measured in all samples as described in the previous section and compared to a snap-frozen sample from each group:

- a. After processing (cutting and washing), samples were stored at 4°C for 1, 2, 4, 6 and 18 hours then frozen (-80°C) after each of the corresponding time points (3 samples were collected at each time point).
- b. For the second group, the tissue was snap-frozen after processing and then left to thaw in the refrigerator for 1, 2, 4, 6 and 18 hours (3 samples were collected at each time point).



**FIGURE 2-6:** SCHEMATIC DIAGRAM OF THE EXPERIMENTAL SET-UP USED TO ASSESS THE EFFECT OF STORAGE TIME ON MEGALIN mRNA EXPRESSION.

Moreover, we studied the effect of *a priori* processing on megalin mRNA expression. The purpose of this study is to simulate the conditions that the tissue will be exposed to during storage. The placental tissues might be stored intact or in large pieces in the refrigerator before being sent to our laboratory for processing and further analysis.

Ideally, the samples should be processed (cut and washed) as soon as possible after delivery. Therefore, we performed this study to determine the effect of storage conditions on mRNA stability. Term placental tissue samples were collected from 3 subjects. Tissue pieces of each subject were divided in two parts according to two different processing methods (a and b), then Megalin mRNA levels were measured in all samples as described in the previous section and compared to a snap-frozen sample:

- a. Samples were processed using the conventional methods of cutting and washing then they were stored in refrigerator for 1, 2, 4, 6, 10, 24, and 48 hours. After that, samples were frozen after each time point.
- b. For the second group, samples did not go any processing and tissue was left as such for 1, 2, 4, 6, 10, 24, and 48 hours. Thereafter, samples were processed and frozen after each time point.

### C. EFFECT OF GESTATIONAL AGE ON MEGALIN EXPRESSION

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Megalin mRNA expression was measured in 10 term placental samples and 5 preterm placentas that were collected, cut into placental villous tissues and snap-frozen.

## RESULTS

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### A.

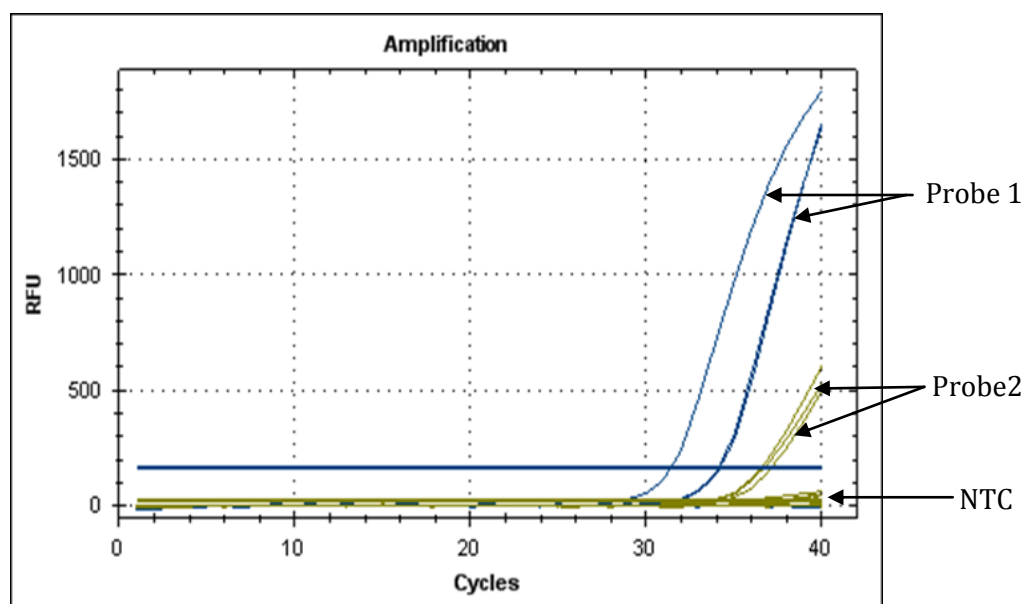
#### MEGALIN mRNA EXPRESSION

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Total mRNA was measured in all samples, and the sample chosen for analysis had 260/280 nm of 1.7-2.1. This ratio indicates the purity of the RNA preparation, a ratio less than that indicates that the sample is contaminated by a substance that absorbs strongly at 280 nm such as protein or phenol. q-PCR analysis showed the megalin is expressed in term and preterm placenta villous tissue samples (Figure 2-7). Probe 1 (rs\_2229263) was chosen for

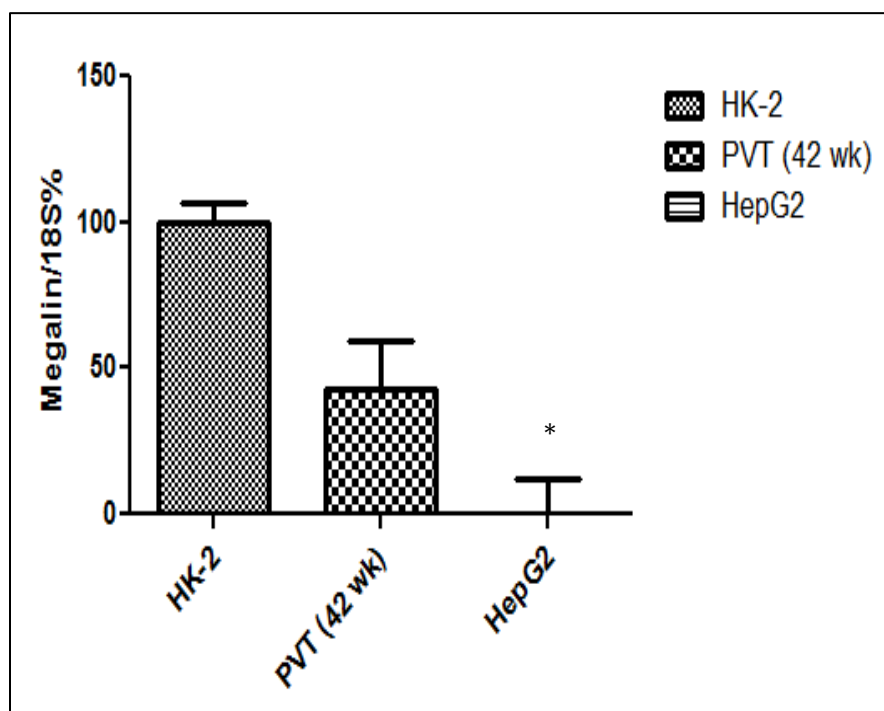
quantification for it has shown higher sensitivity than probe 2. Non template control samples (NTC) showed negligible amplification. NTC samples showed Ct values  $\geq 37$  for the target gene assay. For the 18S assay NTC values were  $\geq 25$  but it was always at least 10 cycles apart from the positive signal. Figure 2-8 compares the expression of megalin mRNA in human placental villous tissue to that of human kidney cells (HK-2) used as a positive control and human hepatocellular carcinoma cells (HepG-2) used as a negative control.

The expression of megalin mRNA in the HK-2 cells was significantly higher than that of placental villous tissue and HepG-2 cells ( $p < 0.05$ ). Megalin mRNA expression was significantly higher in the placental villous tissue than that of HepG-2 cells ( $p < 0.05$ ) (Figure 2-8).



**FIGURE 2-7: MEGALIN mRNA EXPRESSION IN TERM PLACENTAL TISSUE SAMPLES.** The blue lines represent the signal produced by probe 1 (rs\_2229263) and the green lines represent signal from probe 2 (rs\_2225252).

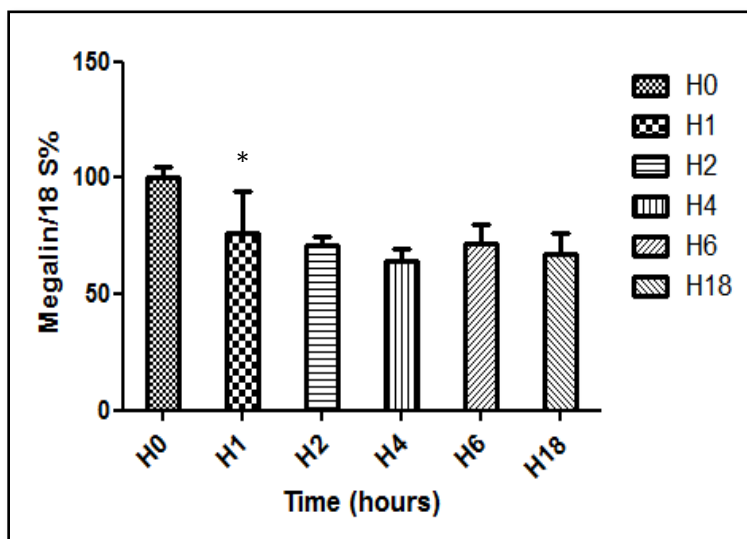




**FIGURE 2-8:** MEGALIN mRNA EXPRESSION IN HUMAN KIDNEY CELLS (HK-2), PLACENTAL VILLOUS TISSUE (PVT) AND HepG-2 CELLS. Expression is expressed as normalized ratio multiplied by 100%. (BeWo cells were used as the calibrator; data represent mean  $\pm$  SD). Data represent mean  $\pm$  SD; \* $p < 0.05$ .

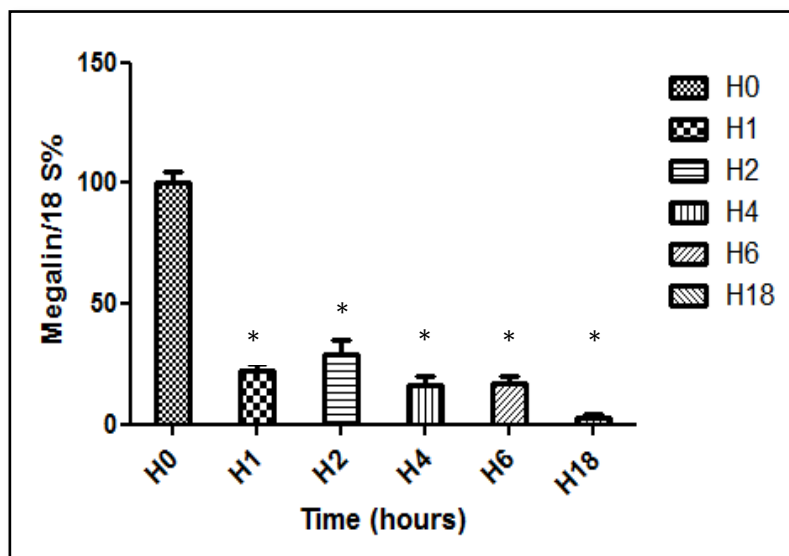
#### B.MEGALIN mRNA STABILITY STUDY

This study was done to examine the effect of the storage time and tissue processing on megalin mRNA expression. Term placental villous tissues from six subjects were stored in the refrigerator (4°C) for up to 18 hours before freezing and then the mRNA expression was compared to that of a snap-frozen sample. One-way ANOVA analysis followed by post-hoc Tukey's test showed that there is a significant difference in relative megalin mRNA expression in snap-frozen samples vs. samples stored at 4°C for 2, 4, 6 and 18 hours prior freezing ( $P < 0.05$ ) (Figure 2-9). Expression in the H0 sample was approximately 1.5-fold (difference ~30%) than that in the other samples. After the first hour there was a slow (if any) decline in the expression and there was no significant difference in mRNA expression among different samples.



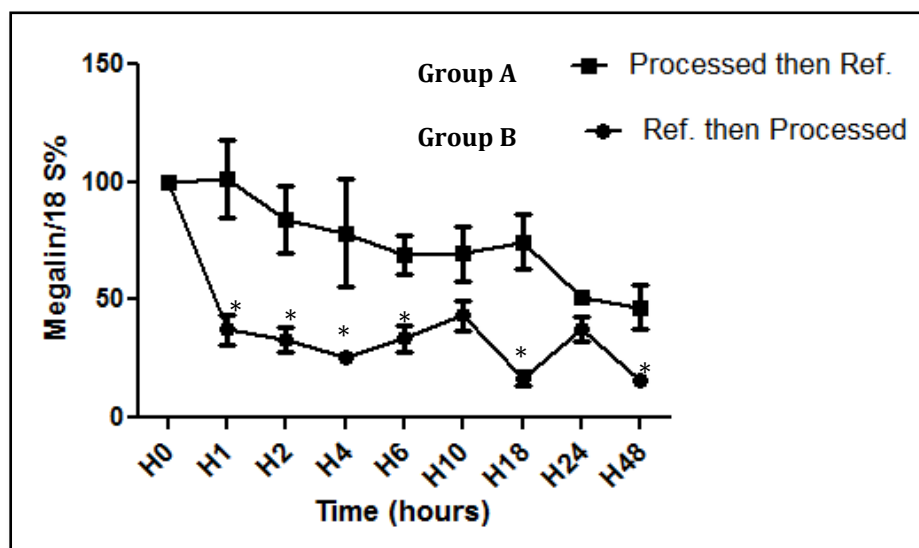
**FIGURE 2-9:** THE EFFECT OF TIME (HOURS) OF STORAGE AT 4°C ON MEGALIN mRNA EXPRESSION. Expression is expressed as normalized ratio multiplied by 100%. (Snap-frozen samples were used as the calibrator; data represent mean  $\pm$  SD; n=3); \*p<0.05.

When samples were all snap-frozen at the same time, and then thawed at 4°C for 1, 2, 4 and 18 hours post freezing, however, the expression was significantly less in samples thawed compared to that than that of snap frozen samples (p<0.05). Post-hoc Tukey's test showed that mRNA expression in the snap-frozen sample was significantly higher than all other samples where the expression in the H0 samples was 4.5, 3.4, 6.25 and 33.3 fold higher than that measured at 1, 2, 4, 6, and 18 hours; respectively (Figure 2-10).



**FIGURE 2-10:** THE EFFECT OF THAWING TIME IN HOURS (AT 4°C) ON MEGALIN mRNA. EXPRESSION. Expression is expressed as normalized ratio (x100%) (H0 represents the snap-frozen sample and was used as the calibrator; data represent mean  $\pm$  SD; n=3); \*p<0.05.

The effect of *a priori* processing of samples on the expression was studied between two groups. First group (Group A) is composed of samples which have been processed first (cut and washed with normal saline and buffer), then left at 4°C before it was frozen at 1, 2, 4, 6, 10, 18, 24 and 48 hours. In the second group (Group B), samples were left unprocessed in refrigerator before being frozen (Figure 2-11). Two-way ANOVA to compare effects of the *priori* processing showed a significant interaction between *priori* processing and mRNA expression; mRNA expression was significantly higher at each time point (1, 2, 4, 6, 10, 18, 24 and 48 hours) in the group A than that of group B (p<0.05)(Figure 2-11). This means that *priori* processing has a favorable effect on mRNA stability. Appendix D shows the cycle threshold values (Ct) for the 18S rRNA.

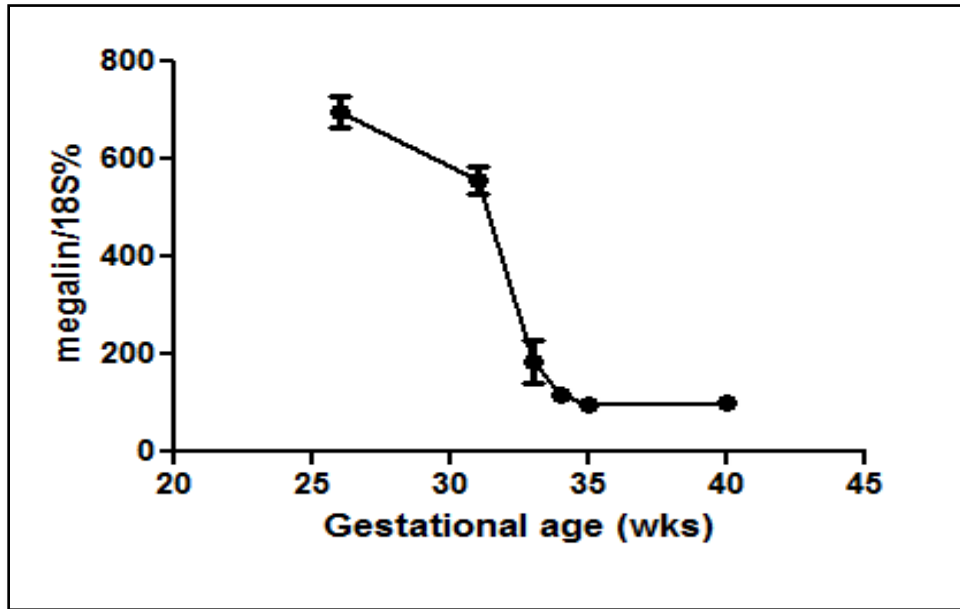


**FIGURE 2-11:** THE EFFECT OF *PRIORI* PROCESSING OF SAMPLES ON MEGALIN mRNA EXPRESSION. Expression is expressed as normalized ratio (x100%). (H0 represents the snap-frozen sample and was used as the calibrator (\*  $p < 0.05$ ;  $n = 3$ ))

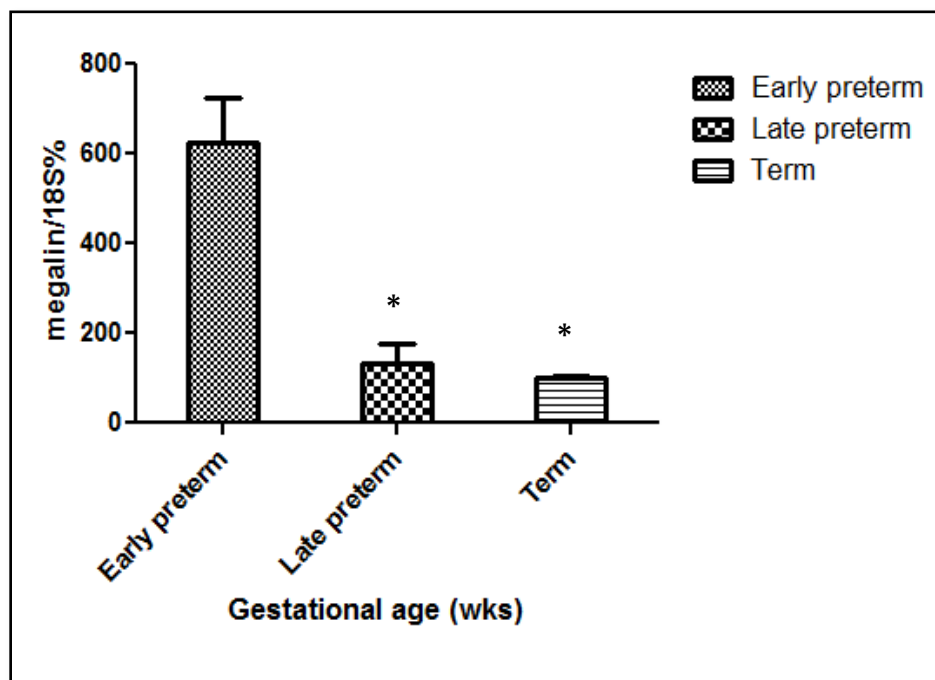
### C. EFFECT OF GESTATIONAL AGE ON MEGALIN mRNA EXPRESSION

Effect of gestational age was studied by comparing the relative megalin mRNA expression (normalized to 18S) in term and preterm placental villous tissues. Pearson correlation test between gestational age (weeks) and expression showed significant correlation between gestational age and megalin mRNA expression ( $r = -0.86$ ;  $P < 0.05$ ) (Figure 2-12). More accurately, there was a biphasic relationship between gestational age and megalin mRNA expression so that there was a steep decrease the expression until 31 weeks then it plateaued thereafter. Furthermore, when preterm samples were divided to clinically relevant groups (early preterm  $< 32$  weeks, moderate preterm: 32 to 35 weeks), one-way ANOVA analysis showed that mRNA expression is significantly greater in early pre-term tissues when compared to those of late preterm and term ( $p < 0.05$ ). Tukey's multiple comparison test showed a mean difference of 495% and 526% in mRNA expression between early pre-term with late preterm and term; respectively Tissues from

early preterm had megalin expression that is about 6-fold higher than that of late preterm and term tissues (Figure 2-13).



**FIGURE 2-12:** THE RELATIONSHIP BETWEEN GESTATIONAL AGE (WEEKS) AND MEGALIN mRNA EXPRESSION. Data represent mean $\pm$ SD for 3 replicates of n=1 sample at each gestational age except 40 weeks. At 40 weeks, data represent the grand mean of all the 10 term samples.



**FIGURE 2-13:** MEGALIN mRNA EXPRESSION IN EARLY PRE-TERM (n=2), LATE PRE-TERM (n=3) AND TERM PLACENTAS (n=10). Data represent mean  $\pm$  SD; \*p<0.05.

## DISCUSSION AND CONCLUSIONS

The expression of megalin mRNA was confirmed by q-PCR methods in both term and preterm placental tissues. The correlation analysis between gestational age and expression was statistically significant. There was a steep decline in megalin mRNA expression until 31 weeks then it plateaued thereafter. Theoretically, and based on the physiologic relevance of megalin function in placenta, we would expect a correlation. Megalin is involved in placental uptake of endogenous substances that are necessary for fetal growth and development such as HDL-C and vitamin B<sub>12</sub>. Therefore, megalin mRNA expression in preterm placentas is expected to be greater than that of term placentas. Furthermore, when the pre-term group was divided into early preterm (<32 weeks) and moderate (32-35 weeks) [according to the World's Health Organization (WHO) definition],

we could see that megalin mRNA expression levels in early preterm are significantly higher than their late preterm and term counterparts. This result matches with the fetal nutritional requirements. The early period of fetal growth is characterized by fast growth and consequently more nutritional needs. Therefore, it would be expected for a receptor like megalin, which contributes to supplying fetuses with vitamins and nutrients, to have higher levels of expression in the early phases of fetal growth (<32 weeks of gestation). Nevertheless, it would be preferable to have more preterm tissue samples so we can compare two groups with equal sample sizes.

Interestingly, data from the mRNA stability study demonstrated that ***processed*** placental tissue samples can be stored for up to 18 hours in the refrigerator before freezing, and that megalin mRNA expression in the stored samples does not differ significantly from fresh-frozen samples. Our data agree with previous studies that have shown an intact total mRNA of Tumor necrosis factor  $\alpha$  and cyclooxygenase 2 over 48 hours in human placental samples stored at 4°C.<sup>119</sup> This interesting phenomenon can be explained by the expression of endogenous RNase inhibitor in human placenta, a protein that inhibits the activity of ribonucleases and consequently protects mRNA from degradation. Human placental RNase inhibitor was purified from placenta by ion-exchange and affinity chromatography in 1977<sup>120</sup> and it was found to abolish both the angiogenic and ribonucleolytic activities of angiogenin toward 18S and 28S rRNAs.<sup>121</sup> Currently, RNase inhibitors that are isolated from human placenta are commercially available as such or in PCR kits to protect RNA against RNases A, B and C (RNasin® plus RNase inhibitor from Promega). Based on the above, we conclude that megalin mRNA is expressed in human placenta. More samples are needed to more accurately assess the effect of gestational age on expression, especially in

the early preterm group. Placental megalin mRNA is interestingly stable when samples are stored at 4°C up to 18 hours. Thawing samples had detrimental effect on megalin mRNA expression. This result is consistent with a previous study that examined the effect of thawing on the mRNA expression of B-cell CLL/lymphoma 2; v-fos FBJ murine osteosarcoma viral oncogene homolog; hypoxia-inducible factor 1 $\alpha$  subunit, proliferating cell nuclear antigen; and transforming growth factor. They found out that RNA degradation can start as early as few minutes after thawing the tissue and then variably decline over 16 hours of thawing.<sup>122</sup> There is no explicit explanation for the effect of thawing on the RNA stability, but it could be due to loss of placental RNase activity after freezing. In addition, the *a priori* processing of samples tend to have a favorable effect on mRNA stability of megalin. It seems that tissue cutting and washing can reduce the tissue RNase activity.

As previously mentioned in this chapter, there are some challenges associated with the collection of samples from placental tissues. Delivery time cannot be predicted accurately, making it difficult to standardize the time from delivery until sample collection. The validity and reproducibility of q-PCR data analysis are strongly correlated with the stability of the isolated mRNA. The finding that megalin mRNA can be stable up to 18 hours provides researchers with flexibility in placental tissue samples collection, particularly in cases where immediate tissue processing and collection is not feasible. Still, researchers need to be careful to avoid over-extrapolating these stability data, because they might be both tissue- and transcript (megalin)-specific and it could not be related solely on the expression of RNase inhibitors. One study found that RNase inhibitors were found to be ubiquitously expressed by northern blotting in 16 human tissues with the lowest expression levels being detected in kidney and brain tissues.<sup>123</sup>



## CHAPTER 3 : THE FUNCTIONAL ACTIVITY OF MEGALIN IN IN VITRO HUMAN PLACENTAL MODELS

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### I. PRELIMINARY STUDIES WITH THE BEWO CELLS

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#### INTRODUCTION

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In chapter 2, we showed by q-PCR techniques that megalin mRNA is expressed in placental villous tissues isolated from term and preterm placentas. However, the fact that megalin is expressed in placenta does not necessarily mean that it is involved in aminoglycoside uptake. Studies therefore need to be conducted to determine whether the megalin protein is involved in the transport of aminoglycoside antibiotics across the placenta. Consequently, the uptake kinetics of gentamicin (an aminoglycoside) were assessed using *in vitro* human placental models.

