DISSERTATION

# IMPACTS OF MATERNAL DIETARY FAT INTAKE ON FETAL DEVELOPMENT IN AN OVINE MODEL

Submitted by

Asma Khalifa Omar Alashibi

Department of Biomedical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2025

Doctoral Committee:

Advisor: Adam J. Chicco

Quintin Winger
Elaine M. Carnevale
Chris Melby

# ABSTRACT

## IMPACTS OF MATERNAL DIETARY FAT ON FETAL DEVELOPMENT IN AN OVINE MODEL

Maternal diet during pregnancy can influence fetal development and may have lasting effects on offspring health and disease risk trajectory. Intake of dietary fatty acids has been a major focus in the biomedical and epidemiological literature given concerns about the adverse effects of excessive fat intake on cardiometabolic risk and the putative health benefits of supplementing specific fats such as omega-3 polyunsaturated fatty acids (*n*3-PUFA). However, a thorough understanding of how maternal dietary fats impact fetal development and metabolism has been limited by ethical and logistical challenges of such studies in humans, highlighting the need for affordable large animal models of human pregnancy for evaluating these interventions. The primary aim of this dissertation was to establish the utility of the pregnant sheep for this purpose given its well-established similarities to human pregnancy in terms of gestational length, primarily singleton or twin births, and substantial mid-gestational fetal size. A series of three projects were carried out in pregnant white-faced ewes to investigate 1) the effects of maternal high-fat diet on fetal skeletal muscle metabolism; 2) the effects of maternal dietary *n*3-PUFA supplementation on fetoplacental fatty acid composition, transport, and metabolism; and 3) the role of placental fatty acid transporter-4 (FATP4) on fetoplacental lipid composition and metabolism. These studies demonstrate close parallels with available data from similar dietary interventions in humans and non-human primate models, including 1) a metabolic shift favoring the use of fat over carbohydrate in fetal skeletal muscle during high-fat pregnancies, and 2) enrichment of fetal tissues with *n*3-PUFA along with diverse impacts on lipid metabolism

following maternal docosahexaenoic acid (DHA) supplementation, and 3) novel links between FATP4 expression and fetoplacental fatty acid metabolism at mid-gestation. Taken together, these studies establish the utility of an ovine model for investigating the impacts of maternal dietary fatty acid intake on fetal physiology during pregnancy, thereby facilitating future studies of the longer-term impacts of maternal diet on offspring metabolic health and disease risk.

# ACKNOWLEDGEMENTS

I would like to express my greatest thanks to everyone who has supported me throughout my PhD journey. I am especially thankful to my advisor Dr. Adam Chicco for his support, expert guidance and constant encouragement. His advice and feedback have truly shaped this dissertation. I also want to thank my committee members, Dr. Winger, Dr. Carnevale and Dr. Melby for their valuable insights and precious suggestions and comments, which helped refine my work. Also, I would like to thank my previous committee members, Dr. Bouma and Dr. Abollel, for their valuable contribution and precious guidance. A special thank go to my sponsors: The University of Benghazi, the Colorado Agricultural Experiment Station and College of Veterinary Medicine and Biomedical Sciences Research Council, and the USDA National Institute of Food and Agriculture.

I would like to thank my lab mates, for their great contribution, Dr. Lance Li Puma, and Luke Whitcomb. Also, I would like to thank my undergraduate students who were great helpers in performing many lab experiments, among which I would like to thank Briana Risk, Aria Witt, Cheyanne Izon and Dorcas Kareng.

Last but not least, I would like to show my great gratitude to my Libyan friends, my husband and my kids who supported me through this long journey and were there for me when I felt disappointed or homesick.

# DEDICATION

I dedicate this dissertation to the soul of my beloved father, Khalifa, how I wish you were here today to see your little daughter confidently taking steps toward earning her PhD. If it weren't for you, for your unwavering support and constant encouragement since I was a young child, I would not have reached where I am today.

To my dearest mother, Halima, who encouraged me to continue my studies, provided me with the perfect environment to study and instilled in me a love for knowledge from a young age. No matter how far we are, you are always in my heart and mind. May God bless you with a long life, good health and happiness.

To my dear husband, Ahmed, I will never forget how you left your job in Libya to travel and live abroad with me so I can pursue my graduate studies. Thank you for your constant support and for standing by my side during the difficult times.

To my beloved kids, your innocent smiles have always been the healing balm for me during the difficult times I faced. Your presence by my side has been my greatest motivation to continue my journey and complete what I started.

TABLE OF CONTENTS

ABSTRACT……ii

ACKNOWLEDGEMENTS……iv

DEDICATION……v

LIST OF TABLES……..……..x

LIST OF FIGURES……..……..xi

CHAPTER I: HYPOTHESES AND EXPERIMENTAL AIMS……1

Overarching Hypothesis…….……..……1

Specific Aims...……2

1. Investigate the effects of maternal high-fat diet during pregnancy on fetal skeletal muscle metabolism and insulin signaling ……..…….……...2

2. Investigate the effect of maternal DHA supplementation on placental and fetal tissue metabolism ...……..…….……..2

3. Investigate the effect of placental fatty acid transporter-4 on placental and fetal tissue metabolism……...…….……3

CHAPTER II: HIGH-FAT DIET DURING PREGNANCY PROMOTES FETAL SKELETAL MUSCLE FATTY ACID OXIDATION AND INSULIN RESISTANCE IN AN OVINE MODEL…….…….……...….6

Abstract…….……..…..….. 6

1. Introduction…….…….....…7

2. Materials and Methods……..…….……..9

2.1 Animal Model and Dietary Intervention…..…..…….…..……..9

2.2 Blood and Fetal Tissue Collection……...…..…..…….10

2.3 Serum Analyses……..…..…...10

2.4 Skeletal muscle mitochondrial metabolism…….……11

2.5 Protein Immunoblotting…….…….….12

2.6 RNA isolation and quantitative PCR…….……..13

2.7 Statistics…….……..14

3. Results……...……..…14

3.1 Maternal and Fetal Morphology……..……..……..…...14

3.2 Serum glucose and lipids……..…...…..…..15

3.3 Placental and fetal muscle fatty acid transporter expression……..…….16

3.4 Muscle oxidative metabolism…….…….…..….16

3.5 Muscle triglycerides and metabolic enzyme expression ……..…….…17

3.6 Muscle insulin signaling…….…..…….…..17

3.7 Liver triglycerides and nutrient handling enzymes……..…….……..18

4. Discussion…….…….19

Acknowledgments…….…….23

Disclosures…….……...22

References…….……..…...32

CHAPTER III: OMEGA-3 FATTY ACID INTAKE DURING PREGNANCY ALTERS FETALTISSUE MEMBRANE COMPOSITION AND LIPID METABOLISM IN AN OVINE MODEL…….…….……..37

Abstract……..…….……..37

1. Introduction…….……...…….....38

2. Materials and Methods…….…….……40

2.1 Animal Model and Dietary Intervention…….…….40

2.2 Blood and Fetal Tissue Collection…….…….41

2.3 Analysis of serum triglycerides, total cholesterol and glucose…….…..……42

2.4 Serum and Tissue Phospholipid Fatty Acid Composition…….…….42

2.5 Fetal cardiac and skeletal muscle oxidative metabolism…….……...…….43

2.6 Protein Immunoblotting…….……...…..44

2.7 Statistical analyses…….…….……..…..45

3. Results.…….…….……46

3.1 Maternal and Fetal Morphology…….…….……46

3.2 Serum lipids in the placental circulation…….……46

3.3 Fatty acid composition of phospholipids in the placental circulation…….....47

3.4 Fatty acid composition of fetal tissue phospholipids…….……48

3.5 Placental and fetal tissue fatty acid transporter expression……48

3.6 Maternal DHA feeding promotes greater fetal cardiac fatty acid oxidation capacity……49

3.7 Maternal DHA feeding increases fatty acid oxidation capacity and decreases IRS-1 expression in fetal muscle……50

3.8 Maternal DHA feeding alters hepatic oxidative metabolism and decreases IRS-1 expression ……51

4. Discussion……51

Acknowledgments……56

References……58

CHAPTER IV: IMPACTS OF PLACENTAL FATP4 GENE TARGETING ON FETOPLACENTAL SERUM FATTY ACID COMPOSITION AND FETAL TISSUE METABOLISM……73

Abstract……73

1. Introduction……75

2. Materials and Methods……77

2.1 Experimental Model……77

2.2 Blood and Fetal tissue collection……78

2.3 Serum Lipids and Glucose ……79

2.4 Serum and Tissue Phospholipid Fatty Acid Composition……79

2.5 Skeletal muscle oxidative metabolism……80

2.6 Protein Immunoblotting……82

2.7 RNA isolation and quantitative PCR……82

2.8 Statistical Analyses……83

3. Results and Discussion……84

3.1 Impacts on fetal morphology……84

3.2 FATP4 gene targeting by CRISPR in D9 embryos selectively deleted FATP4 mRNA from the GD75 cotyledon……84

3.3 FATP4 mRNA deletion from the GD75 cotyledon was not associated with lower placental FATP4 protein expression……85

3.4 FATP4 mRNA deletion from the GD75 cotyledon was not associated with upregulation of FATP1 or CD36 in the GD75 placenta……87

3.5 FATP4 gene targeting by CRISPR in D9 embryos alters GD75 fetoplacental serum fatty acid composition…….……87

3.6 Impacts of FATP4 gene targeting by CRISPR in D9 embryos on GD75 serum glucose, cholesterol and triglycerides in the fetoplacental circulation…….…….90

3.7 FATP4 gene targeting by CRISPR in D9 embryos depletes FATP4 protein from GD75 fetal tissues…….……91

3.8 Cas9 protein is undetectable in KO placental and fetal tissue at GD75……..93

3.9 FATP4 deletion from fetal muscle and heart has minimal effects on membrane fatty acid composition……...…..…94

3.10 Deletion of FATP4 triggers upregulation of FATP1 and CD36, but not FABPpm in fetal muscle……..…….……95

3.11 FATP4 deletion is associated with loss of IRS-1 and impaired carbohydrate oxidation in fetal muscle……’……..……..…96

3.12 FATP4 depletion is associated with higher FATP1, loss of IRS-1 and JNK, and triglyceride accumulation in GD75 fetal liver……..……..98

3.13 Summary and Conclusions……..……...…99

References……101

CHAPTER V: CONCLUDING REMARKS AND FUTURE DIRECTIONS……...……104

LIST OF TABLES

CHAPTER II:

Table 1. Impact of maternal HFD on fetal parameters…….…….24

Table 2. Serum glucose and lipids in the uteroplacental circulation…….……25

CHAPTER III:

Table 1: Fetal morphology……65

Table 2. Fatty acid composition of phospholipids in the placental circulation…….66

Table 3. Fatty acid composition of fetal tissue phospholipids……..67

CHAPTER IV

Table 1. Impact of placental FATP4 gene targeting on GD75 fetal morphology…...….83

LIST OF FIGURES

CHAPTER II:

Figure 1. Placental fatty acid transporter expression. ……..……..….26

Figure 2. Fetal skeletal muscle fatty acid transporter expression.…….27

Figure 3. Fetal skeletal muscle oxidative capacities.…….……28

Figure 4. Expression of oxidative metabolism enzymes in fetal skeletal muscle.…...…..29

Figure 5. Fetal muscle insulin signaling……..…..30

Figure 6. Fetal liver triglyceride content and enzyme expression.……31

CHAPTER III

Figure 1: Serum lipids in the placental circulation……..……..……68

Figure 2. Placental and fetal tissue fatty acid transporter expression…….…….…….69

Figure 3: Impacts on fetal cardiac metabolism…….…..……70

Figure 4. Fetal skeletal muscle metabolism…….…….……71

Figure 5. Fetal liver metabolism……..…..……72

CHAPTER IV

Figure 1: Selective deletion of FATP4 mRNA in the GD75 placenta …....……85

Figure 2: Protein expression of FATP4 in the GD75 placenta…….……86

Figure 3. Protein expression of FATP1 and CD36 in GD75 placenta..…...…….……87

Figure 4. Impacts of *FATP4* gene targeting on the serum fatty acid composition of phospholipids in the GD75 fetoplacental circulation……..…..…….…..……89

Figure 5. Impacts of *FATP4* gene targeting on the serum glucose, cholesterol and triglycerides in the GD75 fetoplacental circulation……..…….……90

Figure 6. Impacts of *FATP4* gene targeting on FATP4 protein expression in GD75 fetal tissues …….……..……92

Figure 7. Impacts of *FATP4* gene targeting on the fatty acid composition of phospholipids in the fetal muscle and heart……...……95

Figure 8. Protein expression of fatty acid transporters other than FAP4 in GD75 fetal muscle.…….…..…96

Figure 9. FATP4 gene targeting on D9 embryos impacts GD75 fetal muscle metabolism…….……97

Figure 10. FATP4 gene targeting on D9 embryos impacts GD75 fetal liver metabolism……99

# CHAPTER I: HYPOTHESES AND EXPERIMENTAL AIMS

## Overarching Hypothesis:

Maternal dietary quality has been associated with pregnancy outcomes and the offspring's metabolic health and development in both humans and animal models for decades. However, causal relationships between maternal diet during pregnancy, intrauterine environment supporting fetal development, and subsequent post-natal health trajectory remain poorly understood. Studies in rodent models have revealed a wealth of biological insight into mechanisms that link the intrauterine environment to fetal development and offspring health, but the studies lack many key parallels with human pregnancy and development. Translational studies in non-human primates have begun to shed light on the impacts of maternal diet and metabolic status on fetal metabolism, but they are prohibitively expensive for most research groups and, thus, not widely available. The aim of this dissertation was to establish utility of pregnant sheep for modeling the impacts of maternal dietary fat intake on mid-gestational fetal development and metabolism, with a particular focus on the effects of high-fat feeding and omega-3 fatty acid supplementation. Our overarching hypothesis was that the ovine model will exhibit many of the hallmark effects of these dietary interventions previously reported in humans and non-human primate models and reveal novel biochemical insights that may link fetal metabolism to offspring metabolic health trajectory in future studies. We also sought to demonstrate the feasibility of early embryonic targeting of genes involved in maternal-fetal fatty acid transport to modify fetal metabolism during pregnancy using methodologies recently established in the ovine model by investigators at Colorado

State University. To address these hypotheses and goals, we performed three separate research projects to address three specific aims.

