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THE COMBINATORY EFFECTS OF PEDIATRIC OBESITY AND ONTOGENY ON MONOCARBOXYLATE TRANSPORTER EXPRESSION IN TISSUES OF DRUG DISPOSITION

Michael Ng
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THE COMBINATORY EFFECTS OF PEDIATRIC OBESITY AND ONTOGENY ON MONOCARBOXYLATE TRANSPORTER EXPRESSION IN TISSUES OF DRUG DISPOSITION

By

Michael Ng

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University of the Pacific
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2021
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By

Michael Ng
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I would like to express sincere gratitude and thanks to Dr. Melanie Felmlee for accepting me as her graduate student and mentoring me throughout my PhD. I arrived at University of the Pacific after years away working, Dr. Felmlee helped me transition back to academic life with ease, and always motivated me to excel in every facet of pharmacology and molecular biology research. Her unwavering support and endless knowledge of drug transporters and proteomics made all the difference. I want to thank my lab mates Jieyun Cao, Hao Wei, Qing Zhang, and Tori Nicholson for always willing to offer assistance on my project, and for all the wonderful stories shared together at the laboratory bench. I want to thank my family and graduate friends who pushed me to succeed and, whenever I did not, stood by my side to commiserate and speak of better days and new opportunities just ahead.

For this dissertation I would like to thank all my committee members, Dr. William K. Chan, Dr. Miki Park, Dr. John Livesey, and Dr. Robert Jones for their time, consideration, and great insight. Last but not least, I want to acknowledge the amazing academic faculty and staff for all their support and guidance in my graduate education. The University of the Pacific and Stockton community will always hold a special place in my heart.
Proton-coupled and sodium-dependent monocarboxylate transporters are encoded by the SLC16A and SLC5A gene family of solute carriers, and are responsible for the transport of essential nutrients such as L-lactate, pyruvate, and ketone bodies. Basigin, or CD147, acts as an ancillary protein for MCT1 and MCT4, and is involved in membrane surface expression of transporters. MCTs are also involved in the shuttling of monocarboxylic xenobiotics across cell membranes, including the drugs valproate and gamma hydroxybutyrate. MCTs are also important for normal mammalian development, particularly during embryogenesis and early neonatal life. Previous studies have shown that ketogenic diets increase MCT expression in the brain, and the obesity biomarker leptin increases MCT1 and CD147 expression and colocalization in colonocytes. Clinical studies in post-mortem tissue demonstrated that hepatic MCT1 expression changes nonlinearly from birth to adulthood. We hypothesize that age and high fat dietary intake regulate monocarboxylate transporter and ancillary protein expression in the liver, and other organs of drug disposition during childhood obesity.

The purpose of this study was to elucidate just how diet and ontogeny regulate MCT1, MCT4, CD147, and SMCT1 mRNA and protein expression in the liver, kidney, and ileum. Timed-pregnant rats were fed either normal or high fat diet, and tissue was harvested from the
progeny of both cohorts at predetermined postnatal timepoints. Serum leptin levels were measured, and MCT1, MCT4, CD147, and SMCT1 transcripts were evaluated using real time quantitative PCR. Whole cell and total membrane proteins were extracted and transporter expression was analyzed via western blot.

In summary, we have demonstrated age, diet, and sex dependent regulation of MCT1, MCT4, CD147, and SMCT1 expression in the liver, kidneys, and intestine, and that these effects are tissue specific. Pediatric drug-dosing is both a pressing and understudied clinical field, with the possibility of altered pharmacokinetics in obese children. Changes in hepatic, renal, and intestinal monocarboxylate transporter expressions during mammalian development may affect functional activity of these transporters and lead to altered metabolism and drug disposition. Further studies of this animal model can shine new light on the dynamic and highly-variable nature of drug pharmacokinetics in pediatric obesity.
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<tr>
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<th>Meaning</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism, and excretion</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BLSS</td>
<td>Basolateral sorting sequence</td>
</tr>
<tr>
<td>C/EBPδ</td>
<td>CCAAT/enhancer binding protein delta</td>
</tr>
<tr>
<td>CD147</td>
<td>Basigin</td>
</tr>
<tr>
<td>db/db</td>
<td>Leptin-receptor deficient</td>
</tr>
<tr>
<td>E2, E7</td>
<td>Embryonic stage 2, 7</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GHB</td>
<td>Gamma-hydroxybutyrate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GP70</td>
<td>Embigin</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MDR1a</td>
<td>Multidrug resistance protein 1a</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro Ribonucleic acid</td>
</tr>
<tr>
<td>Na+/K+ ATPase</td>
<td>Sodium–potassium adenosine triphosphatase</td>
</tr>
<tr>
<td>NC</td>
<td>Normal chow</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic anion transporter</td>
</tr>
<tr>
<td>ob/ob</td>
<td>Obese leptin-deficient</td>
</tr>
<tr>
<td>ObR</td>
<td>Leptin receptor</td>
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</tbody>
</table>
PGP  P-glycoprotein
PND  Postnatal Day
rRNA  Ribosomal RNA
SCFA  Short chain fatty acid
SGLT  Sodium-glucose cotransporter
SLC  Solute carriers
Smad3  Mothers against DPP homolog 3
SMCT  Sodium monocarboxylate transporter
SP1  Specificity protein 1
SRY  Sex determining region Y
STAT  Signal transducer of activation
TMB  Tetramethylbenzidine
TMD  Transmembrane domain
TNFα  Tumor necrosis factor alpha
WCE  Whole cell extract
CHAPTER 1: INTRODUCTION

Rapid shuttling of bio-molecular compounds is essential to maintaining physiological homeostasis and promote cellular metabolism. While some molecules readily enter cells by passive diffusion, polar and hydrophilic nutrients, such as lactate and pyruvate, are impermeable and require active transport to cross the cell membrane. Selective permeability for large polar molecules is governed by a class of integral membrane proteins called transporters, which are embedded in the cell surface and in organelle membranes and act in primary and secondary activated transport capacities. Primary transporters like P-glycoprotein (PGP) exclusively use the hydrolysis of ATP as an energy source to mediate active transport, whereas secondary transporters utilize biological conditions, such as extracellular ion gradients and membrane potentials as a driving force, coupled to the physiochemical properties of the trafficked substrates. The classes of transporters can be divided into the membrane protein super-families of ATP-binding cassettes (ABC) and solute carriers (SLC), which are involved in the pharmacokinetic absorption, distribution, metabolism, and excretion of endogenous substrates and pharmaceutical drugs.

The SLC superfamily is comprised of over 400 unique members, which are further divided into 65 distinct families based on sequence homology in the human genome [1]. SLCs utilize both secondary active symport and antiport, as well as equilibrative facilitated transport of solutes across cell barriers for the uptake and efflux of nutrients, drugs, and other xenobiotics. Facilitative SLCs, such as SLC2/GLUT transporters, rely on an electrochemical gradient to catalyze the movement of substrates across membranes, while secondary active SLCs such as the sodium/glucose cotransporter (SLC5) utilize ion gradients generated by ATP-dependent pumps
to transport substrates against a concentration gradient. SLCs transport essential organic and inorganic substrates in the body, and more than 100 SLC transporters have been associated with human genetic disorders [1]. Mutations in SLC loci contribute to several systemic metabolic diseases, including diabetes, elevated blood pressure, and chronic kidney disease [2].

1.1 Pediatric Obesity

Obesity is defined as having a body mass index (BMI) greater than the 95th percentile for a population based on age and gender [3]. There are currently more obese Americans than ones of a healthy bodyweight, and the proportion of obese Americans is projected to account for 51% of the total population by 2030 [4]. The prevalence of childhood obesity has increased to epidemic proportions in recent years, with an estimated 42 million children worldwide under the age of 5 considered overweight [5]. In the United States, 1 in 6 children are classified as obese, with increased risk for developing chronic diseases such as hypertension, coronary artery disease, glucose intolerance, insulin resistance, and type 2 diabetes. The most common cause of adolescent obesity is lack of physical exercise, over-nutrition, and poor diet, although polygenic and endocrine disorders also contribute to the onset of symptoms. Obese children are more likely to require early clinical pharmacotherapy, which includes multiple medications and interventions to treat obesity-related illnesses, such as diabetes, asthma, and sleep apnea [6].

Obesity has been shown to impact drug metabolism and elimination pathways in both adults and children. For example, obese patients have lower clearance for CYP3A4 substrates with negative correlation between BMI and hepatic CYP3A protein expression, while having higher clearance for drugs metabolized by CYP1A2, CYP2C9, CYP2C19 and CYP2D6 [7, 8]. In C57BL/6 ob/ob mice, obesity decreased the hepatic protein and mRNA expression of uptake transporters such as organic anion transporting polypeptides and sodium/taurocholate co-
transporting polypeptides, while decreasing efflux transporters like multidrug resistance-associated proteins [9]. Dysregulation of drug metabolizing enzymes and drug transporter expression in obese patients, along with inter-individual variability, can alter drug pharmacokinetics, leading to unpredictable drug exposure that can result in inadequate pharmacotherapy or lethal drug toxicity.

1.2 Ontogeny of Drug Transporters

Ontogeny is the biological development of a living organism from conception to adulthood. Age affects mRNA and protein expression of drug transporters, with differential maturation pattern and tissue distribution based on individual transporter [10]. ATP-dependent PGP is an essential efflux transporter used in absorption, distribution, metabolism, and excretion of various toxins, carcinogens, and pharmaceutics. Protein studies in the mouse brain and intestine show the mdr1a isoform of PGP increases markedly with postnatal maturation, while in the kidney and liver, PGP levels remained the same between birth and adulthood [11, 12]. Protein expression of OAT 1 and 3 in the kidney are low at birth but gradually increase with age [13]. In rat intestine, the expression of sodium-coupled glucose transporter 1 (GLUT1; SLC5A1) is higher in both weanlings and adults compared to nursing neonates at PND10 [14]. Present drug dosing strategies in obese children are theoretical and gleaned from existing clinical data on obese adults. There is a paucity of information in literature regarding developmental changes of drug transporters in obese and non-obese children, which may affect drug pharmacokinetics and efficacy of current pediatric drug treatment.

1.3 Monocarboxylate Transporters

1.3.1 Proton-dependent monocarboxylate transporters (MCTs). Monocarboxylate transporters belong to the SLC16A family and mediate proton-linked monocarboxylate transport,
with 14 isoforms identified based on sequence homology and conserved motifs. Of these 14 members, MCT1 (SLC16A1), MCT2 (SLC16A7), MCT3 (SLC16A8) and MCT4 (SLC16A3) have been the most extensively studied and characterized in terms of expression, function and substrates. MCTs have a broad substrate specificity and play a pivotal role in pH balance and cell homeostasis. Sodium-coupled monocarboxylate transporters (discussed in section 1.4) belong to the SLC5A family, and comprise of SMCT1 (SLC5A8) and SMCT2 (SLC5A12), with neither having sequence homology to SLC16A [15].

1.3.1.1 Molecular structure of monocarboxylate transporters. Although no 3D X-ray crystal structures are available for MCT, hydrophobicity plots have shown all MCTs contain 12 highly-conserved transmembrane domain (TMD) spanning helices, with two 6-helical bundles connected via a large intracellular loop between TMDs 6 and 7 (Figure 1.1) [16]. MCTs contain intracellular carboxyl and amino termini, and it is postulated that variable sequences in amino acid residues in the C- and N- termini determine substrate specificity and transporter regulation [17]. Site-directed mutagenesis studies of MCT1 has identified that extracellular lysine residue (K38) on TMD1, and aspartate and arginine residues (D302/R306) on TMD8 are essential for the binding of protons and monocarboxylate anions, leading to a change from an open to closed conformational state during the translocation cycle of substrates [18].
1.3.1.2 Tissue distribution of monocarboxylate transporters. The proton-coupled MCT1-4 are expressed throughout the human body in a tissue-specific manner. MCT1 is ubiquitously expressed in almost every organ and cell type, with the exception of beta-islet cells in the pancreas [19]. It is the predominant isoform in most tissues, and its major physiological function is the influx and efflux of L-lactate for energy expenditure in cells, and to regulate intracellular acidification during glycolysis. MCT1 is expressed in the basolateral plasma membranes of the liver and kidney and facilitates gluconeogenesis, while in the brain MCT1 mediates monocarboxylate transport across the blood brain barrier for uptake into neurons to be used in cell respiration and growth [20]. In the small intestine, MCT1 is present on the basolateral membrane where it controls the uptake of acetate, propionate, and butyrate produced as bacterial byproducts in the gut, where substrates are absorbed into blood circulation. MCT2 has more restricted tissue distribution pattern and expression levels than MCT1, although the isoform has higher binding affinity for pyruvate and lactate. In fact, human tissues exhibit
minimal MCT2 expression based on RNA experiments [16], although immunochemistry studies in frozen human tissues have shown some expression across various organs, in particular the colon, kidney, brain, pancreas, and heart [21]. Moreover, MCT2 appears to have differential expression based on species, with more pronounced levels in mouse, rat, and hamster as compared to humans, suggesting less genetic conservation across species compared to MCT1 [22]. MCT3 expression is exclusively localized to the retinal pigment epithelium and choroid plexus epithelia, where it is responsible for the efflux of glycolytically-derived L-lactate from the retina to prevent ocular edema [23]. MCT4 has a lower affinity, and higher substrate binding capacity for L-lactate and pyruvate than MCT1. MCT4 is widely expressed in highly glycolytic tissues, including white skeletal muscle fibers, leukocytes, and astrocytes in the spinal cord and brain. In the liver, kidneys and small intestine, MCT4 is expressed in the basolateral membrane [24] although hepatic expression is more limited compared to MCT1 (Protein Atlas). Other MCT isoforms are expressed in a tissue-dependent pattern (Figure 1.2). The varied tissue and subcellular distribution of MCT isoforms allows for constant shuttling of endogenous substrates between tissues of energy production to tissues that utilize these nutrients for oxidation or gluconeogenesis.
**Figure 1.2.** Tissue protein expression of MCT isoforms in humans [25].

### 1.3.1.3 Ancillary proteins

Proteomic and sequencing studies have shown that MCTs in their native form are non-glycosylated [26]. However, some MCTs require association with an ancillary glycoprotein in order to be functionally expressed at the plasma membrane. Identification of these ancillary proteins via inhibitor studies revealed selective binding depending on MCT isoform. The 70 kD protein embigin has limited expression across tissues but appears to bind preferentially with MCT2. A closely related protein to embigin is basigin, which is widely expressed in most tissues. Basigin, also known as CD147, contains a single transmembrane domain with a conserved glutamate residue that potentiates protein-protein interactions, a large glycosylated extracellular domain, and a short intracellular C-terminus (Figure 1.1) [27]. It is the preferred binding partner of MCT1/3/4, where the glycoprotein forms
a heterodimer by complexing at the TMDs 3 and 6 of the transporter. CD147 contains a simple peptide basolateral sorting sequence (BLSS) that confers polarity to MCT1 which lacks a BLSS, whereas MCT3 and MCT4 contain BLSS in their C-terminal cytoplasmic tails that supersedes the CD147 sequence [28]. It was originally believed CD147 acted as a chaperone protein for trafficking MCTs to the basolateral membrane. However, immunofluorescence and co-immunoprecipitation studies have confirmed that CD147 remains associated to and co-localizes with MCTs even after translocation, and proper transporter activity is predicated on this sustained binding event. Absence of CD147 in mammalian cell lines cause MCT1 to lose cell polarity and remain in the perinuclear space of the Golgi apparatus and endoplasmic reticulum [29], and experiments in CD147 null mice showed reduced plasma membrane expression of MCT1/3/4 in the retinal pigment epithelium and neural retina [30]. It is hypothesized that changes in expression levels and distribution of plasma membrane MCTs may lead to altered monocarboxylic nutrient and drug absorption.

1.3.1.4 Proton-dependent monocarboxylate transporter substrates. Monocarboxylic nutrients and drugs require rapid and effective transport across plasma membrane for absorption by targeted cells, a process catalyzed by monocarboxylate transporters. MCTs transport short-chain fatty acid monocarboxylates including nutrients (L-lactate, butyrate, pyruvate) and ketone bodies (acetoacetate) that are essential fuel molecules produced by carbohydrate, lipid, and amino acid metabolism in the human body. MCTs also traffic pharmaceutical agents that exhibit monocarboxylic properties, including γ-hydroxybutryrate, bumetanide, and salicylic acid. Of the 14 MCTs identified, only MCT1-4 and MCT7 (SLC16A6) have been experimentally demonstrated to transport monocarboxylates, with MCT1-4 exhibiting electroneutral proton-coupled symporter activity with a stoichiometric exchange of 1 proton and 1 monocarboxylate.
MCT6 (SLC16A5) was recently revealed to be a prostaglandin F2 α transporter [31]. MCT8 and MCT10 (SLC16A2/SLC16A10) have been shown to shuttle thyroid hormones T2, rT3, T3, T4, with MCT10 also responsible for transporting aromatic amines [25] [17]. MCT9 (SLC16A9) has demonstrated carnitine specificity [32], and MCT12 (SLC16A12) has been shown to be a creatinine transporter [33]. The endogenous binding substrates and regulatory mechanisms of MCT5, MCT11, MCT13, and MCT14 (SLC16A4/SLC16A11/ SLC16A13/SLC16A14) in humans are not yet fully characterized or understood. A comprehensive review was recently published detailing the substrates of characterized MCTs [31].

1.4 Sodium-Coupled Monocarboxylate Transporters

While there is growing literature regarding the classification and characterization of monocarboxylate transporters, much less is known about sodium-coupled monocarboxylate transporters. SMCT1 (SLC5A8) is an electrogenic transporter that binds sodium and monocarboxylates in a heterodimeric ratio ranging from 4:1 to 2:1 based on substrate [34]. SMCT2 (SLC5A12) is predicted to have an electroneutral 1:1 binding ratio of sodium and monocarboxylates with a lower affinity, higher binding capacity for substrates. The molecular structure of SMCT consists of 7 transmembrane spanning domains with an extracellular amino terminus and an intracellular carboxyl terminus [35]. SMCT1 was first identified as the SLC5A8 gene for iodide transport in thyrocytes, and then as a tumor suppressing gene in human colonic cancer cell lines, where aberrant DNA methylation and silencing of SMCT1 increased cancer cell proliferation [36, 37]. Initial mRNA studies in mice found SMCT1 expression in most epithelial tissue, including lungs, small intestine, esophagus, stomach, and thyroid, with the most expression in kidney [37]. Further protein experiments have shown SMCT1 expression in the kidney cortex and outer medulla, with expression beginning at the S2 and S3 segments of the
proximal tubule, where it resides at the apical membrane [38]. In the intestines, SMCT1 is localized apically, with expression along the microvilli-rich brush-border membrane. SMCT2 is expressed primarily in the intestine and kidneys, with localization to the apical membrane in enterocytes and early S1 renal proximal tubule, and restricted expression in astrocytes and Müller cells in the brain and retina [39, 40]. Endogenous substrates trafficked by SMCT1 and SMCT2 are similar to those of MCTs, including short-chain fatty acids, lactate, pyruvate, and ketone bodies. While drug compounds that bind to SMCT1 include benzoate, γ-hydroxybutyrate and salicylates, the pharmacological significance of SMCT2 is unknown [41]. The expression of SMCT1/2 on the lumen-facing apical membrane, complemented by basolateral expression of MCTs in drug disposition tissues, combine to form the major efflux and reabsorption pathway for monocarboxylate trafficking, as well as regulating bioavailability of monocarboxylic drugs.