During gestation, pregnant women may have various illnesses or can be exposed to different types of medications. Whether the illness is one that emerges during pregnancy, or a chronic medical condition, it needs to be treated, so as to protect the mother's health and, in some instances, save the fetus' life. However, many maternally administered medications cross the placental barrier and could be harmful to the developing child. Therefore, understanding maternal-fetal drug transport is crucial. Studying drug administration during pregnancy can be challenging. From an ethical point of view, large scale human clinical trials in pregnant women, if not impossible, are very difficult to be

approved and implemented. There are many concerns regarding the safety of both the mother and her developing fetus, which might be endangered from the exposure to drugs. On the other hand, studies in pregnant animals, although useful, do not accurately reflect human gestation due to species differences. Specifically, the human placenta has a unique hemomonochorial multivillous nature where maternal blood comes into direct contact with the chorion, and a single layer of trophoblast tissue separates the maternal blood from fetal blood capillaries.<sup>124,125</sup> In addition, there are multiple interspecies differences in placental morphology and the gestational time between animals and human beings.<sup>124,125</sup> For these reasons, placental *in vitro* models that are of human origin are more useful models to study drug transport across human placenta.<sup>125</sup> Over the past decade, several models have been developed to study the transport kinetics of both endogenous substances and drugs across the maternal-fetal barrier. The models and their advantages and disadvantages are briefly outlined below.

1. Tissue-derived placental villous explants<sup>125,126</sup>: this model has the advantage of possibly providing samples from different gestational ages.<sup>126</sup> However, tissues are very heterogeneous and extremely sensitive to environmental conditions,<sup>126</sup> including the culture medium used, oxygen concentration and the composition of the test buffer. These conditions can affect the model stability and validity.<sup>127,128</sup>
2. Isolated membrane vesicles<sup>125,126,21</sup>: although useful for studying transport mechanisms, this model lacks various regulatory factors of human placenta which could result in poor *in vitro-in vivo* correlation.<sup>126</sup>
3. Perfused placental cotyledon: If carefully validated, this model is the most successful in terms of the high extent of *in vitro -in vivo* correlation,<sup>125,126,21</sup> but it cannot be

used to investigate drug transport during earlier gestational age and has limited viability.<sup>125,126,21</sup>

4. Primary cultured cytotrophoblast cells (CTBs)<sup>125,126,129</sup>: primary cytotrophoblasts can be isolated at different gestational ages and can syncytialize spontaneously to provide a reliable model.<sup>129</sup> Unfortunately, the isolation processes available so far have high risk of contamination with other cells.<sup>126</sup> In addition, the resulting trophoblasts have limited viability (less than 1 week) and cannot form a monolayer when grown on a semi-permeable membrane, which results in large intercellular spaces..<sup>129</sup>
5. Cell lines (e.g. choriocarcinoma BeWo, JEG-3 and JAR cells lines)<sup>125,126,129</sup>: These cells form a polarized confluent monolayer that represents the apical side of third-trimester trophoblasts making them a good model to study transplacental transport.<sup>129</sup> From a practical point of view, these cells rapidly replicate and are easily sub-cultured.<sup>126,129</sup> Unlike primary CTBs, BeWo cells *cannot* syncytialize spontaneously and may not have all markers in the cells of origin.<sup>129</sup> Still, the syncytialization can be achieved by forskolin. Cellular interaction contributes to the overall transport of substrates across human placenta. However, BeWo cells only represent the trophoblastic cell layer, not the full barrier which is composed of the syncytiotrophoblast cell layer, connective tissue and the fetal endothelium. Another disadvantage that is inherent to cell line studies in general, is the inability to monitor inter-individual differences. The BeWo cell line only represents one individual which does not account for potential genetic differences in placental protein expression between individuals.<sup>125</sup>

The choice of model depends upon the question(s) being investigated. We therefore chose to utilize the Bewo (human choriocarcinoma) cell model to study the transport function of term placenta. We also explored isolated primary cytotrophoblasts as a secondary model to assess placental drug transport, so that we can get an insight of the model appropriateness compared to BeWo cells and to test the ease of isolation and culture.

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## METHODS

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- **BeWo cell culture:**

BeWo cells (b30 clone, obtained from Dr. Kenneth Audus at the University of Kansas) were maintained by serial passages (37-55) in 25 cm<sup>2</sup> and 75cm<sup>2</sup> Corning plastic tissue culture flasks (Corning Inc., Corning, N.Y.). The cells were fed using Dulbecco's modified Eagle medium (DMEM) which is supplemented with 10% fetal bovine serum, 1% L-glutamine, penicillin-streptomycin, and nonessential amino acids and incubated at 37°C in atmosphere of 95% air and 5% CO<sub>2</sub>. When confluent monolayers are formed, which usually occurs at 5-6 day post-seeding, the cells were sub-cultured by detachment with 0.05% trypsin-EDTA.

- **Primary cytotrophoblasts (CTBs):**

Primary cytotrophoblasts were isolated and collected from freshly collected placental villous tissue using the protocol by le Bellego *et al.*<sup>130</sup> Briefly, tissues underwent four consecutive digestions with trypsin Type I (8,500 U/mg), DNase type IV (2000 U/mg) and dispase suspended in calcium and magnesium-free Hank's buffered salt solution (CMF-

HBSS). Samples were centrifuged and the supernatant was laid on a Percoll® gradient (0-70%). CTBs were aspirated from the 35%-45% portion, which most likely has the primary cells of interest. Cells then were seeded at  $4.5 \times 10^6$ /well in a MeshWell® 6-well plate (Total yield=  $50 \times 10^6$  cells).

- **Uptake experiments:**

The BeWo cells were grown on plastic Corning 12-well plates with growth area of 3.8 cm<sup>2</sup> at a seeding density of about  $2.5 \times 10^4$ /well. After 2 days of seeding, when the cells reached about 90-100% confluence, they were used for the functional assessment. Media was first removed and cells washed twice with pre-warmed 1X Dulbecco's phosphate buffered saline (DPBS) (which contains 136.8 mM sodium chloride, 2.7 mM potassium chloride, 0.9 mM calcium chloride, 1.1 mM magnesium chloride, 1.5 mM monobasic potassium phosphate, 15.2 mM dibasic potassium phosphate, 0.33 mM sodium pyruvate and 5.6 mM dextrose). Then the reaction was initiated by adding a pre-warmed incubation solution containing <sup>3</sup>H-gentamicin (specific activity=200 mCi/g; concentration=1 mCi/ml). Cells were incubated at (5, 15, 45 and 90 minutes) at both 37 and 4° C. Buffer samples were withdrawn for analysis of radioactivity with liquid scintillation counting. The reaction was stopped by rinsing the cells twice with "ice-cold" DPBS. To obtain the cellular content of gentamicin, cells were lysed using 1% sodium dodecyl sulfate (SDS) and 0.1 M of sodium hydroxide (NaOH), and the radioactivity of gentamicin in the lysate was measured by liquid scintillation counting. The uptake was normalized to each well. In addition, we studied gentamicin uptake in the presence or absence of two chloride channel inhibitors, which interfere with megalin-endocytic pathway: 4, 4'-diisothiocyanostilbene-

2,2'-disulfonic acid (DIDS, 1mM) or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 100  $\mu$ M). Similar uptake experiments were conducted in primary cytotrophoblasts at 37° C for 5, 15, 45 and 120 minutes with 1mM gentamicin.

As an alternative, the transport of albumin, an endogenous megalin substrate, was also examined via assessment of the time- and temperature-dependence fluorescein-labeled bovine serum albumin uptake. (FITC-BSA, Fluorescein/Protein (F/P) molar ratio of 5-1; 0.15 mg/ml). The cellular content was assessed by determining its fluorescence quantum yield of a known concentration of BSA via fluorescence microplate reader. (See appendix B for details on the methods).

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## DATA ANALYSIS

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The original output from the liquid scintillation counter (LSC) was in disintegrations per minute (DPM) which was converted to Curies (1 Curie=2.22 X 10<sup>12</sup> DPMS). Using the specific activity of the radiolabeled gentamicin, (corrected for the ratio of labeled and unlabeled substrate) and its molecular weight, Curies were converted to moles.

The significance of differences observed between data means for gentamicin uptake as a function of time was assessed via one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Effects of temperature or inhibitors on <sup>3</sup>H-gentamicin were analyzed by *t*-test (two groups) or ANOVA (> 2 groups). All statistical analyses were performed using GraphPad® Prism 5. The level of significance for all analyses was  $\alpha = 0.05$ . Data points in graphs represent mean  $\pm$  SD.

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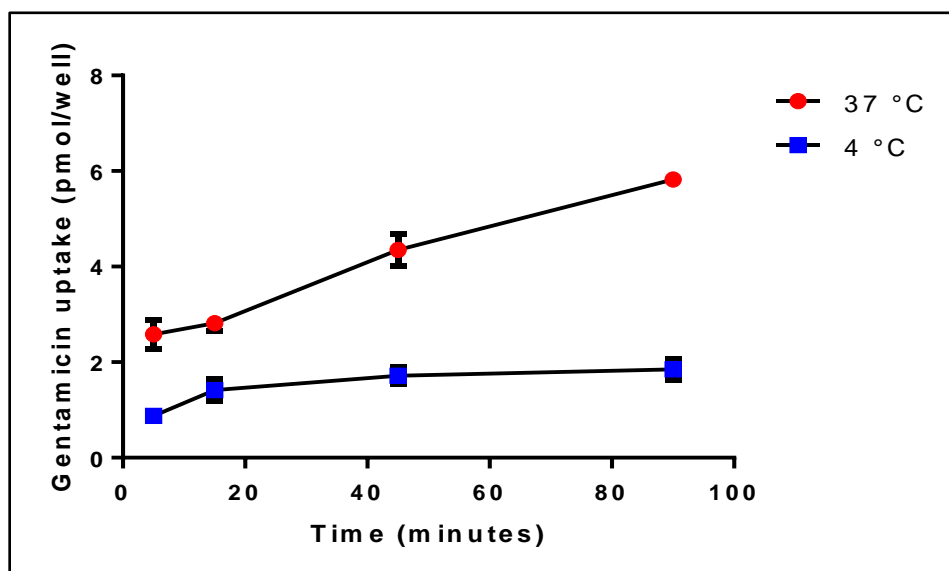
## RESULTS

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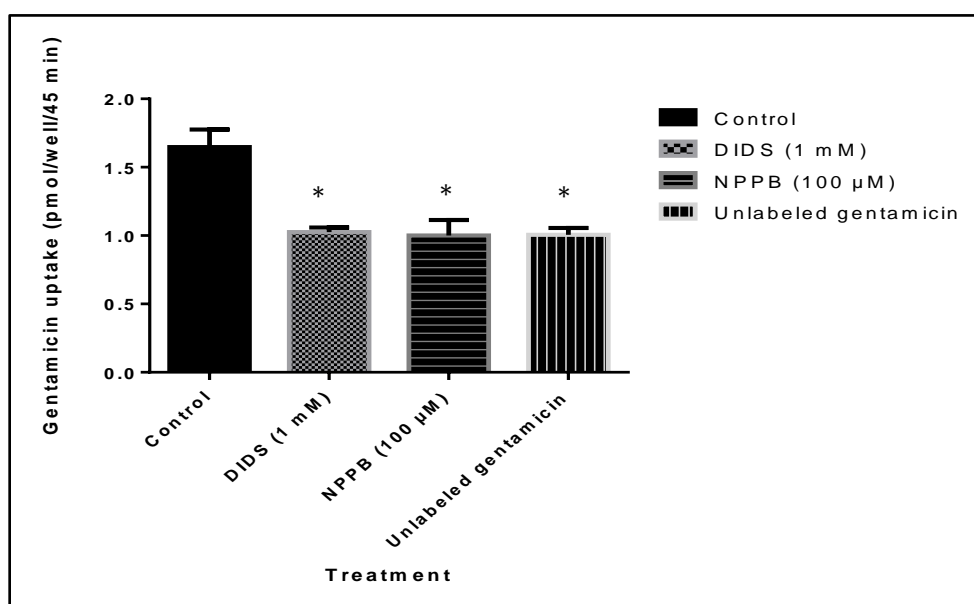
### A. Gentamicin uptake in BeWo cells and CTBs:

In experiments with the human trophoblastic BeWo cell line, cellular gentamicin uptake was time dependent. Mean uptake at 90 minutes was significantly greater than that at 15 minutes ( $5.82 \pm 0.10$  vs.  $2.81 \pm 0.16$  pmol/well, respectively;  $p < 0.05$ ) (Figure 3-1). Gentamicin uptake was significantly ( $p < 0.05$ ) decreased at  $4^{\circ}\text{C}$  vs.  $37^{\circ}\text{C}$  ( $1.72 \pm 0.18$  vs.  $4.35 \pm 0.34$  pmol/45 min/well, respectively) (Figure 3-1). At both temperatures ( $37^{\circ}\text{C}$  and  $4^{\circ}\text{C}$ ), there was a positive Y-intercept (at 2.37 pmol/well). This can be due to non-specific binding of  $^3\text{H}$ - gentamicin to the plastic wells' surface or the cell's surface membrane. To assess the issue of non-specific binding to plastic, we have studied the binding of gentamicin over time in a 12-well plate with no cells. Interestingly, a certain amount of radioactivity could be measured in the lysate samples even in the absence of cells (1.5 pmol/well), which indicates that about 60% of gentamicin measured is due to plastic binding.

Gentamicin uptake was also significantly decreased in the presence of two megalin inhibitors: (DIDS, or NPPB: ( $1.02 \pm 0.03$  or  $1.00 \pm 0.11$  pmol/45 min/well, respectively,  $p < 0.05$ ), and in the presence of unlabeled gentamicin ( $1.01 \pm 0.05$  pmol/45 min/ well,  $p < 0.05$ ) (Figure 3-2). It is noteworthy that DIDS and NPPB cause less than 50% reduction in the apparent uptake of gentamicin compared to control, where we would have expected the uptake to be almost completely inhibited if megalin was the only uptake mechanism involved. This pattern inhibition could be a result of binding artifact.



**FIGURE 3-1:** THE UPTAKE OF  $0.5 \mu\text{M}$   $^3\text{H}$ -GENTAMICIN (pmol/WELL) AT 5, 15, 45 AND 90 MINUTES IN BeWo CELLS. The red dots indicate uptake at 37° C and the blue dots indicate uptake at 4°C. Data represent mean $\pm$  SD.

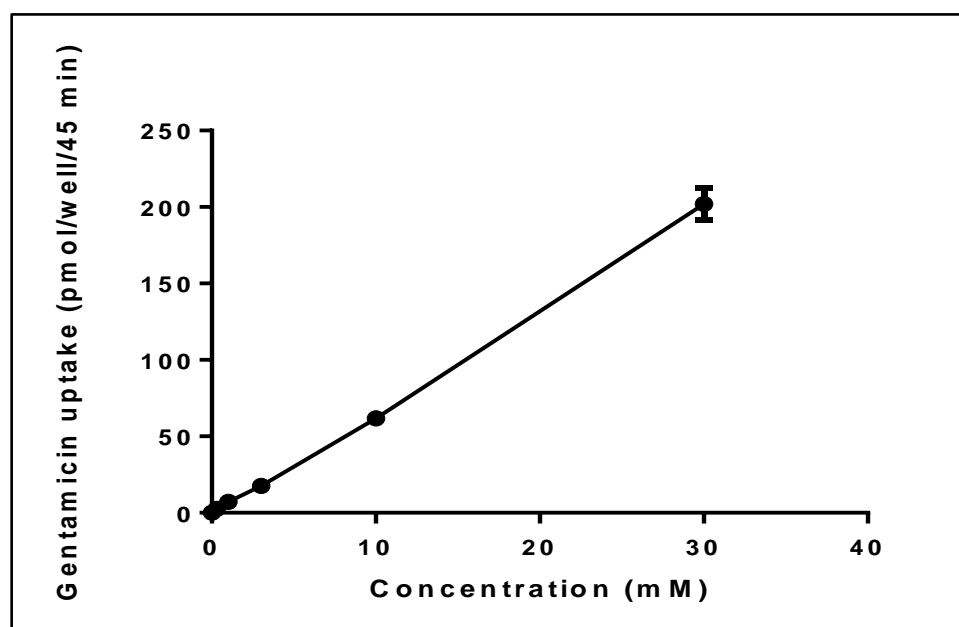


**FIGURE 3-2:** THE EFFECT OF TWO MEGALIN INHIBITORS (DIDS, NPPB) AND UNLABELED GENTAMICIN ON THE UPTAKE OF  $0.20 \mu\text{M}$   $^3\text{H}$ -GENTAMICIN (pmol/45 MIN/WELL) IN BEWO CELLS. Data represent mean  $\pm$  SD; \*  $p < 0.05$ .

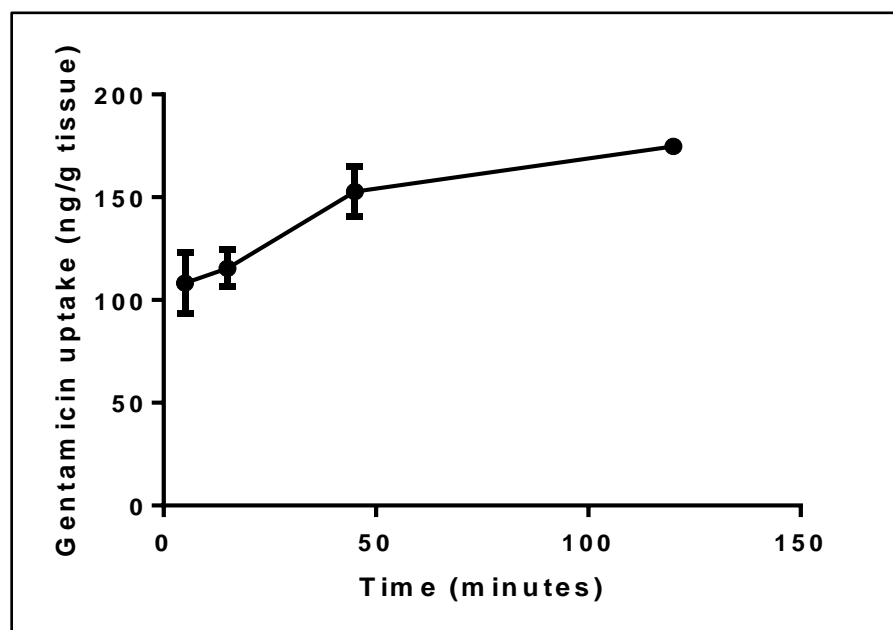


Moreover, the concentration dependency was studied. At a concentration range of 0.01 mM-to 30 mM, the uptake of gentamicin was non-saturable (Figure 3-3), a finding which has been previously reported in other studies using LCC-PK1 cell-line.<sup>131</sup> This suggests either that the process is not receptor-mediated or that another process may be involved in gentamicin uptake.

In primary cytotrophoblasts, gentamicin uptake was time dependent. The mean uptake at 120 minutes was significantly higher than that at 5 minutes ( $175 \pm 2$  vs.  $108 \pm 16$  ng/g tissue, respectively;  $p < 0.05$ ) (Figure 3-4).



**FIGURE 3-3: THE UPTAKE OF  $^3\text{H}$ -GENTAMICIN BY THE BEWO CELLS AT CONCENTRATIONS RANGING FROM 0.01 mM TO 30 mM. Data represent mean  $\pm$  SD; \*  $p < 0.05$ .**



**FIGURE 3-4: THE UPTAKE OF 1 MM GENTAMICIN (ng/g TISSUE) AT 5, 15, 45 AND 120 MINUTES IN PRIMARY CYTOTROPHOBLASTS.** Data represent mean  $\pm$  SD.

#### **B. BSA uptake in the BeWo cells:**

BSA was conjugated with the FITC label according to manufacturer instructions. Unfortunately, the experiments showed uptake values that are barely more than those of no-cells experiments (Appendix B for more details).

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### DISCUSSION AND CONCLUSION

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We expected to see an increase in the uptake of gentamicin with time, and almost complete inhibition at 4°C. Assuming that 4°C is below the *phase transition* temperature for biological membranes, any integral membrane protein mediated-transport process will be inhibited, while simple diffusion may only be slightly decreased.<sup>132</sup> In the concentration study, we expected to observe a saturation profile for the uptake of gentamicin. Since there

is definite number of receptors in the endocytic apparatus, gentamicin accumulation should increase accordingly with the increase the substrate concentration until receptors are saturated at which point the rate of the saturable process plateaus. Moreover, the addition of megalin-mediated endocytosis inhibitors should result in the decrease of gentamicin uptake through the BeWo cells.

*Our preliminary results showed* that the uptake of gentamicin is time- and temperature-dependent, and is partially inhibited by the presence of megalin-mediated endocytosis inhibitors. However, the uptake was non-saturable and with low efficiency ( $1 \times 10^{-6}$  of initial substrate concentration). Therefore, it is important to re-investigate time, temperature and concentration dependence of gentamicin uptake, while ruling out non-specific binding by adding high concentrations of the unlabeled gentamicin. FITC-BSA uptake was very low and it was barely more than nonspecific binding. The FITC labeling includes the binding of the thiocyanate group in the label to the N-terminus of the protein, which could have abolished positive charges on the albumin. Since the ligand binding site of megalin is negatively charged, it is plausible to assume that BSA labeling hindered its active positive site from binding to negatively-charged megalin. Alternatively, this model might lack the functional activity of megalin and therefore did not display any transport of BSA.

## II. THE BEWO CELLS AS AN APPROPRIATE *IN VITRO* MODEL TO STUDY GENTAMICIN TRANSPORT

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### INTRODUCTION

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The results from our preliminary studies were inconclusive. Although there was time-dependency in gentamicin uptake, the uptake was not saturable even at gentamicin concentrations of up to 30 mM. In addition, the uptake had low efficiency comprising of less

than one thousandth of the original mass. This led us to take a step back and re-examine the appropriateness of the BeWo cells as a model to study AG uptake by human placenta. First, we needed to confirm that megalin is indeed expressed in the BeWo cells. A literature review was conducted and no data regarding the neither mRNA nor protein expression of megalin in the BeWo cells was found. However, megalin protein expression had been studied in various other cell lines. In cell lines of porcine and human kidney, there was no evidence of megalin protein expression when these cells when were grown on regular polystyrene plates/flasks. However, megalin was expressed in cells grown on Transwell® inserts which trigger their polarization. These data suggest that megalin protein expression requires three-dimensional environment.<sup>133,134</sup> Based on that, we used BeWo cells grown onto a Transwell® plate to study the uptake of gentamicin. Previous study by Liu *et al*<sup>135</sup> have shown that the transepithelial electrical resistance (TEER) values developed by the BeWo cells grown on Transwell® (seeding density of 100,000 cells/cm<sup>2</sup>) reach their maximum of  $\sim 70 \pm 2 \Omega \cdot \text{cm}^2$  at 5-6 days post-seeding. Monolayer integrity was determined by scanning electron microscope. Permeability of FITC-cyclodextran conjugates (10  $\mu\text{M}$ ) and fluorescein (0.5  $\mu\text{M}$ ) was minimal and decreased with increasing conjugate molecular weight. In agreement with this study, Poulsen *et al*<sup>125</sup> showed that the maximum TEER values of the BeWo cells were achieved 6 days post seeding (TEER =  $51.2 \pm 5.4 \Omega \cdot \text{cm}^2$ ). Minimal transport of fluorescein (100  $\mu\text{M}$ ) and fluorescent Dextran (5  $\mu\text{M}$ ) was observed at those TEER values (1  $\mu\text{M}$  and 0.0005  $\mu\text{M}$  were the concentrations of the substrate measured in the basolateral compartment 30 minutes after incubation with the corresponding substrates; respectively.)

In this section, studies performed to assess the appropriateness of BeWo cells grown on Transwell® plates as a model to study megalin role in AG uptake will be described. In order to demonstrate megalin involvement in gentamicin uptake, the uptake of <sup>3</sup>H-gentamicin was investigated in the presence or absence of a number of inhibitors known to either interfere with the megalin endocytic pathway (EDTA, cytochalasin D), compete with gentamicin on its binding with megalin such as receptor associated protein(RAP), or cause shedding of megalin from the cells where it is expressed (sodium maleate). RAP is a chaperone which has a role in the processing of megalin and is especially useful because it also acts as an antagonist for ligand binding on megalin (reported  $k_d$  values are 8, 42 nM).<sup>29</sup> The binding of most ligands to megalin was found to be calcium-dependent and the addition of the calcium chelating (EDTA, 10 mM) has been found to abolish the binding of purified megalin to its substrates transcobalamin-vitamin B12 complex<sup>37</sup> and vitamin D binding protein.<sup>136</sup> In addition, EDTA (20 mM) completely inhibited the transport of a megalin substrate, neutrophil gelatinase-associated lipocalin (NGAL; 1  $\mu$ M) by rat yolk epithelial cells,<sup>75</sup> and likewise caused more than 50% inhibition of the uptake of 0.7 nM of gentamicin by rabbit renal cortex sections.<sup>76</sup> Therefore the effect of EDTA on gentamicin uptake will be also assessed.