***Specific Aim 1.*** *Investigate the effects of maternal high-fat diet during pregnancy on fetal skeletal muscle metabolism and insulin signaling.*

Maternal high-fat feeding during pregnancy and its effects on fetal metabolism has been previously studied using a non-human primate model, where it altered oxidative metabolism and impaired insulin signaling in fetal skeletal muscle. Therefore, we aimed to establish an ovine model of maternal high-fat feeding and investigate if outcomes were similar in fetal skeletal muscle at mid-gestation. To this end, white-faced ewes were fed a diet supplemented with 6% (w/w) rumen-protected calcium soaps rich in saturated fatty acids or the control base diet from 2 weeks prior to conception through mid-gestation (75 days after conception; GD75). At this point, placental and fetal tissues were collected and analyzed along with serum from the fetoplacental circulation. The results of this study support the hypothesis that maternal high-fat feeding causes impaired insulin signaling and a metabolic shift favoring oxidation of fat over carbohydrate fuel sources in fetal skeletal muscle, which paralleled maternal hyperglycemia, hyperlipidemia and hyperinsulinemia at mid-gestation. Therefore, we demonstrated the feasibility of the ovine model for investigating the impacts of excessive fat intake and its consequent metabolic effects on the uterine environment during pregnancy on fetal health and development, and its potential links to metabolic syndrome later in life.

***Specific Aim 2:*** *Investigate the effect of maternal DHA supplementation on placental and fetal tissue metabolism*

Dietary supplementation with long-chain omega-3 fatty acids such as docosahexaenoic acid (DHA) is recommended to women during pregnancy to prevent pre-term birth and support optimal fetal development. DHA supplementation also decreases circulating triglyceride levels through diverse effects on systemic lipid metabolism, but its impacts on fetal metabolism are largely unknown due to inherent limitations of such investigations during human pregnancy. Therefore, we aimed to establish an ovine model suitable for investigating impacts of prenatal DHA supplementation on fetal development and metabolism. White-faced ewes were fed either a control diet or the same diet supplemented with 3% algae-derived DHA from 2-3 weeks before pregnancy until mid-gestation (GD75). At this stage, serum, placenta and fetal tissues were collected for biochemical analysis of phospholipid fatty acid composition and fatty acid transporter expression, along with a broader characterization of nutrient metabolism of fetal heart, skeletal muscle, and liver. Results confirmed significant DHA enrichment of all six fetal tissues examined from DHA-supplemented pregnancies, which paralleled tissue-specific impacts on fetal fatty acid transporters and metabolism, thereby demonstrating the utility of an ovine model for investigating the biological effects of prenatal DHA supplementation on fetal development and metabolism. Results also revealed novel insight that highlights the need for a better understanding of how maternal DHA supplementation during pregnancy impacts the metabolic health trajectory of the fetus and future offspring.

***Specific Aim 3:*** *Investigate the effect of placental fatty acid transporter FATP4 on placental and fetal tissue metabolism.*

Fetal development depends on the tightly regulated transfer of nutrients from the maternal to the fetal circulation by the placenta. Among the several fatty acid transporters impacted by maternal diet in our previous studies, placental fatty acid transport protein 4 (FATP4) expression has been previously associated with the selective transfer of DHA to the umbilical circulation and increases in response to maternal overnutrition during pregnancy. Therefore, we sought to investigate the influence of placental FATP4 on the fatty acid composition of serum phospholipids in the ovine fetoplacental circulation and fetal tissues, along with the fetal metabolic parameters evaluated in our previous studies. We employed a method for selective deletion of the FATP4 gene from the trophectoderm (pre-placental layer) of Day 9 ewe embryos (hatched blastocysts 9 days following conception) via lentiviral transduction of a CRISPR-Cas9 construct targeting the ovine FATP4 gene. Transfected blastocysts were then implanted into recipient pseudo-pregnant ewes and allowed to develop until GD75 for fetoplacental metabolic analysis. Results demonstrate successful depletion of FATP4 mRNA from the GD75 cotyledon (fetal side of the placenta) and decreased levels of DHA in the umbilical vein relative to the uterine artery, consistent with a role of placental FATP4 in DHA transfer from the maternal to fetal circulation. However, this was not associated with a significant reduction in FATP4 protein in the placenta, perhaps indicating migration of FATP4 transporters from the caruncle (maternal side of the placenta) or the maternal circulation, with unclear functional consequences. Moreover, early embryonic FATP4 gene targeting significantly depleted FATP4 protein expression from all five GD75 fetal tissues examined, which was associated with diverse metabolic effects in fetal muscle and liver. Taken together, results reveal insight into the role of FATP4 during fetoplacental development and highlight the potential pitfalls of CRISPR lentiviral transfection of D9

blastocysts as a method for studying impacts of placenta-specific gene targeting on fetal parameters.

CHAPTER II

# HIGH-FAT DIET DURING PREGNANCY PROMOTES FETAL SKELETAL MUSCLE FATTY ACID OXIDATION AND INSULIN RESISTANCE IN AN OVINE MODEL*


**Abstract**

Maternal diet during pregnancy is associated with offspring metabolic risk trajectory in humans and animal models, but the prenatal origins of these effects are less clear. We examined the effects of a high-fat diet (HFD) during pregnancy on fetal skeletal muscle metabolism and metabolic risk parameters using an ovine model. White-faced ewes were fed standardized diet containing 5% fat w/w (CON), or the same diet supplemented with 6% rumen-protected fats (11% total fat w/w; HFD) beginning two weeks before mating until mid-gestation (GD75). Maternal HFD increased maternal weight gain, fetal body weight, and low-density lipoprotein levels in the uterine and umbilical circulation, but had no significant effects on circulating glucose, triglycerides or placental fatty acid transporters. Fatty acid (palmitoylcarnitine) oxidation capacity of permeabilized hindlimb muscle fibers was >50% higher in fetuses from HFD pregnancies, while pyruvate and maximal (mixed substrate) oxidation capacities were similar to CON. This corresponded to greater triacylglycerol content and protein expression of fatty acid transport and oxidation enzymes in fetal muscle, but no significant effect on respiratory chain complexes or pyruvate dehydrogenase expression. However, serine-307 phosphorylation of insulin receptor substrate-1 was greater in fetal muscle from HFD pregnancies along with c-jun-N terminal kinase activation, consistent with prenatal inhibition of skeletal muscle insulin signaling. These results indicate that maternal high-fat feeding shifts fetal



*This chapter was published in *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* in October 2023. Citation information: Omar AK, Li Puma LC, Whitcomb LA, Risk BD, Witt AC, Bruemmer J, Winger QA, Bouma GJ, Chicco AJ. High-fat diet during pregnancy promotes fetal skeletal muscle fatty acid oxidation and insulin resistance in an ovine model *Am J Physiol Reg Integr Comp Physiol;* 325:R523, 2023. PMID: 37642284; doi: 10.1152/ajpregu.00059.2023

skeletal muscle metabolism toward a greater capacity for fatty acid over glucose utilization and favors prenatal development of insulin resistance, which may predispose offspring to metabolic syndrome later in life.

## 1. Introduction

Maternal overnutrition during pregnancy has been linked to a greater likelihood of offspring developing insulin resistance and obesity in early adulthood [1, 2]. This parallels the longstanding Developmental Origins of Health and Disease (DOHaD) hypothesis originally proposed by Barker [3, 4], which posits that an adverse intrauterine environment can improperly "program" fetal and neonatal tissue responses to nutrient availability and thereby increase the risk of metabolic syndrome and its comorbidities later in life. While epigenetic mechanisms are suggested to play a mechanistic role in metabolic programming [5, 6], understanding how maternal diet impacts fetal metabolism and development in humans is less clear due to inherent logistical and ethical limitations on experimental research in this population. Therefore, robust animal models must be used to investigate this complex process and develop novel interventions that support optimal fetal and offspring health.

The majority of experimental evidence supporting the DOHaD hypothesis has come from rodent models, which have demonstrated impacts of maternal diet and metabolic status on the central regulation of appetite, nutrient handling and metabolism of peripheral tissues, and activation of pro-inflammatory signaling cascades linked to adverse outcomes in the developing fetus and offspring [7-9]. However, significant differences exist between rodent and human pregnancies, including a short (~ 3 week) gestation period and multiple-birth litters compared to

the much longer duration of singleton pregnancies in humans [10], which may impact fetal responses to variations in maternal diet. Studies in non-human primates (NHPs) have provided a highly translatable foundation for understanding the impact of maternal obesity and overnutrition on fetal and offspring metabolism, behavior, and disease risk trajectories [11-13]. Shifts in fetal skeletal muscle nutrient metabolism favoring utilization of fatty acids over glucose have emerged as a signature response to maternal high-fat feeding in NHPs [14], leading to impaired muscle insulin sensitivity in the developing fetus and offspring, even when juveniles were switched to a low-fat diet [11]. This may be due to shifts in the capacity of skeletal muscle mitochondrial capacity to oxidize fatty acid over carbohydrate fuels and reduced efficiency of oxidative phosphorylation that occur secondary to an accumulation of intramuscular lipids when fuel supply exceeds energy demands [14-17]. However, the limited availability and high cost of the NHP model prohibit its use by most of the research community, necessitating development and characterization of other large animal models suitable for studying maternal-fetal programming.

While no animal model fully recapitulates all aspects of human pregnancy and fetal development, the pregnant sheep has been used extensively to investigate maternal-fetal interactions, particularly impacts of placental nutrient transfer and metabolism, which parallel available evidence from human pregnancies [18]. The long ovine gestational period (~5 months) and mostly singleton or twin pregnancies are also well-suited for studies of maternal diet on fetal development. However, dietary interventions are complicated in ruminants, particularly supplementation of fatty acids that are rapidly degraded in the rumen and may be toxic to the endogenous microbiota [19]. Therefore, validation studies are required to establish an ovine model that effectively recapitulates the hallmark findings of high-fat feeding in monogastric animals and humans. The aim of the present study was to establish an ovine model of fetal

metabolic programming by maternal high-fat feeding during pregnancy using commercially available rumen-protected fats. A particular focus was placed on characterizing the aforementioned impacts on fetal skeletal muscle metabolism and insulin sensitivity previously linked to offspring metabolic risk in NHP and rodent models. We hypothesized that maternal high-fat feeding would shift fetal skeletal muscle mitochondria to uptake and oxidize fatty acids over carbohydrate fuels and diminish the capacity or efficiency of oxidative phosphorylation, supporting the potential role of muscle mitochondria in fetal metabolic programming.

## 2. Materials and Methods

*2.1 Animal Model and Dietary Intervention.* All animal procedures were approved by Colorado State University Animal Care and Use Committee in accordance with recommendations from the Declaration of Helsinki and the Guiding Principles on the Care and Use of Animals adopted by the American Physiological Society. White-faced ewes were purchased from a local vendor and housed in outdoor pens with free access to food and water. Heat cycles were determined using a vasectomized ram during the typical sheep breeding season (late October-November). Two weeks before pre-determined conception dates, ewes were randomly assigned to receive the standard control diet (CON, $n$ = 5; Show-rite NewCo Lamb Feed; 17% protein, 5% Fat; approximately 12% Kcal from fat) or a high-fat diet consisting of the control diet with 6% w/w Rumen-protected fat (Alltech Energy BC, Alltech, Lexington, KY, USA) mixed with a few cups of molasses to facilitate even distribution and palatability (HFD, $n$ = 9; 11% Total Fat w/w; approximately 23% Kcal from fat). The rumen-protected fats were calcium soaps of primarily saturated fatty acids (51.2% stearate, 46.3% palmitate, 0.3% myristate, 1.7% oleate) that are protected from microbial degradation in the rumen without impacting fermentation of dietary

fiber, thus providing an efficient means of increasing energy density of the diet in sheep and other ruminants [20]. Animals on similar heat cycles were synchronized by two 2 mL doses of intramuscular Dinoprost Tromethamine (Lutalyse, Zoetis) two days prior to breeding with the same fertile ram to ensure efficient conception and minimize fetal genetic variations from paternal DNA. Pregnant ewes were maintained on their respective diets until collection of fetal tissues at mid-gestation as described below.

*2.2 Blood and Fetal Tissue Collection*. Seventy-five days following conception (GD75), pregnant ewes (confirmed by ultrasound) were fasted overnight and sedated by intravenous injection of diazepam (2 mL) and ketamine (10 mL) prior to establishing general anesthesia by 2% isoflurane inhalation. The uterus was exposed to enable collection of blood from umbilical and uterine circulations, followed by removal of the placenta and fetus for morphological assessments and tissue collection. Sections of hindlimb skeletal muscle (*M. biceps femoris*) were carefully dissected and either snap frozen in liquid nitrogen and stored at -80°C for future biochemical experiments, or placed in ice-cold BIOPS preservation medium containing (in mM) 10 Ca-EGTA (0.1 μM free calcium), 20 imidazole, 20 taurine, 50 K-MES, 0.5 DTT, 6.56 $MgCl_2$, 5.77 ATP, and 15 phosphocreatine, pH 7.1, for respirometry experiments on freshly prepared permeabilized fiber bundles. Ewes were euthanized following removal of the fetus and placenta under general anesthesia.

*2.3 Serum Analyses.* Serum metabolites were determined in venous and arterial blood from the uterine and umbilical circulations using commercially available assay kits for glucose (Invitrogen, Cat# EIAGLUC), insulin (Eagle Biosciences, Cat#INO91-K01), triglycerides (Abcam, Cat# ab65336), total cholesterol, high-density lipoproteins (HDL), and low/very low-

density lipoproteins (LDL+VLDL) (Abcam, Cat# ab65390) according to the manufacturer's instructions.