1.5 Regulation of Monocarboxylate Transporters

1.5.1 Cell signaling cascades and monocarboxylate transporters. MCT and SMCT expression can be modified due to transcriptional and post-transcriptional regulation mechanisms. Treatment of human colonic epithelial cells with sodium butyrate resulted in concentration- and time-dependent upregulation of MCT1 mRNA and protein via butyrate-induced hyperacetylation of histones, transcriptional activation and RNA stabilization [42]. Similarly, extracellular lactate was shown to increase MCT1 expression in rat skeletal muscle cells due to signaling cascades mediated by reactive oxygen species [43]. Rigorous exercise upregulates MCT1 and MCT4 protein expression in skeletal muscles by activating AMP-activated protein kinase and binding to the MCT promoter sequences [44, 45]. Oxygen deprivation increases MCT4 expression by upregulating hypoxia-inducible factor 1-α, which binds to hypoxia response elements on the MCT4 promoter region during anaerobic glycolysis.
miRNAs miR-29a and miR-29b have been shown to suppress MCT1 expression in a tissue-specific manner, such as in pancreatic beta islet cells [47]. While less is known about SMCT cell signaling compared to MCT, mRNA expression of SMCT1 has been shown to be downregulated by transcription factors Sp1, C/EBPδ, and TNFα, while activin A upregulates SLC5A8 gene via Smad3 [2, 48, 49]. Insulin and SGK1 appear to inhibit SMCT1 functional activity in Xenopus oocytes expressing human SMCT1 [50].

1.5.2 Ontogeny and monocarboxylate regulation. Age and development strongly influence mRNA and protein expression of drug metabolizing enzymes and transporters. MCT1 expression is detected as early as oocyte maturation [51], and knockout of the transporter leads to embryonic lethality in mouse animal models, indicating its critical role in early mammalian development and survival [52]. MCTs undergo ontogeny changes in skeletal muscle, with MCT1 protein expression increasing with age, while MCT4 decreases with age, indicating isoform-specific regulatory pathways based on ontogeny [53]. In contrast to the linear expression patterns observed with PGP, OAT, and SGLT, MCT1 and MCT2 mRNA levels in the mouse brain are low at birth, peak at pre-weaning, and return to baseline levels at sexual maturity (Figure 1.3) [54]. Electron microscopic immunogold studies also found that 17-day old suckling rat pups had 25 times more MCT1 labeling in the blood brain barrier than adults [55]. This dynamic change is necessary to allow the developing brain to use circulating ketone bodies derived from maternal milk as an energy source for neuronal growth [54]. Proteomic analysis of human donor and postmortem livers showed MCT1 expression to be nonlinear, with higher expression in infants compared to neonates or adults [10]. There is limited data on the effects age has on MCT and SMCT expression in other drug disposition tissues, particularly the intestine and kidneys of pediatric and adolescent patients.
1.5.3 Diet and obesity on monocarboxylate regulation. Dietary conditions have a substantial role in regulating MCT and SMCT expression due to the nutrient and fuel sensing nature of the transporters. In fasting mice, MCT1 protein expression was elevated in the liver and kidney with no noticeable change in the intestine. This adaptive shift may compensate for energy deprivation by reabsorbing circulating lactate to undergo essential hepatic and renal gluconeogenesis [56]. Mice on a prolonged high fat diet displayed increased cellular expression of MCT1, MCT2 and MCT4 in the cortex and hippocampus, indicating long-term metabolic status effects on the brain caused by ketogenic induction [57]. Obese leptin-deficient (ob/ob) and diabetic leptin-receptor deficient (db/db) mice exhibited similar elevations of brain MCT1 and MCT2 compared to their wildtype controls under a normal diet, suggesting insulin as the putative signal for neuronal MCT regulation [57]. ob/ob mice also had reduced SMCT1 protein expression in the kidneys [58]. Haploinsufficient MCT1 mice resisted diet-induced obesity
when administered a high fat, high sugar diet compared to the wildtype, revealing MCT1’s significance in energy homeostasis (Figure 1.4) [52].

![Figure 1.4](image)

**Figure 1.4.** Photograph of representative MCT1+/+ and MCT1+/- mice fed with either normal diet (A) or high sugar high fat diet (B) [52].

### 1.5.4 Pregnancy and obesity on epigenetic regulation

Dietary effects are compounded by maternal over-nutrition and prenatal stress. Fetuses absorb lactate as a critical energy source via placental production or transfer from maternal blood [59, 60]. *In situ* hybridization experiments detected elevated MCT1, MCT4, and MCT9 mRNA in mouse placenta at gestational day 11.5 that decreased towards parturition [61]. Pregnant rats maintained on a high fat diet throughout gestation developed high body adiposity and yielded obese offspring with hyperinsulinemia, hyperleptinemia, and glucose intolerance upon weaning [62]. The maternal and biological mechanisms that cause metabolic and obesogenic disorders in offspring are vast
and not completely understood. Microarray gene expression analysis of aortas from the offspring of lard-fed rats identified altered expression of over 200 genomic and mitochondrial-specific mRNA sequences compared with rats on normal controlled diet [63]. One mechanism could be the dysregulation of plasma leptin with relation to MCT expression. Leptin hormone is secreted by adipocytes and enterocytes, and is involved in the central regulation of body weight homeostasis and metabolic balance. Leptin acts mainly on ObR leptin receptors in the arcuate nucleus within the hypothalamic–pituitary–adrenal axis to activate JAK and STAT pathways, which modulate neuropeptides that blunt hyperphagia and stimulate satiety [64]. Maternal rats on high fat diet during gestation and nursing produce offspring with hyperleptinemia and reduced STAT3 activation, indicators of decreased leptin sensitivity [65]. Leptin has been shown to enhance MCT1 and CD147-mediated uptake of short chain fatty acids in human intestine (Figure 1.5) [66]. Additionally, leptin plays a critical role in neurotrophic development of the hypothalamus in neonates, with a postnatal leptin surge at PND5 to PND10 in rodents. Blockage of this neurogenic event results in long-term leptin insensitivity and increased susceptibility to diet-induced obesity [67]. Crosstalk between ontogeny, diet, and hormonal leptin can potentially explain differential expression of MCTs in pediatric patients.
1.6 Dissertation Objectives and Hypothesis

Individual factors such as ontogeny and diet have been shown to regulate the expression of monocarboxylate transporters; however, there is minimal literature detailing how obesity affects MCT and SMCT expression throughout pediatric development, or the potential ramifications on monocarboxylic drug disposition in childhood pharmacotherapy. The expression levels of these drug transporters in organs of drug distribution can illuminate the mechanisms for ADME and lead to better predictive pharmacokinetic models in treating pediatric patients.

The overall objective of this dissertation is to elucidate the role of high fat diet induced obesity on MCT and SMCT expression in tissues of drug disposition in an animal model of childhood obesity. Our hypothesis is that monocarboxylic transporter expression will be affected by age in a tissue-specific manner, and that over-nutrition will increase expression levels compared to normal diet conditions. To evaluate this hypothesis, we will quantify mRNA, total cellular and membrane protein expression of MCT1, MCT4, SMCT1, and CD147 in the
gastrointestinal tract, liver and kidneys of obese male and female Sprague Dawley rats and age-matched non-obese control animals from birth to sexual maturity. Furthermore, we will correlate circulating levels of serum leptin, an obesogenic biomarker and hormone regulator of monocarboxylate transporters, with changes in protein expression.
2.1 Animal Study, Tissue Collection, and Storage Conditions

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of the Pacific. Pregnant female Sprague Dawley (SD) rats (Charles River, Hollister, CA) were delivered to animal facilities on embryonic day 2 (E2) and allowed to acclimatize for 5 days before administering the test diets. Starting on E7 pregnant rats received either normal chow (NC) or high fat diet (HFD), and remained on the diet for the duration of the pregnancy until pup weaning. Tissues from the liver, kidney, and intestine were collected from offspring at post-natal day (PND) 1, 7, 14, 21, 28, 35, and 42 (N = 5-7 per group: total number of rats = 167), snap frozen in liquid nitrogen and stored at -80°C. In males, HFD had near identical bodyweights as their NC counterparts, with the only significant increases at PND 21 and 35 (Figure 2.1A). Females only had significant increases in bodyweight at PND 14 and 21 (Figure 2.1B). Similarly, leptin levels displayed physiological inconsistencies with our experimental design, with minimal increases of leptin at PND 21, 28, 35, and 42 in the HFD group. Leptin levels were also higher in the NC compared to HFD at PND1 (Figure 2.1C). This led us to conclude that the HFD was not inducing obesity in the maternal rats when the diet was administered on E7, and subsequently the offspring were not displaying hallmarks of obesity, including increases in body weight and circulating levels of serum leptin. Thus, the animal protocol was revised to utilize an earlier embryonic day to start administering the test diets. Also notable was the exclusion of PND 1 and 7 rats in the new studies, due to the smaller statures of these animals, which would have limited our downstream transcriptional and translational applications, particularly with studies in the neonatal gastrointestinal tract.
Figure 2.1. Rat bodyweights (A, B) and serum leptin (C) from the 2017 obesity study. Data is presented as mean ± SD (N = 5-7 per group). Leptin was not measured in PND35 females due to insufficient N.

In our revised study design, pregnant female SD rats were procured from Charles River (Hollister, California) at E2 upon arrival. Animals were housed in humidity and temperature-controlled facilities with 12-hour day/night cycles. Rats were fed either TestDiet NC consisting of 10% energy from fat, or TestDiet HFD comprised of 60% energy from fat ad libitum. Following parturition, male and female offspring from both dietary groups continued on their respective diets before being culled at PND 14, 21, 28, 35, or 42. Tissues from the kidney, liver, the segments of the small intestine (duodenum, jejunum, ileum), and colon were collected.
following exsanguination under isoflurane anesthesia, snap frozen in liquid nitrogen and stored at -80°C for further analysis. Animal bodyweights and tissue weights were recorded.

2.2 Serum Leptin Assay

Whole blood was collected via aortic puncture and placed in an Eppendorf tube, then incubated at room temperature for 30 minutes to promote coagulation. Blood was centrifuged at 5,000 x g for 15 minutes, and serum collected and stored at -20°C before analysis. Circulating leptin levels were quantified using Leptin Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s instructions. The assay is specific to natural and recombinant mouse and rat leptin, with 0.24% cross-reactivity with human leptin. Rat serum samples were diluted 10-fold in calibrator diluent and pipetted in duplicate into a 96-well plate pre-coated with immobilized polyclonal antibody that specifically binds to rat leptin, and allowed to incubate at room temperature for 2 hours. After a wash step to remove unbound leptin-antibody complexes, an enzyme-linked polyclonal antibody conjugated with horseradish peroxidase (HRP) was added to each well, and allowed to incubate at room temperature for 2 hours. After another wash step to remove unbound conjugate-leptin complexes, a substrate solution containing stabilized hydrogen peroxide and chromogen tetramethylbenzidine (TMB) was added to each well and allowed to incubate for 30 minutes. The colorless, diamine form of TMB is oxidized by HRP to form a blue-tinted diamine intermediate, where the optical density can be determined using a spectrophotometer at A650 nm. However, due to the instability of the redox reaction, a stop solution containing hydrochloric acid was added to each well, resulting in a yellow color-shift and providing an extended time stable A450 nm absorbance. Serum leptin concentrations were calculated utilizing a standard curve of serial diluted recombinant mouse leptin run on the same plate in duplicate (Figure 2.2).
2.3 qPCR

RNA was isolated using PureLink RNA Mini Kit (Invitrogen) from 20-30 mg of frozen tissue following the manufacturer’s instructions. The tissue was homogenized in Lysis Buffer containing 1% 2-mercaptoethanol and guanidinium isothiocyanate, a chaotropic salt that protects RNA from endogenous RNases. After centrifugation to remove cell sediment, the supernatant was added to an equal volume of 70% ethanol and pipetted onto a silica-based cartridge to bind RNA and DNA. DNase I (Invitrogen) was incubated on the cartridge for 15 minutes at room temperature to remove genomic DNA from the sample. After DNase incubation, Wash Buffer I and Wash Buffer II were applied sequentially to wash the cartridge to remove residual DNA, protein, and chemical contaminants. RNA was recovered from the cartridge by eluting with 50 μL of RNase-free water, and RNA samples were stored at -80°C.

RNA concentration was determined using a Nanodrop (Thermo Fisher Scientific). The absorbance at 260 nm and 280 nm was used to assess RNA purity, with a $A_{260}/A_{280}$ ratio of
approximately 2.0 accepted as pure for RNA. FlashGel (Lonza) electrophoresis was used to verify the integrity of all RNA samples prior to qPCR analysis for mRNA expression. RNA integrity was demonstrated by the presence of two clear nucleic acid bands representing 28S and 18S rRNA with an intensity ratio of 2:1. Representative FlashGel of RNA integrity evaluations are shown in Figure 2.3. Absence of bands or presence of smears (lanes #5-6) on the FlashGel indicated that the isolated mRNA was degraded and not suitable for downstream applications, and new RNA had to be isolated. Two clear bands (lanes #1-4) represented good mRNA stability, which were used for subsequent cDNA synthesis.

![Figure 2.3. FlashGel of male PND42 ileum total RNA.](image)

cDNA was synthesized using iScript cDNA Kit (Biorad). 40 μL reaction mixtures were created per PCR reaction sample using 5x iScript Supermix and 2000 ng of total RNA. Each reaction included 2 μL of Alien RNA (Agilent) diluted at 1:6,000 as an external standard control. Alien RNA transcript is an in vitro-transcribed RNA molecule that has no significant homology
to any known nucleic acids. The thermocycler conditions were as follows: 25°C for 5 minutes (priming), 42°C for 30 min (reverse transcription) and 85°C for 5 minutes (RT inactivation). The cDNA was stored at -20°C for further analysis.

qPCR was performed using iTaq™ Universal SYBR® Green One-Step Kit (Biorad). The genes analyzed were MCT1, MCT4, SMCT1, CD147, and r18S housekeeping gene. qPCR assays were validated by a previous graduate student in our lab [68]. Each 20 μL PCR reaction contained 10 μL of 2x iTaq Universal SYBR Green Supermix, 7 μL of molecular grade water, 1 μL of 100 μM forward primers, and 1 μL of 100 μM reverse primers specific to the gene of interest. 1 μL of diluted cDNA template was added per tube at the end step. All tubes were centrifuged and kept on ice prior to transfer to the CFX Connect Real-Time PCR Detection System (Biorad). A serial dilution of plasmid template expressing the gene of interest was used to generate a standard curve. Direct detection of PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR® Green dye to newly amplified double-stranded DNA. PCR read-out was given as the number of PCR cycles (quantitation cycle, or Cq) necessary to achieve a threshold level of fluorescence. The thermocycler conditions were as follows: 95°C for 35 seconds (denaturation), 60°C for 30 seconds (annealing and elongation), and this process was repeated for a total of 40 cycles. Raw data from qPCR was analyzed using CFX Manager (Biorad).

2.4 Protein Sample Preparation

2.4.1 Whole cell lysate of liver. Rat liver tissue (approximately 30 mg) was placed in 600 μL of ice-cold radio-immunoprecipitation assay buffer (RIPA) containing Protease Inhibitor Cocktail (UltraCruz, Santa Cruz Biotechnology) and homogenized using a microtube homogenizer, then rotated for 15 minutes at 4°C. Samples were then centrifuged at 14,000 x g
for 15 minutes at 4°C to remove excess cell debris. The supernatant was collected for analysis of whole cell extracted protein expression and stored at -80°C until further analysis. Protein concentration was measured using the BCA assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific) following the manufacturer’s instructions.

2.4.2 Membrane protein extraction of liver. Membrane bound protein was extracted from snap frozen liver using a Calbiochem ProteoExtract Native Membrane Protein Extraction Kit (EMD Millipore) following the manufacturer’s instructions. This method forgoes 2-phase partitioning and detergent-based protein extraction, which utilizes intrinsic hydrophobicity of membrane proteins, and relies on differential extraction based on the integral protein’s direct association with cellular membranes. One modification in protocol was that all buffer volumes were halved, to account for the smaller mass of the pediatric specimens and to increase the protein concentrations. 30 mg of tissue was homogenized in 1 mL of the provided Extraction Buffer 1 with protease inhibitor. The sample was rotated at 4°C for 10 minutes on a rotary shaker, then centrifuged at 16,000 x g and 4°C for 15 minutes. The supernatant containing the soluble cytosolic protein fraction was collected, and stored at 80°C for validation assays. 500 μL of Extraction Buffer 2 with protease inhibitor was added to the cell pellet, which was gently re-suspended by pipetting over ice. Samples were rotated at 4°C for 30 minutes on a rotator, and then centrifuged at 16,000 x g and 4°C for 15 minutes. The supernatant was then collected, which contained the membrane fraction enriched in integral membrane and membrane associated proteins, and samples were stored at -80°C for further analysis. Protein concentrations were determined using Pierce BCA Protein Assay Kit (ThermoFisher) with bovine serum albumin as the standard. Complete membrane extraction was validated by running western blots using 10
μg of the cytosolic soluble and membrane fractions for each sample and probed for the presence of Na+/K+ ATPase (Figure 2.4).

![Figure 2.4](image)

**Figure 2.4.** Membrane extraction validation of PND21 male and female liver using Na+/K+ ATPase antibody. MF=membrane fraction, SF=soluble fraction.

### 2.4.3 Membrane protein extraction of kidney

Membrane protein fraction of kidneys was isolated using the same protocol as the liver, with one modification in the tissue preparation. Previous morphology studies have shown that MCT1 and SMCT1 are localized in the proximal tubules in the renal cortex [69, 70]. Thus, snap frozen kidney specimens were bisected longitudinally, with only the cortex excised and added to the extraction buffers.