Furthermore, to determine the role of actin cytoskeleton or microtubules, the uptake of gentamicin will be assessed in the presence of cytochalasin D, an endocytosis inhibitor. Moreover, the effect of disodium maleate on gentamicin uptake will be examined. In animal studies including rats, disodium maleate (400 mg/kg; intravascular) has shown to cause reduction in megalin protein expression creating a megalin-shed model of rats' kidney within 45 minutes of exposure.<sup>137</sup> The mechanism by which disodium maleate can

cause this effect is still unknown. However, it was shown that maleate can lead to a concentration-dependent nephrotoxicity in proximal tubular cells which might be in part due to its ability to interact with and shed megalin.<sup>138</sup>

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## METHODS

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### A. Cell culture:

#### BeWo cells

BeWo cells (clone b30) were maintained as described previously in section 1. The Bewo cells of passages 36-48 were seeded at 100,000 cells/ml in 12-well Transwell® polyester plates (SA=1.12 cm<sup>2</sup>; Corning® catalog number 3460). Medium was changed every other day. Confluence was achieved after 5-7 days (TEER~70-80 Ω.cm<sup>2</sup>).

- ***Growth of BeWo Cells in collagen-coated Transwell® Plates:***

According to Bode et al., a stock solution of the coating material was prepared by dissolving human placental collagen (Sigma) in 0.1% acetic acid solution (1 mg in 0.345 mL) and then stored at 4°C. The working solution of the coating material was made immediately before use by diluting the stock solution in 70% ethanol (1:3). 70 µL of the coating material on the membrane of each well for a 12-well Transwell plate (the volume was adjusted with different size wells), while making sure that the membrane is coated evenly. The plate was dried for 2 hours in a laminar flow hood with lid open then Sterilized for 1 hour under UV light with the lid open. Plates were either used immediately or wrapped in aluminum foil and stored at 4°C (If coated plates were stored in the refrigerator, they were allowed to warm to room temperature for about 30 minutes before use). Membranes were pre-wetted

with the addition 1 mL of pre-warmed PBS (37°C) to the apical chamber and 2 mL to the basal chambers for 30–45 min then aspirated from both apical and basolateral chambers. Then cells were seeded as described above.

**HepG-2 (Human Hepatocellular Carcinoma) cells:**

Used as a negative control, HepG-2 cells (passage numbers from 82 to 85) were maintained by serial passages in 25- cm<sup>2</sup> and 75-cm<sup>2</sup> Corning plastic tissue culture flasks. The cells were fed using Dulbecco's modified Eagle medium (DMEM) which is supplemented with 10% fetal bovine serum, and 1% nonessential amino acids and incubated at 37°C in atmosphere of 95% air and 5% CO<sub>2</sub>. When confluent monolayers were formed, the cells were sub-cultured by detachment with 0.05% trypsin-EDTA. The cells were seeded at 100,000 cells/ml in 12-well Transwell® polyester plates (SA=1.12 cm<sup>2</sup>; Corning). Media was changed every other day. Confluence was achieved after 3-4 days (TEER~40-50Ω.cm<sup>2</sup>).

**Madin-Darby Canine Kidney (MDCK) cells:**

The ATCC-type MDCK cells (passage number used ranges from 22 to 25) were maintained by serial passages in 25- cm<sup>2</sup> and 75-cm<sup>2</sup> Corning plastic tissue culture flasks. The cells were fed using DMEM which is supplemented with 10% FBS, 1% of nonessential amino acids and penicillin-streptomycin and incubated at 37°C in atmosphere of 95% air and 5% CO<sub>2</sub>. When confluent monolayers were formed, the cells were sub-cultured by detachment with 0.05% trypsin-EDTA. The cells were seeded at 100,000 cells/ml in 12-well Transwell® polyester plates (SA=1.12 cm<sup>2</sup>; Corning). Media was changed every other day. Confluence was achieved 3-4days post-seeding (TEER 280-310Ω.cm<sup>2</sup>).

## **B. Model appropriateness**

### **Protein extraction and RNA isolation:**

BeWo cells of passages (36-37) were seeded on 6-well *Transwell*® plates (200,000 cells/cm<sup>2</sup>). Upon confluence, which is achieved at day 5, cells were harvested for protein and mRNA expression studies. **For western blotting:** Growth media was removed and cells were washed twice with ice-cold PBS and then 200-300 µl of ice-cold lysis buffer (150 mM NaCl, 1 mM EDTA and 20 mM of Tris, 1% Triton X-100 plus protease inhibitors; pH=7.4)<sup>96</sup> per well were added. The cells were incubated with the lysis buffer on ice for 30 minutes. After that, cells were further scraped out of the plate and the cell suspension was transferred to a pre-cooled centrifuge tube. The suspension was centrifuged at 12,000 rpm for 20 minutes. The supernatant was used for subsequent analysis (i.e. protein assay and western blotting). **For RNA isolation:** 1 ml of Trizol® was added to pre-washed cells in each well at room temperature. Then it was directly added to a centrifuge tube for further isolation. The following steps of reverse transcription and amplification were performed similarly to those used for placental tissues (Chapter 2; section 3)

## **C. Uptake experiments:**

### **The uptake of bovine serum albumin:**

The uptake of BSA was studied in the BeWo cells. First, the cells were incubated with 10 and 100 µg/ml of FITC-BSA (Sigma Aldrich; Florescein/Protein ratio 7 mole: 1 mole) in DPBS, in addition of 1000-fold of the unlabeled BSA to avoid nonspecific binding. The reaction was stopped by washing the cells 4 times with ice-cold DBPS. The cellular content



was assessed by treating the cells with NP-40 lysis buffer on ice for 30 minutes. Finally, the fluorescein the lysate was measured by BioTek® microplate reader at 485/520 nm. The uptake was normalized to protein content. The uptake was studied at 2, 5, 15, 30 and 60 minutes. Also, the uptake of 100 µg/ml FITC-BSA was measured at 4° C and in presence of 2 mM gentamicin (a megalin ligand). The BeWo cells were pre-incubated with gentamicin for 20 minutes then the uptake was measured after 15 minutes of the co-incubation of gentamicin and BSA. Moreover, the uptake of FITC-BSA was measured in the presence of 2 and 4 mg/ml of sodium maleate (megalin shedding agent). Cells were pre-incubated with 2 and 4 mg/ml of sodium maleate for 3 hours, and then the cells were incubated with FITC-BSA for 15 minutes.

### **Gentamicin uptake:**

Uptake experiments were initiated by adding <sup>3</sup>H-gentamicin (100,000 dpms/ml; 0.5 µM) and 2 mM gentamicin (except for the saturation experiment) in DPBS. Samples from the incubation solution were withdrawn for analysis by liquid scintillation counting. The reaction was stopped by washing cells three times with ice-cold DPBS. Cells were lysed with nonidet-p40 lysis buffer (NP-40 buffer: 150 mM NaCl, 1% NP-40 and 50 mM Tris-HCl; pH=8) for 30 minutes on ice then samples were drawn for analysis by liquid scintillation counting. Uptake was normalized to protein content which was measured using the BCA protein assay kit® (Pierce). Gentamicin uptake was measured over 2, 5, 10, 20, 40, and 60 minutes to assess time-dependence. Saturation studies were performed by exposing the cells to the following rising concentrations of gentamicin: 0.125, 0.25, 0.5, 1, 2, 4, and 6 mM.

The effect of temperature was assessed by examining the uptake of gentamicin at 37°C vs. 4°C. The uptake of gentamicin was also studied in the presence of various inhibitors including: receptor-associated protein (RAP), Cytochalasin D, EDTA, and sodium maleate. BeWo cells were incubated with RAP (1 µM), EDTA (10 mM) and cytochalasin D (5 µg/ml; ~10 µM) for 30 minutes. Cells were pre-incubated with 2 mg/ml and 4 mg/ml of sodium maleate (pH=7.4) for 3 hours. The inhibitors were added to both the apical and basolateral chambers. When the inhibitor is not soluble in DPBS, such as is the case of cytochalasin D, 0.2% v/v of dimethyl sulfoxide (DMSO) was added to the control and test cells, then cells were incubated with gentamicin± the inhibitor for 10 minutes.

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#### DATA ANALYSIS

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For FITC-BSA, a calibration curve (appendix C) of the fluorescence (at 480 nm) vs. BSA concentration (µg/ml) was constructed to calculate cellular contents of BSA in µg, then the uptake was normalized to the protein content of cells (mg). For <sup>3</sup>H-gentamicin, DPMs were converted to mass units as previously mentioned in section 1.

The significance of differences observed between data means for gentamicin uptake as a function of time was assessed via one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparison. Effects of temperature or inhibitors on <sup>3</sup>H-gentamicin were analyzed by t-test (two groups) or ANOVA (>2 groups). Concentration-dependency of gentamicin uptake was fitted to Michaelis-Menten equation using GraphPad Prism 5:

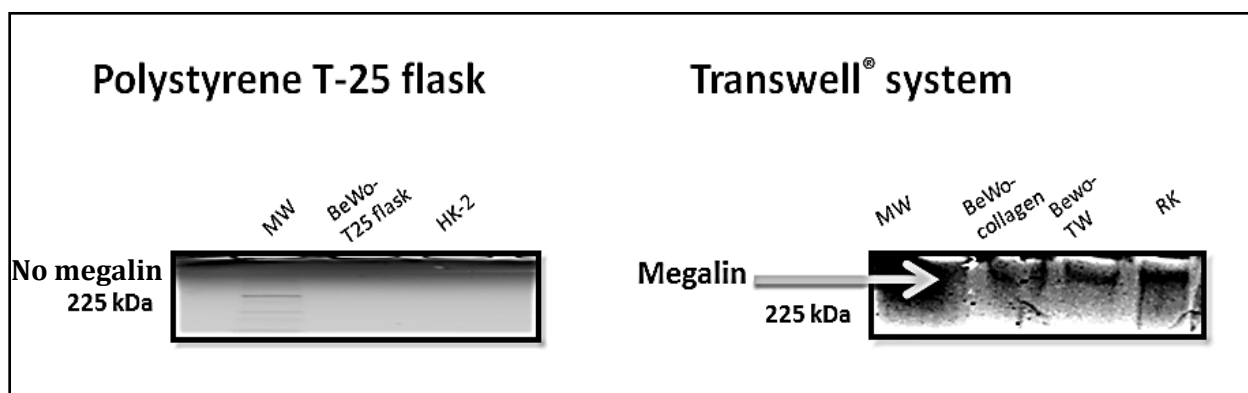
$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

Where  $V$  is the apparent velocity (nmole/mg protein/min);  $V_{\max}$  is the maximum velocity (nmole/mg protein/minute);  $K_m$ : is the concentration of substrate  $[S]$  at which reaction is half of its  $V_{\max}$ . All statistical analyses were performed using GraphPad® Prism 5. The level of significance for all analyses was  $\alpha = 0.05$ . Data points in graphs represent mean  $\pm$  SD.

## RESULTS

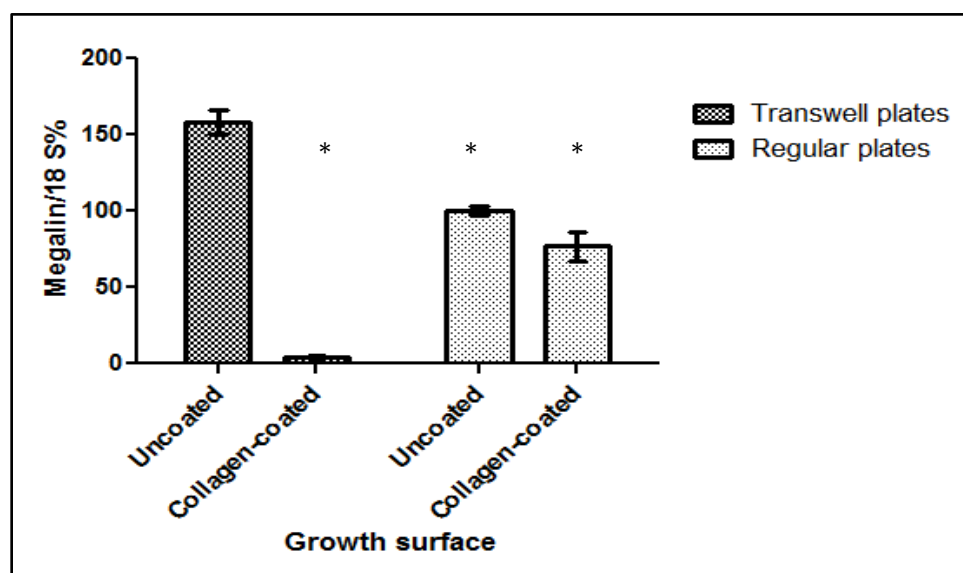
### A. The BeWo cells as an appropriate model to study AG transport:

Immunoblots of the BeWo cells grown on Transwells showed a distinct band at a molecular weight that is higher than 225 kDa which was also present in a rat kidney positive control (Figure 3-5 right). This band was **absent** in a homogenate of BeWo cells and human kidney type 2 (HK-2) cells which were collected from a regular plate or a T-25 flask (Figure 3-5 left).



**FIGURE 3-5:**IMMNOBLOTS OF BeWo CELLS IN T-25 FLASK (LEFT) AND TRANSWELL SYSTEM (LEFT).While the megalin band is absent when BeWo cells are grown on the T-25 flask, we could detect a band when cells were grown on the Transwell plate (TW:Transwell, RK: rat kidney).

In addition, qPCR data demonstrated differential mRNA expression in the BeWo cells when they were grown on different growth surfaces. Megalin mRNA expression varies significantly with the type of growing surface ( $p < 0.05$ ). Considering the BeWo cells grown onto uncoated regular plate as our control (calibrator), megalin expression in uncoated Transwell® plate was 1.5, 2.1 and 150 fold higher than in the control, collagen-coated regular plates and collagen-coated Transwell; respectively (Figure 3-6).

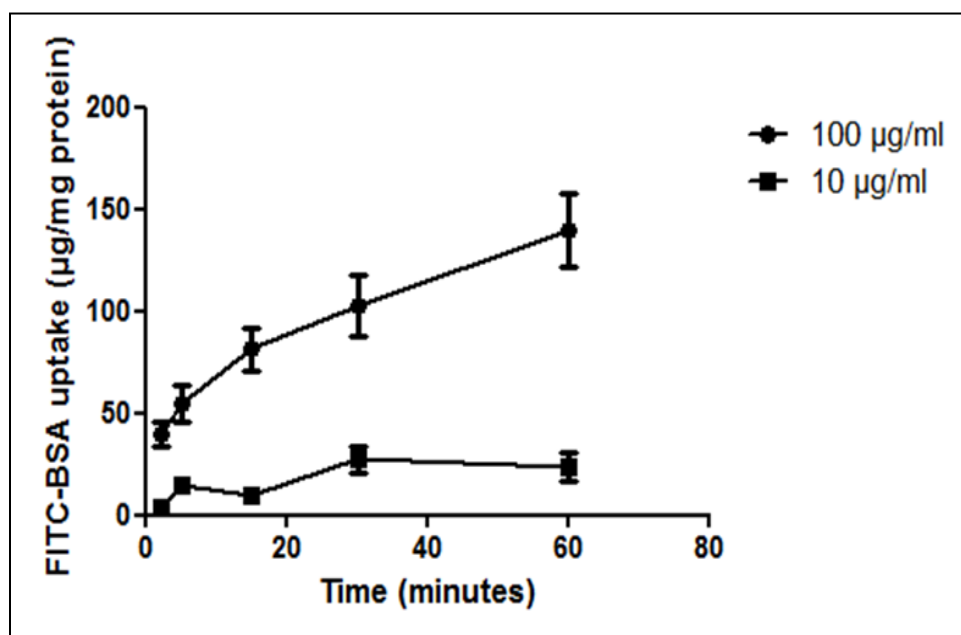


**FIGURE 3-6: MEGALIN mRNA EXPRESSION IN THE BeWo CELLS GROWN ON DIFFERENT GROWTH SURFACES.** Dark columns represent the Transwell plates and the light columns represent the regular plates (Data represent mean  $\pm$  SD; \*  $P < 0.05$  for the statistical difference in megalin expression in the uncoated Transwell relative to other groups).

## B. The uptake of fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) in the BeWo cells:

### Time dependence:

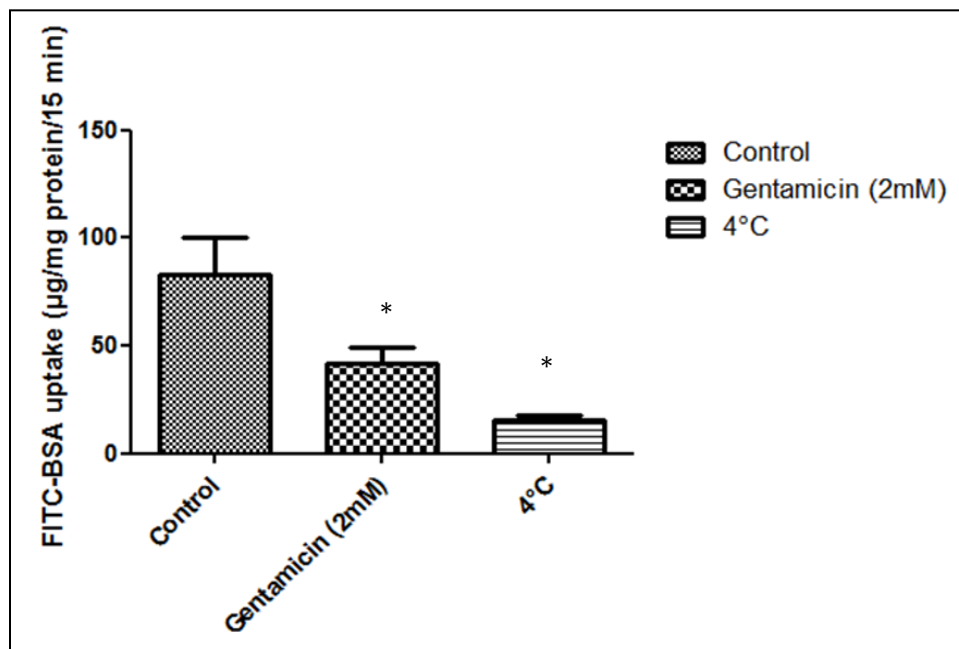
The uptake of two concentrations of BSA at 37° C was studied at 2, 5, 15, 30, and 60 minutes (Figure 3-7). The uptake of FITC-BSA (10 µg/ml) at 60 minutes was significantly higher ( $p < 0.05$ ) than the uptake at 2 and 5 minutes ( $33.83 \pm 6.90$  vs.  $3.52 \pm 0.45$  and  $9.33 \pm 3.50$ ; respectively). The uptake of FITC-BSA (100 µg/ml) was time-dependent, and increased over time and the uptake at 60 minutes was significantly ( $p < 0.05$ ) higher than that at 2 and 5 minutes ( $139.75 \pm 17.73$  µg/ml vs.  $40.00 \pm 6.06$  and  $54.63 \pm 8.66$ ). Also, the uptake at any time point was greater when higher concentration was added ( $p < 0.05$ ).



**FIGURE 3-7:**THE UPTAKE OF 10 AND 100 µg/ml OF FITC-BSA IN THE BeWo CELLS AT 2, 5, 15, 30 AND 60 MINUTES. Data represent mean  $\pm$  SD (n=3).

**The effect of temperature lowering and unlabeled gentamicin:**

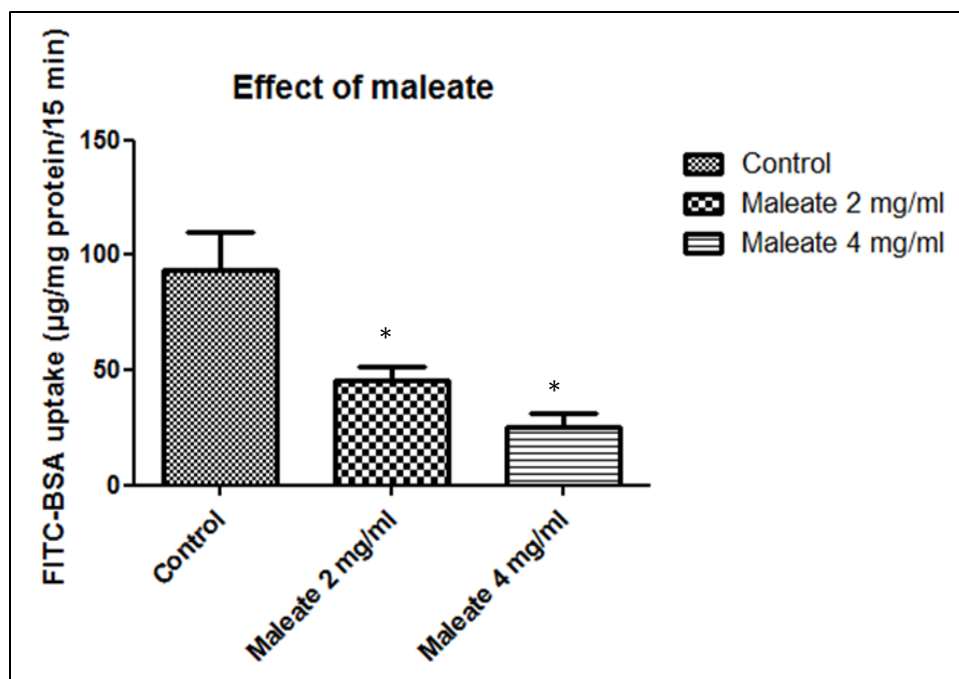
At 15 minutes, the uptake of 100  $\mu\text{g/ml}$  FITC-BSA was measured at 4°C (Figure 3-8) was significantly reduced when compared to that at 37° C ( $82.75 \pm 17.77$  vs.  $15.52 \pm 2.32$ ;  $p < 0.05$ ). In addition, the uptake was reduced by 50% ( $p < 0.05$ ) in the presence of 2 mM of unlabeled gentamicin



**FIGURE 3-8:**THE UPTAKE OF FITC-BSA IN THE PRESENCE OF 2 mM GENTAMICIN AND AT 4 ° C. Data represent mean  $\pm$  SD (n=6) \* $p < 0.05$ .

### The effect of maleate:

The uptake of FITC-BSA was measured after incubation of the cells with 2 and 4 mg/ml of sodium maleate. The uptake was reduced by 50 and 70% ( $p < 0.05$ ); respectively.

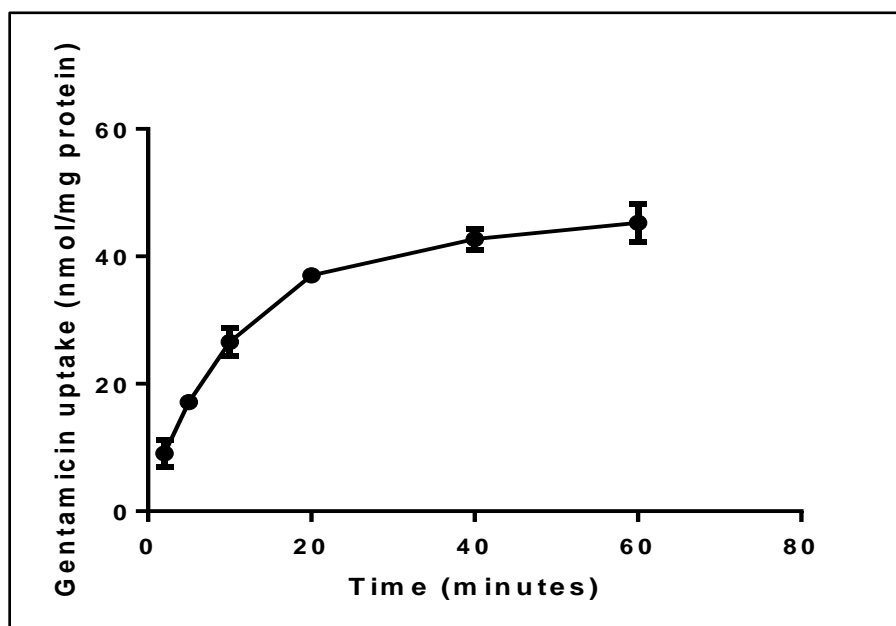


**FIGURE 3-9:**THE UPTAKE OF 100 µg/ml FITC-BSA IN THE PRESENCE OF 2 AND 4 mg/ml OF SODIUM MALEATE. Data represent mean  $\pm$  SD (n=3); \* $p < 0.05$ .

### The uptake of $^3\text{H}$ -gentamicin in the BeWo cells:

#### Time-dependence:

The uptake of 2 mM gentamicin at 37° C was studied at 2, 5, 10, 20, 40 and 60 minutes. The uptake of  $^3\text{H}$ -gentamicin increased steeply from 2 to 20 minutes then it started to level off after 20 minutes (Figure 3-10). The uptake at 60, 40 and 20 minutes was significantly higher than that at 2, 5 and 10 minutes ( $p < 0.05$ ).