*2.4 Skeletal muscle mitochondrial metabolism*. The capacities of fetal skeletal muscle to oxidize fatty acids, pyruvate and mixed substrate combinations were assessed by high-resolution respirometry on permeabilized fiber bundles freshly prepared from hindlimb muscle sections as previously described [21]. Briefly, 10-15 mg of *M. biceps femoris* was trimmed free of connective tissue and gently teased with needle tip forceps in ice-cold BIOPS solution, then incubated with 50 μg/ml saponin for 20 min on ice with gentle rocking to permeabilize cell membranes while leaving mitochondrial membranes intact [22]. Permeabilized fiber bundles were then rinsed in mitochondrial respiration medium (MiR05) containing (in mM) 0.5 EGTA, 3 $MgCl_2$ hexahydrate, 60 lactobionic acid, 20 taurine, 10 $KH_2PO_4$, 20 HEPES, 110 sucrose, and 0.1% BSA, pH 7.1 with KOH by rocking for 2 x 15 min on ice, then gently blotted dry on Whatman paper and weighed immediately before adding 5-6 mg of fiber bundles to the oxygraph chamber for respirometry experiments. Mass-corrected muscle oxidative capacities (per mg fiber bundle) were determined in MiR05 medium at 37°C using an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) by monitoring changes in the negative time derivative of the chamber oxygen concentration signal ($JO_2$) in response to carbohydrate (5 mM pyruvate + 1 mM malate), fatty acid (0.04 mM palmitoylcarnitine + 1 mM malate), or mixed substrate combinations (1 mM malate, 0.04 mM palmitoylcarnitine, 5 mM pyruvate, 10 mM glutamate, 10 mM succinate) following standardized instrumental and chemical background calibrations [23]. Following the addition of substrates, $JO_2$ was recorded in the absence of ADP to assess the capacity for non-phosphorylating "LEAK" $JO_2$ (facilitated by proton leak across the

inner mitochondrial membrane), followed by the addition of 2.5 mM ADP to generate the maximal oxidative phosphorylation (OXPHOS)-linked $JO_2$. The extent of respiratory control by ADP was expressed as the OXPHOS coupling control factor [1-(LEAK/OXPHOS)] for carbohydrate and fatty acid oxidation, where a maximum value of 1.0 represents fully coupled mitochondria (100% control of respiration by ADP), and 0 represents fully uncoupled mitochondria (0% respiratory control). Respirometry chambers were maintained in a narrow hyperoxygenated environment (325–400 μM $O_2$) during experiments to avoid limitations of oxygen diffusion on $JO_2$ of permeabilized fiber bundles [24].

*2.5 Protein Immunoblotting*: Proteins were extracted from snap-frozen sections of placentome and fetal muscle and liver tissue homogenized in lysis buffer containing (in mM): 150 NaCl, 1 EDTA, 1 EGTA, 5 sodium pyrophosphate, 1 sodium orthovanadate, 20 sodium fluoride added to Mammalian Protein Extraction Reagent (Pierce Cat# 78501) with supplemental Protease Inhibitor Cocktail (Sigma Aldrich Cat# P8340). Protein concentrations of 10,000 X g homogenate supernatants were detected by bicinchoninic acid assay (Thermo Scientific, #23225) using a colorimetric microplate reader (VersaMax, Molecular Devices). Proteins (30 μg) were electrophoresed on 4-12% Bis-Tris gels and transferred to PVDF membranes, then blocked for 1 h at room temperature with 5% non-fat milk or bovine serum albumin (for phosphorylated proteins) before incubating with the following primary antibodies (1:1000 dilution) overnight at 4C°: FATP1 (Abcam, ab81875), FATP4 (Abcam, ab200353), CD36 (Abcam, ab133625), OXPHOS complex subunits (Abcam, ab110413), FABPpm (Abcam, ab45966), pPDH-E1α (Abcam, ab92696), PDH-E1α (Abcam, ab110330), PDK4 (Abcam, ab89295), IRS1 (Abcam, ab52167), pIRS1-Ser307 (Abcam, ab5599), pIRS1-Ser1101 (Cell Signaling, 2385S), pJNK

(Abcam, ab47337), JNK (Abcam, ab199380), PKCθ (Abcam, ab52494), PCK1(Abcam, ab28455), HADHA (Abcam; ab-203114), ACADVL (Abcam, ab376239), CPT1β (Abcam ab104662). After three washes with Tris-buffered saline + 1% Tween-20 (TBST), the membrane was incubated in secondary antibodies (goat anti-rabbit-HRP (Abcam, ab6721) or goat anti-mouse-HRP (Abcam, ab6789) at 1:3000 dilution for one hour at room temperature. Blotted proteins were imaged using SuperSignal West Dura Extended Duration Substrate (Lot#VL314742) and a UVP ChemStudio blot imager (Analytik Jena, Germany), normalizing band densities to total protein staining of a 20-30 kDa range of bands near the target protein using AmidoBlack (Sigma A8181), quantified using ImageJ software (NIH).

*2.6 RNA isolation and quantitative PCR*. RNA was isolated from 50 mg fetal muscle using TRIZOL reagent (Sigma, T9424), with purity confirmed by a A260/280 of ≥ 1.8 using a Nanodrop 1000. cDNA was made using Bio-Rad iScript cDNA synthesis kit (cat#1706891) on the same day as qPCR experiments performed on a Roche LightCycler480. Primers against PPARα (Forward: 5'-TCGGCTGAAGCTGGTGTATGA-3'; Reverse: 5'-TGAGATCTGCGGTTTCGGAAT-3'), PPARγ (Forward: 5'-TGACCCGATGGTTGCAGATT-3'; Reverse: 5'-CAATGGCCATGAGGGAGTTG-3') and PGC-1α (5'-TTGATGCGCTGACAGATGGA-3'; Reverse: 5'-TGGTTTGCATGGTTCTGGGTA-3') were synthesized by Fisher Scientific and tested by reverse transcription PCR (Invitrogen, Platinum PCR SuperMix, 12532-016). QPCR was performed using 10 fold serial dilutions of cDNA to obtain a correlation between Log (concentration) of cDNA and Cp (crossing point) for each primer set to determine primer efficiency (1.8 -2.1), and relative expression levels of target mRNAs are presented by plotting mean $2^{-\Delta Cp}$ values using 18S ribosomal RNA as a reference

gene (Forward: 5'-GAGGCCCTGTAATTGGAATGAG -3'; Reverse: 5'-GCAGCAACTTTAATATACGCTATTGG -3').

*2.7 Statistics*: All data are shown as means ± SEM. Sample numbers per group were determined by power calculations using metabolic enzyme expression data from offspring of obese ewes in a previous study [25] and variability in muscle mitochondrial respirometry data from pilot studies in mice. Uneven sample numbers per groups resulted from some pregnancies being unsuccessful, the noted occurrence of twins, and inclusion of more HFD-fed ewes in anticipation of higher variability across ewes and fetuses in this cohort. Accordingly, differences between control and HFD groups were analyzed using independent sample *t*-tests with Welch's corrections to account for uneven sample sizes and variances per group. Analyses of Variance were used to test for main effects of diet on metabolites present in both arterial and venous compartments of the umbilical or uterine circulations, or muscle oxidative metabolism supported by pyruvate or palmitoylcarnitine substrates. All statistical analyses were performed using GraphPad Prism 8 software. Statistically significant differences between groups are reported at both $P < 0.10$ and $P < 0.05$ to increase probability of capturing potentially important trends with modest numbers of animals per group in accordance with guidelines from the American Physiological Society [26].

## 3. Results

*3.1 Maternal and Fetal Morphology*: Pregnant ewes fed the HFD were significantly heavier than CON ewes at GD75 (129 ± 3 vs. 97 ± 5 kg; $P < 0.001$) and gained significantly more weight

during pregnancy (32 ± 4 vs. 16 ± 3 kg; $P < 0.01$), equating to a 32 ± 5 % weight gain in HFD versus 21 ± 5% in CON ($P = 0.04$). Three ewes were found to be pregnant with twins (1 CON, 2 HFD), with no statistically significant impact on fetal weight or organ morphology. Fetuses from HFD pregnancies ($N = 9$; 2 female) were significantly heavier and longer than CON ($N = 6$; 2 female), accounted for largely by heavier viscera (**Table 1**), with no appreciable deposition of fetal adipose tissue in either group.

*3.2 Serum glucose and lipids.* Impacts of maternal HFD on fasting serum glucose, insulin, triglycerides and lipoprotein levels in the umbilical and uterine arteries and veins at GD75 are presented in **Table 2.** Fasting glucose tended to be higher both circulations of animals fed the HFD, but these did not reach statistical significance ($P = 0.10$ - $0.35$). Fasting insulin levels were significantly higher in HFD compared to control in the uterine artery and vein, which corresponded to a higher Homeostatic Model Assessment for Insulin Resistance (HOMA-IR, calculated as (μU/mL insulin × mg/dL glucose)/405). Consistent with data from humans [27], serum insulin was much lower in the umbilical compared to uterine circulation across all animals, and was slightly lower in HFD compared to control animals ($P < 0.05$). Triglycerides were similar between CON and HFD in all vessels sampled, while total lipoprotein cholesterol levels tended to be higher in HFD, reaching statistical significance in the uterine artery and vein ($P < 0.05$). This corresponded to significantly higher LDL+VLDL and HDL levels in the uterine artery and vein of HFD animals ($P < 0.05$), and higher total, LDL+VLDL and (LDL+VLDL)/HDL ratio in the umbilical circulation (main effect of venous and arterial samples by ANOVA; $P < 0.05$).

*3.3 Placental and fetal muscle fatty acid transporter expression*: Given the higher lipid levels observed in the uterine circulation of HFD-fed ewes, we examined effects of maternal diet on placental fatty acid transporter expression by immunoblotting homogenates of cotyledon tissue (fetal side) collected at GD75. In contrast to increases previously reported at mid-gestation in obese pregnant ewes overfed a standard diet [28], we observed no effects of maternal HFD on protein levels of placental fatty acid transport proteins (FATP) 1 and 4, or the CD36 fatty acid translocase (**Figure 1**). However, expression of fatty acid transporters tended to be higher in fetal skeletal muscles from HFD compared to CON pregnancies (**Figure 2**), including FATP1 (33% > CON, $P = 0.07$), FATP4 (26% > CON, $P = 0.04$), CD36 (28% > CON, $P = 0.09$), and plasmalemmal fatty acid binding protein (FABPpm; 43% > CON, $P = 0.08$), suggesting a greater capacity for fatty acid uptake into fetal muscles at mid-gestation.

*3.4 Muscle oxidative metabolism.* To determine the impact of maternal HFD on the capacity of fetal muscle to oxidize fatty acids, carbohydrates, and mixed substrates, we performed high-resolution respirometry on permeabilized muscle fiber bundles prepared from sections of the same *M. biceps femoris* tissues collected for immunoblotting analyses. While carbohydrate oxidation capacity (pyruvate + malate) was similar between CON and HFD in both LEAK and OXPHOS states (**Figure 3A**), muscle capacity to oxidize fatty acids (palmitoylcarnitine + malate) was significantly greater in HFD during both LEAK ($P = 0.04$) and OXPHOS states ($P < 0.01$) compared to CON (**Figure 3B**). This equated to a >2-fold higher capacity of muscle fibers to oxidize palmitoylcarnitine compared to pyruvate in fetuses from HFD versus CON pregnancies (**Figure 3C**). OXPHOS coupling control tended to be lower in HFD compared to

CON with both pyruvate and palmitoylcarnitine substrates (**Figure 3D**), but only reached statistical significance as a main effect by ANOVA ($P = 0.10$). The maximal capacity of muscle fibers to oxidize a combination of carbohydrate, fatty and amino acid substrates during OXPHOS was similar between CON and HFD (**Figure 3E**), indicating that the primary impact of maternal HFD on fetal muscle mitochondrial metabolism is a preferential increase in fatty acid oxidation capacity.

*3.5 Muscle triglycerides and metabolic enzyme expression.* Consistent with results from respirometry experiments, maternal HFD increased fetal muscle expression of enzymes that catalyze mitochondrial long-chain fatty acid uptake (carnitine palmitoyltransferase 1β; CPT1b; $P = 0.02$) and oxidation (very long-chain acyl-CoA dehydrogenase; ACADVL; $P = 0.05$) (**Figure 4A**), while the expression and phosphorylation status of pyruvate dehydrogenase (**Figure 4B**) and relative abundance of OXPHOS Complexes I-V (**Figure 4C**) were similar between groups ($P$ = NS). Consistent with these findings, the relative mRNA levels of peroxisome proliferator activated receptor-gamma (PPARγ; the primary transcriptional regulator of fatty acid uptake and metabolism during fetal development [29]) was higher HFD compared to CON fetal muscle ($P = 0.04$), but not PPARα or PPARγ-coactivator-1α (PGC-1α; a primary regulator of muscle mitochondrial biogenesis) (**Figure 4D**) and the skeletal muscle triglyceride content was higher in HFD compared to CON fetuses ($P = 0.03$; **Figure 4E**).

*3.6 Muscle insulin signaling.* Skeletal muscle insulin resistance is a hallmark feature of metabolic syndrome in humans [30], and has been reported in both fetal and juvenile offspring of

obese or HFD-fed mothers in NHPs [11]. Serine phosphorylation of insulin receptor substrate-1 (IRS-1) is a primary mechanism of insulin resistance resulting from excess lipids in skeletal muscle [31], so was evaluated in fetal muscle tissue in the present study (**Figure 5**). Immunoblotting revealed distinct bands for IRS-1 protein at ~170 and 80 kDa that exhibited similar patterns of variation across samples. The 80 kDa bands were more robust and consistently resolved across all samples and membranes, so were used for quantitative analyses. While expression of IRS-1 protein was ~50% lower in muscle from HFD pregnancies ($P = 0.05$), S307-phosphorylated (p)IRS-1 was ~50% higher ($P = 0.02$), resulting a 3-fold greater S307/IRS-1 ratio compared to CON ($P = 0.02$; **Figure 5A**). The active (phosphorylated) form of c-jun-N terminal kinase 1/2 (JNK1/2), the primary enzyme responsible for S307 phosphorylation of IRS-1 in response to excess lipids and pro-inflammatory signals [31, 32], was 58% higher in HFD than CON ($P = 0.04$), leading to a 69% greater pJNK/JNK ratio ($P = 0.009$; **Figure 5B**). In contrast, S1101-pIRS-1 was similar between HFD and CON (**Figure 5C**), along with levels of protein kinase C-theta (PKC-θ; **Figure 5D**), the major kinase responsible for S1101 phosphorylation of IRS-1 associated with lipid-induced muscle insulin resistance [33]. Taken together, these results demonstrate that maternal HFD induces classic inhibition of insulin signaling at IRS-1 in ovine fetal skeletal muscle, similar to those reported insulin resistant offspring of humans with type-2 diabetes [34].