### 2.4.4 Membrane protein extraction of intestine

Previously, our lab attempted to isolate intestinal membrane using whole tissue from the ileum. However, this resulted in inconsistent extraction efficiencies during the validation step, with high sample-to-sample variation despite careful measurements of both tissue amount and buffer volumes (Figure 2.5A). One postulation was that the inclusion of the entire microanatomy of the ileum during extraction interfered with the accuracy of BCA assays. Inclusion of the various connective tissue layers may have led to overall higher protein concentrations. However, Na+/K+ ATPase is a specific biomarker for membrane proteins, and is expressed in basolateral membrane of enterocytes [71]. By separating only the mucosal layer for protein extraction, we were able to attain higher fidelity in our validation and protein concentrations, ensuring the accuracy of downstream transporter studies (Figure 2.5B).
Figure 2.5. Membrane protein validation in ileum using Na+/K+ ATPase on soluble fraction and membrane fraction of each animal. Whole intestinal tissue yielded incomplete extraction in males 1, 2, and 6 (A), while using enterocyte samples increased the success rate for membrane protein isolation as indicated by enhanced biomarker expression (B).

The membrane protein fraction of ileum was isolated using the same protocol as the liver, with one modification in the tissue preparation. Frozen ileum segments were thawed on a petri dish submerged in ice-cold normal saline solution with protease inhibitor. Surgical scissors were used to cut along the tunica serosa and muscularis, exposing the mucosa layer that was immediately flushed with saline to inhibit enzymatic degradation of tissue. Microscope slides were used to scrape the mucosal lining, which is comprised of intestinal enterocytes. These cells were then added to the extraction buffers for homogenization and membrane isolation.

2.5 Western Blotting

2.5.1 Assay optimization. Western blotting protocols and antibody selection had to undergo rigorous pilot studies to optimize experimentation. An initial obstacle faced was streamlining a western blotting methodology that could account for variabilities in different tissue and species types. For example, one SMCT1 antibody tested yielded vastly different banding patterns based on the aforementioned parameters (Figure 2.6). Lysate preparation also affected the interpretability of western data, with one MCT4 antibody yielding multiple band smears in the whole cell extracts compared to the membrane protein fractions (Figure 2.7).
Figure 2.6. Western blot using SMCT1 antibody probing kidney lysates from different species and protein fractions.

Figure 2.7. Western blot using MCT4 antibody with identical animals, but different protein fractions.

Different batches of commercially available antibody factored into our optimization, necessitating the validation of our antibody inventories to ensure the fidelity of western experiments between lot numbers (Figure 2.8). It was this lot-to-lot variability that required us
to switch MCT4 antibodies between experiments. Buffer conditions also affected western blot outcomes, with SMCT1 antibody diluted in 1X PBST exhibiting singular bands of the target protein compared to the multiple banding patterns when diluted in 1X TBST, likely due to chemically induced nonspecific binding with the tris formulated antibodies (Figure 2.9). Similarly, SMCT1 antibody formulated in SEA BLOCK buffer (Thermofisher) composed of steelhead salmon serum yield western blots with high background artifacts compared to PBST.

Figure 2.8. Western blots of kidney membrane fractions, using MCT4 antibodies from the same vendor and catalog number, but different lot numbers.

Figure 2.9. Western blots using identical ileum samples, with $1^0$ SMCT1 and $2^0$ antibodies diluted in either TBST (A) or PBST (B).
2.5.2 Western blot conditions. Whole cell lysate from the liver (15 μg), and membrane fractions of liver (5 μg), kidney (5 μg), and ileum (5 μg) were mixed with 4X Laemmli sample buffer (BioRad) containing 10% 2-mercaptoethanol and heated at 37°C for 30 minutes. Samples were separated by electrophoresis at 200V on a 10% TGX Stain Free Fast Cast gel (BioRad) and transferred to nitrocellulose membranes at 100V for 25 minutes. Membranes were blocked in 5% milk in 1X PBST for 1 hour at room temperature. Membranes were then incubated with primary antibody on a shaker overnight at 4°C.

The primary antibodies used were rabbit anti-MCT1 at 1:1,350 dilution (AB3540P, EMD Millipore), mouse anti-MCT4 at 1:1,000 dilution (SC-376140, Santa Cruz), rabbit anti-MCT4 at 1:1,500 dilution (AVIVA, OASG04421), goat anti-CD147 at 1:1,000 dilution (SC-9757, Santa Cruz), rabbit anti-SMCT1 at 1:1,000 dilution (ARP44110_P050, AVIVA), and rabbit anti-Na+/K+ ATPase at 1:1,000 dilution (SC-28800, Santa Cruz). The membranes were washed in triplicate for 10 minutes in 1X PBST then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature with gentle rocking. The secondary antibodies used were goat anti-rabbit at 1:10,000 dilution (AB97051, Abcam), donkey anti-goat at 1:10,000 (SC-2056, Santa Cruz), and anti-mouse IgG kappa binding protein at 1:5,000 (SC-516102, Santa Cruz). The membranes were then washed in triplicate for 10 minutes with 1X PBST and incubated with Clarity Western ECL Substrate (BioRad) for 2 minutes. Protein bands were visualized via chemiluminescence using the Chemidoc Touch Imaging System (BioRad). Band density was determined using BioRad Image lab software with Na+/K+ ATPase as a loading control, before transitioning to a stain free method for total protein load.

2.5.3 Protein normalization. Initially, the housekeeping protein Na+/K+ ATPase was used as a reference protein to account for variable sample loading during SDS-PAGE. This
technique worked relatively well in liver membrane protein of ontogeny on a normal lab diet, with minimal changes in Na+/K+ ATPase expression between samples (Figure 2.10A). However, other tissues exhibited differential expression, with linear increases in levels of Na+/K+ ATPase with increasing age in duodenum whole cell extracts (Figure 2.10B). Diet affected Na+/K+ ATPase levels, with slightly higher expression in HFD compared to NC rats in PND 21 ileum (Figure 2.11). Altered expression had been documented in previous obesity studies, with downregulation of plasma membrane Na+/K+ ATPase in mouse jejunum compared to NC [72]. Gender also factored into variable Na+/K+ ATPase expression, with females demonstrating slightly higher levels than males in PND42 kidneys (Figure 2.12).

Figure 2.10. Na+/K+ ATPase protein expression in male liver (A) and duodenum (B) with increasing age.

Figure 2.11. Na+/K+ ATPase protein expression of the ileum in age-matched males on NC and HFD.

Figure 2.12. Na+/K+ ATPase protein expression in kidneys of age-matched males (A) and females (B).
We attempted to use another housekeeping protein, GAPDH, as a loading control, but experienced similarly inconsistent results based on ontogeny (Figure 2.13). GAPDH is largely a cytosolic biomarker with minimal expression or effectiveness in analyzing membranous fractions [73]. Altogether, this showed that despite equal loading of protein derived from our BCA assay calculations, housekeeping proteins are greatly affected by age, diet, gender, and tissue-type, necessitating a more reliable approach for sample normalization.

Figure 2.13. GAPDH protein expression in male duodenum WCE, with increasing age.

Ponceau-S (Santa Cruz Biotechnology) is a reversible staining solution that is used on nitrocellulose membranes after electrophoresis for the detection of proteins. It is used to quantify total protein amount, due to the negatively charged Ponceau-S binding to positively charged amino groups, as well as non-covalent binding to nonpolar protein residues. The limit of detection is around 250 ng of protein, with quick rinses of deionized water to achieve desired staining of proteins, and a wash step in 0.1 N NaOH for complete destaining. The use of Ponceau-S initially yielded favorable results, with similar protein bands per lane, indicating equal loading of protein samples. However, the membranes were difficult to evaluate in Image Lab software due to the faint protein band intensities in the captured image, likely due to the requisite smaller amounts of loaded protein from pediatric samples, and the transient binding nature of the dye to proteins (Figure 2.14A).

Stain-free gels (TGX Stain-Free™ FastCast™ Acrylamide 10%, BioRad) were tested as a method for measuring total protein. Stain-free gels retain a similar chemical composition to
traditional polyacrylamide gels, but contain a proprietary 58 kD trihalo fluorophore moiety in their gel formulation that reacts and covalently binds to tryptophan residues in the protein samples during electrophoresis, with a limit of detection of 20-50 ng protein. This is crucial as 90% of all endogenous proteins in the most commonly studied organisms, such as humans, mice, and rats contain tryptophan, with 10% of the undetectable tryptophan-less proteins having a molecular weight of less than 10 kD. All monocarboxylate transporters and chaperone proteins in these studies have at least one tryptophan residue in their sequence (Uniprot). The trihalo-tryptophan complexes are activated via UV irradiation of the gel, and tryptophan-adducts on nitrocellulose membranes emit fluorescence when exposed to UV light. The captured image could then be analyzed in Image Lab, and observed for total band volume, adjusted total band volume, total lane volume and adjusted total lane volume. Linearity was previously validated by another graduate student using serialized protein loading. Compared to Ponceau-S, the enhanced fluorescence signal using the stain-free method allowed for improved total protein visualization that could be used to accurately and consistently quantitate total protein loaded for normalization of monocarboxylate transporters (Figure 2.14B).

Figure 2.14. SDS-PAGE followed by transfer onto nitrocellulose membrane. Total protein visualization using Ponceau-S (A) or Stain-Free (B).
CHAPTER 3: DEVELOPMENTAL EXPRESSION OF MONOCARBOXYLATE TRANSPORTER 1 AND 4 IN RAT LIVER

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3.1 Introduction

Maturation of drug elimination pathways contributes to the wide range of inter-individual variability in drug disposition observed in pediatric populations. Clinical pharmacokinetic studies have identified post-natal age as a significant factor influencing clearance, with hepatic and renal elimination representing the primary mechanisms of drug clearance [74, 75]. Developmental changes in hepatic drug clearance are governed by maturation of metabolism and drug transport pathways. While maturation of drug metabolizing enzymes [76], and major hepatic drug transporters [77] have been extensively studied in multiple species, there is limited data evaluating the maturation of monocarboxylate transporters in the liver.

Monocarboxylate transporters are involved in the active transport of essential nutrients and pharmaceutical agents, including lactate, pyruvate, butyrate and ketone bodies, nicotinate, valproic acid, nateglinide, salicylate, atorvastatin, and γ-hydroxybutyrate (GHB) [17, 78, 79]. There are two monocarboxylate transporter families, SLC16A and SLC5A; however, only proton-dependent monocarboxylate transporters (MCTs) encoded by SLC16A are expressed in the liver. There are 14 members in the MCT family with MCT1 through 4 (SLC16A1, SLC16A7, SLC16A8 and SLC16A3) involved in proton-coupled transport with overlapping substrate specificity [17, 41]. MCT1 and 4, the primary MCTs expressed in the liver, are sorted and inserted into the basolateral plasma membrane of hepatocytes through their association with the ancillary protein CD147 [38]. MCTs function as bidirectional transporters with directional
flux of MCT substrates governed by the pH gradient [80], with rate of transport also dependent on MCT and CD147 expression and co-localization [29]. Changes in the regulation or trafficking of MCTs leading to differences in membrane expression can alter the hepatic transport of their substrates, leading to alterations in intracellular drug concentrations. Differences in hepatic transport may impact overall drug exposure, which can lead to toxic or sub-therapeutic drug levels during development [75]. It is vital to understand the maturation of these transporters to optimize therapeutic regimens for their substrates.

There is limited information in the literature on the maturation of monocarboxylate transporters in tissues related to drug disposition. MCT1 has transient embryonic expression in the dorsal and lateral anlage of rat cerebral cortex (12), whereas in the gluteus medius muscle in horse, MCT1 increases and MCT4 is stably expressed with age (13), suggesting MCTs are regulated in a tissue-specific manner. MCT expression differs based on species, with minimal expression of MCT1 in hamster liver, whereas MCT1 is the predominant isoform expressed in mouse, rat, and human livers [81]. Proteomic studies of human hepatic MCT1 show nonlinear, age-dependent levels of expression [82]. These studies indicate that MCT maturation should be evaluated in individual tissues to identify tissue-specific maturation patterns of each isoform and the potential impact on drug pharmacokinetics. The objective of the present study is to quantify age-dependent hepatic mRNA, total and membrane protein expression of MCTs and their ancillary protein CD147 in male and female rats, to evaluate their developmental regulation with respect to transcription, translation and membrane trafficking.

3.2 Methods

3.2.1 Animals and tissue collection. Male and female Sprague-Dawley rats (> 21 days of age), pregnant females (E14 on arrival) and lactating females with litters (post-natal day seven
on arrival) were obtained from Envigo (USA). For pups less than 21 days of age, one male and one female pup was removed from each litter at individual post-natal time points. Pups were weaned at 21 days of age. Rats were group housed (except pregnant females) and kept under controlled lighting (12h light/dark cycle) and temperature (20 ± 2°C) conditions with food and water provided ad libitum. Liver samples were collected (N = 3 - 5 per gender per age group) from post-natal day (PND) 1 through 42 following exsanguination under isoflurane anesthesia, and snap frozen in liquid nitrogen with storage at -80°C. Body and liver weights were recorded at time of collection (Figure 3.1). All animal experiments were approved by the Institutional Animal Care and Use Committee of University of the Pacific.
Figure 3.1. Body [A] and liver weights (normalized to body weight) [B] for male and female Sprague-Dawley rats from birth to sexual maturity. Data is presented as mean ± SD (N = 4 – 5 rats per age and sex). *P < 0.05.
3.2.2 Sex determination. Sex was assigned for pups less than PND21 by ano-genital distance at the time of tissue collection and was confirmed by PCR. Liver (25 mg) was utilized to isolate genomic DNA using a PureLink Genomic DNA Mini Kit (Invitrogen) following the manufacturer’s instructions. DNA concentration and purity was evaluated using a Nanodrop (Thermo Scientific). The presence of SRY, a Y specific sequence, and β-actin (PCR control) were evaluated by end-point PCR following a previously published method with minor modifications [83]. Briefly, 2 μl of genomic DNA was combined with AmpliTaq Gold (ThermoFisher) and corresponding buffer, 0.2 mM dNTPs, 0.2 μM forward and reverse primers with a final reaction volume of 25 μl. PCR reactions were run separately for each gene. Samples were run through a thermal cycle using a T100 Thermal Cycler (BioRad) as follows: 1 minute at 95°C, 1 minute at 52°C, and 1 minute at 72°C with the cycle repeated 35 times. PCR products were separated on a 1.4% agarose gel containing SYBR Safe (Invitrogen) in 1X TBE buffer, run at 100 V for 45 minutes and visualized using ChemiDoc Touch imaging system (BioRad).

3.2.3 qPCR. Liver (10 mg) was homogenized and RNA was isolated using a PureLink RNA Mini kit (Invitrogen) according to the manufacturer’s instructions with a DNase treatment step. RNA concentration and purity was assessed using a Nanodrop (Thermofisher). RNA stability was verified by RNA Flash Gel (Lonza) to visualize 18S and 28S rRNA bands. 1000 ng of total RNA was added to each 20 μL cDNA synthesis reaction, which was reverse transcribed using iScript Reverse Transcription Supermix (BioRad). Primer sequences, annealing temperatures, product sizes and assay validation were previously described [68]. Serial dilutions of plasmids (covering 5 to 6 orders of magnitude) containing the gene-specific amplicon were used as standards to confirm qPCR efficiency and to quantify relative gene expression.
Quantitative analyses of MCT1, MCT4, and CD147 were performed with iTaq Universal SYBR Green Supermix as described previously [68] on a CFX96 Connect (BioRad) with a standard curve run on every plate. The external control Alien RNA (Agilent) was utilized for data normalization. All biological samples were run in triplicate. The purity of all qPCR products was determined by melting curve. Data was quantified by $2^{-\Delta\Delta CT}$ method [84] or in arbitrary units based on the standard curve.

### 3.2.4 Western blot analysis

Whole cell extracts were generated from 25 mg of rat liver tissue and 500 μl of ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing UltraCruz Protease Inhibitor Cocktail (Santa Cruz Biotechnology). Samples were briefly homogenized then spun at 13,500 rpm in a 4°C microcentrifuge to collect protein supernatants. Soluble cytosolic and total membrane fractions were isolated from 25 mg liver samples using a Calbiochem ProteoExtract Native Membrane Protein Extraction Kit (EMD-Millipore) following the manufacturer’s instructions. Protein concentrations were calculated using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). MCT1, MCT4 and CD147 protein expression was quantified using western blot analysis of whole cell extract or membrane fraction. Samples (10 μg whole cell extract or 5 μg membrane fraction) were mixed with 2x Laemmli sample buffer (BioRad) and heated at 37°C for 30 minutes, then loaded onto 10% TGX Fastcast acrylamide gels (BioRad) and separated via SDS-PAGE at 200V for approximately 45 minutes. Following transfer onto nitrocellulose membranes at 100V for 25 minutes, membranes were blocked in 5% nonfat milk in phosphate-buffered saline with Tween 20 (PBST) for 1 hour at ambient temperature, then incubated for 16 hours at 4°C under gentle rocking with anti-MCT1 (1:1,350, EMD Millipore), anti-MCT4 (1:1,000, Santa Cruz Biotechnology), anti-CD147 (1:5,000, Santa Cruz Biotechnology), or anti-\(\mathrm{Na}^+\)/\(\mathrm{K}^+\) ATPase (1:1,000, Santa Cruz Biotechnology). All
antibodies were formulated in 1% nonfat milk/PBST. After washing three times for 10 min in PBST, blots were incubated with goat anti-rabbit IgG horseradish peroxidase-conjugated (1:5,000 Santa Cruz Biotechnology) or donkey anti-goat IgG horseradish peroxidase-conjugated secondary antibodies (1:10,000 EMD Millipore) for 1 hour at ambient temperature. Following a final 3×10 min PBST wash, immunoreactive bands were visualized using Clarity Western ECL substrate (Biorad) and band densities determined using ChemiDoc Touch Imaging System (Biorad).

3.2.5 Data analysis. Data is presented as mean ± standard deviation. Data was analyzed in GraphPad Prism 6 software using one-way analysis of variance (ANOVA), followed by Tukey’s HSD post hoc test. Differences of \( p < 0.05 \) in post-hoc comparison were considered to be significant. Pearson correlation analysis in GraphPad Prism 6 was used to evaluate the relationships between whole cell and membrane protein expression of MCT1, MCT4 and CD147.

3.3 Results

3.3.1 Sex determination. Sex was assigned at tissue collection by ano-genital distance for all pups less than PND21 with a target of four males and four females per age group. Based on PCR analysis of the SRY sequence, sex assignments were accurate for all pups at PND5 and later (data not shown). At PND1, the final PCR sex assignments yielded three male and five female pups (Figure 3.2).
3.3.2 MCT1 expression. Males and females had similar nonlinear transcriptional MCT1 profiles during development (Figure 3.3a and 3.3b). Expression generally increased with age, with peak mRNA levels at PND10 in the males and PND14 in females, before decreasing incrementally towards PND42. In males (Figure 3.3c), MCT1 whole cell protein expression was lowest at PND1, before increasing gradually with the highest expression observed at PND14, before reverting to expression levels consistent with neonatal animals at adolescence (PND42). A similar trend was observed in the females (Figure 3.3d), although expression was higher at PND1 and lower at PND42 as compared to the males. Similar patterns were observed for MCT1 membrane expression (Figure 3.3e and 3.3f), with reduced expression at PND1 and PND42, and significantly higher expression at PND14 and PND18 in the males and females, with the males also having significantly increased expression at PND10.