**FIGURE 3-10: UPTAKE OF 2mM GENTAMICIN OVER 2 TO 60 MINUTES IN THE BeWo CELLS** (Data represent mean  $\pm$ SD; n=5-6).

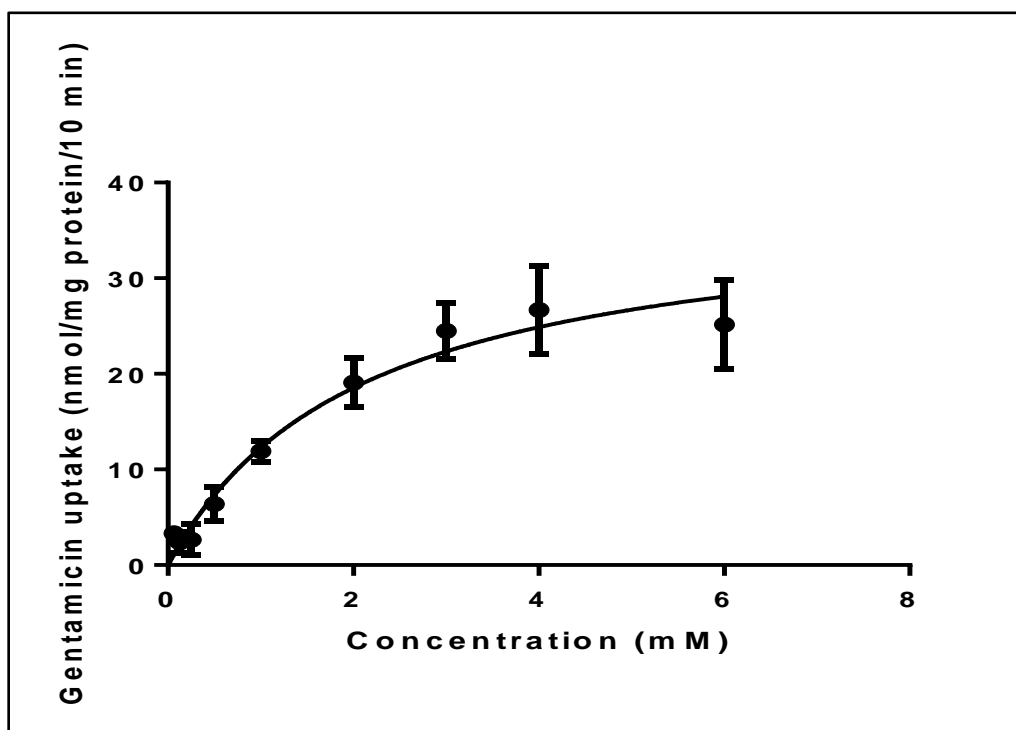
### Saturation kinetics:

The uptake of increasing concentrations of gentamicin was studied after 10 minutes at 37 C. There was a rise in gentamicin uptake as the concentration increases from 0.0625 mM to 2 mM then it plateaued until saturation was achieved at 6 mM of gentamicin (Figure 3-11). Michaelis-Mentenfit of data showed a  $K_m$  of 2.93mM and  $V_{max}$ of 42.3nmol gentamicin/mg protein/minute (Table 3-4).

**TABLE 3-1: MICHAELIS-MENTEN FIT OF GENTAMICIN UPTAKE**

	Best-fit value	SE	95% CI
<b>Vmax(nomle/mg protein/minute)</b>	42.3	4.9	32 to 52
<b>Km (mM)</b>	2.93	0.68	1.5 to 4.3

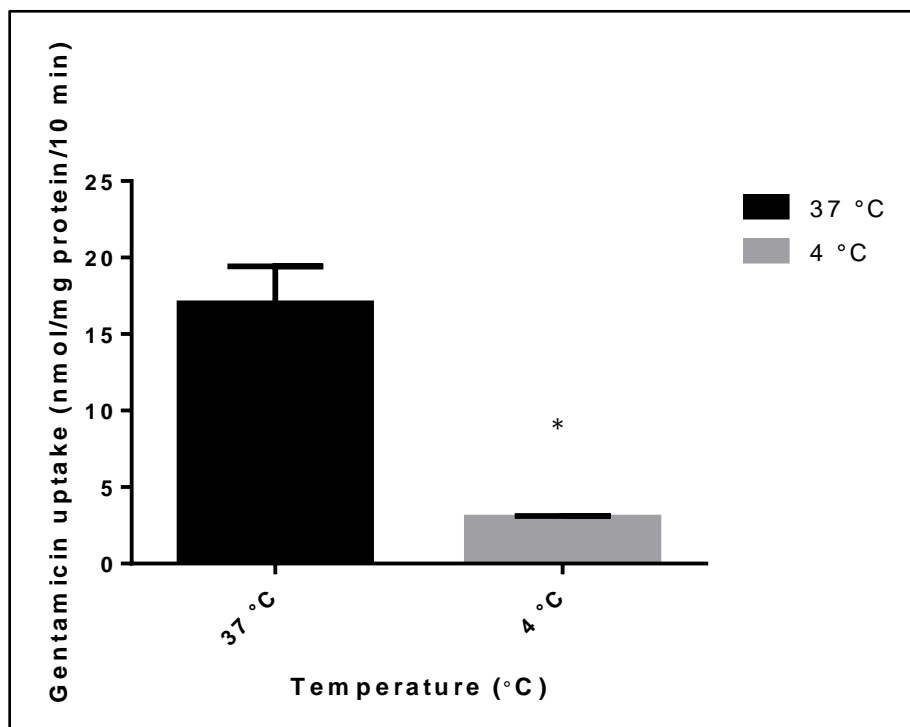




**FIGURE 3-11:** CONCENTRATION-DEPENDENCY OF GENTAMICIN UPTAKE IN THE BeWo CELLS (Data represent mean  $\pm$  SD; n=3-6).

**Temperature-dependence:**

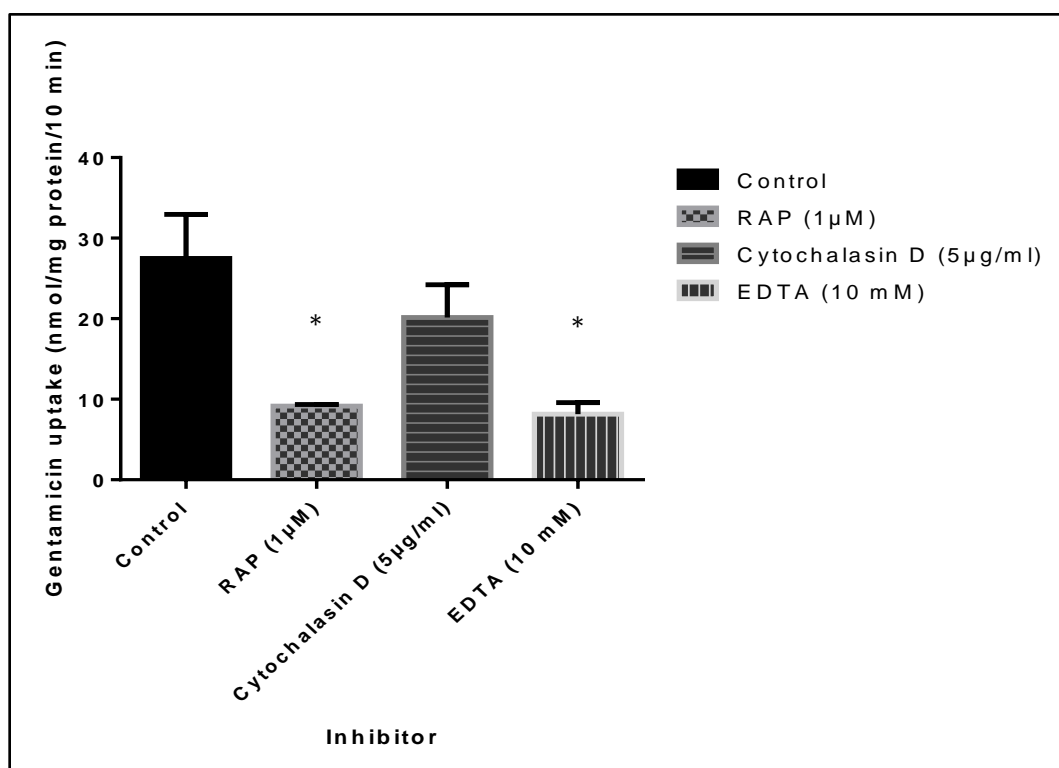
The uptake of  $^3\text{H}$ -gentamicin was temperature dependent at 10 min (Figure 3-12). Unequal variance unpaired t-test showed that the uptake of gentamicin at  $37^\circ\text{C}$  was significantly higher than that at  $4^\circ\text{C}$  ( $p < 0.05$ ).



**FIGURE 3-12:** THE UPTAKE OF GENTAMICIN BY THE BeWo CELLS IN 10 MINUTES AT  $37^\circ$  AND  $4^\circ\text{C}$ . (Data represent mean  $\pm$  SD,  $*p < 0.05$ ;  $n=6$ ).

### Effect of megalin inhibitors:

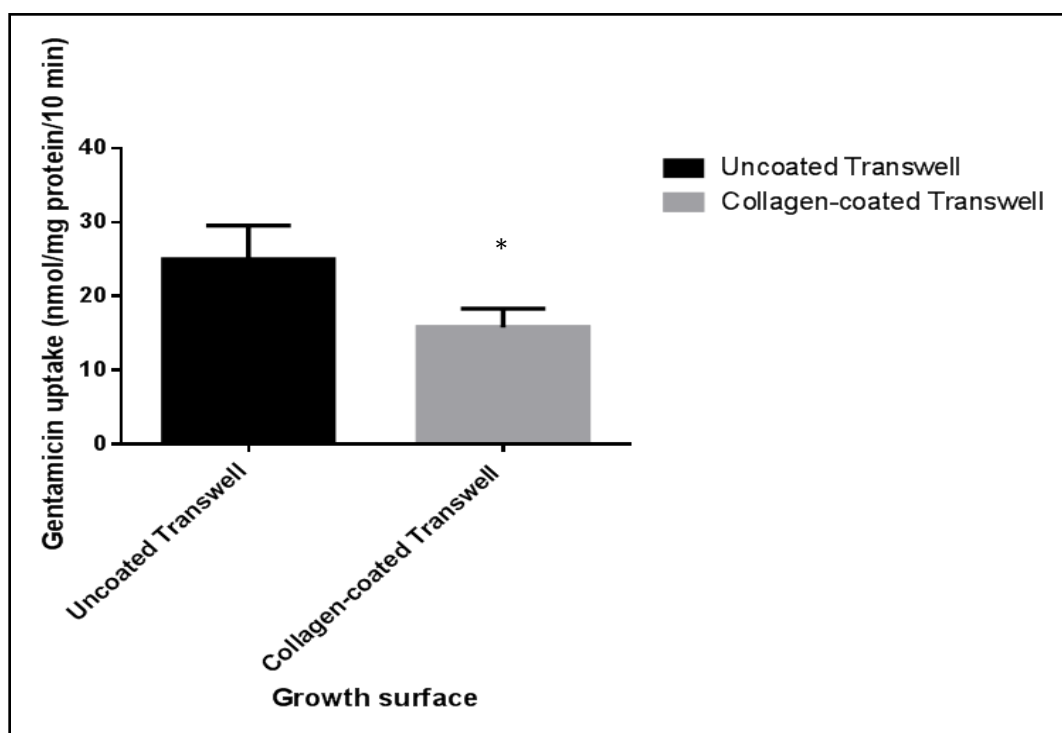
The uptake of  $^3\text{H}$ -gentamicin at  $37^\circ\text{C}$  was evaluated in the presence of three inhibitors; RAP, cytochalasin D, and EDTA. One-way ANOVA followed by the post-hoc Dunnett's test showed that both RAP and EDTA decreased the uptake of gentamicin by 70% ( $p < 0.05$ ). Cytochalasin D, an endocytosis inhibitor, showed a trend toward decreased uptake by 26% (figure 3-13).



**FIGURE 3-13:** THE EFFECT OF MEGALIN INHIBITORS (RAP, CYTOCHALASIN D AND EDTA) ON THE UPTAKE OF  $^3\text{H}$ -GENTAMICIN AT 10 MINUTES (Data present mean  $\pm$  SD, \* $p < 0.05$ ;  $n=3$ )

### The effect of growth surface:

Expression data showed that there is an effect of the growth surface on the mRNA and protein expression of megalin. The BeWo cells grown on uncoated Transwell® plates showed the highest levels of megalin protein and mRNA expression. In agreement with the expression data, the uptake of gentamicin in the uncoated transwell plates was 36% higher (Figure 3-14) than collagen-coated ones ( $p < 0.05$ ).

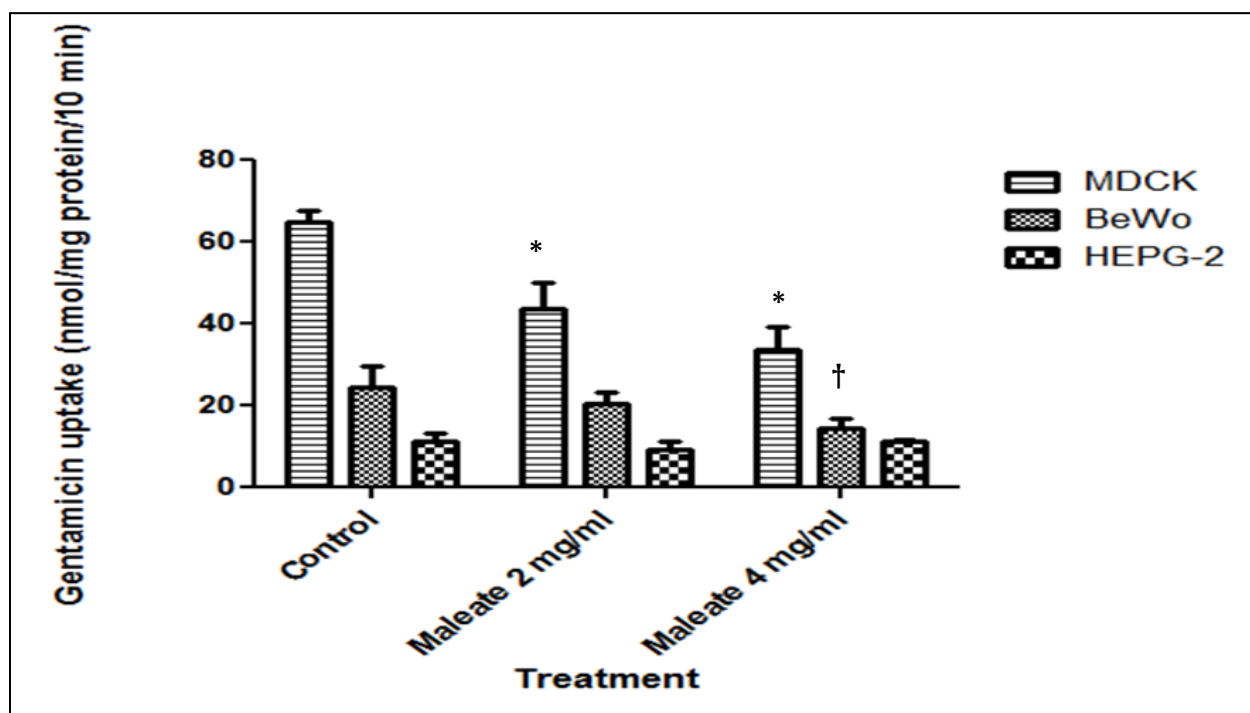


**FIGURE 3-14:** THE EFFECT OF COLLAGEN COATING ON THE UPTAKE OF GENTAMICIN IN THE BeWo CELLS. (Data represent mean  $\pm$  SD;  $p < 0.05$ ,  $n=3$ ).

In both systems (uncoated and collagen-coated Transwell plates), the effect of maleate on the uptake of gentamicin in the Bewo cells was studied. In the uncoated Transwell the uptake of gentamicin in the presence of 2 and 4 mg/ml of sodium maleate (Figure 3-12) was decreased by about 20 % and 45% respectively ( $p < 0.05$ ).

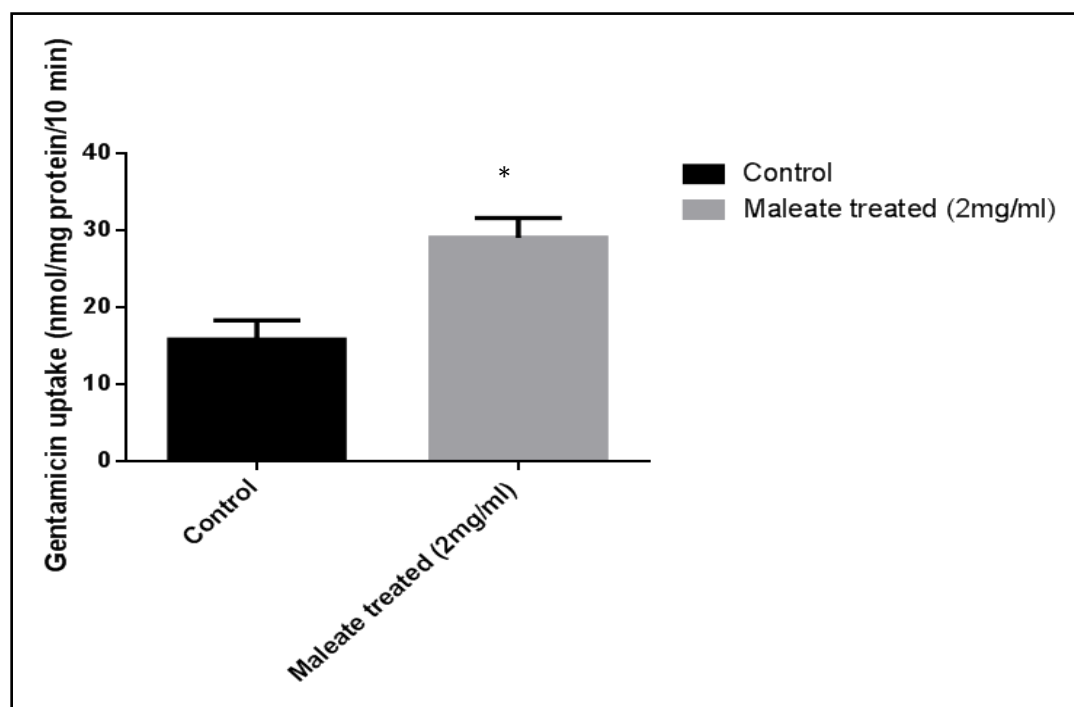
Renal MDCK cells and HepG-2 cells were used as positive and negative control, respectively. In maleate-untreated cells (control group), there was a statistically significant difference of gentamicin uptake among the three cell lines ( $p < 0.05$ ). The uptake of gentamicin by the renal cells was significantly higher than that of the Bewo cells (mean difference= 40.23, 95%CI = 34.92-45.54,  $p < 0.05$ ). The uptake in MDCK and Bewo cells was significantly higher than that of HepG-2 cells (mean difference= 53.45, 13.22; 95%CI =48.14-58.76; 7.909-18.53,  $p < 0.05$ ; respectively).

The uptake of gentamicin by the MDCK cells was significantly decreased by the addition of 2 mg/ml and 4 mg/ml of maleate ( $p < 0.05$ ). The treatment with 2 mg/ml of maleate cause the uptake to drop by 21.29 nmol of gentamicin/mg protein (95% CI: 13.43-29.15,  $p < 0.05$ ). Increasing the maleate concentration resulted in a significant decrease in gentamicin uptake relative to the control and to the 2 mg/ml dose (mean difference=31.23, 9.941; 95% CI: 23.37-39.09, 2.079-17.80;  $p < 0.05$ ; respectively). There was a significant decrease in gentamicin when the BeWo cells were treated with 4 mg/ml (see above). However, there is no effect of maleate treatment on the uptake of gentamicin in the HepG-2 cells as there was not significance change in gentamicin uptake with increasing concentrations of maleate ( $p > 0.05$ ), (Figure 3-15).



**FIGURE 3-15:** THE EFFECT OF SODIUM MALEATE (2 AND 4 mg/ml) ON THE UPTAKE OF GENTAMICIN IN THE MDCK CELLS, BeWo CELLS AND HepG-2 CELLS GROWN ON UNCOATED TRANSWELL PLATES. (Data represent mean  $\pm$  SD; \*  $p < 0.05$  for the effect of maleate on gentamicin uptake in MDCK cells; †  $p < 0.05$  for the effect of maleate in the BeWo cells;  $n=6$ ).

Interestingly, in the collagen-coated plate, a system where megalin mRNA expression is less, the uptake by the cells which were treated with 2 mg/ml of maleate was significantly higher than that of untreated cells ( $t=10.01$ ,  $df=10$ ,  $p<0.05$ ) (Figure 3-16).



**FIGURE 3-16:** THE EFFECT OF 2 MG/ML OF MALEATE ON THE UPTAKE OF GENTAMICIN IN THE BEWO CELLS GROWN ON COLLAGEN-COATED TRANSWELLS (Data represent mean  $\pm$  SD;  $p<0.05$ ;  $n=6$ )

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### *DISCUSSION AND CONCLUSION*

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The molecular mechanisms of AG uptake across human placenta have not been well described to date. Due to ethical and practical purposes, we chose human choriocarcinoma cells (the BeWo cells) as a model to characterize gentamicin uptake via placenta. In accordance with previous literature, megalin protein expression was detected by western blotting when cells were cultured in a three-dimensional environment. When grown on Transwell® plates, megalin mRNA and protein are greater expressed in the BeWo cells. To examine the functional activity of megalin we investigated the uptake of BSA as an

endogenous substrate for megalin. The uptake of BSA by the BeWo cells, grown on Transwell plates, was time dependent, and temperature-dependent. The uptake was significantly reduced in the presence of unlabeled gentamicin and sodium maleate. These findings suggest the role of megalin in BSA uptake across human placenta.

Our data showed that the uptake of gentamicin by the BeWo cells increased with time, and was temperature-dependent. Also, the uptake was saturable and displayed typical Michaelis-Menten kinetics which indicates a transporter-mediated uptake.

To examine megalin involvement in gentamicin uptake, the uptake of gentamicin was studied in the presence of megalin inhibitors; RAP, ETDA, cytochalasin D and maleate. Gentamicin uptake was significantly reduced in the presence of the megalin inhibitors RAP and EDTA (by 70%). RAP is a protein that is required for proper megalin functioning and has been shown to reduce binding of megalin to its ligands including AGs. In agreement with our data, a study by Moestrup *et al*<sup>76</sup> showed that the amount of gentamicin in RAP deficient kidneys was decreased by about 50% as compared with control tissues. In addition, gentamicin excretion in rat perfused proximal tubules model increased to 46.2 % (21.1-81.0%;  $p < 0.01$ ) with addition of 10  $\mu\text{M}$  of RAP, presumably due to decreased renal tubular reabsorption of gentamicin. Considering the high inhibitory affinity of megalin by RAP ( $K_d = 8 \text{ nM}$ )<sup>139</sup> and the high RAP concentration that was used (10  $\mu\text{M}$ ), the results might not represent a real “increase” in gentamicin urinary excretion.

We also showed that cytochalasin D, an endocytosis inhibitor, did not alter gentamicin uptake. A previously published study had shown that cytochalasin D reduced gentamicin uptake in a porcine proximal tubular cells model (LCC-PK1) after 30 minutes of



incubation.<sup>131</sup> It could be that the 30-minute incubation time used in the LCC-PK1 study allowed for more inhibition or that endocytosis contributes to a large extent of gentamicin uptake by renal cells but only partially by placenta.

Sodium maleate, which was shown to shed megalin in renal cells, caused a significant, albeit partial (30-45%) decrease in gentamicin uptake. Our findings match with a previous *in vivo* study by Nagai *et al.*<sup>92</sup> which showed that the administration of intraperitoneal sodium maleate (400 mg/kg) decreased amikacin accumulation by 50% in the renal cortex of wistar rats, three hours after injection. This decrease in the uptake accompanied a simultaneous 50% decrease in megalin protein expression as shown by radiolabeled Calcium ( $^{45}\text{Ca}^{+2}$ ) blotting. The *in vitro* dose of maleate that causes megalin shedding is not known. We used an empirical approach to estimate the *in vitro* concentration proposed in this dissertation. If we consider that the average weight of a Wistar rat is 300 g,<sup>140</sup> and the blood volume is 20 ml,<sup>140</sup> a dose of 400 mg/kg will give a total amount of  $400 \times 0.3 = 120$  mg. Assuming 100% intraperitoneal bioavailability for sodium maleate and a blood volume which equals the volume of distribution (as expected for a very hydrophilic compound such as sodium maleate), then 6 mg/ml (120 mg/20 ml) of maleate is available at the site of action (renal cells). Sodium maleate, however, has shown to be injurious to rat isolate tubules at concentrations higher than 3 mg/ml as assessed by lactate dehydrogenase release.<sup>138</sup> Based on facts from these two studies, we selected our concentrations: 2 mg/ml and 4 mg/ml of maleate. When the BeWo cells were grown onto uncoated plates, the uptake of gentamicin was decreased by ~45% when cells were treated with 4 mg/ml of maleate, which is comparable to the decrease observed in the Nagai study.<sup>92</sup> Interestingly, when 2 mg/ml of maleate was added to the BeWo cells grown

on collagen-coated Transwell®, a system where megalin mRNA expression is inhibited, gentamicin uptake was significantly increased. There might be an efflux mechanism of gentamicin that contributed to that increase such as p-glycoprotein (p-gp)-mediated efflux, but there is no clear reason for this phenomenon and it will require future investigation.