*3.7 Liver triglycerides and nutrient handling enzymes.* Given evidence that maternal HFD induces fetal hepatic steatosis and gluconeogenesis in NHPs [35], which are also associated with metabolic syndrome in humans and animal models [36, 37], we hypothesized that fetal liver triglyceride content along with protein expression of fatty acid transporters, S307-IRS-1, and

phosphoenolpyruvate carboxykinase 1 (PCK1) may also be elevated in our ovine model (**Figure 6)**. Consistent with findings from NHPs, liver triglyceride content was ~20% greater in HFD compared to CON fetuses ($P = 0.03$; **Figure 6A**), despite no differences in the protein expression of FATP1 or FATP4 (**Figure 6B**). While trends generally paralleled those seen in skeletal muscle, there were no significant differences observed in the protein expression or S307 phosphorylation status of IRS-1 in fetal livers from HFD and CON pregnancies (**Figure 6C**), nor any differences in the expression of the gluconeogenesis enzyme PCK1 (**Figure 6D**). Taken together, these results indicate that while fetal liver may also be affected by maternal HFD and insulin resistance during pregnancy, there is a more robust impact on fetal skeletal muscle.

## 4. Discussion

Maternal overnutrition during pregnancy predisposes her offspring to metabolic syndrome later in life [38], but the prenatal biology linking these processes is poorly understood. Skeletal muscle is the principal site of insulin-stimulated glucose disposal in mammals, and consequently a primary driver of whole-body glycemic control in humans [39]. Accordingly, impairment of skeletal muscle insulin sensitivity is a hallmark feature of metabolic syndrome [30], and has been implicated in the pathogenesis of type 2 diabetes [40]. Studies in NHPs have demonstrated that maternal high-fat feeding impairs fetal muscle oxidative metabolism and muscle insulin-stimulated glucose uptake in offspring, even in the absence of obesity [11], suggesting that skeletal muscle may be a primary locus of fetal metabolic programming *in utero*. However, given the high-cost and limited availability of NHPs for biomedical research, additional large animal models are needed to further investigate these maternal-fetal interactions. Herein, we describe a novel ovine model of maternal high-fat feeding during pregnancy utilizing

rumen-protected saturated fats that phenocopies many of the hallmark responses reported in NHPs [11, 14] and offspring of humans with type 2 diabetes [34, 40].

Hyperlipidemia is a primary component of metabolic syndrome and cardiovascular risk in adults, and a common response to high-fat feeding in humans [41] and animals [42]. Lipoproteins are the primary form of circulating lipids in mammals, being formed and released primarily by the liver in response to dietary lipid intake and other metabolic factors [43]. During pregnancy, cholesterol and fatty acids are removed from lipoproteins in the maternal circulation by the placenta, then resynthesized and released into the umbilical vein for transport to the developing fetus [44]. Maternal hyperlipidemia has been linked to fetal aortic LDL accumulation and early atherosclerotic lesion development in childhood [45], indicating that excessive lipids in the maternal circulation are transferred to the developing fetus and increase cardiometabolic risk. Consistent with these observations in humans, we reported higher non-HDL cholesterol and a greater (VLDL+LDL)/HDL ratio in the umbilical circulation during HFD pregnancies, despite no changes in placental fatty acid transporter expression. An excessive supply of lipids to the developing fetus is further reflected by higher triglyceride content in the fetal liver and muscle, and greater expression of fetal muscle fatty acid transporters. These findings are also consistent with reports from NHPs [14, 35], and may be mediated in part by lipid-dependent activation of PPAR-γ, the canonical regulator of fatty uptake and oxidation enzyme expression during development [29]. Indeed, PPAR-γ has been previously reported to be elevated by maternal obesity or HFD in skeletal muscle of fetal sheep [46] and rodent neonates [47, 48], and is postulated be an important mechanistic link between maternal overnutrition and postnatal obesity [49]. While several mechanisms are likely involved, an oversupply of fatty acids relative to energy demands favors a dysregulation of nutrient

metabolism that is associated with hepatic steatosis and skeletal muscle insulin resistance in adolescent humans [30], both of which are primary predictors of type 2 diabetes and cardiovascular disease in adulthood [40, 50].

Fetal exposure to excess blood lipids may impact skeletal muscle substrate metabolism and mitochondrial function [5], which can contribute to dyslipidemia and cardiometabolic risk by altering the distribution of postprandial energy storage later in life [51]. Consistent with findings from NHP fetuses and offspring of obese or HFD-fed mothers [11, 14], the present study suggests that maternal HFD shifts ovine fetal skeletal muscle to favor the uptake and oxidation fatty acids over glucose. However, the extent of fatty acid oxidation *in vivo* is dependent on fetal muscle metabolic demands, which are unlikely to be appreciably higher in HFD pregnancies. When fatty acid supply exceeds metabolic demands, an accumulation of fatty acids can trigger deleterious effects on muscle metabolism, including a loss of OXPHOS coupling efficiency [15, 52], as observed in the present study and in NHPs [14]. While loss of muscle OXPHOS coupling efficiency may protect against muscle insulin resistance [53], the observed trend for lower OXPHOS Complex I levels in HFD fetal muscle has been associated with impaired muscle insulin signaling *in vitro* [54], and also aligns with findings from HFD-fed humans [55] and offspring of rats fed a HFD during pregnancy [56]. Importantly, these changes were not associated with impairment of the maximal OXPHOS-linked respiratory capacity using a combination of substrates, consistent with accumulating evidence that loss of muscle mitochondrial capacity in adults with obesity or type 2 diabetes is likely a consequence of prolonged sedentary activity rather than primary cause of insulin resistance [16, 57].

An oversupply of fatty acids can contribute to skeletal muscle insulin resistance by disrupting insulin signaling through inhibitory serine phosphorylation of IRS-1 [58].

Accumulation of incompletely oxidized lipids within muscle leads to a state of "lipotoxicity" that promotes pro-inflammatory signaling and activation of kinases capable of phosphorylating IRS-1 through multiple mechanisms [59]. Phosphorylation of serine-307 (pS307) is a classic mechanism of IRS-1 inactivation by c-jun-N terminal kinases (JNK1/2) [60], which are activated in response to lipid overload [61] and related pro-inflammatory signals [17, 32] in skeletal muscle. This often precedes loss of muscle IRS-1 protein, further favoring the development of insulin resistance [62]. Activation of this JNK1/2-pS307-IRS-1 axis was observed in fetal muscle from HFD pregnancies in the present study, and is consistent with previous findings in fetal sheep heart [25] and skeletal muscle [46] following maternal overnutrition, as well as mice following prenatal HFD exposure [63]. In contrast, phosphorylation of IRS-1 at S1101, which is accomplished by PKCθ in response to diacylglycerol accumulation in humans [33], was not altered by maternal HFD in fetal skeletal muscle, nor implicated in previous studies. In fetal liver, non-significant trends for activation of JNK1/2-pS307-IRS-1 axis paralleled significant accumulation of triglycerides, further implicating this pathway as a potential link between maternal HFD and prenatal development of insulin resistance resulting from an oversupply of lipids to the development fetus. However, it is important to note that regulation of insulin sensitivity by serine/threonine phosphorylation of IRS proteins is complex and likely differs across tissues and species through many more sites than examined herein [31]. Finally, while umbilical vein insulin levels were very low across all pregnancies and difficult to interpret, the much higher insulin and HOMA-IR in the uterine circulation during HFD pregnancy further supports the development of maternal insulin resistance, which may have contributed to the observed effects on fetal tissues.

In summary, we describe an ovine model of fetal metabolic programming by maternal high-fat feeding that recapitulates many of the hallmark findings reported in NHP models and metabolic syndrome in adolescent humans. Even a modest increase in dietary fat (from 5 to 11% w/w) during pregnancy resulted in maternal dyslipidemia and hyperinsulinemia, fetal hepatic and skeletal muscle lipid accumulation, and a shift in fetal skeletal muscle metabolism that favors greater uptake and oxidation of fatty acids over carbohydrates and impaired insulin signaling at mid-gestation. Additional studies are needed to determine the extent to which these metabolic perturbations persist in the developing offspring and any interactions they may have with postnatal diet and lifestyle modifications. Given the biological and logistical advantages of the pregnant ewe for studying maternal-fetal interactions during human pregnancy, the model described herein provides a strong foundation for addressing these important links between the intrauterine environment and offspring health.

**Acknowledgments**

The authors wish to thank the maintenance crew at Animal Reproduction and Biotechnology Laboratory at CSU, especially Richard Brandes, Greg Harding, and Joel Artzer for their construction and maintenance of the sheep housing facility and assistance with animal care.

**Disclosures**

The authors have no conflicts of interest to disclose. This work was supported in part by grants from the Colorado Agricultural Experiment Station and College of Veterinary Medicine and Biomedical Sciences Research Council to AJC, and Agriculture and Food Research Initiative Competitive Grant no. 2019-67015-29000 from the USDA National Institute of Food and Agriculture.

**Table 1.** Impact of maternal HFD on fetal parameters.

| | CON | HFD |
|---|---|---|
| Body Weight (g) | 206 ± 19 | 253 ± 20* |
| Body Length (cm) | 19.1 ± 0.4 | 20.1 ± 0.3* |
| Heart (g) | 1.6 ± 0.1 | 1.9 ± 0.1* |
| Liver (g) | 14.3 ± 0.9 | 17.8 ± 0.6* |
| Kidneys (g) | 2.2 ± 0.1 | 2.6 ± 0.1* |
| Brain (g) | 6.0 ± 0.3 | 6.7 ± 0.2 |
| Total Visceral Weight (g) | 24.2 ± 1.6 | 29.1 ± 0.7* |
| Visceral/Body Weight (%) | 12.0 ± 0.5 | 11.8 ± 0.6 |

Data are means ± SEM collected from GD75 fetuses obtained from pregnant ewes maintained on a control diet (CON; $N = 5$) or high-fat diet (HFD; $N = 9$). Total visceral weight is the sum mass of the four organs collected. * $P \leq 0.05$ vs. CON.

**Table2**. Serum glucose and lipids in uteroplacental circulation.

| | *Uterine artery* | | *Uterine vein* | | *Umbilical Artery* | | *Umbilical Vein* | |
|---|---|---|---|---|---|---|---|---|
| | **CON** | **HFD** | **CON** | **HFD** | **CON** | **HFD** | **CON** | **HFD** |
| Glucose (mg/dl) | 95 ± 3 | 115 ± 8# | 77 ± 4 | 91 ± 12# | 54 ± 0 | 63 ± 6# | 52 ± 2 | 57 ± 7# |
| Insulin (μIU/ml) | 2.5 ± 1.5 | 19.4 ± 4.7* | 3.5 ± 2.5 | 29.4 ± 9.6* | 0.7 ± 0.1 | 0.3 ± 0.0* | 1.0 ± 0.1 | 0.4 ± 0.1* |
| HOMA-IR | 0.63 ± 0.41 | 6.32 ± 2.04# | 0.66 ± 0.47 | 8.90 ± 3.69# | 0.09 ± 0.01 | 0.05 ± 0.00# | 0.12 ± 0.01 | 0.06 ± 0.00# |
| Triglycerides (mg/dl) | 15.0 ± 0.8 | 13.6 ± 1.3 | 15.3 ± 0.9 | 16.6 ± 1.9 | 18.2 ± 0.8 | 17.3 ± 0.8 | 17.6 ± 1.1 | 17.3 ± 1.1 |
| Total cholesterol (mg/dl) | 313 ± 31 | 474 ± 34* | 286 ± 33 | 429 ± 22* | 130 ± 13 | 166 ± 24# | 107 ± 21 | 125 ± 23# |
| LDL+VLDL (mg/dl) | 118 ± 12 | 177 ± 19* | 103 ± 11 | 157 ± 24* | 105 ± 11 | 121 ± 11# | 92 ± 15 | 104 ± 20# |
| HDL cholesterol (mg/dl) | 196 ± 23 | 296 ± 24* | 182 ± 25 | 232 ± 33 | 42 ± 7 | 44 ± 18 | 38 ± 7 | 29 ± 5 |
| (LDL+VLDL)/HDL ratio | 0.63 ± 0.07 | 0.62 ± 0.08 | 0.61 ± 0.07 | 0.81 ± 0.15 | 3.2 ± 0.9 | 5.2 ± 1.2# | 2.7 ± 0.5 | 5.4 ± 1.6# |

Data are means ± SEM collected at GD75 from fasted anesthetized ewes maintained on a control diet (CON; $N = 5$) or high-fat diet (HFD; $N = 7$). HOMA-IR, Homeostatic Model Assessment for Insulin Resistance, calculated as (μUI/mL Insulin × mg/dL Glucose)/405; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins; * $P < 0.05$ vs. CON in the same compartment. # $P < 0.05$ for main effect of HFD vs. CON in the uterine or umbilical circulation

**Figure 1**

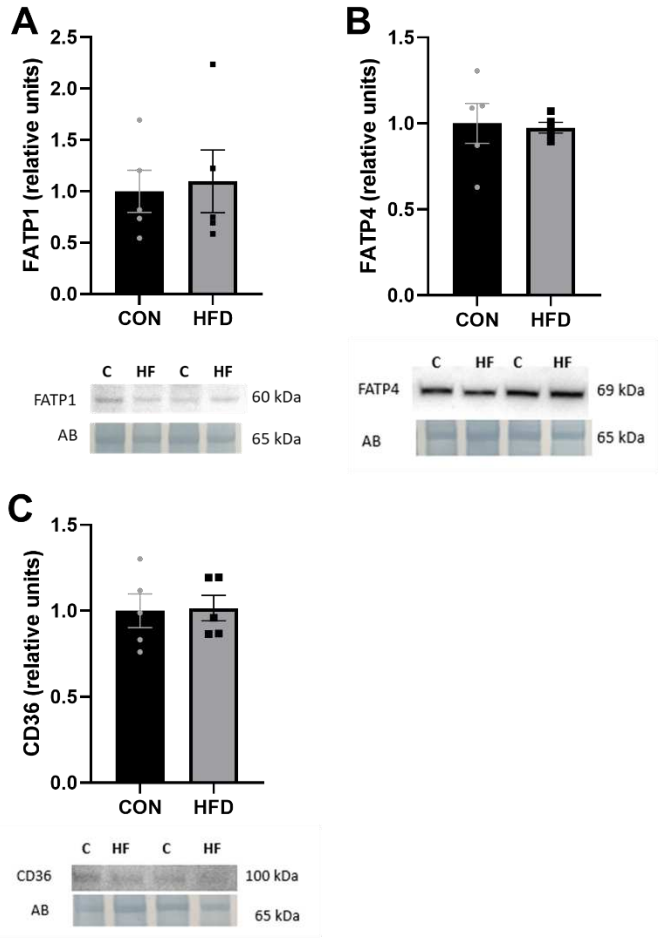



**Figure 1.** Placental fatty acid transporter expression. Maternal high-fat diet (HFD) had no effect on the placental (cotyledon) protein expression of fatty acid transport protein 1 (FATP1; **A**), FATP4 (**B**), or the fatty acid translocase CD36 (**C**) compared to Control (CON). Data are means ± SEM (*n* = 5-6/group) expression normalized to total sample protein by Amido Black (AB) staining.