3.3.3 MCT4 expression. Males and females had similar linear transcriptional MCT4 profiles during development (Figure 3.4a and 3.4b), with the highest levels in neonatal rats at PND1, 3, 5, and 7, before decreasing incrementally with age towards PND42 (adolescents). This
differed from the observed patterns for MCT1 suggesting transporter-specific developmental regulation. MCT4 expression was undetectable in whole cell protein samples over the age range evaluated for both sexes. In males and females, membrane protein was minimally expressed at PND1, before increasing incrementally to peak at PND35, before decreasing drastically at PND42 (Figure 3.4c and 3.4d).
Figure 3.3. MCT1 mRNA, whole cell and membrane protein expression in males [A, C, E] and females [B, D, F] from birth to sexual maturity. A, B. Fold-change in MCT1 mRNA expression in rat liver. C, D. Whole cell MCT1 protein expression in the liver with data normalized to Na\(^+\)/K\(^+\) ATPase expression. A positive control sample (pooled liver whole cell sample from 9 week old male Sprague-Dawley rats) was loaded on each blot. E, F. MCT1 membrane expression in rat liver with data normalized to Na\(^+\)/K\(^+\) ATPase expression. A positive control sample (pooled liver whole cell sample from 9 week old male Sprague-Dawley rats) was loaded on each blot in the last lane. Data is presented as mean ± SD (N = 4 – 5 rats per age and sex). *P < 0.05
Figure 3.4. MCT4 mRNA, whole cell and membrane protein expression in males [A, C] and females [B, D] from birth to sexual maturity. A, B. Fold-change in MCT4 mRNA expression in rat liver relative to PND21 females. C, D. MCT4 membrane expression in rat liver with data normalized to Na⁺/K⁺ ATPase expression. A positive control sample (pooled liver whole cell sample from 9 week old male Sprague-Dawley rats) was loaded on each blot in the last lane. Data is presented as mean ± SD (N = 4 – 5 rats per age and sex). *P < 0.05

3.3.4 CD147 expression. Males and females demonstrated nonlinear developmental expression patterns for both whole cell and membrane protein. In males, CD147 whole protein expression was low at PND1 and increased to peak levels at PND14 and PND18 (approximately three times higher expression), before experiencing a decrease in expression levels towards PND42 (Figure 3.5a). In females, CD147 expression was low at PND1, before increasing significantly to its highest levels at PND14, before decreasing again to neonatal levels at PND42.
(Figure 3.5b). While males and females had similar expression levels at PND1, males had higher whole cell CD147 expression at PND42 compared to PND1, and to females at PND42. Membrane protein expression demonstrated similar expression pattern in both males and females (Figures 3.5b and 3.5c). Males demonstrated the highest membrane CD147 expression at PND14, while females demonstrated significantly higher expression at PND10, PND14 and PND18. Consistent with whole cell CD147 expression at PND42, males demonstrated approximately 2.5 times higher CD147 membrane expression as compared to females.
Figure 3.5. CD147 cell and membrane protein expression in males [A, C] and females [B, D] from birth to sexual maturity. A, B. Whole cell CD147 protein expression in the liver with data normalized to Na\(^+\)/K\(^+\) ATPase expression. A positive control sample (pooled liver whole cell sample from 9 week old male Sprague-Dawley rats) was loaded on each blot. C, D. CD147 membrane expression in rat liver with data normalized to Na\(^+\)/K\(^+\) ATPase expression. A positive control sample (pooled liver whole cell sample from 9 week old male Sprague-Dawley rats) was loaded on each blot in the last lane. Data is presented as mean ± SD (N = 4 – 5 rats per age and sex). *P < 0.05

3.3.5 Correlation analysis. In the male ontogeny (Figure 3.6a), comparisons between membrane and whole cell MCT1 expression displayed a significant positive correlation with an r value of 0.545. The female ontogeny (Figure 3.6b) had a similar significant positive correlation with an r value of 0.587. Correlations on the relationship between MCT1 and CD147 membrane and whole cell protein expression were conducted to investigate membrane trafficking. In males
(Figure 3.6c), there was a significant positive correlation with an r value of 0.585, while females (Figure 3.6d) had a significant positive correlation; however, the r value (0.342) was lower than that observed in males. Correlation analysis between membrane expression of MCT1 and CD147 demonstrated strong significant positive correlations with r values of 0.8318 and 0.8447 in males and females (Figure 3.6e and 3.6f). Relationships between MCT4 whole cell and membrane expression could not be evaluated, as whole cell MCT4 expression was not detectable. Correlation analysis was conducted to evaluate the trafficking relationship between MCT4 and CD147 membrane expression. In contrast to MCT1, MCT4 membrane expression demonstrated a weak negative correlation with membrane CD147 expression; however, this relationship was not significant in male or females (Figure 3.7a and 3.7b).
Figure 3.6. Pearson correlation analysis in males [A, C, E] and females [B, D, F]. A, B. Correlation analysis between MCT1 whole cell protein and membrane expression. C, D. Correlation analysis between whole cell MCT1 and CD147 expression. E, F. Correlation analysis between membrane MCT1 and CD147 expression. (N = 42 males; N = 44 females)
Figure 3.7. Pearson correlation analysis between membrane MCT4 and CD147 expression in males [A] and females [B]. (N = 42 males; N = 44 females)

3.4 Discussion

Monocarboxylate transporters are ubiquitously expressed in the human body, and studies have shown MCTs are associated with the absorption and excretion of monocarboxylate drugs in drug disposition organs such as the liver, kidneys, and intestines. The extent of transport capacity is dependent on MCT membrane protein expression, with changes in expression altering drug disposition in cells and tissues. However, there is limited data regarding the maturation and expression of hepatic MCTs relative to age, particularly during pediatric development in both males and females. A technical obstacle in clinical studies is that hepatic fetal and infant tissues are often derived postmortem, with confounding factors such as race, sex, disease, and polygenic traits, as well as limited sampling size presenting barriers to controlled pediatric studies [82, 85]. To our knowledge, the present work is the first analysis of changes in rat hepatic MCT1, MCT4 and CD147 expression at the mRNA, whole cell, and membrane protein level during early stages of mammalian growth. Here, we show hepatic MCT1 and CD147 expression changes in a nonlinear fashion from birth to sexual maturity with a significant positive correlation between
MCT1 and CD147 membrane expression. The maturation pattern of MCT4 differed from MCT1, with low expressions at birth followed by a surge in membrane expression at PND35. Further, there was a weak negative correlation between MCT4 and CD147 membrane expression.

MCT1 is responsible for nutrient transport and essential for the metabolism of carbohydrates, amino acids, and fats. Consequently, dysregulation of MCT1 expression is associated with metabolic disorders such as obesity and abnormal insulin secretion [86], as well as altered drug transport and pharmacokinetics. MCT1 expression is detected as early as oocyte maturation [51], and knockout of the transporter leads to embryonic lethality in mouse animal models, indicating its critical role in early mammalian development and survival [52]. Comparisons between human fetal (gestational age range of 16.4-37.9 weeks), infant (postnatal age range of 0-11.4 weeks, gestational age at birth range of 27.1-41 weeks), and adult specimen demonstrated nonlinear maturation of MCT1 membrane protein in the liver [82], which is consistent with that pattern of MCT1 maturation observed in the present study. Maturation of mRNA and whole cell protein expression of MCT1 was consistent with membrane protein expression; however, the moderate positive correlation suggests that MCT1 translation does not completely account for the transporter’s expression at the plasma membrane suggesting that other regulatory factors mediate trafficking of MCT1 to the cell membrane during development.

Developmental mRNA expression of MCT4 diverges from the MCT1 profile, with high mRNA expression at birth and decreasing levels towards sexual maturity; however, MCT4 protein data reverses this trend, with low membrane protein expression at early ages until a sudden peak at postnatal age 35. The mechanisms underlying this discrepancy between MCT4 mRNA and protein expression are not completely understood. Other reviews have reported the
historically poor correlation observed between mRNA and \textit{ex-vivo} protein in complex biological samples, which complicates data interpretation for transcriptional-translational model predictions [87]. Previous studies have shown that endogenous microRNAs, miR-29 and miR-124, can degrade MCT1 mRNA or inhibit its translation, while let-b7 downregulates CD147 expression [88], similar mechanisms could explain the discrepancy that we observed between MCT4 mRNA and protein expression. miR-29 in particular is highly expressed in β islet cells, which may explain the diminished amount of MCT1 found in pancreas compared to other tissues [47], and MCT regulation by miRNA may explain the observed tissue differences in expression. While fewer regulation studies have been performed on MCT4, the transcription factor HIF-1α has been shown to upregulate mRNA and protein expression of MCT4 during hypoxia, but not MCT1 [89]. However, the mechanisms regulating MCT4 expression under normoxic conditions remain to be determined [45]. Further experiments identifying possible miRNA activity and other post-transcriptional modifications may explain the dynamic changes in MCT1 and MCT4 expression based on maturation, with subsequent upregulation and downregulation events dependent on MCT isoform, biological sample, and tissue-type [90].

Unlike hepatic transporters and drug metabolizing enzymes, few studies have looked at the age-dependent expression of ancillary proteins in the liver, or their mechanistic roles in drug transporter maturation. The CD147 glycoprotein binds to MCT1 and MCT4 and confers basolateral sorting onto the heterodimer, facilitating the transporter’s localization to the plasma membrane where it becomes functionally active [28]. Studies have shown that increased association between MCT1/4 and CD147 enhances plasma membrane expression of the transporter [91], whereas knockdown or silencing of the chaperone in various cells lines and transgenic animal models causes decreased MCT1/CD147 association and membrane co-
localization, as well as overall altered tissue distribution [92]. CD147 in the rumen of juvenile sheep was found to increase linearly based on age [93]. In our experiment, whole cell CD147 protein is expressed at minimal levels at birth, with increasing and maximum levels at postnatal ages 14/18 in the males and postnatal age 14 in females, then decreasing towards neonatal levels upon sexual maturity. Membrane levels of CD147 reflect the total protein trend, with peak expressions at postnatal age 14 in both sexes, and there exists strong, positive correlation between total and membrane-bound CD147 protein expression. Comparison of total protein MCT1 and CD147 yielded a significant moderate correlation, indicating total translation of the ancillary protein has minimal impact on whole protein expression of its transporter partner. However, we observed a significant strong positive correlation when comparing the membrane expression of MCT1 and CD147, signifying a strong association between the membrane proteins, and suggesting the ancillary protein is required as a chaperone for proper MCT1 trafficking to the cell surface throughout normal mammalian development, with a nonlinear expressional pattern dependent on age. In contrast, MCT4 membrane expression demonstrated a weak negative correlation with CD147 membrane expression suggesting that CD147 does not determine MCT4 membrane expression during development. The present study did not evaluate MCT4 membrane localization, which may be altered if it is no longer associated with CD147.

Previously, our group described the sex hormone dependent regulation and localization of MCT1 and MCT4 in the liver, with changes in expression between male and female rats, as well as in female rats at various stages in their estrus cycle [68]. Consistent with our previous results, we observed variability in hepatic MCT1, MCT4, and CD147 expression between the males and females in both juvenile and sexually mature rats. Males exhibited increased MCT1 membrane expression at PND10 and 18 compared to the females. Similarly, MCT4 and CD147 membrane
protein expression was higher in males compared to the females in juvenile rats, implicating a sex-specific, sex-hormone independent regulatory mechanisms in MCT expression. MCT1, MCT4 and CD147 membrane expression was higher in males than females at PND42, at which age rats will have circulating sex hormones. Increased abundance of MCTs and ancillary protein in males may result in greater hepatic transport capacity for nutrients and pharmaceutical agents compared to females, which can lead to increased drug absorption and reduced hepatobiliary clearance.

Our data is consistent with previous clinical studies on MCT maturation [82] and suggests several avenues for future transporter work. The nonlinearity of hepatic MCT1 expression indicates a clear need to assess the maturation of drug disposition processes to account for inter-individual variability in pharmacokinetics in pediatric populations. Drug uptake and efflux assays in the liver can also evaluate the mechanisms between MCT expression and function during development, as well as between adolescent male and female populations. Furthermore, we can explore the effects of age on the expessional changes of MCTs in other drug disposition tissues such as the intestines and kidney. In conclusion, we have demonstrated differential expression of hepatic MCT1, MCT4, and CD147 during development at the level of mRNA transcription, protein translation, and membrane trafficking in a pediatric animal model.
CHAPTER 4: MONOCARBOXYLATE TRANSPORTER IN THE LIVER-IMPACT OF DIET, ONTOGENY, AND SEX ON TRANSPORTER EXPRESSION

4.1 Introduction

The liver is a versatile organ responsible for gluconeogenesis and the synthesis of glycogen and lipid soluble vitamins, molecules essential for energy metabolism and normal cell function. Along with metabolic homeostasis, the liver plays a critical role in the elimination of toxins in the body. Hepatic elimination pathways comprise of the basolateral uptake of compounds from the extracellular space, basolateral efflux from hepatocytes into the blood, phase I and II biotransformation, and biliary excretion.

Membrane transporters are major determinants of energy metabolism, as well as pharmacokinetics, drug safety and efficacy. It is well established that the quantity of transporter at the membrane is proportional to the transmembrane flux of substrate, and functional activity of transporters is often inferred from protein expression [94]. Monocarboxylate transporters are encoded by the SLC16A gene family, and expressed in the basolateral plasma membrane of hepatocytes [38]. They control the uptake and efflux of a wide range of endogenous and exogenous monocarboxylate nutrients and drug substrates, such as lactate, pyruvate, ketone bodies, and valproic acid [25] [95]. There are 14 members in the SLC16A gene family, where MCT1-4 and 7 are categorized as proton-dependent monocarboxylate transporters. Among these, only MCT1, 2, and 4 are expressed in the human liver [17].

Inter-individual variability seen in drug pharmacokinetics and pharmacodynamics in humans may be influenced by the time-dependent development of drug elimination pathways. Indeed, crucial developmental changes in transporter expression occur early in fetal and infant development, which greatly affects the systemic uptake and clearance of pharmaceutical drugs in
pediatric patients [96] [97]. Maturation of monocarboxylate transporters is also influenced by obesity and over-nutrition, which can dysregulate normal expression patterns throughout ontogeny and adversely affect normal drug pharmacokinetics and drug therapy in obese populations. There is limited clinical data on how over-nutrition and age affect monocarboxylate transporter expression and activity in pediatric patients as they mature.

4.2 Chapter Objective

Monocarboxylate transporters exhibit transcriptional and translational changes based on ontogeny, as previously observed in the rat liver. Dietary changes also affect expression of MCTs; however, the combined effects of both diet and age on hepatic MCT regulation and expression has not been explored. Experiments from other groups have shown intersection between obesity, leptin levels, and increased MCT expression. Previous investigations by our laboratory have shown rodent MCT1 and CD147 protein expression peaks during nursing, but prior to weaning, while MCT4 expression is highest post-weaning [98]. The objective of this study is to compare hepatic MCT expression in a pediatric animal model of diet-induced obesity and age-matched controls, and observe how these patterns shift based on age and sex. We hypothesize that monocarboxylate transporters 1 and 4 would have highest expression pre-weaning and post-weaning respectively, and that the high fat diet cohorts would have increased transporter expression compared to their normal diet counterparts.

4.3 Data Analysis

Data is expressed as mean ± standard deviation. Expression of mRNA was normalized to the mean of the house-keeping genes, 18S and alien RNA, and fold-change was determined using the $2^{-\Delta\Delta CT}$ method. MCT1, MCT4, and CD147 membrane protein expression was normalized to total protein loaded using the Stain Free method. Correlation analysis was
performed to evaluate the relationships between transporter protein expression and leptin, and monocarboxylate transporter expression and CD147. Data was analyzed in GraphPad Prism 7 using two-way analysis of variance (ANOVA) with Tukey’s post hoc test. Differences were considered statistically significant when the p value was less than 0.05.

4.4 Results

4.4.1 Bodyweight and leptin. Bodyweights for all groups are presented in Figure 4.1A. There were statistically significant differences based on diet. Males on high fat diet (HFD) had statistically higher bodyweights at PND28 (p=0.002) and PND42 (p < 0.0001) compared to the normal diet (NC) cohorts. Females on HFD had statistically higher bodyweights at PND21 (p = 0.0303), PND28 (p = 0.0115), and PND35 (p = 0.0065) compared to the NC counterparts. There were statistically significant sex differences, with NC males having higher bodyweights at PND35 (p < 0.0001) and PND42 (p < 0.0001) than their female counterparts. HFD males had statistically higher bodyweights at PND42 (p < 0.0001) compared to females in the same age group.

Leptin levels for all groups are presented in Figure 4.1B. NC males had statistically higher leptin levels based on age at PND14 (p = 0.023) and PND21 (p = 0.0001) compared to PND42 males. HFD males had statistically significant increase in leptin based on age at HFD PND21 (p = 0.001) compared to HFD PND42 males. There was statistical significance based on diet, with HFD males having statistically higher leptin at HFD PND14 (p = 0.0005), HFD PND21 (p < 0.0001), and HFD PND42 (p = 0.011) compared to their age-matched NC counterparts (Figure 4.1C).

NC females on normal diet had statistically higher leptin based on age at NC PND14 (p = 0.0296) and NC PND21 (p = 0.0029) compared to NC PND42 females. HFD females had
statistically increased leptin based on age at HFD PND14 (p = 0.0250) and HFD PND21 (p < 0.0001) compared to HFD PND42 females. There was statistical significance based on diet, with females at HFD PND14 (p = 0.0125), HFD PND21 (p < 0.0001), and HFD PND28 (p = 0.0008) compared to females of the same age on normal diet (Figure 4.1D). There were no sex differences with leptin levels (Figure 4.1E, F).
Figure 4.1. Bodyweights and serum leptin levels. A. Bodyweights of all groups. B. Leptin of all groups. C. Leptin in males. D. Leptin in females. E. Leptin in normal chow groups. F. Leptin in high fat diet groups. Data presented as mean ± SD. (n = 6 rats per group). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.

4.4.2 MCT1 mRNA and membrane protein expression. MCT1 mRNA expression for all groups is presented in Figure 4.2A. MCT1 expression was nonlinear in males (high, low, high, low). In NC males, MCT1 mRNA was significantly higher at PND14 compared to NC
PND42 (p = 0.0024) (Figure 4.2B). MCT1 expression was nonlinear in females (low, high, low, high). Females on high fat diet had significantly higher MCT1 mRNA at PND 21 compared to HFD PND42 (p = 0.0405) (Figure 4.2C).