Megalyn mRNA expression in the BeWo cells was significantly higher than that of HepG2 cells. Even more, megalin protein expression in the liver and mRNA expression in HepG2 cells was almost zero. Expectedly, the uptake of gentamicin in the HepG2 cells was significantly less than that of BeWo cells and MDCK cells. Still, there is some extent of uptake in the liver cells which is not explained by the megalin mRNA expression data alone. This might be due to other mechanisms that are involved with gentamicin uptake by the liver such as passive diffusion or organic cationic transporters (OCT-1). Since the liver is not a physiologic site of AG-induced toxicity or pharmacologic action, the uptake of AGs by hepatocytes has not been studied. The only data we have about the extent of AG uptake into the liver is available from animal pharmacokinetic studies of AG tissue distribution. One study examined the tissue distribution of gentamicin in rabbits.<sup>141</sup> Gentamicin was administered intramuscularly as both single injection of 15/mg and seven daily injections. After the single dose, gentamicin was only detectable in kidney ( $70 \pm 11$  µg/g tissue) but not in the liver (detection limits were not reported). After seven daily injections, gentamicin was detected in the liver ( $18 \pm 5.5$  µg/g tissue), however, it was much lower than those levels achieved in the kidney ( $135$  µg/kg  $\pm$  65). The measurements were taken twenty hours after the last injection, which might lead to underestimation of gentamicin levels in both organs. A relatively more recent study, examined tissue distribution of gentamicin in rats with emphasis on inner ear fluids.<sup>87</sup> After the intramuscular

administration of 100 mg/kg of gentamicin, the peak renal cortex levels were  $4.06 \pm 0.23$   $\mu\text{g}/\text{mg}$  of protein 6 hours after the injection, simultaneously, the liver levels peaked at  $0.06 \pm 0.003$   $\mu\text{g}/\text{mg}$  (detection limits were 0.1-1 ng/mg protein). After continuous infusion, steady state concentrations were 2.1, 5.6, 10  $\mu\text{g}/\text{mg}$  and 10  $\mu\text{g}/\text{mg}$  in the renal cortex, while the parallel liver values were  $0.018 \pm 0.002$ ,  $0.02 \pm 0.002$ ,  $0.033 \pm 0.005$ , and  $0.09 \pm 0.04$   $\mu\text{g}/\text{mg}$  (infusion rates of 1.5  $\mu\text{g}/\text{min}$ , 4.5  $\mu\text{g}/\text{min}$ , 15  $\mu\text{g}/\text{min}$  and 150  $\mu\text{g}/\text{min}$ ; respectively). Although it was obvious from both studies that the uptake of gentamicin in the renal tissue was much higher than that of the liver, there was some degree of detectable uptake in the liver. The latter indicates that AGs can get into the liver, but the mechanisms have not been elucidated.

In renal proximal tubular cells, megalin contributes to the reabsorption of small molecular weight proteins, such as albumin.<sup>142</sup> Therefore, we expected that albumin would be an appropriate positive control in order to demonstrate a functional megalin in the BeWo cells. When we studied the uptake of FITC-BSA in the BeWo cells grown on regular plates, we did not observe time or temperature-dependent profiles (Appendix B). However, when cells are grown on the Transwell® system, which has the greatest level of megalin mRNA and protein expression, the BSA uptake was time-dependent, temperature-dependent and partially inhibited by unlabeled gentamicin. There is only one study that examined the uptake of albumin across human placenta<sup>31</sup> using a placental villous tissue model. It found that clathrin-mediated endocytosis is the major contributor of albumin uptake by placenta. Albumin uptake was significantly decreased by a clathrin-mediated endocytosis inhibitor, chlorpromazine. However, the concentration of chlorpromazine used (1.4 mM) was about 10 fold higher than what is usually used to shut down this pathway

entirely (100 $\mu$ M). In the same study,<sup>31</sup> DIDs, and NPPB, two megalin pathway inhibitors, did not affect the transport of albumin. However, a definitive conclusion about the mechanism of albumin transport in human placenta cannot be made. In addition, the iron-transferrin complex is assumed to cross placenta by megalin-mediated endocytosis,<sup>47</sup> but it was later found that the receptor component is mostly cubilin and not megalin.<sup>47</sup> Another potential substrate is the vitamin B12–transcobalamin complex. It is found to undergo megalin-mediated endocytosis in the *very early* form of placenta which is yolk sac.<sup>37</sup> Consequently, our research was able to demonstrate the validity of albumin as an endogenous control to test megalin functional activity in *term* placenta.

Considering data from our functional studies, we believe that megalin-mediated endocytosis is at least partially involved in the binding and uptake of aminoglycosides by placenta and ultimately fetal delivery of these antibiotics.

## CHAPTER 4 : CONCLUDING REMARKS AND FUTURE DIRECTIONS

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### CONCLUDING REMARKS

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During gestation, pregnant women may develop various types of short- and long-term conditions which should be promptly managed to protect both maternal and fetal health. Medications can be administered with the intention to treat the mother and/or the fetus. One example where both the mother and fetus need to be treated is a group of pregnancy-related infections called intra-amniotic infections. Intra-amniotic infections (IAIs) are common complications of labor and delivery, being more frequent in preterm births. If left unmanaged, these infections can lead to significant morbidity and mortality in the mother and the fetus. Intrapartum aminoglycosides, in combination with penicillin, is the recommended treatment for documented or suspected IAIs. AGs are known to readily cross the placenta and to rapidly achieve bactericidal concentrations in fetal serum. However, the highest and most persistent fetal levels are achieved in renal tissue. Since there is a direct correlation between the rate and extent of AG accumulation in renal tissue and susceptibility to nephrotoxicity, the fetus may be particularly vulnerable to the nephrotoxic effects of AGs. Persistence of AGs in renal tissues after birth also increases the susceptibility of the newborn to injury during the early postnatal period when AGs are routinely administered to prevent or treat infections acquired *in utero*.

Human placenta represents a vital transport organ between the mother and the developing fetus. Many transport mechanisms play a role in nutrient delivery, drug transport, and gas exchange across placenta. These mechanisms include passive and facilitated diffusion, active transport and endocytosis. Megalin, a 600 kDa protein, is endocytic receptor that is expressed in the apical epithelia of several tissues. Megalin is extensively expressed in renal proximal tubules and plays a role in the receptor-mediated endocytosis of aminoglycosides. Being expressed in placenta, megalin may be similarly involved in placental transport of AGs. However, the role of megalin in the placental transport of these antibiotics is unknown. Our project provided an insight of these mechanisms by assessing and *comparing* megalin mRNA expression in term and preterm placental villous tissue samples, in addition to examining the functional activity of megalin by *in vitro* placental models.

Megalin protein expression was assessed via western blotting. Immunoblot analysis showed a band at a molecular weight (higher than 225 kDa and 460 kDa) in term placental villous tissue samples and in rat kidney (positive control). The band was, essentially absent in mouse liver (negative control).. On the contrary, the mass spectroscopy analysis was inconclusive. Due to these controversies of the protein expression results, we could not solely rely on western blotting data to confirm megalin detection. Moreover, we could not semi-quantify the protein using western blotting due to technical difficulties. Therefore, we evaluated the mRNA expression of megalin in human placenta. The expression of megalin mRNA was confirmed by q-PCR methods in both term and preterm placental tissues. Moreover, we found a strong correlation between the gestational age and megalin mRNA expression which is in accordance with the changing fetal nutritional requirements during

different developmental stages and involvement of megalin in the supply of nutrients during these stages.

Interestingly, data from the mRNA stability study demonstrated that placental megalin mRNA expression is not significantly altered when processed placental tissue samples were stored for up to 18 hours at 4 °C. This finding would allow future researchers with flexibility in placental tissue samples collection, particularly in cases where immediate tissue processing and collection is not feasible.

The molecular mechanisms of AG uptake across human placenta have not been well described to date. Due to ethical and practical purposes, we chose human choriocarcinoma cells (the BeWo cells) as a model to characterize gentamicin uptake via placenta. Since both megalin protein and mRNA are expressed in the Bewo cells grown on Transwell® plates, these cells, appear to be a valid model to assess the role of megalin in AG uptake. Our data showed that the uptake of gentamicin by the BeWo cells increased with time, and was temperature-dependent. Also, the uptake was saturable and displayed typical Michaelis-Menten kinetics which indicates a transporter-mediated uptake. Gentamicin uptake was significantly reduced in the presence of the megalin inhibitors RAP and EDTA (by 70%). Sodium maleate, which was shown to shed megalin in renal cells, caused a significant, albeit partial (30-45%) decrease in gentamicin uptake. Considering data from our functional studies, we believe that megalin-mediated endocytosis is at least partially involved in the binding and uptake of aminoglycosides by placenta and ultimately the fetal delivery of these antibiotics.

## RENGTHS AND LIMITATIONS

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The **strengths** of this project include novelty and methodological rigorousness. Data from previous literature have shown that megalin is expressed in human placenta. Nevertheless, neither mRNA nor protein expression had been quantified and the ontogeny of megalin expression had never been studied. Our data not only verify the expression of megalin mRNA in human placenta but further extend the qualitative nature of this piece of information. It is also the first to **compare** the mRNA expression of megalin in human term and preterm placenta villous tissue samples, a model which has not been examined for megalin expression to date.

Furthermore, this research identifies a widely used human trophoblastic cell line (BeWo cells) as an appropriate model to study megalin functional activity which had not yet been established. We were able to detect megalin protein and mRNA expression in the BeWo cells and *compare* the mRNA expression under different growth environments (regular plates, vs. collagen-coated, vs. Transwell® systems). While the role of megalin in the **renal** uptake of aminoglycosides has been widely studied, the involvement of megalin in AG uptake by **placenta** is not known. We were the first to document the involvement of megalin in the binding and/or uptake of aminoglycosides by human placenta through examining the uptake kinetics of these antibiotics in the BeWo cells and comparing AGs uptake in the absence of presence of various megalin inhibitors.

From a methodological point of view, this research also assessed the stability of human megalin mRNA as a function of storage time and storage conditions. Samples which were freshly collected and processed appropriately were chosen for analysis. The use of



high quality tissue samples and a relatively reasonable sample size (10 term placentas, and 5 preterm placentas) are two valuable features of this study. We were the first to show that *placental* megalin mRNA is stable up to 18 hours. This finding would allow future researchers with flexibility in placental tissue samples collection, particularly in cases where immediate tissue processing and collection is not feasible.

In renal proximal tubular cells, megalin contributes to the reabsorption of small molecular weight proteins, such as albumin.<sup>142</sup> Therefore, we expected that albumin would be an appropriate positive control in order to demonstrate a functional megalin in the Bewo cells. When we studied the uptake of FITC-BSA in the BeWo cells grown on regular plates, we did not observe time or temperature-dependent profiles (Appendix B). However, when cells are grown on the Transwell® system, which has the greatest level of megalin mRNA and protein expression, the BSA uptake was time-dependent, temperature-dependent and partially inhibited by unlabeled gentamicin (evidence of saturability). There is only one study that examined the uptake of albumin across human placenta<sup>31</sup> using a placental villous tissue model. It found that clathrin-mediated endocytosis is the major contributor of albumin uptake by placenta. Consequently, our research was able to demonstrate the validity of albumin as an endogenous control to test megalin functional activity in *term* placenta.

Nevertheless, the project is not void of drawbacks. One limitation is the inability to use the western blotting technique to compare the protein expression in term and preterm placenta. Although megalin mRNA expression was confirmed and compared as a function of gestational age, this assessment cannot eliminate the need to quantify megalin protein

expression. As a large protein which undergoes some post-translational modifications, mRNA expression might not be related proportionally to protein expression. Due to difficulties in quantifying the protein by western blotting, we could not study the correlation between mRNA and protein expression. Furthermore, we *could not* compare megalin functional activity in term and preterm placenta. The BeWo cell line represents trophoblasts from human *term* placenta. The *in vitro* models of preterm placenta can be either obtained from placental villous tissues, or isolated primary cytotrophoblasts. Due to the practical limitations of these models (i.e., tissue heterogeneity and high susceptibility to contamination) accompanied with lack of preterm tissues with timely availability, we could not assess megalin function in preterm placental samples. Also, the functional studies are limited by their lack of ability to distinguish binding and uptake.

#### OPPORTUNITIES FOR FUTURE RESEARCH

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Our research not only extends the existing knowledge about receptor-mediated endocytosis via human placenta, but also provides venues for future studies to be conducted in this area. Methods to quantify megalin **protein** in placenta should be developed and optimized. Moreover, the use of a known substrate to assess megalin functional activity in the BeWo cells would significantly enhance the current data. Examples include, but are not limited to: HDL-C, vitamin B12-transcobolamin complex and potentially albumin. Examination of gentamicin uptake separately from binding is also warranted. This could be achieved through immunohisto/cyto-chemical studies which include double detection of gentamicin and megalin, which will enable the assessment of the subcellular distribution of gentamicin. In addition, AG uptake in preterm placental

samples should be investigated. This might involve the development of more efficient techniques to isolate primary cytotrophoblasts other than the Percoll® gradient method. Laminin-coated magnetic beads have been successfully used to isolate cytotrophoblasts from term villous tissue with high purity.<sup>143,144</sup> In addition, negative selection with anti CD9 immunomagnetic separation had been described for isolation of CTBs from first trimester chorionic villi.<sup>145</sup>

While our research strongly suggests that placental megalin is involved in the uptake AG, it did not delineate the other mechanisms that might be playing a role in AGs maternal-fetal transport across the placenta which can include passive diffusion, organic cationic transport, etc. Further studies should pursue these other mechanisms, and examine the transport of gentamicin at the basolateral (fetal side) of placenta. As shown in chapter 3, there was some extent of gentamicin uptake in the liver cells which was not explained by the megalin mRNA expression data alone. This uptake might be due to other uptake mechanisms of gentamicin in the liver. Although the liver is not a physiologic site of AGs-induced toxicity or pharmacologic action, hepatocytes can be utilized as a model to investigate other mechanisms which are involved in the uptake of gentamicin by other tissues.

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#### MEGALIN mRNA SILENCING AS A POTENTIAL TECHNIQUE TO STUDY THE ROLE OF MEGALIN IN PLACENTAL UPTAKE OF AMINOGLYCOSIDES

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In order to confirm and to determine the extent to which megalin is involved in the transport of aminoglycosides in the BeWo cells, RNA interference or silencing of the LRP2 gene (low-density lipoprotein related- protein2; megalin) would provide valuable information regarding the extent of megalin's involvement of AG uptake.

The approaches utilizing siRNAs or shRNAs to silence our gene of interest, which is the LRP2, are feasible options to study megalin's role in the placental transport of gentamicin. LRP2 silencing approach has been performed in many cell lines the aim of which was to assess the role of megalin in the endocytosis of endogenous proteins such as albumin,<sup>146</sup> myeloma light chains,<sup>99</sup> and insulin-like growth factor.<sup>147</sup> The availability of literature further provides an initial guidance on how to develop and optimize our methods beyond what has been published in the providers' protocol. Moreover, human choriocarcinoma cells (the BeWo cells) would be an appropriate model to employ the RNA silencing technique. The BeWo cells have been widely used as model to study placental transfer of drugs. BeWo cells also have good tendency to accept DNA and RNA through various delivery paradigms, hence, achieving reasonable transfection efficiency.<sup>148</sup> The silencing technique will provide an evidence of the extent of megalin involvement in AG uptake. Given the broad substrate specificity profiles for megalin, the use of a single chemical inhibitor can be difficult to interpret. Therefore, we used several approaches to inhibit megalin endocytosis. RNA interference, therefore, offers a distinct advantage over the use of chemical inhibitors.

In terms of expression, western blot analysis of BeWo cells and placental tissues detected a high molecular weight band the identity of which couldn't be accurately determined by amino acid sequencing. The use of megalin silencing in this case provides a negative control sample (cells where megalin expression is suppressed).

In spite of these advantages, gene silencing will not allow us to further explore or delineate other mechanisms involved in AGs uptake. The RNA silencing-dependent

approach will only distinguish megalin as a major endocytic receptor but will not illustrate the role of other molecules associated with megalin in the endocytic apparatus.

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#### POTENTIAL STRATEGIES TO PROTECT FETAL KIDNEY

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It is important to understand the mechanisms involved in the placental transport of aminoglycosides so that strategies can be developed to limit accumulation in fetal kidney without comprising placental transfer. Megalin-targeted strategies to protect kidney from AG-induced nephrotoxicity is based on the assumption that megalin blockade will prevent AG endocytosis and accumulation into proximal tubular cells hence, protecting renal tissue from the accompanying concentration-dependent nephrotoxicity. Megalin ligands such as lysozyme, aprotinin, cytochrome C inhibited renal accumulation of intravascularly administered gentamicin in rat renal cortex in experimental animals. For example, when cytochrome C was co-administered with gentamicin, the gentamicin-induced nephrotoxicity was attenuated as estimated by the reduced urinary secretion of *N*-acetyl- $\beta$ -D-glucosaminidase (NAG).<sup>151</sup>

In this project, the perspective is to maternally administer AGs (intrapartum) for the treatment of maternal/ fetal infections (or the intra-amniotic infections) with the aim of treating both the mother and fetus because both of them are infected. Based on our data, we are more inclined to conclude **that multiple transport mechanisms, including megalin-mediated endocytosis are involved in AGs uptake by placenta.** What these mechanisms exactly are is beyond the scope of this dissertation. Say that megalin-mediated endocytosis and passive diffusion are both involved in AGs uptake, and then we could use a megalin blocker that is also known to cross placenta. The blocker is to be used in high

concentration so as to saturate megalin binding sites. AGs now can escape megalin binding and get transported to fetus by passive diffusion and the blocker too. A concern that is raised using this strategy is that using the blocker in a high concentration might not be safe for the fetus. Nevertheless, not all the amount of the blocker will arrive to fetus because part of it will be occupying megalin. In any case, before using any megalin blockade strategy in clinical setting, it is very important that the clearance of AG should be evaluated in the presence and absence of megalin inhibitors to fully characterize the effect of megalin inhibition on the maternal to fetal transfer clearance of AGs. Also the effect of megalin inhibition during late gestation in animal models must be investigated to see if there are any adverse developmental effects associated with megalin inhibition in the late gestation fetus.

## CLINICAL IMPLICATIONS

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The investigation of the ontogeny of megalin will have significant implications for our understanding of the developmental maturation of the body's ability to handle aminoglycosides. As previously shown in chapter 2, we found that megalin expression in preterm placenta is significantly higher than that of term placentas. Knowing that megalin is involved in the transport of AGs cross human placenta, this higher level of expression might potentially result in a higher extent of AGs transport across human preterm placenta, so more of the antibiotic will reach fetal serum and be available at the sites of aminoglycosides-induced toxicity (renal proximal tubular cells), Nevertheless, the levels of megalin expression and function in the fetal kidney will dictate the extent of AG accumulation in renal proximal tubules, hence the risk of AG-induced nephrotoxicity.

Therefore, the investigation of the ontogeny of megalin expression in renal tissue is also warranted. Moreover, preclinical studies of AG distribution in experimental animals would be very helpful in assessing the extent to which this variation in protein expression in either tissue (placenta or kidney) could impact the distribution kinetic profiles of AGs, including their ability to cross human placenta and accumulate in fetal renal tissues. Other factors that increase the risk of aminoglycosides induced nephrotoxicity should be taken in consideration such as the co-administration of nephrotoxic medications (such as vancomycin and corticosteroids) or other nephrotoxic agents, sepsis, dose and frequency of AG treatment.<sup>152</sup> If the risk is high, antibiotics other than AG-containing regimens could be considered such as broad spectrum  $\beta$ -lactams (ticarcillin, ceftriaxone) or vancomycin, clindamycin or erythromycin in cases of penicillin allergy. The choice should be based on the susceptibility of the infecting microorganisms, cost, and fetal safety.

Yet, it is still important to keep in mind that pregnancy-related infections are more common in preterm births. So, it is more likely that preterm infants had been already exposed to these antibiotics *in utero* or that they will continue to receive AGs during the early postnatal period as they are routinely administered to prevent or treat those infections. For all these reasons, preterm neonates with immature developing kidney are expected to be more prone to AG-induced nephrotoxicity.

Accordingly, the differential megalin expression according to the gestational age, in addition to other risk factors, would contribute to decisions with regard to the therapeutic dosing of AGs for preterm infants. Since AGs were found to induce nephrotoxicity in fetal rats that were exposed *in utero*, the pregnancy risk category of AGs is C. Information about

age-dependent changes of megalin function and expression in placenta and kidney could result in the change of AG pregnancy risk category according to the time of gestation (trimester) during which the drug is administered, which will ultimately modify clinical decisions of AG use, dose adjustment and therapeutic drug monitoring.

This research is the first to examine the effect of gestational age on megalin expression. It also demonstrates a placental cell line (BeWo cells) as a model to assess the role of megalin in the uptake of AGs by human placenta. Since we showed that megalin is at least partially involved in AG uptake by placenta, this knowledge will help future researchers to develop novel strategies to reduce/prevent fetal renal accumulation and the associated nephrotoxicity. In addition, it will affect clinical decisions of AG use for the treatment and prevention of infections acquired *in utero* according to the gestational age.



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## APPENDIX A: IRB PROTOCOL

### IRB APPROVAL NOTICE FOR PROTOCOL NUMBER (HM04212)

# VCU Memo

V i r g i n i a   C o m m o n w e a l t h   U n i v e r s i t y

Office of Research  
Office of Research Subjects Protection  
BioTechnology Research Park  
800 East Leigh Street, Suite 114  
P.O. Box 980568  
Richmond, Virginia 23298-0568  
  
(804) 827-0868  
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DATE: June 1, 2012

TO: Phillip M. Gerk, Pharm.D., Ph.D.  
School of Pharmacy  
Box 980533

FROM: Lea Ann Hansen, Pharm D *Lea Ann Hansen, Pharm D / EP*  
Chairperson, VCU IRB Panel D  
Box 980568

RE: **VCU IRB #: 04212**  
**Title: Placental Drug Transporters in Pregnancy**

On May 31, 2012, this research study was approved for continuation by expedited review according to 45 CFR 46.108(b) and 45 CFR 46.109(e) and 45 CFR 46.110 Category 3. This determination reflects the revisions received in the Office of Research Subjects Protection on May 30, 2012.

**PROTOCOL: Placental Drug Transporters in Pregnancy**

- Research Plan (Version 6, dated May 7, 2008; received by ORSP May 1, 2012)

**ADDITIONAL DOCUMENTS:**

- VCU IRB Study Personnel Roster (Version Date: 4/25/2012; received by ORSP May 1, 2012)

In addition, changes to your research study were approved in accordance with 110 (b) (2). This approval includes the following items reviewed by this Panel:

**HIPAA PROCESS:**

The following pathways for accessing and/or using PHI have been approved:

- Signed Authorization combined with Informed Consent

**VCU IRB APPROVED CONSENT/ASSENT FORMS (attached):**

- Research Subject Information and Consent Form (English, Version 3, dated May 16, 2012, 4 pages; received by ORSP May 30, 2012)
- El Formulario de Información y Consentimiento (Spanish, la versión 3, dated 30 de Mayo 2012, 5 pages; received by ORSP May 30, 2012)

**This approval expires on April 30, 2013.** Federal Regulations/VCU Policy and Procedures require continuing review prior to continuation of approval past that date. Continuing Review report forms will be mailed to you prior to the scheduled review.

The Primary Reviewer assigned to your research study is Ranjodh S. Gill, MD. If you have any questions, please contact Dr. Gill at [rgill@mcvh-vcu.edu](mailto:rgill@mcvh-vcu.edu) or 828-5323; or you may contact Elicia Preslan, IRB Coordinator, VCU Office of Research Subjects Protection, at [IRBPanelD@vcu.edu](mailto:IRBPanelD@vcu.edu) or 827-0899.