**Figure 2**

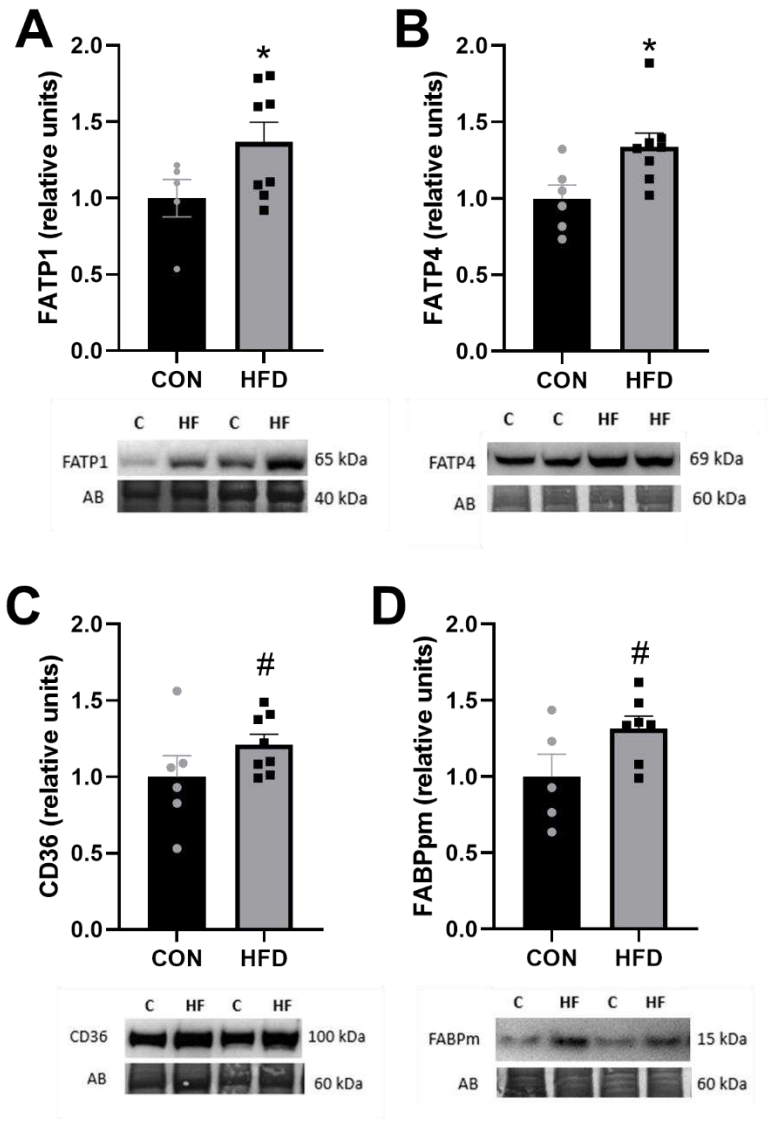



**Figure 2.** Fetal skeletal muscle fatty acid transporter expression. Maternal high-fat diet (HFD) led to greater fetal *M. biceps femoris* protein expression of fatty acid transport protein 1 (FATP1; **A**), FATP4 (**B**), fatty acid translocase/CD36 (**C**) and the plasmalemmal fatty acid binding protein (FABPpm; **D**) compared to control (CON) fetuses. Data are means ± SEM ($n$ = 5-9/group) of relative expression normalized to total sample protein by Amido Black (AB) staining. $^{\#}P < 0.10$, $^{*}P < 0.05$.

**Figure 3**

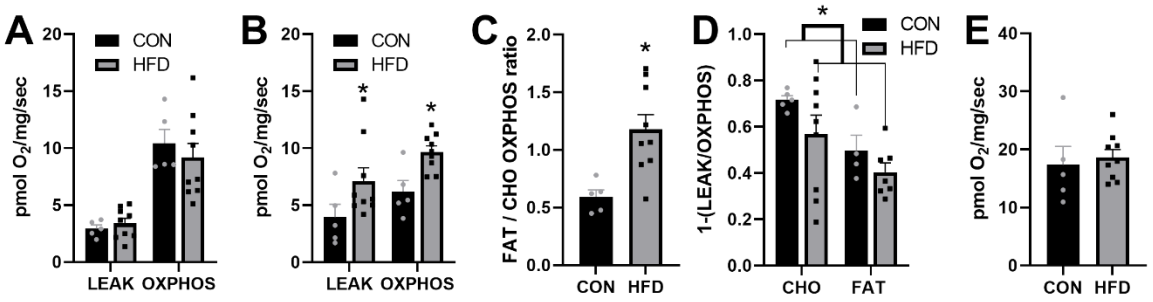



**Figure 3**. Fetal skeletal muscle oxidative capacities. Maternal high-fat diet (HFD) had no significant effect on the capacity of permeabilized fetal *M. biceps femoris* fibers to oxidize carbohydrates (pyruvate + malate; CHO) during OXPHOS (+ ADP) or LEAK states (no ADP) (**A**), but increased capacities to oxidize fatty acids (palmitoylcarnitine + malate; FAT) during both states (**B**), which equates to a much greater relative capacity to oxidize fats over carbohydrates during OXPHOS (**C**). Maternal HFD decreased OXPHOS coupling control with both substrates (main effect of diet by ANOVA); **D**), but had no effect on maximal OXPHOS-linked oxidative capacity supported by a combination of CHO, FAT and amino acid substrates (**E**). Data are means ± SEM ($n$ = 5-9/group) of oxygen consumption rates expressed per mg of permeabilized muscle tissue. *$P < 0.05$.

**Figure 4**

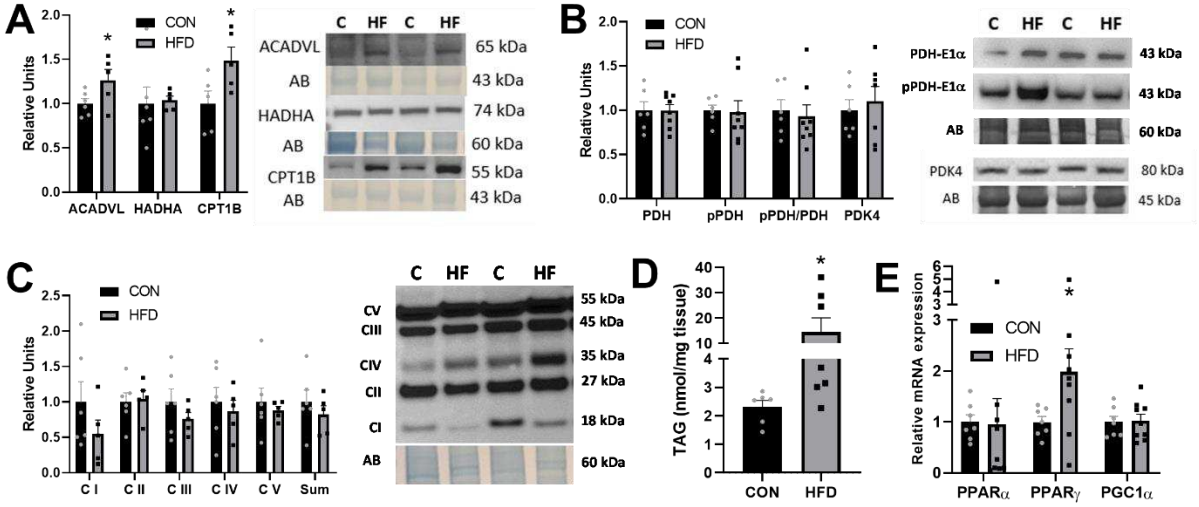



**Figure 4.** Expression of oxidative metabolism enzymes in fetal skeletal muscle. Maternal high-fat diet (HFD) led to greater fetal *M. biceps femoris* expression of enzymes involved in the transport and oxidation of long-chain fatty acids (**A**), but had no effect on the expression or phosphorylation of pyruvate dehydrogenase (**B**), or electron transport chain complexes (**C**). Muscle triacylglycerol content (**D**) and mRNA expression of PPARγ, a transcriptional regulator of fetal muscle fatty acid metabolism (**E**), were also greater in HFD compared to CON fetuses. ACADVL, very long-chain acyl-CoA dehydrogenase; CI-V, respiratory chain complexes I-V; CPT1B, carnitine palmitoyltransferase-1β; HADHA long-chain hydroxyacyl-CoA dehydrogenase-A; (p)PDH, (phospho) pyruvate dehydrogenase; PDK1, PDH kinase-1; PPAR, peroxisome proliferator activated receptor; PGC1α, PPAR gamma coactivator 1-apha. TAG, triacylglycerol. Data are means ± SEM ($n$ = 5-9/group) of relative expression normalized to total sample protein by Amido Black (AB) staining. $^{\#}P < 0.10$, $^{*}P < 0.05$.

**Figure 5**

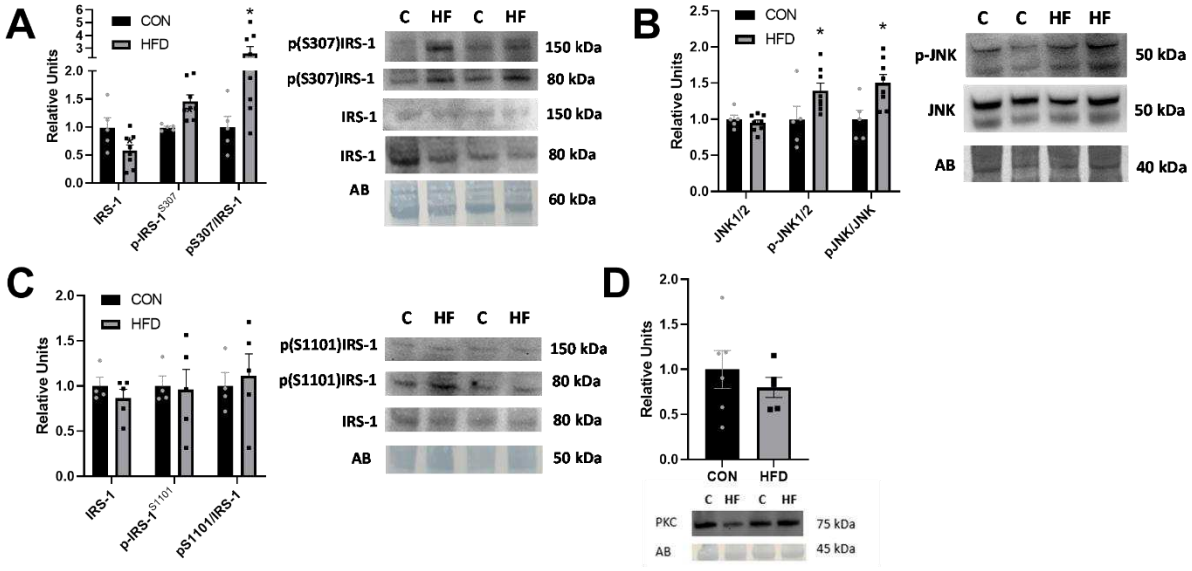



**Figure 5**. Fetal muscle insulin signaling. Maternal high-fat diet (HFD) decrease protein expression of insulin receptor substrate-1 (IRS-1), but increased its phosphorylation at serine-307 (**A**) along with the phosphorylated (active) c-Jun N-terminal kinase (JNK1/2) (**B**). In contrast, maternal HFD had no effect on IRS-1 phosphorylation at serine-1101 (**C**) or protein kinase C (PKC; **D**). Data are means ± SEM ($n$ = 5-9/group) of relative expression normalized to total sample protein by Amido Black (AB) staining or ratio of phosphorylated:total enzyme expression.*$P < 0.05$.

**Figure 6**

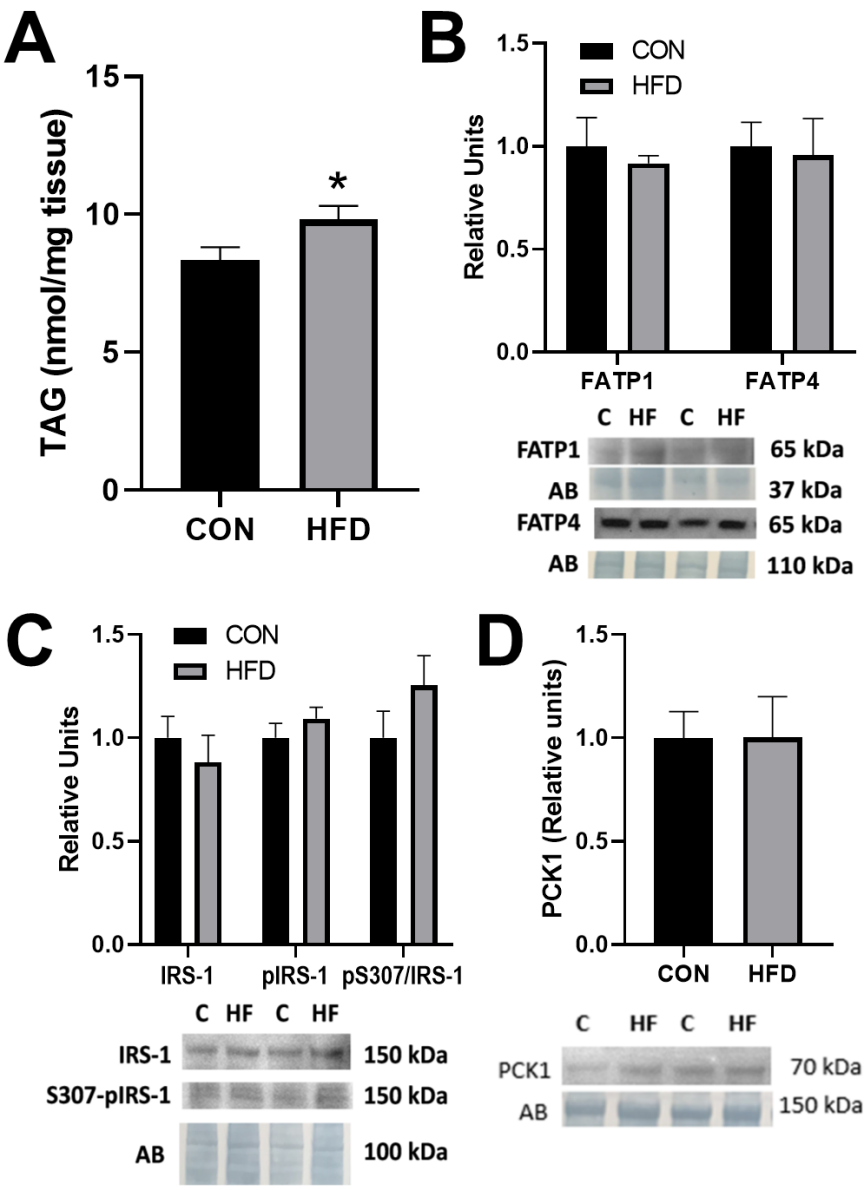



**Figure 6.** Fetal liver triglyceride content and enzyme expression. Maternal high-fat diet (HFD) increased fetal liver triglyceride content (**A**), but had no effect on protein expression of fatty acid transporter-1 (FATP1) or FATP4 (**B**), expression or serine-307 phosphorylation of insulin receptor substrate-1 (IRS-1; **C**), or the gluconeogenesis enzyme phosphoenolpyruvate carboxykinase-1 (PCK1; **D**). Data are means ± SEM ($n$ = 5-9/group) of relative expression normalized to total sample protein by Amido Black (AB) staining or ratio of phosphorylated:total enzyme expression.*$P < 0.05$.