MCT1 protein expression for all groups is presented in Figure 4.3A. In NC males, MCT1 protein expression was significantly different at PND14 (higher, p = 0.0043) and PND28 (lower, p = 0.0438) compared to NC PND42. Males on high fat diet had significantly different expression at PND14 (higher, p < 0.0001), PND21 (p = 0.0067), PND28 (lower, p = 0.0005), and PND35 (lower, p = 0.0018) compared to HFD PND42. In both male normal and high fat diets, MCT1 protein levels peaked at PND14, decreased with age, before increasing at PND42, and protein expression was nonlinear (high, low, high). There was significant dietary difference, with males at HFD PND14 exhibiting higher MCT1 expression than the normal diet cohort (p = 0.0288). There was an observed, although not significant increase in expression in male HFD PND21, PND28, and PND42 compared to their normal diet counterparts (Figure 4.3B).

In NC females, MCT1 membrane protein expression was significantly higher at PND14 (p = 0.0067) and PND21 (p < 0.0001) compared to NC PND42. Similarly, HFD females had significantly higher expression at PND14 (p=0.0038) and PND21 (p=0.0173) compared to HFD PND42. There were significant dietary differences, with females at NC PND21 exhibiting higher MCT1 expression than the age-matched HFD females (p = 0.0001). There was an observed, though not significant increase in expression in female HFD PND14, PND35, and PND42 compared to their NC counterparts (Figure 4.3C). NC females had nonlinear MCT1 protein expression (low, high, low), while HFD females had a separate nonlinear pattern (high, low, high, low).
There were significant sex differences in NC animals. Males had significantly higher MCT1 expression than females at NC PND14 (p < 0.0001) and 42 (p = 0.0024); however, females had significantly higher expression than males at NC PND21 (p < 0.0001) (Figure 4.3D). There were significant sex differences in HFD animals, with males exhibiting higher MCT1 expression than females at HFD PND14 (p < 0.0001) and 42 (p < 0.0001) (Figure 4.3E).
Figure 4.2. mRNA Expression of MCT1 in the liver.  A. MCT1 fold change in all groups.  B. MCT1 fold change in males.  C. MCT1 fold change in females.  D. MCT1 fold change in normal chow groups.  E. MCT1 fold change in high fat diet groups.  Fold change relative to female post-natal day 35 group, normalized to Alien and 18S RNA.  Data presented as mean ± SD.  (n = 3-6 rats) *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes.  Statistical significance determined if P < 0.05.
Figure 4.3. Membrane protein expression of MCT1 in the liver, normalized to total loaded protein. 
A. MCT1 protein in all groups. B. MCT1 protein in males. C. MCT1 protein in females. D. MCT1 protein in normal chow groups. E. MCT1 protein in high fat diet groups. F. Representative western blot, MCT1 in PND42 males. Data presented as mean ± SD. (n = 6 rats). 
*Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
4.4.3 MCT4 mRNA and membrane protein expression. MCT4 mRNA for all groups is presented in Figure 4.4A. In NC males, MCT4 mRNA was nonlinear (high, low, high), with significantly higher levels at PND14 compared to NC PND42 ($p = 0.0419$). HFD males had nonlinear expression (low, high, low), with significantly higher MCT4 mRNA at PND14 ($p = 0.0238$) and PND21 ($p = 0.0025$) compared to HFD PND42 (Figure 4.4B). HFD females had significantly higher MCT4 mRNA at PND14 ($p = 0.0173$) and PND21 compared to HFD PND42 ($p=0.0002$) (Figure 4.4C). Females had nonlinear MCT4 mRNA expression (low, high, low, high), with no statistical significance.

MCT4 membrane protein expressions for all groups is presented in Figure 4.5A. There was significantly higher MCT4 expression in males at NC PND28 compared to NC PND42 ($p < 0.0001$). HFD males had significantly higher MCT4 expression at HFD PND28 compared to HFD PND42 ($p < 0.0001$). In both normal and high fat diets, MCT4 expression was nonlinear (low, high, low). MCT4 levels were low at PND14, peaked at PND28, before decreasing at PND42. There was significant dietary difference in males, with NC PND28 males having higher MCT4 expression than their age-matched HFD counterparts ($p = 0.0008$) (Figure 4.5B).

Female MCT4 membrane protein expression was nonlinear (high, low, high). In NC females, there was significantly higher MCT4 expression at NC PND14 compared to NC PND42 ($p < 0.0001$). HFD females had significantly lower MCT4 expression at HFD PND21 compared to HFD PND42 ($p = 0.0012$). In females on both diets, MCT4 expression peaked at PND14. There was significant dietary difference at PND14, with females on normal diet having higher MCT4 expression than their HFD counterparts ($p = 0.0361$) (Figure 4.5C).

There were significant sex differences in MCT4 expression in NC and HFD animals. NC males had significantly higher MCT4 mRNA levels than females at PND14 ($p=0.0212$) (Figure
Females had significantly higher MCT4 membrane expression than males at NC PND14 (p < 0.0001). In contrast, males had higher MCT4 membrane expression than females at NC PND28 (p < 0.0001) (Figure 4.5D). Females had higher MCT4 membrane expression than males at HFD PND14 (p = 0.0016) and PND42 (p = 0.0076), while males had higher expression than females at HFD PND21 (p = 0.0383) (Figure 4.5E).
Figure 4.4. mRNA Expression of MCT4 in the liver. A. MCT4 fold change in all groups. B. MCT4 fold change in males. C. MCT4 fold change in females. D. MCT4 fold change in normal chow groups. E. MCT4 fold change in high fat diet groups. Fold change relative to female post-natal day 42 group, normalized to Alien and 18S RNA. Data presented as mean ± SD. (n = 3-6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
4.4.4 CD147 mRNA and membrane protein expression. CD147 mRNA for all groups is presented in Figure 4.6A. NC males had nonlinear CD147 mRNA (high, low, high, low),
while HFD males had linear mRNA (high, low). NC females had nonlinear CD147 mRNA (high, low, high), while HFD females had nonlinear mRNA (low, high, low, high, low). At the mRNA level, the only statistical significance was higher CD147 transcription in males at NC PND14 compared to NC PND42 (p = 0.0250) (Figure 4.6B).

CD147 membrane protein expression for all groups is presented in Figure 4.7A. In males, CD147 is expressed nonlinearly at the membrane (high, low, high). In NC males, there was no significant differences in CD147 protein expression throughout ontogeny compared to PND42. HFD males had significant differences in expression at HFD PND14 (higher, p = 0.0018), PND21 (lower, p = 0.0008), PND28 (lower, p = 0.0109), and PND35 (lower, p = 0.0018) compared to HFD PND42. In both diets, male CD147 expression peaked at PND14, decreased with age, before increasing at PND42. There was significant dietary difference in males, with HFD PND14 (p = 0.0001) and HFD PND42 (p = 0.0126) males having higher CD147 expression than their age-matched NC counterparts (Figure 4.7B).

NC females had significantly higher CD147 membrane expression at NC PND21 compared to NC PND42 (p < 0.0001), with a nonlinear pattern (high, low, high). HFD females had stable CD147 expression at PND14-28, with linear pattern (high, low) (Figure 4.7C). Peak CD147 expression was at NC PND21 and HFD PND14-28.

There were statistically significant sex differences in animals fed NC, with males having higher CD147 membrane expression at NC PND14 compared to females (p = 0.0475). In contrast, females at NC PND21 had significantly higher expression than their age-matched male counterparts (p < 0.0001) (Figure 4.7D). There were significant sex differences in animals on HFD, with males at PND14 (p < 0.0001) and PND42 (p < 0.0001) having higher CD147 expression than age-matched females (Figure 4.7E).
Figure 4.6. mRNA Expression of CD147 in the liver. A. CD147 fold change in all groups. B. CD147 fold change in males. C. CD147 fold change in females. D. CD147 fold change in normal chow groups. E. CD147 fold change in high fat diet groups. Fold change relative to female post-natal day 28 group, normalized to Alien and 18S RNA. Data presented as mean ± SD. (n = 3-6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
**Figure 4.7.** Membrane protein expression of CD147 in the liver, normalized to total loaded protein. 
*A.* CD147 protein in all groups.  
*B.* CD147 protein in males.  
*C.* CD147 protein in females.  
*D.* CD147 protein in normal chow groups.  
*E.* CD147 protein in high fat diet groups.  
*F.* Representative western blot, CD147 in PND42 males.  Data presented as mean ± SD. (n = 6 rats).  
*Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes.  
Statistical significance determined if P < 0.05.

### 4.4.5 Pearson correlation
Membrane protein expression was analyzed and compared to determine direction and degree of association. Positive correlation represents co-expression of two proteins and a possible link in upregulation, whereas negative correlation indicates
independent or indirect expression. Pearson correlation of MCT1-CD147 was statistically significant ($p < 0.0001$), with an $r$ value of 0.8606 (Figure 4.8A). Pearson correlation of MCT4-CD147 was statistically significant ($p < 0.0001$), with an $r$ value of -0.3595 (Figure 4.8B).

Membrane protein expression and leptin concentrations were compared to determine if leptin levels were associated with increased or decreased translation. Positive correlation demonstrates that leptin upregulates protein expression, while negative correlation could implicate leptin’s suppressive role in translational regulation. Pearson correlation of MCT1-Leptin was statistically significant ($p = 0.0015$), with an $r$ value of 0.2865 (Figure 4.8C). Pearson correlation of MCT4-Leptin was statistically significant ($p = 0.0012$), with an $r$ value of -0.2925 (Figure 4.8D). Pearson correlation of CD147-Leptin was statistically significant ($p = 0.0436$), with an $r$ value of 0.1846 (Figure 4.8E).
4.5 Discussion

The current study evaluated the effect of age, diet, and sex on the regulation of MCT transporters and the ancillary protein CD147 in the liver. To our knowledge, this is the first
study assessing the influence of overnutrition on MCT and CD147 expression in the liver utilizing an animal model for pediatric obesity. Here we demonstrate that transporter expression changes with age, with high fat diet altering expression at distinct postnatal time points. Additionally, sex affected protein expression in an isoform specific manner.

We have previously shown how monocarboxylate transporter mRNA expression in the liver is affected by ontogeny, with nonlinear patterns in MCT1 and MCT4 and minor differences in mRNA expression based on sex [98]. In this study, we observed statistical significance based on age in MCT1, MCT4 and CD147 expression at the mRNA level; however, there was no significant changes based on diet or sex. mRNA transporter expression was often different than the observed protein expression pattern. For example, females on HFD had observed higher MCT4 mRNA at PND14 and PND21 compared to the NC group, but this pattern was reversed with membrane protein expression. Transcriptional and membrane expression often exhibited inverse relationships at PND42. For example, PND42 males had lowest levels of MCT1 mRNA, but second highest MCT1 protein expression. This could indicate high inter-individual variability between each animal, and the relative volatility and dynamic expression of mRNA compared to more stable membrane protein localization. For example, the standard deviation of MCT4 mRNA expression was 1.0432 and 2.0014 in NC PND21 and HFD PND21 females respectively, while in the paired membrane protein the standard deviation was 0.0033 and 0.0035. Other studies have explored the relatively poor association between mRNA and protein expression, with some Pearson correlation coefficients as low as 0.40 [99]. Another explanation for the discrepant transcriptional and translational expression is the existence of microRNA’s that may target transporter mRNA and post-transcriptionally regulate gene expression [47]. However, effects of microRNA and transcription can only be compared in total protein
expression, and not membrane expression as in this study. While our previous study showed strong, positive correlation between total protein and membrane protein, future experiments must include analyses of whole cell extracts [98].

Age affects male and female MCT1 protein expression differently, particularly in early age groups at PND 14 and 21. These patterns were similar to the results in our previous liver paper, although we were not able to capture protein expression at earlier timepoints [98]. MCT1 changes nonlinearly with age in males on both normal chow and high fat diet (high, low, high). MCT1 ontogeny is also nonlinear in females, although the pattern is distinct from age-matched males. In addition, HFD females have a different expression pattern (low, high, low) than NC females (high, low, high), and over-nutrition seems to disrupt normal MCT1 expression patterns in the liver. Males exhibit similar nonlinear changes in CD147 expression, while HFD females had a linear decrease, while a nonlinear pattern (low, high, low) was observed in NC females. MCT4 ontogeny changes nonlinearly in both males and females in both dietary groups (low/high/low in males, vs high/low/high in females). Together, this data shows that hepatic monocarboxylate transporters and ancillary protein expression are affected by age, and that these expression patterns are isoform specific.

In general, HFD upregulated MCT1 membrane protein expression in both males and females, with the exceptions of NC females at PND21. These results are consistent with findings of leptin on MCT1 activity in colonocytes, as well as obesity in haploinsufficient MCT1 mice [52, 66]. HFD suppresses MCT1 expression in females at PND21 compared to females on a normal diet; a combination of obesity and female hormones may be involved in this female cohort mimicking the male cohort on high fat diet. Interestingly, high fat diet has a suppressive effect on MCT4 expression, which aligns with our diet observations of MCT expression in the
ileum (Chapter 6). This dietary effect extended to CD147 expression patterns, although HFD appeared to suppress ancillary protein levels at PND21 and PND28 in females. Studies in hepatic CD147 knockout mice fed high fat diet showed reduced body weight, liver weight, serum aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT), and liver steatosis [100]. To our knowledge, this study is the first to elucidate the patterns of CD147 expression based on ontogeny and obesity.

In both dietary groups, sex affected MCT1 and CD147 protein expression at specific postnatal ages, indicating a link between sex and ontogeny. In NC males, MCT1 membrane expression declined from PND14 to PND21, while expression in females increased, indicating a dynamic sex-linked effect on protein expression at these early timepoints. Previous studies have shown that prepubescent male rats have half the circulating levels of testosterone than in late adolescent males, while females have low levels of testosterone throughout ontogeny. Both prepubescent males and females have low levels of progesterone pre-weaning. However, estradiol is elevated at around PND10 in both sexes, suggesting different levels of sex hormones may affect MCT expression in males and females [101]. There was no further significance until PND42, where males and females reach sexual maturity and developmental expression is influenced by circulating sex hormones. In HFD, males had higher MCT1 levels than females at PND14 and 42. CD147 mirrors the MCT1 trend with sex-linked differences in membrane protein expression at the same ages. MCT4 was similarly affected by sex difference, although the patterns of expression were distinctly different from MCT1 and CD147. NC females had statistically higher MCT4 protein at PND14, while NC males had higher expression than females at PND28. HFD females had statistically higher MCT4 at PND14 and 42, while males had higher expression at PND21. In our group’s previous sex difference study in 8-week old rats fed
normal diet, males had observed higher MCT1 and CD147 membrane protein than females, which matches the current study. However the sex difference study also demonstrated that adult males had higher MCT4 protein than females, data that contradicts the current ontogeny study in NC PND42 rats, which indicates dynamic shifts in MCT4 expression between sexual maturity and adulthood [68].

MCT1-CD147 membrane expression have a significant positive correlation, indicative of an association between increasing protein expression and membrane localization dependent on age, diet and sex. This is integral in that the complexing of transporter and ancillary protein are essential for MCT expression and activity at the cell membrane [102]. In contrast, hepatic MCT4-CD147 have a significant negative correlation, implicating indirect association and expression. This result is opposite of previous studies reporting tight MCT4-CD147 association in rat lymphoblastic Y3 cells and adult rats, and indicates less complexing of ancillary protein with the partner transporter in the pediatric liver, increasing subcellular residency and potentially inhibiting the translocation to the cell surface [29, 68]. Interestingly, the negative correlation aligns with our previous liver paper discussed in Chapter 3, indicating ontogeny may dramatically affect the role of ancillary proteins and MCT4 expression [98]. An alternative reasoning could be a difference in MCT4’s stoichiometric binding of CD147 compared to MCT1, where fewer molecules of ancillary protein are required for transporter activation. Interestingly, the hepatic MCT4-CD147 correlation is the opposite in the ileum (Chapter 6), signifying tissue specific differences in transporter-ancillary protein complexes based on tissue distribution and endogenous functions.

In our studies, leptin concentrations were significantly increased stemming from over-nutrition with a high fat diet, which resulted in an increase in body weight. Leptin was highest at
PND14 and PND21 in both dietary groups, evidence of a postnatal leptin surge often seen in developing neonates, which diminishes upon maturation [67]. Both MCT1 and CD147 exhibit significant positive correlations with leptin indicating an inducing effect on transporter and ancillary protein membrane expression and corroborating the upregulation seen in intestinal cells [66]. Leptin has been shown to also upregulate HIF-1α, MCT4, and CD147 in prostate cancer cells [103]. In contrast, MCT4 had a significant and negative correlation with leptin in our study. Leptin may selectively induce expression based on MCT isoform, the mechanism of which may be separate for the ancillary protein. Obesity may affect the tissue expression of transporters in a tissue specific manner, with each drug-disposition organ displaying variable expression levels despite constant levels of circulating leptin. Leptin and protein expression correlation does not necessarily equate to causation, and there is the possibility that other obesogenic factors play a role in MCT expression. Thus, additional experiments need to be performed confirming the mechanistic role of leptin and pediatric MCT regulation.

We have demonstrated the extent that over-nutrition and obesity can affect normal hepatic monocarboxylate transporter expression in an animal model of pediatric obesity. High fat diet and the resulting increased leptin appear to increase hepatic MCT1 and CD147 membrane expression; however, this effect is variable over different stages of development and between sexes. Moreover, over-nutrition stymies MCT4 membrane protein expression, with inconsistencies based on developmental stage and sex. Cross-talk in expressional patterns across different organs may be a mechanism of shuttling monocarboxylic substrates and nutrients between tissues of production to organs that readily utilize them for cell metabolism and gluconeogenesis [104, 105]. While we have documented the expression patterns in our data, the physiological ramifications of these findings on functional activity and drug substrate-binding
remain unclear. Furthermore, the expression findings underscore the need for pharmacokinetic experiments evaluating monocarboxylic substrates such as valproate and GHB in animal models of pediatric obesity.
5.1 Introduction

The kidneys are the primary excretory organs in the human body and regulate the body’s fluid composition. The functional unit of the kidney is the nephron, which is subdivided into the renal corpuscle, which contains bowman capsule and glomerulus, proximal convoluted tubule, loop of Henle, and the distal convoluted tubule. Blood undergoes glomerular filtration, and as the filtrate is traveling through the proximal tubules and loop of Henle, water and essential solutes are reabsorbed to blood circulation, while metabolic wastes, toxins, and excess ions and minerals in the filtrate are converted into and excreted as urine at the distal tubule and collecting duct. The kidneys also regulate blood volume, blood pressure, extracellular fluid pH and blood acidity[106].