Attachment – Conditions of Approval

***Conditions of Approval:***

In order to comply with federal regulations, industry standards, and the terms of this approval, the investigator must (*as applicable*):

1. Conduct the research as described in and required by the Protocol.
2. Obtain informed consent from all subjects without coercion or undue influence, and provide the potential subject sufficient opportunity to consider whether or not to participate (unless Waiver of Consent is specifically approved or research is exempt).
3. Document informed consent using only the most recently dated consent form bearing the VCU IRB "APPROVED" stamp (unless Waiver of Consent is specifically approved).
4. Provide non-English speaking patients with a translation of the approved Consent Form in the research participant's first language. The Panel must approve the translated version.
5. Obtain prior approval from VCU IRB before implementing any changes whatsoever in the approved protocol or consent form, unless such changes are necessary to protect the safety of human research participants (e.g., permanent/temporary change of PI, addition of performance/collaborative sites, request to include newly incarcerated participants or participants that are wards of the state, addition/deletion of participant groups, etc.). Any departure from these approved documents must be reported to the VCU IRB immediately as an Unanticipated Problem (see #7).
6. Monitor all problems (anticipated and unanticipated) associated with risk to research participants or others.
7. Report Unanticipated Problems (UPs), including protocol deviations, following the VCU IRB requirements and timelines detailed in VCU IRB WPP VIII-7:
8. Obtain prior approval from the VCU IRB before use of any advertisement or other material for recruitment of research participants.
9. Promptly report and/or respond to all inquiries by the VCU IRB concerning the conduct of the approved research when so requested.
10. All protocols that administer acute medical treatment to human research participants must have an emergency preparedness plan. Please refer to VCU guidance on <http://www.research.vcu.edu/irb/guidance.htm>.
11. The VCU IRBs operate under the regulatory authorities as described within:
  - a) U.S. Department of Health and Human Services Title 45 CFR 46, Subparts A, B, C, and D (for all research, regardless of source of funding) and related guidance documents.
  - b) U.S. Food and Drug Administration Chapter I of Title 21 CFR 50 and 56 (for FDA regulated research only) and related guidance documents.
  - c) Commonwealth of Virginia Code of Virginia 32.1 Chapter 5.1 Human Research (for all research).

## VCU RESEARCH PLAN TEMPLATE

Use of this template is required to provide your VCU Research Plan to the IRB. Your responses should be written in terms for the non-scientist to understand. If a detailed research protocol (e.g., sponsor's protocol) exists, you may reference specific sections of that protocol. **NOTE: If that protocol does not address all of the issues outlined in each Section Heading, you must address the remaining issues in this Plan. It is NOT acceptable to reference a research funding proposal.**

**ALL Sections of the Human Subjects Instructions must be completed with the exception of the Section entitled "Special Consent Provisions."** Complete that Section if applicable. When other Sections are not applicable, list the Section Heading and indicate "N/A."

**NOTE: The Research Plan is required with ALL Expedited and Full review submissions and MUST follow the template, and include version number or date, and page numbers.**

### **DO NOT DELETE SECTION HEADINGS OR THE INSTRUCTIONS.**

#### I. TITLE

Assessment of drug, chemical and endogenous substance transport across human placenta

#### II. RESEARCH PERSONNEL

##### A. PRINCIPAL INVESTIGATOR

List the name of the VCU Principal Investigator

Phillip M. Gerk



**B. STUDY PERSONNEL****NOTE:**

1. Information pertaining to each project personnel, including their role, responsibilities, and qualifications, is to be submitted utilizing a *VCU IRB Study Personnel Information and Changes Form*. This form is available at <http://www.research.vcu.edu/forms/vcuirb.htm>.
2. A roster of all project personnel, including the principal investigator, medically responsible investigator, and non-VCU personnel, is to be maintained as a separate study document which is retained with the Research Plan, and is to be updated as necessary. This template document, entitled *VCU IRB Study Personnel Roster*, is available at <http://www.research.vcu.edu/forms/vcuirb.htm>.

**C. Describe the process that you will use to ensure that all persons assisting with the research are adequately informed about the protocol and their research-related duties and functions.**

All personnel will be provided with a copy of the study protocol and other forms. Prior to study initiation, the Principal Investigator will also meet with all study personnel to review the protocol and answer questions.

**III. CONFLICT OF INTEREST**

**Describe how the principal investigator and sub/co-investigators might benefit from the subject's participation in this project or completion of the project in general. Do not describe (1) academic recognition such as publications or (2) grant or contract based support of VCU salary commensurate with the professional effort required for the conduct of the project**

Other than the generation of new scientific knowledge, there are no other benefits (financial or otherwise) to the Principal Investigator for the subject's participation in this project or completion of the project in general

**IV. RESOURCES**

**Briefly describe the resources committed to this project including: (1) time available to conduct and complete the research, (2) facilities where you will conduct the research, (3) availability of medical or psychological resources that participants might require as a consequence of the research (if applicable), and (4) financial support.**

The clinical, personnel and laboratory resources available to the Principal Investigator are more than adequate to ensure the successful completion of the proposed research.

- (1) **Time available:** The Principal Investigator (PI) has sufficient time to conduct research such as that proposed in this application.
- (2) **Facilities:** Samples will be collected from the Labor and Delivery Unit in VCU Medical Center. Sample analyses will be performed in the Principal Investigator's laboratory located on the 3<sup>rd</sup> floor of the Smith Building.
- (3) **Medical or psychological resources needed:** N/A

**Financial Support:** Partial support for this study is provided by a grant from the Thomas F. and Kate Miller Jeffress Trust and the VCU School of Pharmacy.

## V. HYPOTHESIS

**Briefly state the problem, background, importance of the research, and goals of the proposed project.**

Insufficient literature is available describing the mechanisms by which drug and other substances are transported across the human placenta. Human placental tissue culture models are reliable and efficient mechanisms to evaluate placental transfer of various substances *in vitro*. However, the lack of readily available placental tissue samples, significantly limits our ability to develop and prepare an appropriate *in vitro* model when needed for a particular study. Therefore, the primary goals of the proposed research are to (1) investigate drug transport and metabolism across human placenta using *in vitro* models created from placentas collected immediately after delivery and (2) create a repository of placental tissue samples that can be used to investigate mechanisms of transplacental transport and metabolism. Specifically, the stored placental samples will be utilized in experiments seeking to generate mechanistic data toward understanding and predicting the placental handling of therapeutically important drugs, chemicals and/or other endogenous substances. The information generated from these investigations will ultimately allow us to maximize maternal and fetal health during pregnancy and after birth by optimizing maternal and fetal exposure to drugs and endogenous substances. Given that they will help us characterize the mechanisms involved in the transport of substances across placenta and their metabolism, these investigations will also aid in the development of strategies that may protect the fetus from harm occurring as a result of *in utero* drug exposure.

## VI. SPECIFIC AIMS

**Specific Aims:** The overall goals of this project are to (1) obtain human placentas that can be

used to evaluate transport functions *in vitro* and (2) create a repository of stored placental tissue samples that can be used in future investigations designed to evaluate the role of novel or known placental transporters, enzymes, receptors, and/or ion channels in mediating the uptake of substances during pregnancy. These goals will be achieved through the following specific aims:

1. Collect placentas from mothers, 18-45 year old, who deliver at VCU Hospital.
2. Prepare *in vitro* models (e.g., placental villous tissue fragments, intact placental perfusion model, and primary cytotrophoblast cells) from freshly collected tissue samples and perform mechanistic evaluations of trans-placental transport and metabolism.
3. Process freshly collected placental tissue and store at -80°C for future quantitative analyses (e.g., transporter, enzyme, receptor and/or ion channel expression).

## VII. BACKGROUND AND SIGNIFICANCE

**Include information regarding pre-clinical and early human studies. Attach appropriate citations.**

During gestation, the fetus is continuously exposed to a wide variety of drugs, chemicals and endogenous substances via transport from the maternal circulation. Exchange of substances between the fetal and maternal circulations is mediated by the placental chorionic villus. The outer layer of the chorionic villus is composed of multinucleated syncytiotrophoblast cells joined together by tight intercellular junctions. Unlike many other species, the apical (i.e., outside or maternal-facing) surface of the syncytiotrophoblasts in human placenta is directly bathed in maternal blood. The syncytiotrophoblast layer forms the main barrier to the free passage of drug molecules between maternal and fetal blood.

Within the membranes of the syncytiotrophoblasts there are various enzymes, receptors and transporters that are differentially distributed throughout the tissue. These include integral membrane proteins located on the apical surface of syncytiotrophoblasts that use ATP as an energy source to drive the efflux of substances away from the fetus. Other membrane proteins, such as the organic anionic transporters (OATs), mediate the uptake and/or the transport of substances across the syncytiotrophoblasts. This coordinated process is critical to ensure the transport of various nutrients (vitamins, minerals), proteins (albumin, immunoglobulins) and enzymes necessary for the proper growth and development to the developing fetus. There is great concern, however, that the fetus may be inadvertently exposed to drugs and/or other potentially harmful chemicals ingested by the mother, leading to uncertainty regarding the safety of medication use during pregnancy. Nevertheless, it is often necessary for women to take medications during pregnancy to treat chronic medical conditions (e.g., diabetes, seizures) and/or medical problems arising during pregnancy (e.g., hypertension, infections). There are also some instances (e.g., infections) where medications are administered to the mother for the purpose of treating the fetus *in utero* and achieving "therapeutic" fetal drug levels.

Much of the uncertainty regarding the safety of medication use in pregnancy is related to the lack of information regarding the extent of fetal exposure occurring with maternal drug treatment. Given the difficulty in conducting maternal-fetal pharmacokinetic studies, it is often not possible to obtain this information directly from pregnant patients. However, human placental tissue samples and *in vitro* models derived from such tissues (e.g., placental villous tissue fragments, perfused placenta) are useful tools to characterize the mechanisms involved in and extent of transplacental transport of drugs, chemicals and endogenous substances. Unfortunately, availability of placental samples is often a limiting factor in the ability to conduct these types of investigations. Collecting placental samples via this protocol will greatly facilitate our ability to develop *in vitro* models (e.g., placental villous tissue, explants or placental cotyledons) that we can use to conduct transport and/or metabolism experiments. Establishing a repository of placental tissue samples will also give us ready access to human samples that can be used for future quantitative analyses.

#### **VIII. PRELIMINARY PROGRESS/DATA REPORT**

**If available.**

**Not applicable**

#### **IX. RESEARCH METHOD AND DESIGN**

**Include a brief description of the project design including the setting in which the research will be conducted and procedures. If applicable, include a description of procedures being performed already for diagnostic or treatment purposes.**

The Principal Investigator will be responsible for maintaining the integrity of all stored samples and data. Other individuals such as students, trainees and collaborators at VCU may have access to the samples. The role of these individuals will include the use of placental tissue samples to perform experiments that are related to this particular research and related research. A code containing the protocol number will be assigned to each tissue sample and released to collaborating investigators. The code will also be used to associate the tissue sample with non-identifiable clinical sample data (Maternal age and parity, height & weight, drug treatment regimens, prior and current medical conditions, obstetric history, fetal gestational age, birth weight and height, Apgar scores and time of birth will be recorded from the maternal and neonatal charts. If received intact, placental weight and gross placental abnormalities (if any) will be documented). Identifiable

data will only be used temporarily until the non-identifiable clinical data are obtained from the medical records. Identifiable data include: patient name, date of birth, date of admission, date of delivery, and medical record number. The identifiable data will be stored only on paper (not electronically) in a locked cabinet (to which only Dr. Gerk has the key) in Dr. Gerk's office. After the non-identifiable data are obtained, the identifiable data will be promptly and confidentially destroyed. Samples will be stored in the principal investigator's laboratory in the Smith building on the VCU Medical Center campus, will be kept at -70°C (Smith room 514) or -80°C (Smith room 321) and will be labeled with a non-identifiable code specific for each sample. All personnel who gain access to the stored samples will be appropriately trained and qualified. There will be no electronic storage format that includes identifiable data.

**Specific Aim 1: Collect placentas from mothers who deliver at VCU Hospital. Women ages 18 to 45 years presenting for delivery at VCU Medical Center will be eligible for enrollment.** The principal investigator will coordinate with the medically responsible investigator (Dr. Susan Lanni) in the Labor and Delivery Department of VCUHS to obtain the placentas. After delivery, the PI and/or the student will be contacted by the responsible nurse to obtain the resultant placenta. Placentas that would otherwise be discarded as medical waste will be collected within 60 minutes of delivery. If necessary, the physician may send the placenta to Pathology for further examination, as commonly occurs for preterm births. If the placenta is sent to Pathology, a sample of the placental tissue may be obtained from Pathology with permission. All placentas will be visually inspected and any gross abnormalities will be recorded along descriptive parameters (e.g., weight, condition of the maternal and fetal surface). Information, including maternal age and parity, height & weight, drug treatment regimens, prior and current medical conditions, obstetric history, fetal gestational age, birth weight and height, Apgar scores and time of birth will be recorded from the maternal and neonatal charts.

**Specific Aim 2: Prepare in vitro models (e.g., placental villous tissue fragments, intact placental perfusion model or primary cytotrophoblasts cells) from freshly collected tissue samples and perform mechanistic evaluations of trans-placental transport.** Freshly collected placental tissue samples will be processed as required to prepare various in vitro transport models including but not limited to placental villous tissue fragments and intact placental perfusion models. For investigations into regulatory mechanisms of substance transport, tissue will be minced and explants will be cultured as described and incubated with selected transporter probe compounds and/or other useful probe compounds and clinically relevant drugs to investigate transport. Concentrations of these agents will be measured in the buffer or medium surrounding the tissue, as well as in homogenized tissue, using either HPLC or microplate detection methods. Expression and/or function of various proteins such as nuclear receptors and transcription factors, drug metabolizing enzymes, placental transporters, or cellular housekeeping proteins will also be evaluated. Transport of radio- or fluorescein-labeled substrates will be examined as function of time, temperature and concentration. Moreover, the uptake of substrates will be assessed in the presence or absence of known receptor inhibitors.

**Specific Aim 3: Process freshly collected placental tissue and store at -80°C for future quantitative analyses (e.g., transporter, receptor and/or ion channel expression).** Freshly collected placental tissue samples will be frozen at -80°C for future quantitative analyses of transporter, enzyme, receptor and/or ion channel expression. Protein expression will be

assessed using techniques such as Western blot. Transcription of mRNA for proteins of interest will also be determined by isolating RNA from placental tissue and performing quantitative PCR.

#### **X. PLAN FOR CONTROL OF INVESTIGATIONAL DRUGS, BIOLOGICS, AND DEVICES.**

**Investigational drugs and biologics:** IF Investigational Drug Pharmacy Service (IDS) is not being used, attach the IDS confirmation of receipt of the management plan.

**Investigational and humanitarian use devices (HUDs):** Describe your plans for the control of investigational devices and HUDs including:

- (1) how you will maintain records of the product's delivery to the trial site, the inventory at the site, the use by each subject, and the return to the sponsor or alternative disposition of unused product(s);**
- (2) plan for storing the investigational product(s)/ HUD as specified by the sponsor (if any) and in accordance with applicable regulatory requirements;**
- (3) plan for ensuring that the investigational product(s)/HUDs are used only in accordance with the approved protocol; and**
- (4) how you will ensure that each subject understands the correct use of the investigational product(s)/HUDs (if applicable) and check that each subject is following the instructions properly (on an ongoing basis).**

Not applicable

#### **XI. DATA ANALYSIS PLAN**

**For investigator-initiated studies.**

For quantitative experiments, normalized band intensities for each protein or mRNA product will be compared by two-tailed Student's t-test,  $p < 0.05$ . The significance of differences observed amongst data means for substrate transport or metabolism will be assessed via two-way analysis of variance (ANOVA) followed by an appropriate post-hoc test. Effects of variables on

parameters will be analyzed by ANOVA followed by post-hoc tests appropriate. Saturation experiments will be performed to determine the kinetic parameters  $K_m$  and  $V_{max}$  by nonlinear regression. Statistical testing will be performed using GraphPad Prism 5.0 or other suitable software.

## **XII. DATA AND SAFETY MONITORING**

- If the research involves greater than minimal risk and there is no provision made for data and safety monitoring by any sponsor, include a data and safety-monitoring plan that is suitable for the level of risk to be faced by subjects and the nature of the research involved.
- If the research involves greater than minimal risk, and there is a provision made for data and safety monitoring by any sponsor, describe the sponsor's plan.
- If you are serving as a Sponsor-Investigator, identify the Contract Research Organization (CRO) that you will be using and describe the provisions made for data and safety monitoring by the CRO. Guidance on additional requirements for Sponsor-Investigators is available at <http://www.research.vcu.edu/irb/wpp/flash/X-2.htm>

The proposed research is does not involves no more than minimal risk to participants.

## **XIII. MULTI-CENTER STUDIES**

If VCU is the lead site in a multi-center project or the VCU PI is the lead investigator in a multi-center project, describe the plan for management of information that may be relevant to the protection of subjects, such as reporting of unexpected problems, project modifications, and interim results.

Not applicable

## **XIV. INVOLVEMENT OF NON-VCU INSTITUTIONS/SITES (DOMESTIC AND FOREIGN)**

1. Provide the following information for each non-VCU institution/site (domestic and foreign) that has agreed to participate:

- Name of institution/site
- Contact information for institution/site
- Engaged in Research or not (if YES AND the research involves a DIRECT FEDERAL AWARD made to VCU, include FWA #). See OHRP's guidance on "Engagement of Institutions in Research" at <http://www.hhs.gov/ohrp/policy/engage08.html>.
- Request for the VCU IRB to review on behalf of the Non-VCU institution? See requirements found at <http://www.research.vcu.edu/irb/wpp/flash/XVII-6.htm>.
- See VCU WPPs:  
<http://www.research.vcu.edu/irb/wpp/flash/XVII-6.htm> and  
<http://www.research.vcu.edu/irb/wpp/flash/XVII-11.htm>.

		Engaged (Y/N)	Request for VCU IRB
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Name of Institution	Contact Information for Site	and FWA # if applicable	to review on behalf of the non-VCU institution (Y/N)*

\*NOTE: If a Non-VCU site is engaged in the research, the site is obligated to obtain IRB review or request that the VCU IRB review on its behalf.

**2. Provide a description of each institution's role (whether engaged or not) in the research, adequacy of the facility (in order to ensure participant safety in the case of an unanticipated emergency), responsibilities of its agents/employees, and oversight that you will be providing in order to ensure adequate and ongoing protection of the human subjects. You should only identify institutions that have agreed to participate. If additional institutions agree to participate at a later time, they must be added by amendment to the protocol.**

**Not applicable**

#### **XV. HUMAN SUBJECTS INSTRUCTIONS**

**ALL sections of the Human Subjects Instructions must be completed with the exception of the section entitled "Special Consent Provisions." Complete that section if applicable.**

##### **A. DESCRIPTION**

**Provide a detailed description of the proposed involvement of human subjects or their private identifiable data.**

Placentas that would otherwise be discarded as medical waste will be collected from subjects within 60 minutes of delivery and used to perform investigations regarding the maternal-fetal transport of drugs, chemical and endogenous substances. Some placental tissues will be obtained from Pathology. The Principal Investigator will be responsible for maintaining the integrity of all stored samples and data. Other individuals such as students, trainees and collaborators at VCU may have access to the samples. The role of these individuals will include the use of placental tissue samples to perform experiments that are related to this particular research and related research. A code containing the protocol number will be assigned to each tissue sample and released to collaborating investigators. The code will also be used to associate the tissue sample with non-identifiable clinical sample data (Maternal age and parity, height & weight, drug treatment regimens, prior and current medical conditions, obstetric history, fetal gestational age, birth weight and height, Apgar scores and time of birth will be recorded from the maternal and neonatal charts. If received intact, placental weight and gross placental abnormalities (if any) will be documented).

Identifiable data will only be used temporarily until the non-identifiable clinical data are obtained from the medical records. Identifiable data include: patient name, date of birth, date of admission, date of delivery, and medical record number. The identifiable data will be stored only on



paper (not electronically) in a locked cabinet (to which only Dr. Gerk has the key) in Dr. Gerk's office. After the non-identifiable data are obtained, the identifiable data will be promptly and confidentially destroyed. Samples will be stored in the principal investigator's laboratory in the Smith building on the VCU Medical Center campus, will be kept at -70°C (Smith room 514) or -80°C (Smith room 321) and will be labeled with a non-identifiable code specific for each sample. All personnel who gain access to the stored samples will be appropriately trained and qualified. There will be no electronic storage format that includes identifiable data.

## **B. SUBJECT POPULATION**

**Describe the subject population in terms of sex, race, ethnicity, age, etc., and your access to the population that will allow recruitment of the necessary number of participants. Identify the criteria for inclusion or exclusion of all targeted populations and include a justification for any exclusions. Explain the rationale for the involvement of special cases of subjects, such as children, pregnant women, human fetuses, neonates, prisoners or others who are likely to be vulnerable. If you plan to allow for the enrollment of Wards of the State (or any other agency, institution, or entity), you must specifically request their inclusion and follow guidance in VCU IRB WPP XV-3: Wards and Emancipated Minors available at <http://www.research.vcu.edu/irb/wpp/flash/XV-3.htm>.**

A minimum of 100 women ages 18 to 45 years presenting for delivery at VCU Medical Center will be eligible for enrollment. Pregnant women will be the only subjects included since placental tissue samples are to be collected. The ethnic makeup will reflect that of the patient population at VCU Medical Center Obstetrics Unit.

## **C. RESEARCH MATERIAL**

**Identify the sources of research material obtained from individually identifiable living human subjects in the form of specimens, records, or data. Indicate whether the material or data will be obtained specifically for research purposes or whether use will be made of existing specimens, records, or data.**

This study will only use existing specimens, records and clinical data. Placentas that would otherwise be discarded as medical waste will be collected from subjects within 60 minutes of delivery or obtained from Pathology and used to perform investigations regarding the maternal-fetal transport of drugs, chemical and endogenous substances. The Principal Investigator will be responsible for maintaining the integrity of all stored samples and data. Other individuals such as students, trainees and collaborators at VCU may have access to the samples. The role of these

individuals will include the use of placental tissue samples to perform experiments that are related to this particular research and related research. A code containing the protocol number will be assigned to each tissue sample and released to collaborating investigators. The code will also be used to associate the tissue sample with non-identifiable clinical sample data (Maternal age and parity, height & weight, drug treatment regimens, prior and current medical conditions, obstetric history, fetal gestational age, birth weight and height, Apgar scores and time of birth will be recorded from the maternal and neonatal charts. If received intact, placental weight and gross placental abnormalities (if any) will be documented).

Identifiable data will only be used temporarily until the non-identifiable clinical data are obtained from the medical records. Identifiable data include: patient name, date of birth, date of admission, date of delivery, and medical record number. The identifiable data will be stored only on paper (not electronically) in a locked cabinet (to which only Dr. Gerck has the key) in Dr. Gerck's office. After the non-identifiable data are obtained, the identifiable data will be promptly and confidentially destroyed. Samples will be stored in the principal investigator's laboratory in the Smith building on the VCU Medical Center campus, will be kept at -70°C (Smith room 514) or -80°C (Smith room 321) and will be labeled with a non-identifiable code specific for each sample. All personnel who gain access to the stored samples will be appropriately trained and qualified. There will be no electronic storage format that includes identifiable data.

#### **D. RECRUITMENT PLAN**

**Describe in detail your plans for the recruitment of subjects including:**

- (1) how potential subjects will be identified (e.g., school personnel, health care professionals, etc),**
- (2) how you will get the names and contact information for potential subjects, and**
- (3) who will make initial contact with these individuals (if relevant) and how that contact will be done.**

**If you plan to involve special cases of subjects, such as children, pregnant women, human fetuses, neonates, prisoners or others who are likely to be vulnerable, describe any special recruitment procedures for these populations.**

Patients meeting the inclusion/exclusion criteria will be recruited for participation in this study. Potential subjects will be identified from using data available in their medical records, where we can also find names and contact information for the potential participants (Knowing that after the non-identifiable data are obtained, the former data will be promptly and confidentially destroyed). Given that the study involves no more than minimal risk, no special recruitment procedures will be utilized for subjects' recruitment.

## E. PRIVACY OF PARTICIPANTS

**NOTE:** Privacy refers to individuals and their interests in controlling access to their identities, their physical person, and how and what kind of information is obtained about them. Privacy also encompasses the interests of defined communities (e.g. those with a certain diagnosis or social circumstance) in controlling access to the group identity and information about the group or individuals as part of the group.

Describe how the privacy interests of subjects (and communities, if appropriate) will be protected including:

- (1) in the research setting (e.g., in the identification, recruitment, and intervention settings) and
- (2) with the information being sought and the way it is sought. For example, providing drapes or barriers, interviewing in a private room, and collecting only the amount of sensitive information needed for identification, recruitment, or the conduct of the study.

Every effort will be made to protect the confidentiality of the subject during the screening and enrollment process.

Identifiable data will only be used temporarily until the non-identifiable clinical data are obtained from the medical records. After the non-identifiable data are obtained, the identifiable data will be promptly and confidentially destroyed. . There will not be electronic storage of identifiable data.

## F. CONFIDENTIALITY OF DATA

**NOTE:** Confidentiality refers to the way private, identifiable information about a subject or defined community is maintained and shared.