## References


1. Lawlor, D.A., et al., Epidemiologic evidence for the fetal overnutrition hypothesis: findings from the mater-university study of pregnancy and its outcomes. Am J Epidemiol, 2007. 165(4): p. 418-24.
2. Whitaker, R.C., Predicting preschooler obesity at birth: the role of maternal obesity in early pregnancy. Pediatrics, 2004. 114(1): p. e29-36.
3. Barker, D.J., The origins of the developmental origins theory. J Intern Med, 2007. 261(5): p. 412-7.
4. Barker, D.J. and C. Osmond, Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet, 1986. 1(8489): p. 1077-81.
5. Heerwagen, M.J., et al., Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. Am J Physiol Regul Integr Comp Physiol, 2010. 299(3): p. R711-22.
6. Zhu, Z., F. Cao, and X. Li, Epigenetic Programming and Fetal Metabolic Programming. Front Endocrinol (Lausanne), 2019. 10: p. 764.
7. Alfaradhi, M.Z. and S.E. Ozanne, Developmental programming in response to maternal overnutrition. Front Genet, 2011. 2: p. 27.
8. Rajia, S., H. Chen, and M.J. Morris, Maternal overnutrition impacts offspring adiposity and brain appetite markers-modulation by postweaning diet. J Neuroendocrinol, 2010. 22(8): p. 905-14.
9. Williams, L., et al., Animal models of in utero exposure to a high fat diet: a review. Biochim Biophys Acta, 2014. 1842(3): p. 507-519.
10. Soncin, F., et al., Comparative analysis of mouse and human placentae across gestation reveals species-specific regulators of placental development. Development, 2018. 145(2).
11. Campodonico-Burnett, W., et al., Maternal Obesity and Western-Style Diet Impair Fetal and Juvenile Offspring Skeletal Muscle Insulin-Stimulated Glucose Transport in Nonhuman Primates. Diabetes, 2020. 69(7): p. 1389-1400.
12. Ganu, R.S., et al., Maternal diet: a modulator for epigenomic regulation during development in nonhuman primates and humans. Int J Obes Suppl, 2012. 2(Suppl 2): p. S14-S18.
13. Wood, E.K. and E.L. Sullivan, The Influence of Diet on Metabolism and Health Across the Lifespan in Nonhuman Primates. Curr Opin Endocr Metab Res, 2022. 24.
14. McCurdy, C.E., et al., Maternal obesity reduces oxidative capacity in fetal skeletal muscle of Japanese macaques. JCI Insight, 2016. 1(16): p. e86612.
15. Rial, E., et al., Lipotoxicity, fatty acid uncoupling and mitochondrial carrier function. Biochim Biophys Acta, 2010. 1797(6-7): p. 800-6.
16. Affourtit, C., Mitochondrial involvement in skeletal muscle insulin resistance: A case of imbalanced bioenergetics. Biochim Biophys Acta, 2016. 1857(10): p. 1678-93.

17. Martins, A.R., et al., Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. Lipids Health Dis, 2012. 11: p. 30.

18. Barry, J.S. and R.V. Anthony, The pregnant sheep as a model for human pregnancy. Theriogenology, 2008. 69(1): p. 55-67.

19. Behan, A.A., et al., Effects of Supplementation of Rumen Protected Fats on Rumen Ecology and Digestibility of Nutrients in Sheep. Animals (Basel), 2019. 9(7).

20. Sklan, D., L. Nagar, and A. Arieli, Effect of feeding different levels of fatty acids or calcium soaps of fatty acids on digestion and metabolizable energy in sheep. Animal Production, 1990. 50(1): p. 93-98.

21. Li Puma, L.C., et al., Experimental oxygen concentration influences rates of mitochondrial hydrogen peroxide release from cardiac and skeletal muscle preparations. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2020. 318(5): p. R972-R980.

22. Pesta, D. and E. Gnaiger, High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. Methods Mol Biol, 2012. 810: p. 25-58.

23. Chicco, A.J., et al., Adaptive remodeling of skeletal muscle energy metabolism in high-altitude hypoxia: lessons from AltitudeOmics. Journal of Biological Chemistry, 2018. 293(18): p. 6659-6671.

24. Li Puma, L.C., et al., Experimental oxygen concentration influences rates of mitochondrial hydrogen peroxide release from cardiac and skeletal muscle preparations. Am J Physiol Regul Integr Comp Physiol, 2020. 318(5): p. R972-R980.

25. Wang, J., et al., Overnutrition and maternal obesity in sheep pregnancy alter the JNK-IRS-1 signaling cascades and cardiac function in the fetal heart. FASEB J, 2010. 24(6): p. 2066-76.

26. Curran-Everett, D., D.J. Benos, and S. American Physiological, Guidelines for reporting statistics in journals published by the American Physiological Society. Am J Physiol Endocrinol Metab, 2004. 287(2): p. E189-91.

27. Thomas, K., M. de Gasparo, and J.J. Hoet, Insulin levels in the umbilical vein and in the umbilical artery of newborns of normal and gestational diabetic mothers. Diabetologia, 1967. 3(3): p. 299-304.

28. Zhu, M.J., et al., Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe. Am J Physiol Regul Integr Comp Physiol, 2010. 299(5): p. R1224-31.

29. Rees, W.D., C.J. McNeil, and C.A. Maloney, The Roles of PPARs in the Fetal Origins of Metabolic Health and Disease. PPAR Res, 2008. 2008: p. 459030.

30. Cree-Green, M., et al., Muscle Insulin Resistance in Youth with Obesity and Normoglycemia is Associated with Altered Fat Metabolism. Obesity (Silver Spring), 2019. 27(12): p. 2046-2054.

31. Copps, K.D. and M.F. White, Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia, 2012. 55(10): p. 2565-2582.

32. Aguirre, V., et al., The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem, 2000. 275(12): p. 9047-54.

33. Szendroedi, J., et al., Role of diacylglycerol activation of PKCtheta in lipid-induced muscle insulin resistance in humans. Proc Natl Acad Sci U S A, 2014. 111(26): p. 9597-602.

34. Morino, K., et al., Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest, 2005. 115(12): p. 3587-93.

35. McCurdy, C.E., et al., Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. J Clin Invest, 2009. 119(2): p. 323-35.

36. Petersen, M.C., D.F. Vatner, and G.I. Shulman, Regulation of hepatic glucose metabolism in health and disease. Nat Rev Endocrinol, 2017. 13(10): p. 572-587.

37. Yki-Jarvinen, H., Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol, 2014. 2(11): p. 901-10.

38. Eitmann, S., et al., Maternal overnutrition impairs offspring's insulin sensitivity: A systematic review and meta-analysis. Matern Child Nutr, 2020. 16(4): p. e13031.

39. Sylow, L., et al., The many actions of insulin in skeletal muscle, the paramount tissue determining glycemia. Cell Metab, 2021. 33(4): p. 758-780.

40. DeFronzo, R.A. and D. Tripathy, Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care, 2009. 32 Suppl 2(Suppl 2): p. S157-63.

41. Chiu, S., P.T. Williams, and R.M. Krauss, Effects of a very high saturated fat diet on LDL particles in adults with atherogenic dyslipidemia: A randomized controlled trial. PLoS One, 2017. 12(2): p. e0170664.

42. Wong, S.K., et al., Animal models of metabolic syndrome: a review. Nutr Metab (Lond), 2016. 13: p. 65.

43. Cohen, D.E. and E.A. Fisher, Lipoprotein metabolism, dyslipidemia, and nonalcoholic fatty liver disease. Semin Liver Dis, 2013. 33(4): p. 380-8.

44. Madsen, E.M., et al., Human placenta secretes apolipoprotein B-100-containing lipoproteins. J Biol Chem, 2004. 279(53): p. 55271-6.

45. Napoli, C., et al., Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. J Clin Invest, 1997. 100(11): p. 2680-90.

46. Yan, X., et al., Up-regulation of Toll-like receptor 4/nuclear factor-kappaB signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. Endocrinology, 2010. 151(1): p. 380-7.

47. Bayol, S.A., B.H. Simbi, and N.C. Stickland, A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. J Physiol, 2005. 567(Pt 3): p. 951-61.

48. Kelly, A.C., et al., Transcriptomic responses are sex-dependent in the skeletal muscle and liver in offspring of obese mice. Am J Physiol Endocrinol Metab, 2022. 323(4): p. E336-E353.

49. Muhlhausler, B.S., J.L. Morrison, and I.C. McMillen, Rosiglitazone increases the expression of peroxisome proliferator-activated receptor-gamma target genes in adipose tissue, liver, and skeletal muscle in the sheep fetus in late gestation. Endocrinology, 2009. 150(9): p. 4287-94.

50. Steinberger, J., et al., Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). Circulation, 2003. 107(10): p. 1448-53.

51. Petersen, K.F., et al., The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc Natl Acad Sci U S A, 2007. 104(31): p. 12587-94.

52. Schrauwen, P., et al., Mitochondrial dysfunction and lipotoxicity. Biochim Biophys Acta, 2010. 1801(3): p. 266-71.

53. Li, B., et al., Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. Nat Med, 2000. 6(10): p. 1115-20.

54. Hong, J., et al., Mitochondrial complex I deficiency enhances skeletal myogenesis but impairs insulin signaling through SIRT1 inactivation. J Biol Chem, 2014. 289(29): p. 20012-25.

55. Sparks, L.M., et al., A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes, 2005. 54(7): p. 1926-33.

56. Pileggi, C.A., et al., Maternal High Fat Diet Alters Skeletal Muscle Mitochondrial Catalytic Activity in Adult Male Rat Offspring. Front Physiol, 2016. 7: p. 546.

57. Holloszy, J.O., "Deficiency" of mitochondria in muscle does not cause insulin resistance. Diabetes, 2013. 62(4): p. 1036-40.

58. Hotamisligil, G.S., et al., IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science, 1996. 271(5249): p. 665-8.

59. Samuel, V.T. and G.I. Shulman, The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. J Clin Invest, 2016. 126(1): p. 12-22.

60. Aguirre, V., et al., Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem, 2002. 277(2): p. 1531-7.

61. Solinas, G., et al., Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. Proc Natl Acad Sci U S A, 2006. 103(44): p. 16454-9.

62. Henstridge, D.C., et al., Skeletal muscle-specific overproduction of constitutively activated c-Jun N-terminal kinase (JNK) induces insulin resistance in mice. Diabetologia, 2012. 55(10): p. 2769-2778.

63. Turdi, S., et al., Interaction between maternal and postnatal high fat diet leads to a greater risk of myocardial dysfunction in offspring via enhanced lipotoxicity, IRS-1 serine phosphorylation and mitochondrial defects. J Mol Cell Cardiol, 2013. 55: p. 117-29.

# CHAPTER III:

# OMEGA-3 FATTY ACID INTAKE DURING PREGNANCY ALTERS FETAL TISSUE MEMBRANE COMPOSITION AND LIPID METABOLISM IN AN OVINE MODEL*


**Abstract**

Dietary supplementation with long-chain omega-3 fatty acids such as docosahexaenoic acid (DHA) is recommended to women during pregnancy to prevent pre-term birth and support optimal fetal development. DHA supplementation also decreases circulating triglyceride levels through diverse effects on systemic lipid metabolism, but its impacts on fetal metabolism are largely unknown due to inherent limitations of such investigations during pregnancy. The aim of the present study was to establish a large animal model suitable for investigating impacts of prenatal DHA supplementation on fetal development and metabolism. White-faced ewes were fed either a control diet (Show-rite NewCo Lamb Feed) or a DHA-supplemented diet (control diet + 0.3% algae-derived DHA) from 2-3 weeks before pregnancy until mid-gestation (75 days), after which serum, placenta and fetal tissues were collected for biochemical analysis of phospholipid fatty acid composition and fatty acid transporter expression, and a broader characterization of nutrient metabolism of fetal heart, skeletal muscle, and liver. Prenatal DHA supplementation reduced maternal serum triglycerides and significantly enriched all six fetal tissues examined. These results were paralleled by tissue-specific impacts on fetal fatty acid transporters and metabolism, a greater capacity of fetal muscle and heart to oxidize lipids over carbohydrate substrates, and reduced expression of insulin receptor substrate-1 in fetal muscle and liver. In conclusion, this study demonstrates the utility of an ovine model for investigating


*This chapter was submitted to the *Journal of Nutiritional Biochemistry* in May 2025 and is currently under review. Citation information: Omar AK, Li Puma LC, Risk BD, Witt AC, Izon CS, Whitcomb LA, Kareng DJ, Winger QA, Bouma GJ, Chicco AJ. Omega-3 fatty acid intake during pregnancy alters ovine fetoplacental membrane composition and lipid metabolism.

the biological effects of prenatal DHA supplementation on fetal development and metabolism and highlights the need for a better understanding of its impacts on fetal and offspring metabolic health trajectory.