The liver and kidney comprise the two main clearance and elimination pathways in humans. Nephron tubules transport molecules via simple and facilitated diffusion, active transport, symport, and osmosis. Renal epithelial cells are polarized and consist of an apical membrane, which separates the lumen of the nephron from the cytoplasm and is directed towards the luminal filtrate, and a basolateral membrane, which forms the outer wall of the nephron and faces the interstitial fluid and bloodstream. The lateral surfaces of epithelial renal cells form tight junctions that are bound to the surfaces of other cells of the nephron. Essential endogenous molecules, such as sodium, potassium, and glucose undergo tubular secretion and reabsorption mediated by membrane transporter proteins, which are also involved in the transport of drugs and xenobiotics. The renal transport of monocarboxylate nutrients, such as lactate and pyruvate, and drugs including γ-hydroxybutyric acid, are regulated by monocarboxylate transporters.
MCTs, like most renal drug transporters, are primarily located in the renal proximal tubules, and play an important role in tubular secretion and reabsorption of monocarboxylic drug molecules in the kidney [107]. Among the proton-coupled transporters, MCT1 is expressed in the basolateral membrane of epithelial cells, while MCT2 is localized to the thick ascending limb of the Loop of Henle and the distal convoluted tubule [69, 108]. MCT4 is expressed in the basolateral membrane of renal proximal tubule epithelial cells [109]. Protein experiments have shown sodium-coupled SMCT1 expression in the kidney cortex and outer medulla, with expression beginning at the S2 and S3 segments of the proximal tubule, where it resides at the apical membrane, likely facilitating the uptake of substrates from the urinary ultrafiltrate [70]. In this study, Sprague Dawley rats were challenged with both normal and high fat diets to observe changes in renal MCT1, MCT4, CD147 and SMCT1 expression during mammalian development.

5.2 Chapter Objective

In humans, the developing kidney undergoes dramatic structural and functional changes, where nephrogenesis occurs between 8 and 36 weeks gestation [110]. Renal tubular development continues from birth to adulthood, and contributes to increased plasma flow, glomerular filtration rate, renal clearance, and tubular secretion [111]. Monocarboxylate transporters exhibit transcriptional and translational changes based on ontogeny, as previously observed in the rat liver [98]. There is increased interest in age-specific pharmacological information in order to formulate effective pediatric drug regimen beyond inferred pharmacokinetics in adult populations, or empirical data in adolescent patients. While literature exists detailing the hepatic and intestinal expression of several drug transporters based on ontogeny, there is a paucity of data analyzing MCT expression in the developing mammalian
kidney [96]. Dietary changes also affect expression of MCTs, with a ketogenic diet increasing MCT1 in the rat brain, yet the combined effects of both diet and age on renal MCT regulation and expression has not been explored [112]. The objective of this study is to investigate renal MCT expression in a diet-induced model of pediatric obesity and age-matched normal weight controls, and observe how these developmental patterns shift based on age and sex. We hypothesize that there will be a distinct, tissue-specific change in transporter expression compared to the liver and intestine, and that expression will be predicated on age. Furthermore, obesity and circulating leptin will be correlated with upregulated MCT expression in the kidney compared to subjects on a normal diet.

5.3 Data Analysis

Data is expressed as mean ± standard deviation. Expression of mRNA was normalized to the mean of house-keeping gene 18S and alien RNA, and fold-change was determined using the $2^{-\Delta\Delta CT}$ method. MCT1, MCT4, CD147, and SMCT1 membrane protein expression was normalized to total protein loaded using the Stain Free method. Correlation analysis was used to evaluate the relationships between membrane expression and leptin, and transporter expression and CD147. Data was analyzed in GraphPad Prism 7 using two-way analysis of variance (ANOVA) with Tukey’s post hoc test. Differences were considered statistically significant when the p value was less than 0.05.

5.4 Results

5.4.1 MCT1 mRNA and membrane protein expression. MCT1 mRNA for all groups is presented in Figure 5.1. There were no significant differences in MCT1 mRNA levels based on age, sex, or diet. Male MCT1 mRNA increases linearly, while female MCT1 mRNA was expressed nonlinearly with age (low/high/low).
MCT1 membrane protein expression for all groups is presented in Figure 5.2A. MCT1 expression was nonlinear in males (high, low, high). In NC males MCT1 protein expression was significantly lower at NC PND14 (p = 0.0030), PND21 (p < 0.0001), PND28 (p < 0.0001), and PND35 (p < 0.0001) compared to NC PND42. HFD males had significantly lower expression at HFD PND14 (p < 0.0001), PND21 (p < 0.0001), and PND28 (p < 0.0001) compared to HFD PND42. There was a significant difference in dietary expression at PND42, with the HFD males exhibiting a 2-fold increase in protein expression compared to NC males (p < 0.0001). Both NC and HFD males had peak MCT1 expression at PND42 (Figure 5.2B). MCT1 expression was nonlinear in females (low, high, low, high), with significantly lower MCT1 expression at HFD PND14 (p = 0.0007), PND21 (p = 0.0004), and PND35 (p = 0.0161) compared to HFD PND42. Both NC and HFD females had highest MCT1 expression at PND28 and PND42 (Figure 5.2C).

There were observed, though not significant, sex differences in MCT1 membrane protein expression in NC animals with males at PND14 and PND35 having higher protein expression than the age-matched NC females. However, NC PND42 males had significantly higher expression than NC PND42 females (p < 0.0001) (Figure 5.2D). HFD males had significantly higher MCT1 expression than HFD females at PND14 (p = 0.0216) and PND42 (p < 0.0001) (Figure 5.2E).
Figure 5.1. mRNA Expression of MCT1 in the kidney. A. MCT1 fold change in all groups. B. MCT1 fold change in males. C. MCT1 fold change in females. D. MCT1 fold change in normal chow groups. E. MCT1 fold change in high fat diet groups. Fold change relative to female postnatal day 14 group, normalized to Alien and 18S RNA. Data presented as mean ± SD. (n = 5 - 6 rats) *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
Figure 5.2. Membrane protein expression of MCT1 in the kidney, normalized to total loaded protein. Data presented as mean ± SD. (n = 6 rats). A. MCT1 protein in all groups. B. MCT1 protein in males. C. MCT1 protein in females. D. MCT1 protein in normal chow groups. E. MCT1 protein in high fat diet groups. F. Representative western blot, MCT1 in PND42 males. Data presented as mean ± SD. (n = 6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
5.4.2 MCT4 mRNA and membrane protein expression. MCT4 mRNA expression for all groups is presented in Figure 5.3A. There was a statistically significant sex difference only in MCT4 mRNA at PND14 in NC animals, with females exhibiting higher mRNA levels compared to males ($p = 0.0339$) (Figure 5.3D). MCT4 mRNA in males was stably expressed throughout ontogeny, while MCT4 maturation in females was nonlinear (high, low, high).

MCT4 membrane protein expression for all groups is presented in Figure 5.4A. Males exhibited nonlinear MCT4 expression (low, high, low). In NC males, MCT4 expression was significantly higher at NC PND28 compared to NC PND42 ($p = 0.0016$). HFD males had significantly higher expression at HFD PND28 compared to HFD PND42 ($p = 0.0021$). Both NC and HFD males had peak MCT4 expression at PND28 (Figure 5.4B). Females exhibited nonlinear MCT4 expression (low, high, low, high), with significantly lower MCT4 expression at HFD PND14 compared to HFD PND42 ($p = 0.0046$), and in both female dietary groups MCT4 expression peaked at PND21 (Figure 5.4C).

There were observed, though not significant, sex differences in MCT4 membrane protein expression in NC animals, with males at PND14 and PND21 having higher expression than the age-matched NC female cohort. However, NC PND28 males had significantly higher MCT4 expression (2.5-fold higher) than NC PND28 females ($p < 0.0001$) (Figure 5.4D). HFD males had observed increases at PND14 and PND35 compared to HFD females, with statistically significant increase (3-fold) at HFD PND28 ($p < 0.0001$) (Figure 5.4E).
Figure 5.3. mRNA Expression of MCT4 in the kidney. A. MCT4 fold change in all groups. B. MCT4 fold change in males. C. MCT4 fold change in females. D. MCT4 fold change in normal chow groups. E. MCT4 fold change in high fat diet groups. Fold change relative to female PND42 group, normalized to Alien and 18S RNA. Data presented as mean ± SD. (n = 3 - 6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
Figure 5.4. Membrane protein expression of MCT4 in the kidney, normalized to total loaded protein. Data presented as mean ± SD. (n = 6 rats). A. MCT4 protein in all groups. B. MCT4 protein in males. C. MCT4 protein in females. D. MCT4 protein in normal chow groups. E. MCT4 protein in high fat diet groups. F. Representative western blot, MCT4 in PND42 males. Data presented as mean ± SD. (n = 6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
5.4.3 CD147 mRNA and membrane protein expression. CD147 mRNA expression for all groups is presented in Figure 5.5A. There were no statistically significant differences in CD147 mRNA levels based on age, sex, or diet. CD147 mRNA expression was nonlinear in both males and females (high, low, high, low).

CD147 membrane protein expression for all groups is presented in Figure 5.6A. CD147 expression was nonlinear in males (high, low, high). In NC males, CD147 expression was significantly lower at NC PND21 (p < 0.0001), PND28 (p = 0.0012) and PND35 (p = 0.0149) compared to NC PND42. HFD males had significantly lower expression at PND14 (p < 0.0001), PND21 (p < 0.0001), PND28 (p < 0.0001), and PND35 (p < 0.0001) compared to PND42. There was a significant dietary difference in males at PND42, with the HFD males exhibiting a 2-fold higher expression than the NC males (p < 0.0001). In both NC and HFD males, peak CD147 expression occurred at PND42 (Figure 5.6B). In females, CD147 expression was nonlinear (low, high, low, high), with statistically lower CD147 protein expression at NC PND14 (p = 0.0335), PND28 (p = 0.0196), and PND35 (p = 0.0197) compared to NC PND42, while expression was significantly lower at HFD PND14 (p = 0.0060) and PND35 (p = 0.0154) compared to HFD PND42. In both dietary groups, peak MCT4 expression occurred at PND42 (Figure 5.6C).

There were observed, though not significant, sex differences in CD147 expression in NC animals, with males at NC PND14, PND28, and PND35 having higher expression than the age-matched NC females. However, NC PND42 males had statistically significantly higher CD147 expression than NC PND42 females (p=0.0172). Females had observed higher MCT expression than males at NC PND21 (Figure 5.6D). There were significant sex differences in the HFD cohort, with PND14 and PND42 males having higher expression compared to the age-matched female cohort (p< 0.0001) (Figure 5.6E).
Figure 5.5. mRNA Expression of CD147 in the kidney. A. CD147 fold change in all groups. B. CD147 fold change in males. C. CD147 fold change in females. D. CD147 fold change in normal chow groups. E. CD147 fold change in high fat diet groups. Fold change relative to female post-natal day 28 group, normalized to Alien and 18S RNA. Data presented as mean ± SD. (n = 3-6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
5.4.4 SMCT1 mRNA and membrane protein expression. SMCT1 mRNA expression for all groups is presented in Figure 5.7A. SMCT1 mRNA expression decreased linearly with age in both males and females. At the mRNA level, there was statistically higher expression in males at NC PND21 compared to PND42 (p = 0.0475) (Figure 5.7B).
SMCT1 membrane protein expression for all groups is presented in Figure 5.8A. In NC males, SMCT1 expression was nonlinear (low, high, low), with significantly higher levels at NC PND21 ($p = 0.0468$) and PND28 ($p < 0.0001$) compared to NC PND42. HFD males had peak SMCT1 expression at PND14 (linear maturation; high to low). There was a statistically significant dietary difference in males at PND28, with NC males having a 2-fold higher protein expression of SMCT1 compared to HFD males ($p < 0.0001$) (Figure 5.8B). There were no significant differences in NC or HFD SMCT1 protein expression in the females; however, both diets had the highest SMCT1 expression at PND14 (nonlinear maturation; high, low, high, low) (Figure 5.8C).

Similarly, there were no significant sex differences in NC animals. In females we observed increases in SMCT1 expression at NC PND14, while males exhibited higher expression than females at NC PND21 and PND28 (Figure 5.8D). There was a statistically significant sex difference in the HFD animals, with females at HFD PND14 having higher expression of SMCT1 than males ($p = 0.0005$) (Figure 5.8E).
Figure 5.7. mRNA Expression of SMCT1 in the kidney. A. SMCT1 fold change in all groups. B. SMCT1 fold change in males. C. SMCT1 fold change in females. D. SMCT1 fold change in normal chow groups. E. SMCT1 fold change in high fat diet groups. Fold change relative to female PND35 group, normalized to Alien and 18S RNA. Data presented as mean ± SD. (n = 5 - 6 rats) *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.
Figure 5.8. Membrane protein expression of SMCT1 in the kidney, normalized to total loaded protein. A. SMCT1 protein in all groups. B. SMCT1 protein in males. C. SMCT1 protein in females. D. SMCT1 protein in normal chow groups. E. SMCT1 protein in high fat diet groups. F. Representative western blot, SMCT1 in PND42 males. Data presented as mean ± SD. (n = 6 rats). *Significant compared to NC PND42; †significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.

5.4.5 Pearson correlation. Transporter and ancillary protein expression were compared to determine direction and degree of association. Positive correlation represents co-expression of
two proteins and a possible link in upregulation, whereas negative correlation indicates independent or indirect expression. Pearson correlation of MCT1-CD147 was positive and statistically significant ($p < 0.0001$), with an $r$ value of 0.9078 (Figure 5.9A). Pearson correlation of MCT4-CD147 produced an $r$ value of 0.1168 (Figure 5.9B). Pearson correlation of SMCT1-CD147 was negative and statistically significant ($p = 0.0428$), with an $r$ value of -0.1853 (Figure 5.9C).

Membrane protein and leptin concentrations were compared to determine if leptin levels were associated with increased or decreased translational expression. Leptin and bodyweights were previously presented in Chapter 4. Positive correlation associates leptin with upregulation of protein expression, while negative correlation could suggest a role for leptin suppressing translational regulation and/or membrane trafficking of MCT. No statistically significant correlations were observed between leptin concentrations and renal membrane expression of monocarboxylate transporters. Pearson correlation of MCT1-Leptin produced an $r$ value of -0.05980 (Figure 5.9D). Pearson correlation of MCT4-Leptin produced an $r$ value of 0.1012 (Figure 5.9E). Pearson correlation of CD147-Leptin produced an $r$ value of -0.03604 (Figure 5.9F). Pearson correlation of SMCT1-Leptin produced an $r$ value of -0.07401 (Figure 5.9G).
Figure 5.9. Pearson correlation of renal transporters to ancillary protein and leptin.  

A. MCT1-CD147 correlation.  

B. MCT4-CD147 correlation.  

C. SMCT1-CD147 correlation.  

D. MCT1-Leptin correlation.  

E. MCT4-Leptin correlation.  

F. CD147-Leptin correlation.  

G. SMCT1-Leptin correlation.  

(n = 120 rats).  

Statistical significance determined if P < 0.05.
5.5 Discussion

We have previously demonstrated MCT1, MCT4, and CD147 express nonlinearly in the maturing rat liver, which resembles clinical data in hepatic ontogeny \[85, 98\]. The current study aims to evaluate age, diet, and sex on the regulation of monocarboxylate transporters and the ancillary protein CD147 in the kidney. Here we demonstrate that renal transporter expression changes nonlinearly with pediatric age based on isoform, in a distinctly different pattern than the rat liver. High fat diet can both upregulate and downregulate protein expression at distinct postnatal time points. Sex also affected protein expression in an MCT isoform specific manner, with sex differences determined by age.

While ontogeny data for other drug transporters have been previously reported, there is minimal data on renal MCT expression in mammalian development \[96\]. Higher MCT membrane protein expression could represent increased functional activity in renal drug and nutrient transport based on age, and similar patterns in ancillary protein expression suggests co-expression at these timepoints. MCT1 has nonlinear membrane protein expression in NC and HFD males, (high, low, high), while females exhibit near linear expression increase through sexual maturity. Males appear to have higher MCT1 levels in early adolescence, which offers a wide range of biological implications, including increased cell growth and metabolism. CD147 displays a similar pattern in development to MCT1, an indication of coupling and close association between the ancillary protein and transporter throughout ontogeny. Both sexes exhibit nonlinear MCT4 patterns (low, high, low, high) based on age. Interestingly, male MCT4 levels peak at PND28, while female levels peak at PND21. SMCT1 has nonlinear expression in males (low, high, low) and females (high, low, high, low, high), the vast difference in pattern
could indicate females utilize the SMCT1 protein more dynamically in ontogeny, possibly for nutrient absorption in critical life stages.

In the kidney, diet had varied effects on MCT1 protein expression in both sexes, although males had significant increases at HFD PND42 compared to NC males of the same age. CD147 protein was similarly significantly higher in HFD males. Females actually had an observed increase in MCT1 and CD147 in NC PND21 compared to HFD females. These incongruous effects of high fat diet on protein expressions likely reflects the same pattern seen between leptin and MCT1 correlation, indicating that obesity and leptin alone do not regulate MCT and CD147 expression. MCT4 was minimally impacted by diet-induced obesity, and both dietary cohorts shared near identical expression levels. In SMCT1, high fat diet appeared to slightly suppress membrane expression, and HFD males even had significantly lower SMCT1 compared to their normal diet counterparts at PND28.

Sex also regulates renal transporters. Previous studies have shown sexual dimorphism in Na+/H+ exchanger isoform 3, Na+/Pi cotransporter 2, claudin-2, aquaporin 1, Na+/Cl− cotransporter, and epithelial Na+ channel protein expression in the mouse kidney [113]. Males had significantly higher MCT1 and CD147 protein in both diets than females at PND42, and higher levels at HFD PND14. MCT1 has a prominent role in mammalian growth, and these two time-points may signify the importance of the transporter for development in early adolescents and sexual maturity in males compared to females. Similarly, nutrient and drug substrates taken up by MCT1 would likely affect males more than females at these post-natal ages. Males generally had higher MCT4 levels than females, with significant peak at PND28. Interestingly, females had significantly higher MCT4 mRNA levels than males at NC PND14; however, this pattern was reversed at the protein level. The dichotomy between transcriptional and
translational data may involve post-transcriptional modification of mRNA, including microRNA’s which may suppress and alter MCT4 protein expression. Females have higher expression of SMCT1 early in development at PND14, however males match or surpass their expression levels later in ontogeny, a clear link between sex and age in transporter regulation.