Check all of the following precautions that will be used to maintain the confidentiality of identifiable information:

- ☒ Paper-based records will be kept in secure location and only accessed by authorized study personnel
- ☐ Electronic records will be made available only to those personnel in the study through the use of access controls and encryption
- ☐ Identifiers will be removed from study-related data (data is coded with a key stored in a

separate secure location)

- ☐ For research involving web-based surveys, data is secured via passwords and encryption
- ☐ Audio or video recordings of subjects will be transcribed and then destroyed to prevent audio or visual identification. Note the date of destruction (e.g., 3 months from close of study; after transcription is determined to be error free).
- ☐ Obtaining a Certificate of Confidentiality
- ☒ Other precautions: The Principal Investigator will be responsible for maintaining the integrity of all stored samples and data. Other individuals such as students, trainees and collaborators at VCU may have access to the samples. The role of these individuals will include the use of placental tissue samples to perform experiments that are related to this particular research and related research. A code containing the protocol number will be assigned to each tissue sample and released to collaborating investigators. The code will also be used to associate the tissue sample with non-identifiable clinical sample data. Identifiable data will only be used temporarily until the non-identifiable clinical data are obtained from the medical records. The identifiable data will be stored only on paper (not electronically) in a locked cabinet (to which only Dr. Gerk has the key) in Dr. Gerk's office. After the non-identifiable data are obtained, the identifiable data will be promptly and confidentially destroyed. Samples will be labeled with a non-identifiable code specific for each sample. All personnel who gain access to the stored samples will be appropriately trained and qualified. There will be no electronic storage format that includes identifiable data.

#### **G. POTENTIAL RISKS**

**Describe potential risks (physical, psychological, social, legal, or other) and assess their likelihood and seriousness. Where appropriate, describe alternative treatments and procedures that might be advantageous to the subjects.**

The study utilizes existing specimens and data and involves no more than minimal risk to study participants.

#### **H. RISK REDUCTION**

**Describe procedures for protecting against or minimizing potential risk. Where appropriate, discuss provisions for ensuring necessary medical or professional intervention in the event of adverse events to the subjects. Describe the provisions for monitoring the data collected to ensure the safety of subjects, if any.**

The study utilizes existing specimens and data and involves no more than minimal risk to study participants.

#### **I. ADDITIONAL SAFEGUARDS FOR VULNERABLE PARTICIPANTS**

**Describe any additional safeguards to protect the rights and welfare of participants if you plan to involve special cases of subjects such as children, pregnant women, human fetuses, neonates, prisoners or others who are likely to be vulnerable.**

**Safeguards to protect the rights and welfare of participants might relate to Inclusion/Exclusion Criteria:** (“Adults with moderate to severe cognitive impairment will be excluded.” “Children must have diabetes. No normal controls who are children will be used.”) **Consent:** (“Participants must have an adult care giver who agrees to the participant taking part in the research and will make sure the participant complies with research procedures.” “Adults must be able to assent. Any dissent by the participant will end the research procedures.”) **Benefit:** (“Individuals who have not shown benefit to this type of drug in the past will be excluded.”).

The study uses existing specimens and clinical data and the risk to participants is no more than minimal risk. No special provision for pregnant subjects are therefore required.

#### **J. RISK/BENEFIT**

**Discuss why the risks to participants are reasonable in relation to the anticipated benefits to subjects and in relation to the importance of the knowledge that may reasonably be expected to result. If a test article (investigational new drug, device, or biologic) is involved, name the test article and supply the FDA approval letter.**

Subjects will not benefit directly from the research. However, the information gained from the conduct of this work will allow women and their health care providers to make safer, more informed medication decisions regarding medication use during pregnancy. Since data are greatly lacking regarding the extent and mechanisms of drug transfer across the human placenta, and since the risks to the subject are no more than minimal, the benefits of participation outweigh the potential risks.

#### **K. COMPENSATION PLAN**

**Compensation for participants (if applicable) should be described, including possible total compensation, pro-rating, any proposed bonus, and any proposed reductions or penalties for not completing the project.**

Subjects will not be compensated for their participation in the study.

**1. CONSENT PROCESS**

Indicate who will be asked to provide consent/assent, who will obtain consent/assent, what language (e.g., English, Spanish) will be used by those obtaining consent/assent, where and when will consent/assent be obtained, what steps will be taken to minimize the possibility of coercion or undue influence, and how much time will subjects be afforded to make a decision to participate.

Not applicable

**2. SPECIAL CONSENT PROVISIONS**

If some or all subjects will be cognitively impaired, or have language/hearing difficulties, describe how capacity for consent will be determined. Consider using the VCU Informed Consent Evaluation Instrument available at <http://www.research.vcu.edu/irb/guidance.htm>. If you anticipate the need to obtain informed consent from legally authorized representatives (LARs), please describe how you will identify an appropriate representative and ensure that their consent is obtained. Guidance on LAR is available at <http://www.research.vcu.edu/irb/wpp/flash/XI-3.htm>.

Not applicable

**3. ASSENT PROCESS**

If applicable, explain the Assent Process for children or decisionally impaired subjects. Describe the procedures, if any, for re-consenting children upon attainment of adulthood. Describe procedures, if any, for consenting subjects who are no longer decisionally impaired. Guidance is available at <http://www.research.vcu.edu/irb/wpp/flash/XV-2.htm> and <http://www.research.vcu.edu/irb/wpp/flash/XVII-7.htm>.

Not applicable

**4. REQUESTS FOR WAIVERS OF CONSENT (COMPLETE IF REQUESTING ANY TYPE OF WAIVER OF CONSENT OR ASSENT)**

**4-B. REQUEST TO WAIVE DOCUMENTATION OF CONSENT:** A waiver of documentation occurs when the consent process occurs but participants are not required to sign the consent form. Guidance is available at [http://www.research.vcu.edu/irb/wpp/flash/wpp\\_guide.htm#XI-2.htm](http://www.research.vcu.edu/irb/wpp/flash/wpp_guide.htm#XI-2.htm). One of the following two conditions must be met to allow for consenting without signed documentation. Choose which condition is applicable and explain why (explanation required):

☐ The only record linking the participant and the research would be the informed consent form. The principal risk to the participant is the potential harm resulting from a breach of confidentiality. Each participant will be asked whether he/she wants documentation linking the participant with the research and the participants wishes will govern. → Explain how your study fits into the category:

☐ The research presents no more than minimal risk of harm to participants & involves no procedures for which signed consent is normally required outside of the research context. → Explain how your study fits into the category:

**4-C. REQUEST TO WAIVE SOME OR ALL ELEMENTS OF ASSENT FROM CHILDREN  $\geq$  AGE 7 OR FROM DECISIONALLY IMPAIRED INDIVIDUALS:** A waiver of assent means that the IRB is not requiring the investigator to obtain assent OR the IRB approves an assent form that does not include some/all of the required elements. Guidance is available at <http://www.research.vcu.edu/irb/wpp/flash/XV-2.htm>.

**4-C.1. Explain why a waiver or alteration of informed consent is being requested.**

**In order for the IRB to approve a request for waiver of assent, the conditions for 4-C.2, 4-C.3, OR 4-C.4 must be met. Check which ONE applies and explain all required justifications.**

**4-C.2.** ☐ Some or all of the individuals age 7 or higher will not be capable of providing assent based on their developmental status or impact of illness. → Explain how your study meets this criteria:

**4-C.3.** ☐ The research holds out a prospect of direct benefit not available outside of the

**research.** → Explain how your study meets this criteria:

**4-C.4. ☐ Describe how this study meets ALL FOUR of the following conditions:**

- The research involves no more than minimal risk to the participants. → Explain how your study meets this criteria:
  
- The waiver or alteration will not adversely affect the rights and welfare of participants. → Explain how your study meets this criteria:
  
- The research could not practicably be carried out without the waiver or alteration. → Explain how your study meets this criteria:
  
- Will participants be provided with additional pertinent information after participation?  
☐ Yes  
☐ No → Explain why not:

**4-D. REQUEST TO WAIVE CONSENT FOR EMERGENCY RESEARCH:** Describe how the study meets the criteria for emergency research and the process for obtaining LAR consent is appropriate. See guidance at <http://www.research.vcu.edu/irb/wpp/flash/XVII-16.htm>.

**5. GENETIC TESTING**

**If applicable, address the following issues related to Genetic Testing.**

**5-A. FUTURE CONTACT CONCERNING FURTHER GENETIC TESTING RESEARCH**

**Describe the circumstances under which the subject might be contacted in the future concerning further participation in this or related genetic testing research.**

Not applicable



**5-B.FUTURE CONTACT CONCERNING GENETIC TESTING RESULTS**

If planned or possible future genetic testing results are unlikely to have clinical implications, then a statement that the results will not be made available to subjects may be appropriate. If results might be of clinical significance, then describe the circumstances and procedures by which subjects would receive results. Describe how subjects might access genetic counseling for assistance in understanding the implications of genetic testing results, and whether this might involve costs to subjects. Investigators should be aware that federal regulations, in general, require that testing results used in clinical management must have been obtained in a CLIA-certified laboratory.

Not applicable

**5-C.WITHDRAWAL OF GENETIC TESTING CONSENT**

Describe whether and how subjects might, in the future, request to have test results and/or samples withdrawn in order to prevent further analysis, reporting, and/or testing.

Not applicable

**5-D.GENETIC TESTING INVOLVING CHILDREN OR DECISIONALLY IMPAIRED PARTICIPANTS**

Describe procedures, if any, for consenting children upon the attainment of adulthood. Describe procedures, if any, for consenting participants who are no longer decisionally impaired.

Not applicable

**5-E.CONFIDENTIALITY OF GENETIC INFORMATION**

Describe the extent to which genetic testing results will remain confidential and special

precautions, if any, to protect confidentiality.

Not applicable

VCU IRB

## APPENDIX A: HIPAA FOR RESEARCH

<b>PRINCIPAL INVESTIGATOR:</b>	Phillip M. Gerk, Pharm.D., Ph.D.
<b>EMAIL:</b>	pmgerk@vcu.edu
<b>RESEARCH COORDINATOR:</b>	
<b>EMAIL:</b>	
<b>P.O. BOX #:</b>	980533
<b>STUDY TITLE:</b>	Assessment of drug, chemical and endogenous substance transport across human placenta

## SECTION A: GENERAL INFORMATION

### 1. Describe the health information that will be obtained or used in this research.

To link a placental tissue sample with medical records, it will be necessary to temporarily record the patient's name, date of birth and/or admission, as well as medical records number. This will facilitate the collection of the following sample information: Maternal age and parity, height & weight, drug treatment regimens, prior and current medical conditions, obstetric history, fetal gestational age, birth weight and height, Apgar scores and time of birth will be recorded from the maternal and neonatal charts. If received intact, placental weight and gross placental abnormalities (if any) will be documented. A code containing the protocol number will be assigned to each tissue sample and released to collaborating investigators. The code will also be used to associate the tissue sample with non-identifiable clinical sample data. Identifiable data will only be used temporarily until the non-identifiable clinical data are obtained from the medical records. The identifiable data will be stored only on paper (not electronically) in a locked cabinet (to which only Dr. Gerk has the key) in Dr. Gerk's office. After the non-identifiable data are obtained, the identifiable data will be promptly and confidentially destroyed. Samples will be labeled with a non-identifiable code specific for each sample.

### 2. Indicate the source(s) of the health information. (check all that apply)

- ☒ VCUHS medical records
- ☐ Non-VCUHS health care provider medical records
- ☐ PHI held by a component of the VCU ACE (other than VCUHS)
- ☐ Directly from the research participant (e.g., physical exams, diagnostic results, interviews and questionnaires)
- ☐ Records open to the public
- ☐ Other (please specify):

**3. Explain how the PHI collected or used in this research is the minimum necessary to accomplish the research.**

Every effort will be made to protect the confidentiality of the subject during the screening and enrollment process.

No identifiable medical information will be retained. There will not be electronic storage of identifiable data.

We do not need to retain identifiable data from the patients for this research; we only need access to the medical records and the information listed in Part 1. After obtaining non-identifiable data from the medical records, all identifiable data will be promptly and confidentially destroyed.

**4. Select all of the identifiers that will be used in this research.**

- |                                                                                               |                                                                                |                                                                              |
|-----------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|------------------------------------------------------------------------------|
| <input checked="" type="checkbox"/> Names                                                     | <input type="checkbox"/> Social security numbers                               | <input type="checkbox"/> IP addresses                                        |
| <input checked="" type="checkbox"/> Dates (e.g., birth, admission, death)                     | <input checked="" type="checkbox"/> Medical record numbers                     | <input type="checkbox"/> License numbers                                     |
| <input type="checkbox"/> Phone numbers                                                        | <input type="checkbox"/> Health plan beneficiary numbers                       | <input type="checkbox"/> Internet URLs                                       |
| <input type="checkbox"/> Fax numbers                                                          | <input type="checkbox"/> Device identifiers & serial numbers                   | <input type="checkbox"/> Vehicle ID & serial numbers                         |
| <input type="checkbox"/> Ages $\geq 89$                                                       | <input type="checkbox"/> Full-face photos or comparable                        | <input type="checkbox"/> Biometric identifiers                               |
| <input type="checkbox"/> Geographic subdivisions smaller than state (e.g., city, county, zip) | <input type="checkbox"/> Account numbers (e.g., bank, invoice#, credit card #) | <input type="checkbox"/> Other unique identifying #, code, or characteristic |

☐ None of the above

**5. Select all pathways this research will employ or use to access PHI.**

- ☐ De-identified data [FINISHED WITH THIS FORM AFTER THIS QUESTION]
- ☐ All identifiers removed (safe harbor)
- ☐ Statistical analysis verifying no possibility of re-identification [SUBMIT ATTESTATION FROM STATISTICIAN WITH THIS FORM]
- ☐ Limited Data Set (may ONLY include city, state, zip code, dates, and ages) [COMPLETE DATA USE AGREEMENT]
- ☒ Waiver of Authorization [COMPLETE SECTION B]
- ☐ Partial Waiver of Authorization for Recruitment (allows access to PHI to contact potential participants who will sign consent and authorization upon enrollment) [COMPLETE SECTION C]
- ☐ Signed Authorization from participants in a combined Informed Consent and Authorization form [FINISHED WITH THIS FORM]
- ☐ Signed Authorization from participants in a separate Authorization form [FINISHED WITH THIS FORM]

**SECTION B: WAIVER OF AUTHORIZATION**

**1. Describe how the use of PHI in this study poses no greater than minimal risk to participants' privacy.**

We will not publish any PHI, nor will we provide it to others or use it beyond when it is required.

**2. When will identifiers be destroyed? (Identifiers must be destroyed at earliest opportunity)**

- ☐ End of the study
- ☐ \_\_\_\_\_ years after the end of the study (enter # of years)
- ☒ Other (please specify): After samples are labeled with a code related to non-identifiable data, the identifiable data will be promptly and confidentially destroyed.

**3. Other than the PI and research personnel, who else will have access to the health information?**

Nobody.

**4. Explain why this research cannot practicably be conducted without the use of PHI.**

We need only temporary access to the medical information in order to facilitate collection of research data described above.

**5. Explain why this research cannot practicably be conducted without a waiver of authorization.**

It is not possible to consent patients outside of usual business hours; thus a need to obtain informed consent would greatly inhibit this research.

**Assurances**

In applying for a waiver of authorization, I agree to the following:

- A) The identifiers used for this research study will not be used for any other purpose or disclosed to any other person or entity (aside from members of the research team identified in the research application), except as required by law.
- B) If at any time I want to reuse this information for other purposes or disclose the information to other individuals, I will seek approval from the IRB.
- C) I will comply with VCU HIPAA policies and procedures and with the use and disclosure restrictions described above.
- D) I assume responsibility for all uses and disclosures of the PHI by members of the study team.

**SIGNATURE OF PRINCIPAL  
INVESTIGATOR OR  
DESIGNEE:**

**DATE OF  
SIGNATURE:**

**SECTION C: PARTIAL WAIVER OF AUTHORIZATION**

**1. Describe how the use of PHI for recruitment poses no greater than minimal risk to participants' privacy.**

**2. When will identifiers be destroyed? (Identifiers must be destroyed at earliest opportunity)**

- ☐ Following participant contact
- ☐ Following participant enrollment
- ☐ Upon reaching study accrual objectives
- ☐ Other (please specify):

**3. Other than the PI and research personnel, who else will have access to the health information?**

**4. Explain why this recruitment cannot practicably be conducted without the use of PHI.**

**5. Explain why the recruitment cannot practicably be conducted without the partial waiver of authorization.**

#### **Assurances**

In applying for a partial waiver of authorization, I agree to the following:

- A) The identifiers used for this research study will not be used for any other purpose or disclosed to any other person or entity (aside from members of the research team identified in the research application), except as required by law.
- B) If at any time I want to reuse this information for other purposes or disclose the information to other individuals, I will seek approval from the IRB.
- C) I will comply with VCU HIPAA policies and procedures and with the use and disclosure restrictions described above.
- D) I assume responsibility for all uses and disclosures of the PHI by members of the study team.

**SIGNATURE OF PRINCIPAL  
INVESTIGATOR:**

**DATE OF  
SIGNATURE:**

## IRB APPROVAL NOTICE FOR PROTOCOL NUMBER (HM14035)

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# VCU Memo

V i r g i n i a C o m m o n w e a l t h U n i v e r s i t y

Office of Research  
Office of Research Subjects Protection

BioTechnology Research Park  
800 East Leigh Street, Suite 114  
P.O. Box 980568  
Richmond, Virginia 23298-0568

(804) 828-0868  
Fax: (804) 827-1448

DATE: May 4, 2012

TO: Phillip M. Gerk, Pharm.D., Ph.D.  
Pharmaceutics  
Box 980533

FROM: Lea Ann Hansen, Pharm D *Lea Ann Hansen, PharmD /EP*  
Chairperson, VCU IRB Panel D  
Box 980568

RE: **VCU IRB #: HM14035**  
**Title: Assessment of Drug, Chemical, and Endogenous Substance Transport Across Human Placenta**

On May 3, 2012, the following research study was approved by expedited review according to 45 CFR 46.110 Category 5. This approval reflects the revisions received in the Office of Research Subjects Protection on April 10, 2012. This approval includes the following items reviewed by this Panel:

**PROTOCOL: Assessment of Drug, Chemical, and Endogenous Substance Transport Across Human Placenta**

- Research Plan (Version 4, dated 4/10/2012; received by ORSP April 10, 2012)

**HIPAA PROCESS:**

The following pathways for accessing and/or using PHI have been approved:

- Waiver of Authorization: The three criteria for waiver of authorization have been met [45 CFR 164.512(i)(1)(i)]

**CONSENT/ASSENT:**

- All four conditions for waiver of consent have been met. See §45 CFR 46.116(d). The IRB Panel has waived all elements of consent.

**ADDITIONAL DOCUMENTS:**

- VCU IRB Study Personnel Roster (Version date: 2; received by ORSP April 10, 2012)

**This approval expires on April 30, 2013.** Federal Regulations/VCU Policy and Procedures require continuing review prior to continuation of approval past that date. Continuing Review report forms will be mailed to you prior to the scheduled review.

The Primary Reviewer assigned to your research study is Amy Ladd, PhD. If you have any questions, please contact Dr. Ladd at [acladd@vcu.edu](mailto:acladd@vcu.edu) or 827-4427; or you may contact Elicia Preslan, IRB Coordinator, VCU Office of Research Subjects Protection, at [IRBPanelD@vcu.edu](mailto:IRBPanelD@vcu.edu) or 827-0899.

Attachment – Conditions of Approval



***Conditions of Approval:***

In order to comply with federal regulations, industry standards, and the terms of this approval, the investigator must *(as applicable)*:

1. Conduct the research as described in and required by the Protocol.
2. Obtain informed consent from all subjects without coercion or undue influence, and provide the potential subject sufficient opportunity to consider whether or not to participate (unless Waiver of Consent is specifically approved or research is exempt).
3. Document informed consent using only the most recently dated consent form bearing the VCU IRB "APPROVED" stamp (unless Waiver of Consent is specifically approved).
4. Provide non-English speaking patients with a translation of the approved Consent Form in the research participant's first language. The Panel must approve the translated version.
5. Obtain prior approval from VCU IRB before implementing any changes whatsoever in the approved protocol or consent form, unless such changes are necessary to protect the safety of human research participants (e.g., permanent/temporary change of PI, addition of performance/collaborative sites, request to include newly incarcerated participants or participants that are wards of the state, addition/deletion of participant groups, etc.). Any departure from these approved documents must be reported to the VCU IRB immediately as an Unanticipated Problem (see #7).
6. Monitor all problems (anticipated and unanticipated) associated with risk to research participants or others.
7. Report Unanticipated Problems (UPs), including protocol deviations, following the VCU IRB requirements and timelines detailed in VCU IRB WPP VIII-7:
8. Obtain prior approval from the VCU IRB before use of any advertisement or other material for recruitment of research participants.
9. Promptly report and/or respond to all inquiries by the VCU IRB concerning the conduct of the approved research when so requested.
10. All protocols that administer acute medical treatment to human research participants must have an emergency preparedness plan. Please refer to VCU guidance on <http://www.research.vcu.edu/irb/guidance.htm>.
11. The VCU IRBs operate under the regulatory authorities as described within:
  - a) U.S. Department of Health and Human Services Title 45 CFR 46, Subparts A, B, C, and D (for all research, regardless of source of funding) and related guidance documents.
  - b) U.S. Food and Drug Administration Chapter I of Title 21 CFR 50 and 56 (for FDA regulated research only) and related guidance documents.
  - c) Commonwealth of Virginia Code of Virginia 32.1 Chapter 5.1 Human Research (for all research).

## APPENDIX B: FITC-BSA CONJUGATION AND UPTAKE STUDIES

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This appendix describes the process of albumin conjugation and functional assays of albumin in the BeWo cells.

### **Methods:**

#### **A. BSA FITC conjugation:**

FITC labeling of BSA was done according to the manufacturer's instructions using the FluroTag™ FITC conjugation kit (Sigma-Aldrich). Basically, 5 mg/ml of BSA was prepared in NaHCO<sub>3</sub> buffer (pH=9). Then the stock of FITC solution was reconstituted with NaHCO<sub>3</sub> to make 3 dilutions (20:1, 10:1, and 5:1). The protein solution was added drop wise to each dilution. The reaction vials were protected from light and incubated for 2 hours with gentle stirring.

#### **B. BSA isolation:**

The Large scale column (which is provided in the kit) was equilibrated with PBS then the reaction mixture prepared previously was added to the column. The column was eluted with 10 ml PBS and ten 1 ml-fractions were collected. Fractions which have the conjugate (3-5) were pooled if their A<sub>280</sub> is  $\geq 0.4$ .

#### **C. Determination of Fluorescein/Protein Molar Ratio (F/P):**

The F/P molar ratio is defined as the ratio of moles of FITC to moles of protein in the conjugate. To determine this ratio, the absorbance readings (A<sub>280</sub> and A<sub>495</sub>) of the conjugate sample are used according to the equation:

**Molar F/P =  $A_{495} \times C / A_{280} - [(0.35 \times A_{495}) \dots]$  Equation 1**

(C is a constant value given for a protein)

Where:  $C = MW \times E^{0.1\%}_{280} / (389 \times 195)$

MW is the molecular weight of the protein.

389 is the molecular weight of FITC.

195 is the absorption  $E^{0.1\%}$  of bound FITC at 490 nm at pH 13.0.

$(0.35 \times A_{495})$  is the correction factor due to the absorbance of FITC at 280 nm

$E^{0.1\%}$  is the absorption at 280 nm of a protein at 1 mg/ml

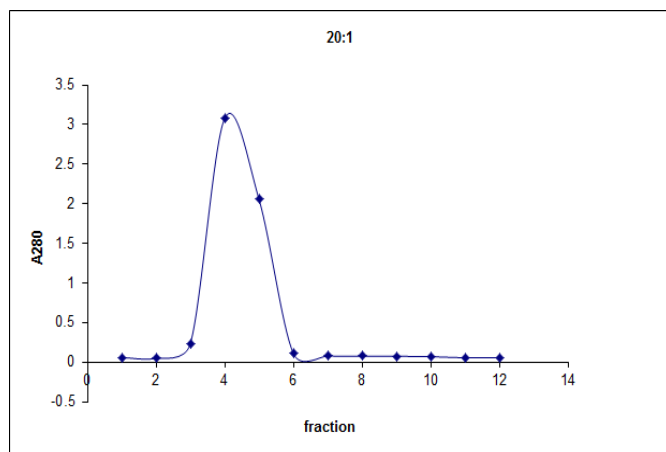
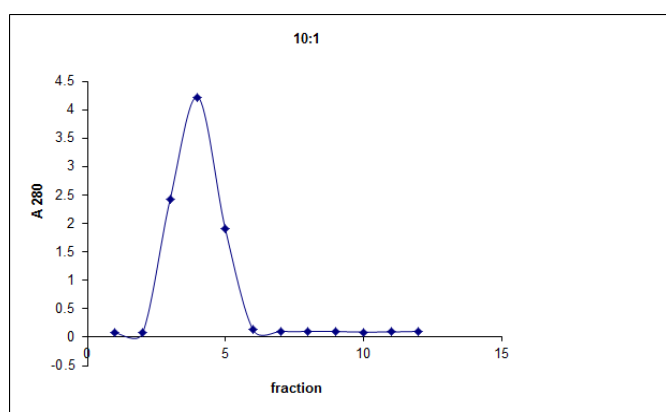
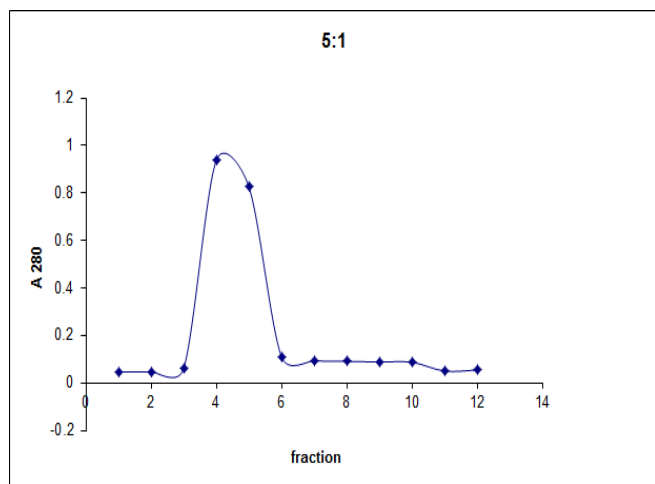
**D. Uptake experiments:**

The BeWo cells were pre-incubated with 0.5 mg/ml with unlabeled BSA for 30 minutes. Then they were incubated with 0.25 mg/ml of FITC-labeled BSA at 5, 10, 30 and 60 minutes. Reaction was stopped by washing the cells twice with ice-cold DPBS then 0.5% w/v of unlabeled BSA. For analysis 0.5 ml of water was added to each well and wells were frozen overnight. Fluorescence was measured in the microplate reader at 480 nm.