## 1. Introduction

Long-chain omega-3 polyunsaturated fatty acids (*n*3-PUFA), specifically docosahexaenoic acid (DHA; 22:6*n*3) and eicosapentaenoic acid (EPA; 20:5*n*3), are among the most widely consumed dietary supplements in the world [1,2]. Purported benefits range from prevention of cardiovascular disease, diabetes and cancer to optimization of prenatal development and neurocognitive function [3,4]. The majority of evidence-based support for *n*3-PUFA supplementation in humans comes from studies of its effects on cardiovascular risk parameters, where despite some controversy over the extent of benefit and outcomes affected [5,6], there is longstanding evidence for dose-dependent decreases in circulating triglycerides (TAGs) [7,8]. This likely results from multiple effects of *n*3-PUFA on systemic lipid metabolism [9], with additional putative benefits arising from their anti-inflammatory derivatives or displacement of *n*6-PUFA in biological membranes [10]. However, widespread discrepancies in the findings of clinical and epidemiological studies highlight the need for more basic and translational research to better understand the biological effects of dietary *n*3-PUFA on systems relevant to human health and disease.

Intake of *n*3-PUFA as 350-1000 mg of DHA per day is currently recommended for women during pregnancy [11], based primarily on consistent evidence that consumption at these levels reduces risk of preterm birth [12,13]. DHA is an essential component of retinal and brain phospholipids [14,15], leading to suggestions that dietary supplementation during pregnancy

may also be important for optimizing offspring cognitive and visual development [16,17]. However, randomized clinical trials (RCTs) have generally found evidence supporting these benefits to be lacking or inconclusive in children aged 18 months to 7 years [18–20]. Consistent with prevention of pre-term births, prenatal DHA supplementation can increase infant birthweight [21], particularly in populations with routinely low *n*3-PUFA intake [22], which may persist as a higher body mass index (BMI) into early childhood [23]. While not seen in all studies [24], recent observational follow-ups of two separate RCTs investigating impacts of prenatal *n*3-PUFA supplementation reported greater offspring fat mass at 24 months [25] and higher metabolic syndrome scores at 10 years of age [26]. These studies suggest that in addition to affecting maternal lipid metabolism, *n*3-PUFA supplementation during pregnancy may have lasting effects on fetal metabolism that impact offspring metabolic risk trajectory. However, establishing causal links between these outcomes is difficult due to inherent technical and ethical limitations of experimental research during fetal and early childhood development in humans, highlighting the need for studies in relevant animal models.

While no animal model fully recapitulates all aspects of human pregnancy and fetal development, the pregnant sheep has been used extensively to investigate placental nutrient transfer and maternal-fetal interactions, the results of which have generally paralleled available evidence from humans [27]. In contrast to rodent models, the long ovine gestational period (~5 months) and predominantly singleton or twin pregnancies are also well suited for studies of maternal diet on fetal development. As in humans, *n*3-PUFAs prevent preterm delivery in sheep [28], and perinatal dietary supplementation with rumen-protected *n*3-PUFA calcium salts led to their enrichment of some fetal and offspring tissues [29,30]. However, the broader impacts of prenatal *n*3-PUFA supplementation on maternal and fetal metabolism during ovine pregnancy

are unknown. Therefore, the aim of the present study was to establish an ovine model for investigating the impacts of prenatal *n*3-PUFA supplementation on fetal development and metabolism, with a particular focus on the extent of *n*3-PUFA distribution to several fetal tissues and impacts on fetoplacental fatty acid transport and metabolism.

## 2. Materials and Methods:

### *2.1 Animal Model and Dietary Intervention*

All animal procedures were approved by Colorado State University Animal Care and Use Committee in accordance with recommendations from the Declaration of Helsinki and the Guiding Principles on the Care and Use of Animals adopted by the American Physiological Society. White-faced ewes were purchased from a local vendor and housed in outdoor pens with free access to food and water. Heat cycles were determined using a vasectomized ram during the typical sheep breeding season (late October-November). Two weeks before pre-determined conception dates, ewes were randomly assigned to receive the standard control diet (CON; Show-Rite NewCo Lamb Feed D22.7; Mankato, MN, USA; containing 17% crude protein, 12% fiber, and 5% Fat) or a DHA-supplemented diet (DHA; control diet + 0.32% DHA w/w). DHA was added to feed as 3% w/w All-G-Rich™ (Alltech Inc., Nicholasville, KY), a commercial source of unextracted DHA from dehydrated, heterotrophically grown, whole-cell microalgae (*Aurantiochytrium limacinum*; CCAP 4087/2) containing 16% DHA w/w, 37% palmitic acid and 11% crude protein. Algae-sourced DHA is increasingly popular as an environmentally sustainable vegetarian *n*3-PUFA supplement that is equally effective at increasing DHA status in humans [31,32]. Moreover, All-G-Rich has been previously shown to be well-tolerated and effective at raising serum DHA levels in ruminants [33] by largely escaping the lipolysis and biohydrogenation of directly ingested PUFA oils by rumen bacteria [34]. Ewe weights prior to

dietary intervention averaged 83 ± 5 and 86 ± 5 kg in CON and DHA groups, respectively (N = 5 per group), and 3 kg of feed was provided daily to meet recommendations for nutrient requirements for pregnant sheep during early gestation [35]. Animals on similar heat cycles were synchronized by two 2 mL doses of intramuscular Dinoprost Tromethamine (Lutalyse, Zoetis) two days prior to breeding with the same fertile ram to ensure efficient conception and minimize fetal genetic variations from paternal DNA. Pregnant ewes were maintained on their respective diets until collection of fetal tissues at mid-gestation as described below.

*2.2 Blood and Fetal Tissue Collection*

Seventy-five days following conception (GD75), pregnant ewes (confirmed by ultrasound) were fasted overnight and sedated by intravenous injection of diazepam (2 mL) and ketamine (10 mL) prior to establishing general anesthesia by 2% isoflurane inhalation. A Caesarean-section was performed to enable collection of blood from umbilical and uterine circulations, followed by removal of the placenta and fetus for morphological assessments and tissue collection. Blood was stored on ice for at least 60 min to enable clotting before being centrifuged at 10000 × *g* at 4°C for 10 min to generate serum, which was subsequently extracted and stored at -80°C for future analyses. Sections of heart (left ventricle) and hindlimb skeletal muscle (*M. biceps femoris*) were carefully dissected and either snap frozen in liquid nitrogen and stored at -80°C, or placed in ice-cold BIOPS preservation medium containing (in mM) 10 Ca-EGTA (0.1 μM free calcium), 20 imidazole, 20 taurine, 50 K-MES, 0.5 DTT, 6.56 $MgCl_2$, 5.77 ATP, and 15 phosphocreatine, pH 7.1, for mitochondrial respirometry experiments on freshly prepared permeabilized fiber bundles. Sections of placental (cotyledon) tissue and fetal liver, kidney and brain (frontal cortex) were also frozen in liquid nitrogen and stored at -80°C for future analyses. Ewes were euthanized following removal of the fetus and placenta under general anesthesia.

*2.3 Analysis of serum triglycerides, total cholesterol and glucose*

To determine the impact of DHA supplementation on circulating metabolites relevant to cardiometabolic health in the maternal and feto-placental circulation, commercially available assay kits were used to measure levels of total triglycerides (TAGs, Abcam, Cat# ab65336) and total cholesterol (lipoproteins; Abcam, Cat# ab65390) according to the manufacturer's instructions. Serum was extracted from venous and arterial blood that was collected from the uterine and umbilical circulations.

*2.4 Serum and Tissue Phospholipid Fatty Acid Composition*

The impact of maternal DHA supplementation on the fatty acid composition of the serum, placenta (cotyledon), fetal muscle, heart, liver, kidney, and brain was determined by gas chromatography of fatty acid methyl esters (FAMEs) hydrolyzed from the total phospholipid fraction as previously described [36]. Briefly, 10 mg of tissue was homogenized in 800 µL of precooled methanol in a glass homogenizer to solubilize the total phospholipid fraction [37]. For serum, 50 µL was directly added to 600 µL of precooled methanol in a glass tube. After vortexing samples for 30 seconds, tubes were centrifuged at 900 × g for 5 min to pellet the non-phospholipid fraction. The supernatant (containing phospholipids) was transferred to a fresh glass tube, to which 25 µL of methoxide solution was added to synthesize methyl esters from hydrolyzed phospholipids. The reaction was stopped after 3 min by adding 75 µL of methanolic HCl, and FAMEs were extracted by adding 700 µL of hexane, transferring the upper hexane layer to 2 mL chromatography vials. FAMEs were dried under nitrogen flow and resuspended in hexane for gas chromatography (GC) analysis using an Agilent Technologies DB-225 30m x 0.250mm x 0.25µm column (model 122-2232, J&W Scientific) on an Agilent 6890 Series Gas

Chromatograph with a flame ionization detector [36]. A fatty acid standard solution of 1 mg/mL was injected into the GC every 6-7 runs to recalibrate the fatty acid peaks on the chromatograph, enabling relative quantitation of up to 18 fatty acids commonly found in mammalian membranes expressed as a percentage of total phospholipid fatty acids. In addition, total levels of saturated fatty acid (SFA) monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) were calculated, along with total N3-PUFA, N6-PUFA and their ratio.

*2.5 Fetal cardiac and skeletal muscle oxidative metabolism*

The capacities of fetal cardiac and skeletal muscle to oxidize fatty acids, pyruvate, and mixed substrate combinations were assessed by high-resolution respirometry on permeabilized fiber bundles freshly prepared from hindlimb muscle or myocardial tissue sections as previously described [38]. Briefly, 10-15 mg of *M. biceps femoris* or 1-2 mg of left ventricular tissue was trimmed free of connective tissue and gently teased with needle tip forceps in ice-cold BIOPS solution, then incubated with 50 μg/ml saponin for 20 min on ice with gentle rocking to permeabilize cell membranes while leaving mitochondrial membranes intact [39]. Permeabilized fiber bundles were then rinsed in mitochondrial respiration medium (MiR05) containing (in mM) 0.5 EGTA, 3 $MgCl_2$ hexahydrate, 60 lactobionic acid, 20 taurine, 10 $KH_2PO_4$, 20 HEPES, 110 sucrose, and 0.1% BSA, pH 7.1 with KOH by rocking for 2 x 15 min on ice, then gently blotted dry on Whatman paper and weighed immediately before adding 5-6 mg of muscle or 1-2 mg of cardiac fiber bundles to the oxygraph chamber for respirometry experiments. Mass-corrected oxidative capacities (per mg fiber bundle) were determined in MiR05 medium at 37°C using an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) by monitoring changes in the negative time derivative of the chamber oxygen concentration signal

(rate of sample oxygen consumption; $JO_2$) following standardized instrumental and chemical background calibrations [40]. Maximal oxidative phosphorylation (OXPHOS)-linked $JO_2$ supported by carbohydrate (CHO; 5 mM pyruvate + 1 mM malate), fatty acid (FA; 0.05 mM palmitoylcarnitine + 1 mM malate), and mixed substrate combinations (CHO + 10 mM glutamate, 10 mM succinate with and without FA) was measured in the presence of 2.5 mM adenosine diphosphate (ADP) to determine the absolute and relative capacities of fetal heart and skeletal muscle mitochondria to oxidize carbohydrate- versus lipid-derived substrates. Respirometry chambers were maintained in a narrow hyperoxygenated environment (325–400 μM $O_2$) during experiments to avoid limitations of oxygen diffusion on $JO_2$ of permeabilized fiber bundles [38].

*2.6 Protein Immunoblotting*

Proteins were extracted from snap-frozen sections of placenta and fetal tissues homogenized in lysis buffer containing (in mM): 150 NaCl, 1 EDTA, 1 EGTA, 5 sodium pyrophosphate, 1 sodium orthovanadate, 20 sodium fluoride added to Mammalian Protein Extraction Reagent (Pierce Cat# 78501) with supplemental Protease Inhibitor Cocktail (Sigma Aldrich Cat# P8340). Protein concentrations of supernatants, following centrifugation of homogenates at 10,000 × *g*, were detected by bicinchoninic acid assay according to the manufacturer's instructions (Thermo Scientific, #23225) using a colorimetric microplate reader (VersaMax, Molecular Devices). Extracted proteins (30 μg) were electrophoresed on 4-12% Bis-Tris gels and transferred to PVDF membranes, then blocked for 1 h at room temperature with 5% non-fat milk or bovine serum albumin (for phosphorylated proteins) before incubating with the following primary antibodies overnight at 4C°: Fatty acid transfer protein-1 (FATP1; Abcam, ab81875), FATP4 (Abcam, ab200353), the fatty acid translocase cluster of differentiation 36 (CD36; Abcam, ab133625), a

cocktail of antibodies recognizing a subunit of each of the four mitochondrial respiratory chain complexes and ATP Synthase (OXPHOS; Abcam, ab110413), plasmalemmal fatty acid binding protein (FABPpm; Abcam, ab45966), pyruvate dehydrogenase subunit E1α (PDH-E1α; Abcam, ab110330), phosphorylated PDH-E1α (pPDH-E1α (Abcam, ab92696), PDH kinase-4 (PDK4; Abcam, ab89295), insulin receptor substrate-1 (IRS1; Abcam, ab52167), and serine-307 phosphorylated IRS1 (pIRS1-Ser307; Abcam, ab5599). All antibodies were applied at 1:1000 dilution based on optimization in our previous study in sheep [41]. After three washes with Tris-buffered saline + 1% Tween-20 (TBST), the membrane was incubated with goat anti-rabbit-HRP (Abcam, ab6721) or goat anti-mouse-HRP (Abcam, ab6789) secondary antibodies (1:3000 dilution) for one hour at room temperature. Blotted proteins were imaged using SuperSignal West Dura Extended Duration Substrate (Lot#VL314742) and a UVP ChemStudio blot imager (Analytik Jena, Germany), normalizing band densities to total protein staining of a 20-30 kDa range of bands near the target protein using AmidoBlack (Sigma A8181), quantified using ImageJ software (NIH).

*2.7 Statistical analyses*

All data are presented as means ± SEM. Data from CON and DHA groups were compared using independent samples *t*-tests using GraphPad Prism 8 software, with statistically significant differences between groups being reported at both #$P < 0.10$ and *$P < 0.05$ to increase probability of capturing potentially important trends with modest numbers of animals per group, in accordance with guidelines from the American Physiological Society [42].