MCTs themselves are not glycosylated, and require transmembrane ancillary proteins to both enhance expression and to become activated [19]. MCT1-CD147 have significant positive correlation, indicative of an association between increasing membrane expression dependent on age, diet and sex. The complexing of transporter and chaperone ancillary protein are essential for MCT1 expression and activation at the cell membrane [29]. In contrast, the correlation of renal MCT4 and CD147 was not significant, implicating minimal association and influence on MCT4 membrane expression. This indicates less complexing of ancillary protein with the partner MCT4 transporter, with decreased translocation to the cell surface. Embigin, or GP70, a chaperone protein that primarily binds to MCT2, but may associate with MCT4 at different postnatal timepoints [114], should be evaluated in future studies. An alternative reasoning could be a difference in MCT4’s stoichiometric binding of CD147 compared to MCT1, where fewer molecules of ancillary protein are required for transporter activation. MCT4 has a strong, intrinsic basolateral sorting signals (BLSS) which are redundant to the weak BLSS located in CD147 [28]. SMCT1-CD147 have significant negative correlation, implicating indirect association and expression. A likely explanation is that SMCT1 expresses independently and does not require binding to ancillary proteins in order to be functionally active at the cell membrane. Supporting experiments demonstrated CD147 to exhibit BLSS quintessential to MCT1 chaperoning and expression at the basolateral membrane, whereas SMCT1 is exclusively expressed at the apical membrane [28, 115]. To date, no studies have shown an association
between SMCT1 and other ancillary proteins [116]. Kidney MCT1-CD147 correlation is similar to the hepatic and intestinal correlations, but renal MCT4-CD147 is completely different in the liver and ileum, signifying spatial differences in transporter-ancillary protein complexes based on tissue distribution and endogenous functions.

The role of leptin in obesity and upregulation of monocarboxylate transporter expression have been explored previously [52, 66, 117]. In our studies, leptin is significantly and positively correlated with increased bodyweight in rats stemming from over-nutrition with high fat diet. We evaluated the correlation between circulating leptin and renal transporter expression. MCT1, CD147, and SMCT1 exhibited negative correlations with leptin, while MCT4 showed a positive correlation (all correlations were not statistically significant), an indication that the hormone has minimal impact on transporter expression in the kidney. This result was in sharp contrast to leptin’s role in the regulation MCT1 expression in the liver (Chapter 4) and ileum (Chapter 6), which saw positive and negative correlations. Leptin appears to regulate transporter expression in a tissue-specific manner, and the ability for leptin to upregulate or suppress MCT membrane expression is isoform-specific, which underscores the physiological and dynamic importance of MCT expression throughout maturation.

There are limitations to our renal studies. The majority of nephrogenesis occurs during gestation, and a significant portion of MCT expression and the effects of maternal over-nutrition may occur at these early life-stages. The biomarker leptin does not preclude other hormones’ roles in renal MCT expression during maturation. Testosterone enhances both SMCT1 mRNA and protein expression in mouse kidney, whereas progesterone suppresses SMCT1 protein levels in the same tissue, and these hormones may alter MCT expression between sexes throughout ontogeny and diet-induced obesity. mRNA and membrane protein expression are not a substitute
for functional activity experiments for monocarboxylic substrate transport and renal clearance of
drugs, and additional assays need to be executed to measure activity in these samples, and how
they correlate with increased or decreased translational transporter expression. These activity
assays can also determine if overexpression of MCTs aid in the extrusion or increase uptake of
substrates in cells. Lastly, we need to compare our animal model of pediatric obesity to clinical
data, either from literature or by studying biopsied tissues for cell culture.

We have demonstrated that over-nutrition from high fat diet can dysregulate normal
monocarboxylate transporter expression in the kidney. Obesity affects MCT expression in an
isoform-specific and tissue-dependent manner, with pediatric ontogeny and sex impacting
transporter expression. Future work will include researching clinical literature to compare
models of MCT expression in pediatrics, and measuring transporter activity utilizing labeled
monocarboxylic substrates in vivo. Pharmacokinetic studies will further link expressional data to
the renal clearance of monocarboxylic drugs in this new animal model of pediatric obesity.
CHAPTER 6: MONOCARBOXYLATE TRANSPORTERS IN THE ILEUM-IMPACT OF DIET, ONTOGENY, AND SEX ON TRANSPORTER EXPRESSION

6.1 Introduction

The intestinal tract is a major organ for nutrient absorption and drug metabolism. Enteric bacteria in the intestines ferment undigested carbohydrates and dietary fibers to produce short chain fatty acids (SCFAs) such as propionate, butyrate and acetate, which comprise the major fuel for cell respiration in the gastrointestinal tract. SCFA’s directly affect the intestinal walls by regulating colonic blood flow and stimulating fluid and electrolyte uptake [118]. Butyrate acts as the principal energy source for epithelial colonocytes to perform various cell processes, including cell proliferation, differentiation and apoptosis [119]. The uptake of SFCA’s in the intestine is mediated by simple diffusion, as well as by proton- and sodium-coupled monocarboxylate transporters (MCTs and SMCTs) [120].

Along with nutrient absorption, the intestines are also an important location for drug disposition. Together with the liver, the intestine comprises the first pass effect for drug bioavailability in the circulatory system via the hepatic portal vein. The intestinal epithelium expresses transporters that mediate short chain fatty acid and oral drug absorption, with monocarboxylate transporters involved in the uptake and efflux of monocarboxylate drug substrates, including GHB, gabapentin enacarbil, β-lactam antibiotics, and valproic acid, the last of which is a first-line drug for childhood epilepsy and seizures [19, 121-123]. Changes in intestinal MCT and SMCT expression and membrane localization may disrupt cell energy homeostasis and drug absorption in the human body [45]. Expression variability of these membrane transporters can alter drug pharmacokinetics, which could lead to toxic or sub-therapeutic plasma concentrations altering therapeutic outcomes in patients [85, 96].
The small intestine is subdivided into the duodenum, jejunum, and ileum, and these segments have varying expression of MCT and SMCT transporters [38, 124, 125]. MCT1 is well characterized and the predominantly expressed isoform in the intestine. Immunohistochemistry and immunoblotting of intestinal epithelial cells have shown MCT1 localized at the apical membrane in human, rats and the Caco2 cell line [66, 125, 126]. Other groups have shown basolateral localization of MCT1 in the digestive tract of mouse, rat, and humans, underscoring the wide-ranging data for MCT membrane localization in mammals [127]. MCT4 is expressed basolaterally in the small intestine, albeit at lower levels compared to MCT1. Both MCT1 and MCT4 mRNA and protein expression have been shown to increase distally in the gastrointestinal tract, with the highest levels in the human ileum and colon [125]. There is minimal data of intestinal SMCT1 expression in humans. SMCT1 mRNA in mice has intense expression in the distal ileum and colon, and immunohistochemical studies have demonstrated SMCT1’s selective localization at the brush border of the apical plasma membrane [128]. SMCT1 has high apical expression in the rat duodenum, with minimal expression in the mid-jejunum [124].

6.2 Chapter Objective

Monocarboxylate transporters exhibit transcriptional and translational changes based on ontogeny, as previously observed in the rat liver and kidney. The ileum has been shown to express the highest levels of MCTs, which are involved with metabolic balance and drug disposition [125]. While the ontogeny of intestinal drug transporters has been extensively studied, the ontogeny patterns of monocarboxylate transporters, particularly at the protein level, are largely unknown [85, 96]. Dietary changes also affect expression of MCTs; the obesity biomarker leptin has been shown to increase MCT1 expression in the Caco-2 intestinal cell line.
However, the combined effects of both diet and age on intestinal MCT regulation and expression has not been explored. The objective of this chapter is to observe how age and obesity affect MCT expression in the ileum. We hypothesize that MCT and ancillary protein expression in the intestine will change nonlinearly based on age as observed in the liver of animal model on normal diet, and that obesity induced by high fat diet will increase protein expression. We expect SMCT1 protein to express nonlinearly in the ileum, as previously reported in the kidney (Chapter 5).

6.3 Data Analysis

Data is expressed as mean ± standard deviation. mRNA experiments were not performed due to low correlation between transcriptional and translational data in the intestinal tract. This may be related to stability issues with the digestive enzyme-rich intestinal lumen, which prevented optimal extraction of GI mRNA (detailed in Chapter 2). MCT1, MCT4, CD147, and SMCT1 membrane protein expression was normalized to total protein loaded using the Stain Free method. Correlation analysis was used to evaluate the relationships between membrane protein expression and leptin, and membrane expression of transporters and CD147. Data was analyzed in GraphPad Prism 7 using two-way analysis of variance (ANOVA) with a Tukey’s post hoc test. Differences were considered statistically significant when the p value was less than 0.05.

6.4 Results

6.4.1 MCT1 membrane protein expression. MCT1 membrane expression for all groups is presented in Figure 6.1A. In NC males, MCT1 expression increased linearly with age with peak expression at PND42 (low, high). HFD males had similar levels of MCT1 expression from PND14-35, before doubling expression to peak at PND42. There was an observed, though
not significant dietary difference, with NC males exhibiting higher MCT1 levels than their HFD cohorts at PND28-35, before equilibrating to comparative levels at PND42 (Figure 6.1B).

In NC females, MCT1 levels increased linearly from PND14 to peak levels at PND21-42 (low, high). HFD females had similar MCT1 levels throughout ontogeny. There was observed, though not significant dietary difference, with NC females exhibiting higher MCT1 levels than their HFD cohorts at PND21-42. HFD PND14 MCT1 was higher than NC PND14 (Figure 6.1C).

There were observed, though not significant sex difference in normal diet, with males having higher MCT1 expression at PND14, while females had higher expression from PND21-35, before equilibrating with male cohort (Figure 6.1D). There were observed, though not significant sex differences in high fat diet, with females having higher MCT1 levels than males, while at PND42 this pattern was reversed (Figure 6.1E).
Figure 6.1. Membrane protein expression of MCT1 in the ileum, normalized to total loaded protein. 
A. MCT1 protein in all groups. B. MCT1 protein in males. C. MCT1 protein in females. D. MCT1 protein in normal chow groups. E. MCT1 protein in high fat diet groups. F. Representative western blot, MCT1 in PND21 females. Data presented as mean ± SD. (n = 6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.

6.4.2 MCT4 membrane protein expression. MCT4 membrane protein expression for all groups is presented in Figure 6.2A. In NC males, MCT4 expression was linear (low, high), with significantly lower levels at PND14 (p < 0.0001), PND21 (p < 0.0001), PND28 (p <
0.0001), and PND35 (p < 0.0001) compared to NC PND42. Male NC expression peaked at PND42. HFD males had nonlinear expression (low, high, low, high), with significantly different MCT4 membrane expression at PND14 (lower, p = 0.0002) and PND21 (higher, p = 0.0042) compared to HFD PND42. Male HFD expression peaked at PND21. There was statistically different dietary difference in males, with HFD PND21 having higher MCT4 membrane expression than their NC age-matched counter parts (p = 0.0002). In contrast, NC PND42 males had significantly higher MCT4 level than their HFD cohort (p < 0.0001) (Figure 6.2B).

Females on both diets had nonlinear MCT4 expression (low, high, low, high). In NC females, there was statistically lower MCT4 membrane expression at PND14 (p < 0.0001), PND28 (p < 0.0001), and PND35 (p < 0.0001) compared to NC PND42. Female NC expression peaked at PND42. HFD females had statistically lower expression at PND14 compared to HFD PND42, with peak expression levels at sexual maturity (PND42; p = 0.0001). There were observed, though not significant dietary differences with females, with NC PND21 and PND42 having increased MCT4 membrane expression compared to their HFD age-matched counterparts (Figure 6.2C).

There were statistically significant sex differences in NC animals, with female NC PND21 having higher expression than the male cohort (p = 0.0001). NC females had higher, though not significantly higher, MCT4 levels at PND14, PND28, PND35, and PND42 compared to age-matched males (Figure 6.2D). There were statistically significant sex differences in HFD animals, with female HFD PND42 having higher expression than age-matched males (p = 0.0177). Females had observed higher, though not significantly higher, MCT4 levels at HFD PND14, PND28, and PND35 compared to age-matched males (Figure 6.2E).
Figure 6.2. Membrane protein expression of MCT4 in the ileum, normalized to total loaded protein.  
A. MCT4 protein in all groups.  
B. MCT4 protein in males.  
C. MCT4 protein in females.  
D. MCT4 protein in normal chow groups.  
E. MCT4 protein in high fat diet groups.  
F. Representative western blot, MCT4 in PND42 males.  
Data presented as mean ± SD. (n = 6 rats).  
*Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes.  
Statistical significance determined if P < 0.05.
6.4.3 CD147 membrane protein expression. CD147 membrane protein expression for all groups is presented in Figure 6.3A. Males on both diets had linear maturation of CD147 expression (low, high). In NC males, CD147 expression was significantly lower at PND14 (p < 0.0001), PND21 (p < 0.0001), PND28 (p < 0.0001) and PND35 (p < 0.0001) compared to NC PND42. Male NC expression peaked at PND42. HFD males had significantly lower CD147 levels at PND14 (p < 0.0001), PND21 (p < 0.0001), PND28 (p = 0.0001) and PND35 (p < 0.0001) compared to HFD PND42 (peak expression). There was a statistically significant dietary difference in males, with NC PND42 having higher expression compared to the age-matched HFD males (p = 0.0142). There was an observed, though not significant dietary difference in males, with NC PND14, PND28, and PND35 having increased CD147 levels compared to age-matched HFD males (Figure 6.3B).

In NC females, CD147 expression was nonlinear (low, high, low), with statistically lower CD147 expression at PND14 (p = 0.0009) and PND21 (p = 0.0009) compared to NC PND42. Female NC expression peaked at PND35. HFD females had linear expression (low, high), with statistically lower expression at PND14 compared to HFD PND42 (p = 0.0001). Female HFD expression peaked at HFD PND35-42 (Figure 6.3C).

There was a statistically significant sex difference in NC animals, with male NC PND42 (p < 0.0001) having higher expression than age-matched females (Figure 6.3D). Similarly, HFD PND42 males had statistically significantly higher CD147 expression than age-matched females (p = 0.0007) (Figure 6.3E).
Figure 6.3. Membrane protein expression of CD147 in the ileum, normalized to total loaded protein.  
A. CD147 protein in all groups.  
B. CD147 protein in males.  
C. CD147 protein in females.  
D. CD147 protein in normal chow groups.  
E. CD147 protein in high fat diet groups.  
F. Representative western blot, CD147 in PND21 males.  Data presented as mean ± SD.  (n = 6 rats).  *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes.  Statistical significance determined if P < 0.05.
6.4.4 SMCT1 expression. SMCT1 membrane protein expression for all groups is presented in Figure 6.4A. Males on both diets had nonlinear expression (low, high, low, high). In NC males, there was statistically lower SMCT1 expression at PND14 (p < 0.0001) and PND21 (p < 0.0001) compared to NC PND42. Male NC expression peaked at PND28 and 42. Similarly, HFD males had statistically lower SMCT1 expression at PND14 (p < 0.0001) and PND21 (p < 0.0001) compared to HFD PND42, with peak expression at HFD PND28 (Figure 6.4B).

Females on both diets had nonlinear SMCT1 expression (low, high, low, high). In NC females, there was statistically lower SMCT1 expression at PND14 (p < 0.0001), PND21 (p = 0.0004), and PND35 (p = 0.0097) compared to NC PND42. Female NC expression peaked at PND42. In HFD females there was statistically lower expression at PND14 (p = 0.0024) compared to HFD PND42, where HFD expression also peaked (Figure 6.4C).

Females at NC PND21 had higher SMCT1 levels than age-matched males, while males at NC PND35 had higher expression than age-matched females (Figure 6.4D). Females had higher SMCT1 levels at HFD PND14 and PND21 compared to age-matched males, while males had higher expression at HFD PND28 and PND42 than age-matched females (Figure 6.4E). There were no statistically significant sex differences in either normal or high fat diet.
Figure 6.4. Membrane protein expression of SMCT1 in the ileum, normalized to total loaded protein. A. SMCT1 protein in all groups. B. SMCT1 protein in males. C. SMCT1 protein in females. D. SMCT1 protein in normal chow groups. E. SMCT1 protein in high fat diet groups. F. Representative western blot, SMCT1 in PND42 females. Data presented as mean ± SD. (n = 6 rats). *Significant compared to NC PND42; +significant compared to HFD PND42; #significant between diets; ^significant between sexes. Statistical significance determined if P < 0.05.

6.4.5 Pearson correlation. Membrane protein expression was compared to determine direction and degree of association. Positive correlation represents co-expression of two proteins.
and a possible link in upregulation, whereas negative correlation indicates independent or indirect expression. Pearson correlation of MCT1-CD147 was statistically significant ($p = 0.0012$), with an $r$ value of 0.2927 (Figure 6.5A). Pearson correlation of MCT4-CD147 was statistically significant ($p = 0.0001$), with an $r$ value of 0.3441 (Figure 6.5A). Pearson correlation of SMCT1-CD147 was statistically significant ($p < 0.0001$), with an $r$ value of 0.4731 (Figure 6.5C).

Protein and leptin concentrations were compared to determine if leptin levels were associated with increased or decreased membrane expression. The positive correlation suggests leptin upregulates protein expression or increased trafficking to the plasma membrane, while negative correlation could implicate leptin’s suppressive role in translational regulation or membrane translocation. Leptin and bodyweights were detailed in Chapter 4. Pearson correlation of MCT1-Leptin was statistically significant ($p = 0.0095$), and produced an $r$ value of -0.2359 (Figure 6.5D). Pearson correlation of MCT4-Leptin produced an $r$ value of -0.1510 (Figure 6.5E). Pearson correlation of CD147-Leptin was statistically significant ($p = 0.0003$), with an $r$ value of -0.3217 (Figure 6.5F). Pearson correlation of SMCT1-Leptin was statistically significant ($p = 0.0002$), with an $r$ value of -0.3347 (Figure 6.5G).
Figure 6.5. Pearson correlation of transporters to ancillary protein and leptin. 

A. MCT1-CD147 correlation.  

B. MCT4-CD147 correlation.  

C. SMCT1-CD147 correlation.  

D. MCT1-Leptin correlation.  

E. MCT4-Leptin correlation.  

F. CD147-Leptin correlation.  

G. SMCT1-Leptin correlation.  

(n = 120 rats). Statistical significance determined if P < 0.05.
6.5 Discussion

The current study aims to evaluate the influence of age, diet, and sex on the regulation of monocarboxylate transporters and the ancillary protein CD147 in the ileum. Changes in MCT expression levels are an important factor influencing drug absorption and bioavailability [121]. Increased MCT expression in the intestinal enterocyte membrane may result in increased oral absorption of monocarboxylic drugs, leading to higher plasma concentrations. Dysregulation of MCTs may also affect nutrient reabsorption and metabolism. Here we demonstrate that intestinal transporter expression changes in patterns different than those observed in the liver and kidney, with mostly linear increases in MCT1, and nonlinear changes in MCT4 and SMCT1. High fat diet either increases or decreases expression at distinct postnatal time points. Sex affected protein expression in an isoform specific manner in the gastrointestinal tract, with females exhibiting generally higher MCT1 and MCT4 levels than males.

There is limited information on the how monocarboxylate transporter expression in the intestinal tract is influenced by ontogeny. MCT1 increases linearly with age in males in both normal weight and obese animals. NC females demonstrated slight linearity in MCT1 expression; however, this effect is altered in the obese females, where MCT1 levels remain static through maturation. This suggests high fat diet alters normal MCT1 expressional patterns during growth, and this effect may involve physiological sex hormones present in females. MCT4 increases linearly with age in males on normal diet. However, in females MCT4 displays nonlinear expression patterns (low, high, low, high) in both dietary groups, and males on high fat diet exhibit the same nonlinear MCT4 expression. In this instance, high fat diet induces males to mimic the same MCT4 patterns as females, which suggests age and diet-related interactions on MCT4 expression independent of circulating sex hormones. Males and females had nonlinear
expression of SMCT1 in the ileum (low, high, low, high). Together, this shows that monocarboxylate transporters are affected by age, and that these expressional patterns are isoform specific.