**Results:**

**A. Conjugation of BSA with FITC:**

After the elution of the column with 10 ml PBS, Ten 1 ml-fraction of the elute were collected. For each dilution i.e. 5:1, 10:1 and 20:1, the  $A_{280}$  for each fraction was measured (Figure 1-3) Fractions with  $A_{280} \geq 0.4$  were selected for further analysis.



**Figure A-B-1:** A280 of fractions collected from FITC-conjugation at 5:1, 10:1 and 20:1 dilutions.

## B. Determination of molar F/P ratio

C value for BSA was calculated then F/P ratios were calculated according to equation 1:

$$C_{BSA} = 66400 \text{ Da} \times 0.6 / (389 \times 195) = 0.53$$

- Molar F/P (20:1) =  $3.302 \times 0.53 / 2.512 - (0.35 \times 3.302) = 1.3$
- Molar F/P (10:1) =  $2.6 \times 0.53 / 1.6 - (0.35 \times 2.6) = 2$
- Molar F/P (5:1) =  $2.185 \times 0.53 / 1.428 - (0.35 \times 2.185) = 1.75$

Fractions from the dilutions 10:1 and 5:1 achieved the expected molar F/P ratio. The dilution 5:1 was selected for the uptake experiments.

## C. Uptake experiments:

In order to calculate cellular content of FITC-BSA in the cells, a calibration curve of fluorescence at 480 nm vs. FITC-BSA concentration was constructed (Figure 1):

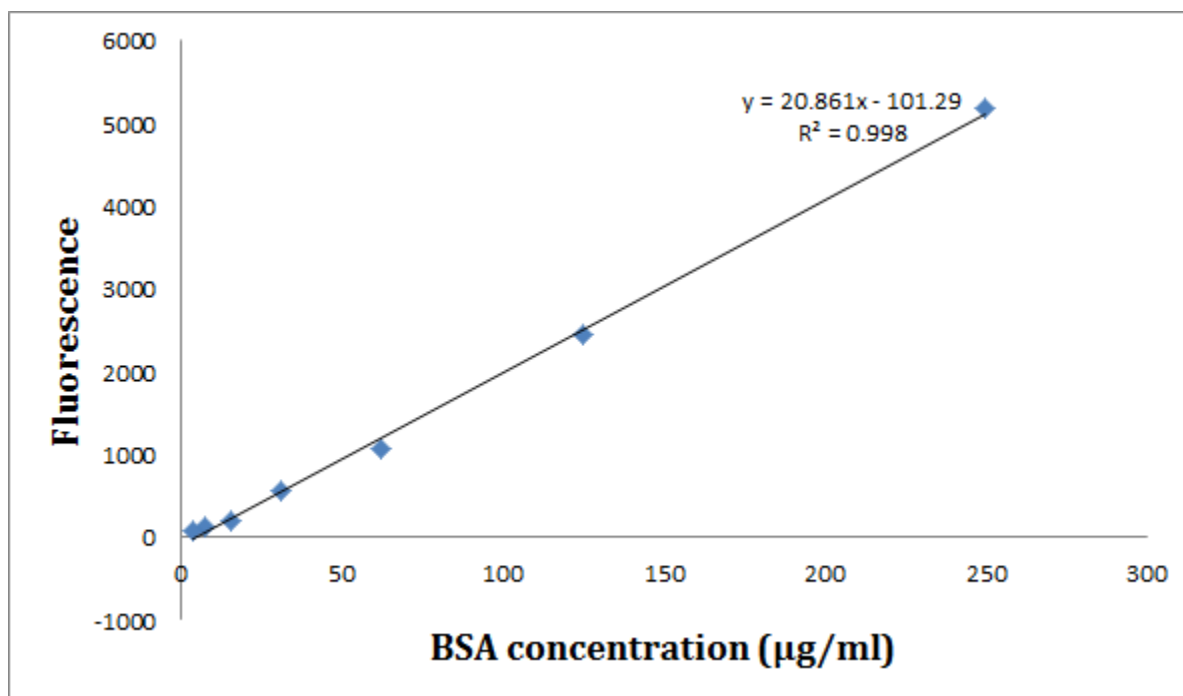
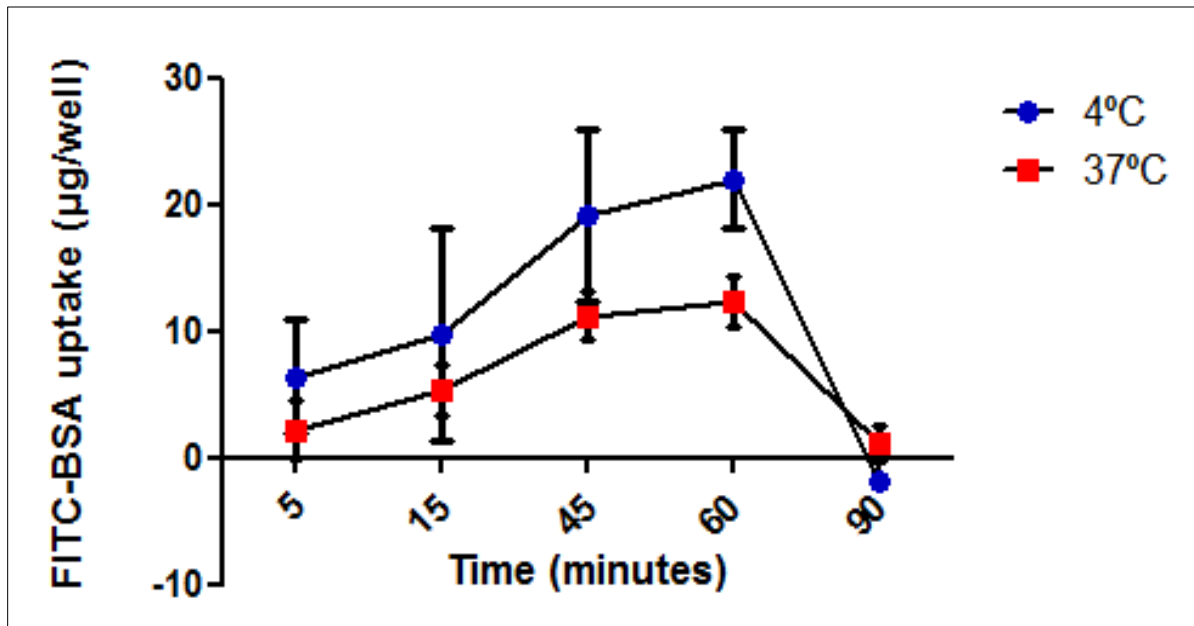


Figure A-B-2-1: Calibration curve (Fluorescence vs. BSA concentration).

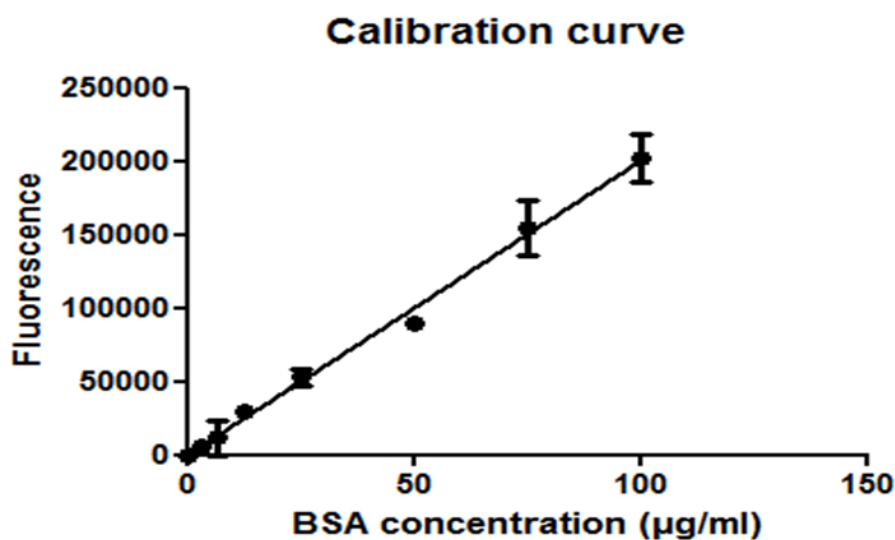
At 37 °C, the cellular content of BSA barely increased over 60 minutes then there was a sharp drop 90 minutes, and no significant difference in the uptake between 4 °C and 37 °C (Figure 2). There was an apparent more uptake at 4 °C ( $p>0.05$ ).



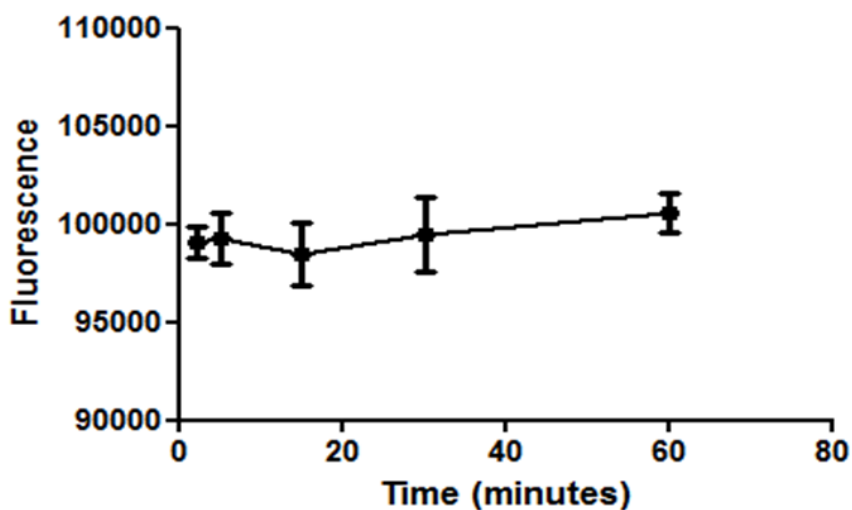
**Figure A-B-3:** The uptake of FITC-BSA by the BeWo cells at 37 and 4 °C (data represents mean  $\pm$ SD; n=6)

## APPENDIX C: VALIDATION OF THE BSA UPTAKE ASSAY

This appendix describes the process of BSA uptake assay validation (chapter 3 section II)



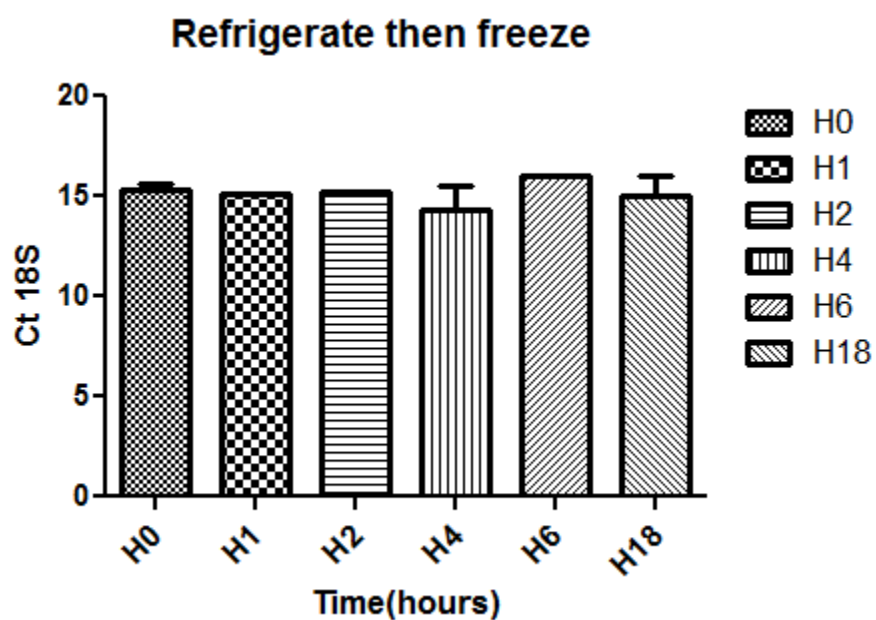
**Figure A-C-1:** Calibration curve of fluorescence (480 nm) vs. FITC-BSA concentrations ranging from 1.56 to 100 µg/ml in NP-40 (mean  $\pm$  SD; n=2).



**Figure A-C-2:** Stability of Fluorescence (480 nm) of FITC-BSA (100 µg/ml) in DPBS (mean  $\pm$  SD ; n=3).

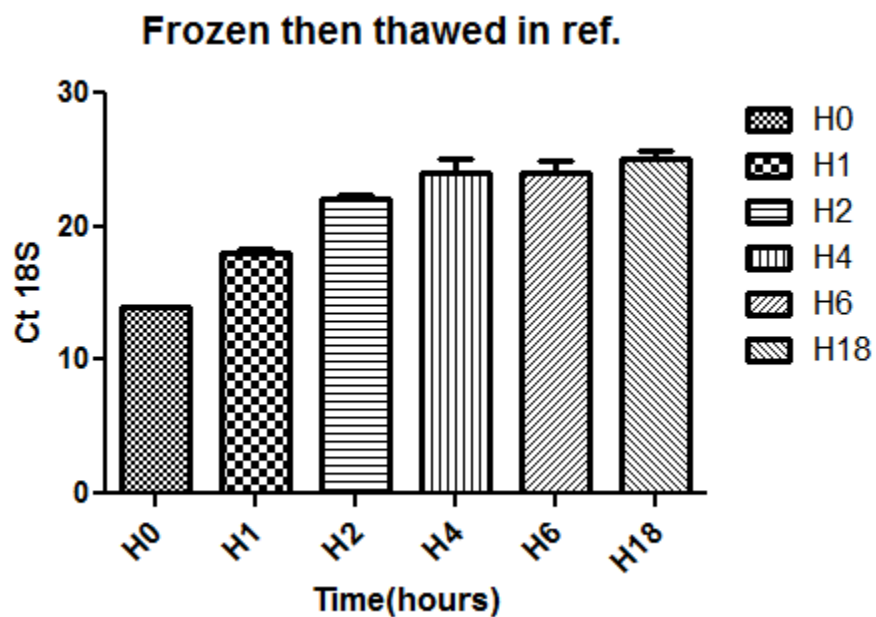
## APPENDIX D: CYCLE THRESHOLD VALUES (CT) FOR PLACENTAL VILLOUS TISSUE SAMPLES

This appendix shows Ct values of the reference gene (18S rRNA) for mRNA stability studies:

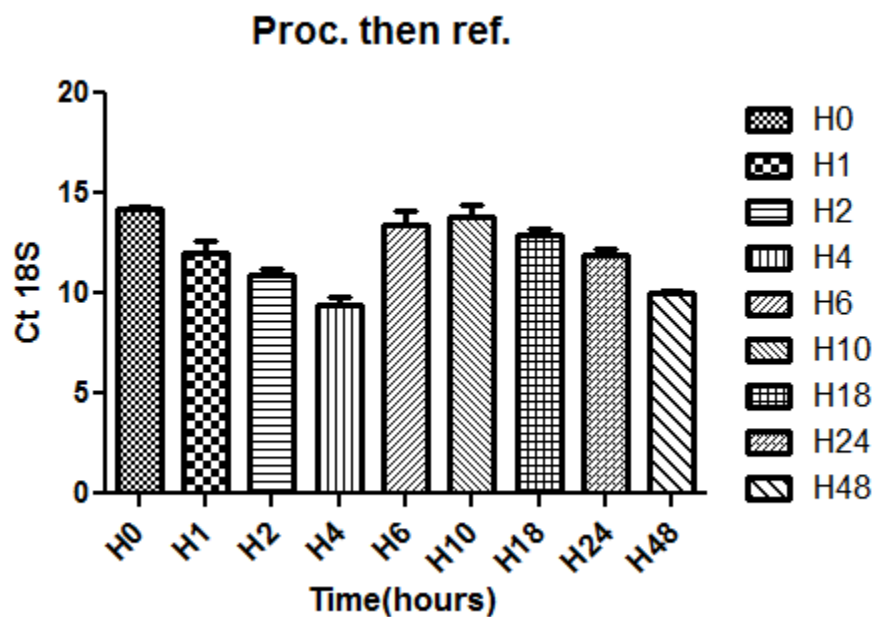


**Figure A-D-1:** Ct values of the 18S rRNA vs. time in hours in term placental villous tissue samples (gestational age=39 weeks) that were refrigerated for 0 to 18 hours (Data points represent mean  $\pm$  SD; n=3)

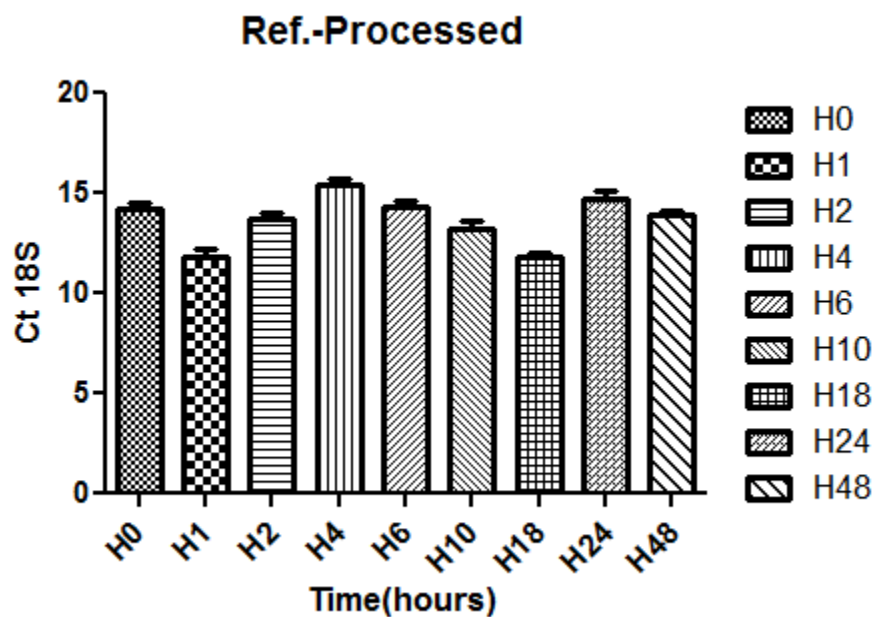




**Figure A-D-2:** Ct values of the 18S rRNA vs. time in hours in term placental villous tissue samples (gestational age =39 weeks) that were frozen then thawed at 4° C for 0 to 18 hours (Data points represent mean  $\pm$  SD; n=3)



**Figure A-D- 3:** Ct values of the 18S rRNA vs. time in hours in term placental villous tissue samples (gestational age =41 weeks) that were processed then refrigerated for 0 to 48 hours (Data points represent mean  $\pm$  SD; n=3)



**Figure A-D-4:** Ct values of the 18S rRNA vs. time in hours in term placental villous tissue samples (gestational age =41 weeks) that were left unprocessed in the refrigerator for 0 to 48 hours (Data points represent mean  $\pm$  SD; n=3)

## VITA

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### Amal Akour

307 N. Boulevard apt. 1 ● Richmond, VA ● (804) 549-2815 ● akoura@vcu.edu

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#### EDUCATION

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Virginia Commonwealth University– Richmond, VA  
**PhD candidate**

**Degree expected 12/2012**

- Pharmacotherapy and Outcomes Science. PhD Program. In translational therapeutics. Focus: Transport of drugs across placenta, specifically aminoglycoside transport.

The University of Jordan-Amman, Jordan

**B.Sc. Pharmacy**

**2002-2007**

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#### LABORATORY SKILLS

---

Virginia Commonwealth University

**Graduate Student**

**2009-Current**

- Adept in western blotting and Immuno-fluorescence techniques.
- Adept in Trizol® RNA isolation from tissues and cell lines and quantitative polymerase chain reactions (qPCR).
- Adept in performing uptake studies in cell lines and the subsequent analysis via radiolabeled substrates.
- Good experience in collecting human placental samples from pregnant subjects.
- Moderate experience in analyzing data in Prism®, JMP® 8.0.2 and JMP® 9.0.2, Scientist®, and NONMEM®.

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**TEACHING EXPERIENCE**


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Virginia Commonwealth University

**Teaching Assistant****8/2011-12/2011**

- **Lecture: Allergic drug reactions: “emphasis on antibiotics”:** Teaching P3 students concepts in allergic drug reactions as opposed to adverse drug reactions
- Handled Foundations IV lab sections, composed and administered exams and graded students’ work.

The University of Jordan

**Teaching assistant****2007 to 2008**

- Handled pharmacology and therapeutics lab sections, explained related theory, composed and administered exams and graded all students’ work.
- Handled workshops in pharmacokinetics, explained related theory, composed and administered exams and graded all students’ work.

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**ORGANIZATIONAL MEMBERSHIP**


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- Member of the American College of Clinical Pharmacology (ACCP) **2011**
- Member of the American Society of Clinical Pharmacology and Therapeutics (ASCPT) **2009-**  
**Current**
- Member of Jordanian Pharmacy Association (JPA) **2002-**  
**Current**

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**AWARDS AND HONORS**


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Virginia Commonwealth University, Richmond, VA **2012**

- Victor A. Yanchik Award for distinction in research, teaching and scholarship.

Virginia Commonwealth University, Richmond, VA **2011**

- Certificate in "Preparation of Future Faculty" awarded after completing a 4-session workshop and an internship of teaching for P3 students in the same University.

College of Pharmacy, University of Jordan, Amman, Jordan <3 **2008-current**

- Full scholarship that includes living expense and tuition to pursue PhD in the US.

Jordanian National Ranking **2007**

- Ranked second in the Pharmacy Qualification Exam amongst all Jordanian pharmacy students who graduated in June 2007.

The University of Jordan, Amman, Jordan **2002-2007**

- Full Bachelor Degree Scholarship

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**ORAL PRESENTATIONS**


---

Virginia Commonwealth University, Richmond, VA

- Assessment of Megalin Expression in Human Placental Models **2012**
- The Role of Megalin in the Transplacental Transport of Aminoglycosides **2011**
- Intrapartum Aminoglycosides and Neonatal Nephrotoxicity **2010**
- The Effect of Contemporary Oral Contraceptive Pills on Carbohydrate Metabolism in Women with Metabolic Risk Anomalies: Review of literature **2009**

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**POSTER PRESENTATIONS**


---

Virginia Commonwealth University, Richmond, VA **2012**

VCU Institute of Women Health

- Assessment of Megalin Expression in Human Placental Models

American Society of Clinical Pharmacology and Therapeutics (ASCPT) Meeting **2011**

Dallas, Texas

- Evidence that megalin-mediated endocytosis is involved in the placental uptake of Aminoglycosides.

Virginia Commonwealth University, Richmond, VA **2010**

Research and Career Day

- Transplacental Transport of Aminoglycosides Across human trophoblasts.

Virginia Commonwealth University, Richmond, Virginia **2010**

Graduate Research Symposium

- Characterization of Megalin Expression in Human Term and Preterm Placenta.

Virginia Commonwealth University, Richmond, VA Public Health Research Forum **2009**

- The Effect of Moderate Physical Activity on the Prevalence of Self-reported Depression

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**PUBLISHED ABSTRACTS**


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Evidence That Megalin-Mediated Endocytosis is involved in the **2011**

Placental Transport of Aminoglycosides

- A. Akour, A. Landsberg, P.M. Gerck, M.J. Kennedy. Clinical Pharmacology and Therapeutics. (2011): 89 (1): S63-S64.

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**IN PROGRESS**

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Receptor-Mediated Endocytosis across Human Placenta: emphasis on

Aminoglycosides

**2012**

- By: A. A. Akour, M.J. Kennedy ,and P.M. Gerk
  - Submitted to Molecular Pharmaceutics
-