In general, high fat diet downregulated MCT1 expression in both males and females, which the exceptions of males and females at PND14 where high fat diet had higher protein levels. This effect was similar in CD147, although the exception occurred at PND21. High fat diet downregulated MCT4 expression in males except at PND21, while MCT4 was decreased in high fat diet females at all times points. These results are contradictory to findings of leptin on MCT1 activity and membrane expression in colonocytes [66], as well as obesity in haplo-insufficient MCT1 mice [52]. While mutagenic studies offer insight into overall functional role of MCT1 on energy metabolism, whole body knockdown may mask the physiological effects of high fat diet on endogenous transporter expression in the ileum. Similarly, cultured colonocytes may have mRNA and protein patterns of MCT expression distinct from the ileum and may not serve as a representative surrogate for MCT1 activity in the gastrointestinal tract. The effect of a high fat diet on SMCT1 was highly variable in both males and females. Noticeably, the SMCT1 levels fluctuated dependent on postnatal time-point, suggesting an interaction between age and diet.

Females generally had higher MCT1 levels than males except NC PND14 and HFD PND42. This pattern was similar in CD147, although males exhibited significantly higher expression at NC PND42. As ancillary proteins are essential for MCT trafficking and translocation, the higher CD147 in males at sexual maturity may be a compensatory mechanism for MCT1 regulation. In our group’s previous study, 8-week old males had greater CD147 membrane expression in the ileum than females of the same age range, which aligns with our
current findings (unpublished intestine manuscript). Females exhibited higher MCT4 expression than males overall, comparable to MCT1 levels. SMCT1 expression between males and females was highly variable. Males had higher SMCT1 expression at PND21, however SMCT1 between the two sex groups approached similar levels at sexual maturity. This was similar to our previous sex difference study, where SMCT1 ileal membrane expression in males was higher than females at various estrous stages, except in comparison to the estrus group (unpublished intestine manuscript).

The ancillary protein CD147 interacts with MCTs by stabilizing their localization at the cell membrane. Dysregulated expression of CD147 has been linked to reduced surface expression of MCTs, along with loss of function phenotypes in animal models. Ancillary proteins expression changes with age. Males exhibit linear increases in CD147 expression, while females had linear increases on high fat diet, but observed nonlinear (low, high, low) patterns in normal chow. Over-nutrition may cause dysregulation in normal ancillary protein expression, which can affect the chaperoning activity and membrane expression of MCTs. We wanted to investigate the relationship between ancillary protein and transporters and observe patterns of interaction within the ileum. MCT1-CD147 and MCT4-CD147 have significant positive correlation, indicative of an association between protein expressions dependent on age, diet and sex. Interestingly, SMCT1 has stronger, positive correlation with CD147 than either MCT1 or MCT4. There is currently no literature indicating SMCT1 complexes with ancillary protein CD147 or embigin, and more co-localization and activity studies need to be performed to determine the significance of this correlation in the intestine.

The role of leptin in obesity and upregulation of monocarboxylate transporter expression in colonocytes have been explored previously. In a study comparing wildtype and MCT1
haplodeficient mice on high fat diet, leptin levels and MCT1 protein expression was significantly higher in the liver, brain, heart, and white adipose of WTs compared to the heterozygotes [52, 66]. In our studies, leptin is significantly and positively correlated with increased bodyweight in rats stemming from over-nutrition with high fat diet. We next wanted to observe the correlation between circulating leptin and protein expression in the ileum. MCT1, SMCT1 and CD147 exhibited significant and negative correlation with leptin, suggesting a suppressive effect on transporter and ancillary protein expression. MCT4 had similar negative correlation, although this interaction did not reach significance. These results were opposite the leptin correlation in the liver, which saw positive interaction between leptin, MCT1 and CD147, indicating a tissue-specific role leptin plays in MCT regulation.

We have shown how ontogeny, overnutrition, and sex can affect intestinal monocarboxylate transporter expression. Obesity appears to both increase and decrease MCT1 and MCT4 regulation in the ileum based on specific age. The most distinct example of this effect was with MCT4 expression in males, where HFD increased protein levels at PND21, but decreased protein levels at PND42 with respect to the NC cohort. The lack of dietary differences in SMCT1 may reflect high inter-individual variability of this transporter’s expression in the obese animal model. There are limitations to our current work. Although duodenal and jejunal tissues were collected, these segments were not analyzed due to time and reagent restrictions, and we are unable to present a complete overview of MCT expression in the gastrointestinal tract. Whole protein in the ileum was not isolated nor investigated, which would shine light on the fraction of membrane MCTs trafficked to the cell surface. Unlike the liver and kidneys, ileal mRNA was not measured due to sub-optimal extraction conditions and poor correlation, and improved protocols need to be developed. However, the emerging data on the ontogeny of MCT
expression in the liver, kidney and intestine provides new insight on the potential impact in pediatric pharmacokinetics and oral drug administration. Higher expression of MCTs at sexual maturity may warrant changes in clinical drug regimen in younger adolescents, and obese patients may necessitate higher doses of monocarboxylic drugs due to decreased MCT expression in this understudied population. Females would require careful observation for optimal therapeutic drug dosing, as young girls express MCTs at higher levels than boys within the same age group, and may experience increased drug toxicity and side effects. However, there may be species differences between human and rat MCT regulation and expression; longitudinal clinical studies in humans are needed to confirm the correlative reliability of preclinical animal models. Further investigations are required to complete these maturation studies, including transcriptional and translational expression patterns in the duodenum, jejunum, and colon, and future experiments should include gut permeability assays measuring monocarboxylic substrate transport.
Monocarboxylate transporters are involved in the shuttling of monocarboxylic substrates such as ketone bodies, lactate and pyruvate, as well as drugs such as GHB. Proton-coupled monocarboxylate transporters are ubiquitously present in tissues throughout the human body, located in the apical and basolateral membranes of organs involved with nutrient and drug disposition, while sodium-dependent transporters have more restricted expression in the liver, kidneys, and intestine. Dysregulation of MCT protein expression may disrupt essential biological processes like metabolic homeostasis, and impair pharmaceutical drug clearance. Ontogeny has been shown to influence MCT levels in an age-dependent manner, and variability in MCT activity could play a critical role in effective pharmacotherapy in children. MCTs have also been implicated in controlling the rate of obesity in animal models on a high fat diet. Previous studies in our group have shown sex differences in MCT expression, including dynamic changes during the female estrous cycle [68]; however, it is unknown how age, over-nutrition, and sex affect MCTs/SMCTs and the ancillary protein CD147 in tissues involved in drug disposition. The purpose of this study was to measure the mRNA and protein expression of MCT1, MCT4, CD147, and SMCT1 in the liver, kidney, and gastrointestinal tract in an animal model of pediatric obesity with the inclusion of both male and female groups.

In Chapter 3, monocarboxylate transporters were expressed differentially based on age. We have demonstrated age-dependent regulation of MCT1, MCT4, and CD147 in the liver on a normal diet. Hepatic MCT1 and ancillary CD147 protein expression in an animal model of ontogeny matched clinical protein expression patterns of MCT1 from the literature, while MCT4 had an entirely different expression profile in our current study [10]. MCT1 total protein
expression (nonlinear; low, high, low) is highest pre-weaning, which is consistent with membrane protein expression at the cell surface. The ancillary protein CD147 follows the translational pattern of MCT1, while hepatic MCT4 membrane protein expression (linear; low, high) was highest post-weaning. mRNA expression had poor association with MCT1 and CD147 protein levels, and MCT4 mRNA was highest at early neonatal stages before decreasing to baseline levels at sexual maturation, which was opposite to its protein expression pattern. In latter studies and chapters, we were unable to gather data at earlier time-points of postnatal day 1 and 7 due to insufficient sample volumes. Thus, we investigated expressional patterns starting at PND14 through sexual maturity.

In Chapter 4, pregnant rats were fed a high fat diet during gestation/lactation, and gave birth to pups that had higher bodyweights and circulating serum leptin than rats on a normal chow diet. Males on both diets had highest hepatic MCT1 and CD147 protein expression at PND14 (nonlinear; high, low, high), while NC females had highest MCT1 and CD147 at PND21 (nonlinear; low, high, low). HFD females had peak MCT1 expression at PND14 (nonlinear; high, low, high, low), while CD147 expression was highest at PND14-28 (linear; high, low). Males on both diets had highest MCT4 protein levels at PND28 (nonlinear; low, high, low), and in females at PND14 (nonlinear; high, low, high). Diet affects transporter and ancillary protein expression liver. Age and sex differences determine whether high fat diet upregulates or suppresses MCT1 and MCT4 and CD147 protein expression in the liver, as seen in PND14 males (HFD > NC in MCT1/CD147) and PND21 females (HFD < NC in MCT1/CD147).

In Chapter 5, MCT1 and CD147 protein expression in both males and females were highest at sexual maturity (PND42) in the kidney. MCT1 expression was nonlinear in males (high, low, high) and females (low, high, low, high). Females reach higher expression of MCT4
at PND21 (nonlinear, low, high, low, high), compared to PND28 in males (nonlinear; low, high, low). SMCT1 protein expression varies based on age and diet. HFD males had peak SMCT1 expression at PND14 (linear; high, low), while NC males had highest expression later at PND28 (nonlinear; low, high, low). Females on both diets had highest SMCT1 expression at PND14 (nonlinear; high, low, high, low). Sex played a role in MCT expression, with males having higher MCT1 (NC and HFD PND42, and HFD PND14), MCT4 (NC and HFD PND28), and CD147 (NC and HFD PND42, and HFD PND14) protein expression than females, while females had higher SMCT1 protein expression than males at HFD PND14. High fat diet had an inconsistent effect on membrane protein expression based on isoform, elevating MCT1 and CD147 expression in males at PND42, while suppressing SMCT1 expression in males at PND28.

In Chapter 6, the ileum served as a surrogate for MCT expression in the mammalian intestine. MCT1 and CD147 protein expression is highest at PND42 in males (linear; low, high); females on normal chow have sustained MCT1 levels from PND21 to PND42 (linear; low, high), while transporter expression is stable throughout development in HFD animals. Interestingly, female CD147 membrane expression did not mirror MCT1 expression patterns as observed in the liver and kidney. In females, CD147 increased with age and peaked at NC PND35 (nonlinear; low, high, low), and HFD PND35 and PND42 (linear; low, high). MCT4 protein expression was highest in males at NC PND42 (linear; low, high), and HFD PND21 (nonlinear; low, high, low, high), while females had highest expression at PND42 in both diets (nonlinear; low, high, low, high). In the ileum, SMCT1 protein expression was highest at PND28 and PND42 in males (nonlinear; low, high, low, high), while in females SMCT1 peaked at PND42 (nonlinear; low, high, low, high). High fat diet had varied effects on protein expression based on
isoform and age; over-nutrition upregulates MCT4 protein in males at PND21, while suppressing MCT4 levels at PND42. Sex also affected protein expression based on isoform and ontogeny, where females had lower CD147 levels than males at NC and HFD PND42, while exhibiting higher MCT4 levels at NC PND21 and HFD PND42. Though not reaching significance, females generally had higher MCT1 and MCT4 protein levels at most timepoints compared to age-matched males.

Drugs have different pharmacokinetic profiles between children and adults, and there is a paucity of information investigating effective drug treatment regimens in children. Infants and adolescents undergo rapid developmental changes in biochemical processes throughout maturation compared to adults [129]. As a result, pediatric patients experience a wider range of inter-individual physiology and pharmacotherapy, which can expose them to a greater risk for sub-therapeutic or toxic drug dosing. Variability in drug absorption, distribution, and excretion can be caused by changes in membrane transporter expression in organs of drug disposition [130, 131]. Since most drugs prescribed to children are administered orally, the intestine is a major site of absorption, followed by hepatic first-pass metabolism, which contribute to drug bioavailability [132]. The expression patterns of monocarboxylate transporters in the liver, kidney, and ileum demonstrate the intersectionality of ontogeny, diet and sex on protein regulation. For example, high levels of renal SMCT1 and MCT1 in PND14 males may indicate higher transporter activity, apical reabsorption, and basolateral uptake of substrates compared to PND35 males, where MCT expression is lower at the same age. PND14 females have lower MCT1 protein expression compared to males, but have 2-fold higher SMCT1 at the same time-point, which indicates sex difference in protein regulation and may confer a compensatory mechanism for drug or nutrient uptake.
Obesity in adults can result in altered drug pharmacokinetics of monocarboxylate drugs. Similarly, childhood obesity due to genetics or over-nutrition can alter drug absorption, distribution, metabolism, and elimination, which can lead to the lack of therapeutic efficacy or even drug toxicity when prescribing pharmaceutics to pediatric patients. In the present study, a high fat diet had varied effects on MCT and SMCT expression across all organs. For example, HFD induces renal MCT1 and CD147 expression at PND28 in males; however, HFD suppresses SMCT1 membrane protein expression at the same age. This effect appears to mitigate the potential for enhanced intracellular uptake of drug substrates by inhibiting the absorption of drugs and nutrients in the glomerular filtrate. HFD also has no impact on MCT4 protein expression compared to NC, indicating MCT4 is not regulated by biological mechanisms of obesity, and may be regulated independently of MCT1/SMCT1 drug uptake in the kidneys in obese patients. Thus, monocarboxylate transporters must be scrutinized by isoform at specific postnatal day time-points to measure the significance of obesity on overall expression.

mRNA and protein expression patterns did not correlate in tissues of drug disposition. MCT1, CD147 and MCT4 mRNA expression had the reverse protein expression in multiple postnatal day time-points in the liver (Chapter 4). Additionally, these inconsistencies were observed in SMCT1 mRNA expression in the kidney (Chapter 5). It is unclear what specific biochemical mechanisms are regulating these shifts in transcriptional and translational regulation. One possible explanation for the incongruous relationship between mRNA and protein expression is the post-transcriptional modification of MCT1, MCT4, CD147, and SMCT1 mRNA in the regulation of membrane trafficking. miRNAs are short hairpin RNAs that can bind to the 3’ untranslated region of target genes. Once bound, miRNAs either inhibit translation or destabilize and lead to the programmed destruction of the target mRNA.
miR-29a and miR-29b have been shown to suppress MCT1 expression in a tissue-specific manner, such as in pancreatic beta islet cells [47]. The presence of miRNAs specifically binding and blocking MCT mRNA translation may explain the discrepancy between RNA and protein expression throughout ontogeny.

There are limitations to our study and scope of work. In chapter 3, Na+ /K+ ATPase was used as a protein loading control for western blot analysis. However, in latter chapters there was substantial inter-individual variability based on both ontogeny and diet in the kidneys and ileum. This was observed by other research groups, where relative protein expression of Na+ /K+ ATPase was lower in neonates and infants compared to adults [133]. Adjusting our protocol to quantify total protein loaded solved this discrepancy (detailed in Chapter 2). We were unable to extract and analyze whole cell protein in the kidneys and ileum due to low starting tissue volumes, a frequent hindrance of experimenting with pediatric samples. This information would help elucidate the translation of MCTs/SMCTs in these organs of interest, and determine the translocation cycle of proteins to the cell surface during maturation. Transporter expression in the gastrointestinal tract in our pediatric animal model of obesity requires further investigation. The method of extracting enterocytes in these samples is highly-involved, yet yields a relatively low amount of protein, which limits both total and membrane-bound protein analyses. More optimal protein extraction protocols should be explored, and LC-MS proteomics may be a viable alternative to western blot for quantification of protein expression as this technique is more sensitive [134]. Furthermore, only the ileum was studied for MCT protein expression, due to the time, material, and sample volume restrictions in our study, and no mRNA experiments were performed in the ileum. In a recent study, miR-29a, b, and c have been shown to downregulate SMCT1 expression in mouse intestinal epithelial cells, which may explain differential expression
of these transporters along the length of the gastrointestinal tract [135]. Spatial expression in the remaining duodenum, jejunum, and colon must be explored for a complete overview of MCT/SMCT mRNA and protein expression in the intestine.

We have shown that pregnant rats fed a high fat diet during gestation give birth to pups with increased bodyweights and circulating levels of leptin, a biomarker of obesity. However, serum leptin had mixed correlation with overall MCT/SMCT protein expression across different tissues. The whole-body effects of obesity, associated co-morbidities, and the multitude of biochemical signals involved may mask the role of endogenous leptin in MCT regulation. *In vitro* studies should be conducted using hepatic, renal, and intestinal primary cells to determine how leptin influences MCT expression based on sex and ontogeny. Other metabolic biomarkers of obesity can be studied in the existing serum samples to determine possible correlations with MCT regulation. For example, obesity leads to the infiltration of macrophages into expanded adipose tissue, resulting in increased levels of proinflammatory cytokines interferon-gamma and tumor necrosis factor-alpha [136]. IFN-γ and TNFα can downregulate MCT1 and SMCT1 in the intestines [49, 137]. Testosterone has been shown to increase lactate transport, MCT1 and MCT4 expression in rat skeletal muscle, and obesity is known to lower testosterone levels due to insulin resistance and disruption of the hypothalamic-pituitary-testicular axis [138, 139]. Although sex hormones are not strong contributory factors to physiological development until sexual maturity, measuring the circulating levels of testosterone and estrogen in early adolescence may provide insight on other biochemical signals that control MCT/SMCT expression in obese pediatric patients.

We have shown throughout that MCT/SMCT protein expression is regulated by age, sex, and diet in a tissue and isoform specific manner; however, the impact on transporter activity is
still unknown. Higher expression of renal MCT1 and SMCT1 in one postnatal time-point should theoretically lead to increased substrate transport, yet the current proteomic work cannot validate this claim. In addition, we must investigate the developmental expression of other MCT isoforms in this mammalian model, including MCTs 2, 5, 6, 8, 10-14, and SMCT2. Future studies will involve culturing primary cell lines from pediatric tissues and measuring functional activity of MCTs and comparing to expressional data. *In situ,* single-pass intestinal perfusion assays will be used to measure oral drug absorption, and jugular cannula blood sampling to determine substrate uptake. qPCR and immunoblotting of the remaining monocarboxylic transporters will further characterize the expression of MCT and SMCT in mammalian development and drug tissue maturation. In conclusion, we have generated an insightful new animal model of pediatric obesity that demonstrates the dynamic and unique expression patterns of monocarboxylate transporters in the liver, kidney, and intestine. This model can be evaluated for additional drug transporters and drug metabolizing enzymes in future proteomic and pharmacokinetic studies of ontogeny.
REFERENCES