## 3. Results

### *3.1 Maternal and Fetal Morphology*

Pregnant ewes fed the DHA diet were similar in body weight to CON ewes at GD75 (108 ± 7 vs. 109 ± 9 kg; $P$ = NS), representing a 26% and 31% weight gain in both groups, respectively (22 ± 5 vs. 26 ± 6 kg; $P < 0.05$ vs. baseline in DHA and CON), with no significant difference between groups. One ewe from the DHA group was found not to be pregnant, and multiple birth pregnancies occurred in four ewes (2 CON, 2 DHA), resulting in collection of six fetuses from DHA-supplemented pregnancies, and seven from CON pregnancies. Birth multiplicity had no significant effect on fetal weight, length or organ morphology within either group, and mean values for all morphological outcomes were similar between groups ($P$ = NS; **Table 1**).

### *3.2 Serum lipids in the placental circulation*

**Fig. 1A** illustrates the distinct maternal (uterine) and fetal (umbilical) sides of the placental circulation from which serum was collected to measure levels of metabolites relevant to cardiometabolic health. As predicted, DHA supplementation during pregnancy significantly decreased serum TAGs in the maternal circulation (uterine artery and vein; $P < 0.05$) but had no effect on levels in the umbilical circulation (**Fig.1B**). DHA feeding tended to decrease total cholesterol (lipoprotein) levels in both circulations, but this only reached statistical significance in the umbilical artery ($P < 0.05$; **Fig. 1C**). Taken together, these results demonstrate that

prenatal DHA supplementation significantly impacts levels of lipids in the maternal and fetal circulation, potentially reflecting impacts on the fetal supply and/or uptake of lipids during pregnancy.

*3.3 Fatty acid composition of phospholipids in the placental circulation*

PUFAs are predominantly esterified to phospholipids found in cellular membranes and circulating lipoproteins *in vivo* [43]. Therefore, we analyzed the fatty acid composition of total phospholipids extracted from serum and placental (cotyledon) tissue to determine the effects of maternal DHA supplementation on the relative abundance of PUFA in the fetoplacental circulation (**Table 2**). As expected, DHA supplementation led to significantly higher DHA levels in maternal circulation (uterine artery and vein) and placental tissue ($P < 0.05$). Interestingly, DHA feeding did not result in higher DHA levels in the umbilical circulation, but DHA feeding led to >2-fold increases in its metabolic precursor, eicosapentaenoic acid (EPA, 20:5 *n*3), in the umbilical artery and vein ($P < 0.05$). Similar increases in serum EPA were seen in the uterine artery ($P < 0.05$) and vein ($P < 0.10$), but not placental tissue. Levels of the *n*6-PUFA linoleic acid (LA, 18:2 *n*6) and its downstream desaturation-elongation product, dihomo-gamma linolenic acid (DGLA, 20:3 *n*6), were also ~2-fold greater in the umbilical circulation of DHA supplemented ewes ($P < 0.05$) Taken together, these effects resulted in significantly higher total PUFA levels in the uterine and umbilical arteries ($P < 0.05$), but the serum omega-3/6 ratio was only increased in the umbilical artery ($P < 0.05$). Higher circulating levels of PUFAs were accounted for largely by proportionate decreases in levels of saturated fatty acids (SFA), particularly palmitic acid (16:0) and stearic acid (18:0), reaching statistical significance in the umbilical artery and vein ($P < 0.05$). In contrast, SFAs tended to increase in placental tissue ($P = 0.07$) due largely to a significant increase in 16:0 ($P < 0.05$). Collectively, these results

demonstrate that maternal DHA feeding during pregnancy significantly increases long-chain *n*3-PUFA (DHA and/or EPA) in the uterine and umbilical circulation, with potentially relevant impacts on levels of *n*6-PUFA and SFAs in the fetal circulation.

*3.4 Fatty acid composition of fetal tissue phospholipids*

The impact of maternal DHA supplementation on the fatty acid composition of total phospholipids extracted from fetal muscle, heart, liver, kidney and brain (frontal cortex) tissue is presented in **Table 3**. Most notably, DHA feeding during pregnancy led to significantly higher levels of DHA in all six fetal tissues examined ($P < 0.05$), and higher EPA ($P < 0.05$) in all tissues except brain, where levels were nearly undetectable. This accounted for significantly higher total N3-PUFA levels in all fetal tissues except brain, which was associated with trends for reciprocally lower monounsaturated fatty acids (largely oleic acid, 18:1 *n*9) across all tissues, reaching statistical significance in all tissues except liver ($P < 0.05$). DHA feeding also tended to decrease levels of total *n*6-PUFA driven largely by lower levels of ARA (20:4 *n*6) in all tissues, reaching statistical significance for both outcomes in fetal heart, kidney and brain ($P < 0.05$). These trends led to a significantly higher phospholipid *n*3/*n*6-PUFA ratio in all fetal tissues except liver from DHA supplemented pregnancies ($P > 0.05$), with variable effects on the proportion of SFAs across tissues. Taken together, these results demonstrate a significant DHA enrichment of mid-gestation fetal tissues following maternal DHA supplementation, which together with higher EPA, tend to displace *n*6-ARA and MUFA from tissue phospholipids. While generally consistent with our serum analyses and results from other species, variations across tissues and serum compartments suggest changes in the uptake and/or metabolism of fatty acids by the placenta and fetal tissues that were further investigated below.

*3.5 Placental and fetal tissue fatty acid transporter expression*

Maternal DHA supplementation differentially affected the protein expression of the four major fatty acid transport proteins FATP1, FATP4, CD36 and FABPpm in placenta and fetal tissues at GD75 (**Fig. 2**). In placenta, maternal DHA feeding tended to suppress expression of fatty acid transporters (**Fig. 2A**), but this only reached statistical significance for FATP4 ($P < 0.05$). Consistent with its exclusive expression on the maternal facing membranes of the human placenta [44], FABPpm was not detected in the fetal (cotlydeon) placental tissue. In fetal skeletal muscle (**Fig. 2B**), maternal DHA feeding led to lower expression of FATP1 and FATP4 ($P < 0.05$) but tended to increase levels of CD36 and FABPpm ($P < 0.10$). A similar trend for higher CD36 and FABPpm was seen in fetal heart (**Fig. 2C;** $P < 0.10$), while FATP4 tended to decrease ($P < 0.10$) and FATP1 was unaffected. CD36 was also higher in fetal liver (**Fig. 3D**; $P < 0.05$), while FATP4 tended to be higher ($P < 0.10$) and FATP1 lower ($P < 0.10$). There were no significant effects of maternal DHA feeding on expression of any of these four fatty acid transporters in fetal kidney (**Fig. 2E**) or brain (**Fig. 2F**). Taken together, these results indicate that DHA supplementation during pregnancy differentially impacts expression of fatty acid transporters across the fetoplacental unit in a tissue-specific manner, with the most robust effects suggesting changes in the uptake and/or handling of fatty acids by the fetal heart, skeletal muscle and liver.

*3.6 Maternal DHA feeding promotes greater fetal cardiac fatty acid oxidation capacity*

To determine if DHA supplementation during pregnancy altered cardiac energy metabolism, we determined the OXPHOS-linked respiratory capacity of permeabilized cardiac muscle fibers

supported by saturating concentrations of substrates derived from carbohydrate catabolism (CHO OXPHOS; pyruvate + malate), lipolysis/fatty acids (FA OXPHOS; palmitoylcarnitine + malate), and mixed substrate combinations (CHO + glutamate + succinate) in the presence or absence of palmitoylcarnitine (Max OXPHOS ± FA). CHO OXPHOS was significantly lower in fetal cardiac fibers from DHA pregnancies ($P < 0.05$), while FA OXPHOS was higher ($P < 0.10$), with similar non-significant trends for Max OXPHOS in the presence and absence of FA, respectively (**Fig. 3A**). Consequently, the ratio of FA:CHO OXPHOS capacity was significantly higher in DHA cardiac fibers ($P < 0.05$), which was paralleled by a higher ratio of Max OXPHOS capacity in the presence:absence of FA ($P < 0.10$) (**Fig. 3B**). Consistent with the greater expression of the sarcolemmal fatty acid transport proteins CD36 and FABPpm (**Fig. 2C**), DHA feeding also increased expression of CPT-1β involved in mitochondrial fatty acid uptake ($P < 0.05$), but had no significant effects on regulators of pyruvate oxidation (**Fig. 3C**). There were also no differences in the expression of OXPHOS Complexes I-V between groups (**Fig. 3D**), indicating specific effects of DHA feeding on myocardial utilization pathways that favors a greater capacity for fatty acid over carbohydrate oxidation.

*3.7 Maternal DHA feeding increases FA OXPHOS and decreases IRS-1 in fetal muscle*

Similar to results in cardiac muscle, DHA supplementation during pregnancy increased FA OXPHOS capacity in skeletal muscle ($P < 0.05$; **Fig. 4A**), but had no significant effect CHO OXPHOS or Max OXPHOS in the presence or absence of FA, and no effect on ratios of these indices (data not shown). Similarly, consistent with higher expression of CD36 and FABPpm (**Fig. 2B**), DHA feeding increased CPT-1β expression in fetal muscle tissue ($P < 0.05$), but had

no significant effects on regulators of pyruvate oxidation (**Fig. 4B**) or OXPHOS complex expression (**Fig. 4C**). However, given the importance of skeletal muscle in whole-body glucose homeostasis and insulin sensitivity [45], we also examined effect of maternal DHA feeding on fetal muscle insulin receptor substrate-1 (IRS-1), and found >50% lower IRS-1 protein expression compared to CON, with no impact on $Ser^{307}$-phosphorylation status (**Fig. 4D**). Collectively, these findings suggest that DHA supplementation during pregnancy promotes a greater capacity to utilize fatty acid oxidation over glucose catabolism to meet energy demands.

*3.8 Maternal DHA feeding alters hepatic oxidative metabolism and decreases IRS-1*

In contrast to fetal cardiac and skeletal muscle, DHA supplementation during pregnancy led to significantly lower protein expression of CPT-1β in liver tissue ($P < 0.05$), with no significant effect on PDH or PDH kinase expression (**Fig. 5A**). Despite inducing higher FATP4 and CD36 expression (**Fig. 2D**), DHA feeding had no effect on liver triglyceride content (**Fig. 5B**). Interestingly, hepatic protein expression of OXPHOS Complexes I-IV were significantly higher in DHA vs. CON fetuses, but not Complex V (ATP synthase) (**Fig. 5C**). Finally, similar to findings in fetal muscle, DHA feeding decreased hepatic IRS-1 protein expression by >50% compared to CON, with no impact on $Ser^{307}$-phosphorylation status (**Fig. 5D**).

## 4. Discussion

The primary aim of this study was to establish the feasibility of using a large animal model for investigating the impacts of prenatal *n*3-PUFA supplementation on fetal development and metabolism. The ovine model was selected based on an extensive literature of studies on

placental and fetal development demonstrating similarities with human pregnancy and placental nutrient transport [27,46], including investigations of maternal diet on fetal and offspring metabolism [41,47,48], and studies modeling dozens of human diseases [49,50]. We found that dietary microalgae-sourced DHA supplementation during pregnancy significantly decreased triglycerides and increased DHA in the ovine maternal circulation, replicating the signature effects of DHA supplementation in humans [8]. This was associated with DHA enrichment of phospholipids in the placenta and all five fetal tissues examined, thereby validating the efficacy of this model for studying impacts of prenatal DHA supplementation on fetal development and related pregnancy outcomes. Consistent with broad impacts of *n*3-PUFA on systemic lipid metabolism [9], our studies revealed diverse tissue-specific effects of DHA supplementation on fetoplacental fatty acid transporter expression and oxidative metabolism. These changes collectively favor greater uptake and oxidation of fatty acids over glucose in fetal heart and skeletal muscle, with distinct effects on fetal liver enzyme expression that suggest alterations in fetal nutrient metabolism. Taken together, these results provide a foundation for use of the ovine model to further investigate the mechanisms and longer-term implications of prenatal DHA supplementation on offspring health and disease risk parameters.

Consistent with evidence from human pregnancies [51,52], our study demonstrates an important regulatory role of the placenta in the selective transfer of long-chain PUFAs from the ovine maternal to fetal circulation. This process, known as "biomagnification" [53], is demonstrated by the ~3-fold enrichment of DHA and ARA in serum phospholipids in the umbilical (fetal) compared to uterine (maternal) circulation in CON ewes (**Table 1**), and results from the complex interplay of placental fatty acid transport and metabolism enzymes that remain incompletely understood [54,55]. Evidence from humans and sheep indicates that the expression

of these proteins can be modified by maternal metabolism and supply of lipids [41,56,57], and perhaps DHA in particular [29,58], in part via regulation of peroxisome proliferator activated receptor-gamma activity [30,59]. While elucidating these mechanisms is beyond the scope of the present study, it is worth noting that DHA supplementation tended to decrease placental protein expression of fatty acid transporters, particularly FATP4 that has been previously associated with DHA enrichment in human cord blood [58]. This may reflect a suppressive effect of hypolipidemia in the uterine circulation induced by DHA feeding on placental lipid transfer mechanisms [56], which also tended to decrease lipoprotein levels in the umbilical circulation (**Fig 1**). However, despite these effects and no augmentation of phospholipid-bound DHA in the umbilical circulation, DHA supplementation still significantly enriched fetal tissue phospholipids with DHA. This aligns with previous evidence from humans for retention of DHA levels in cord blood despite significant downregulation of placental *FATP1*, *CD36* and *FABP3* in adolescent pregnancies [60], and emphasizes the complexity of mechanisms that regulate placental transfer and fetal accretion of DHA in response to maternal supplementation. Interestingly, DHA feeding also increased phospholipid 20:5*n*3 (eicosapentaenoic acid; EPA) concentrations in both the uterine and umbilical circulations in the present study, but not in the placenta. This likely reflects a slowing of endogenous maternal EPA metabolism rather than retro-conversion of DHA to EPA [61], and perhaps its preferential shunting to the fetus, further highlighting the diverse impacts of DHA feeding on maternal lipid metabolism and placental *n*3-PUFA transport. Finally, since DHA supplementation was initiated prior to conception, we cannot rule out lasting biological effects on the oocyte on the observed effects in mid-gestation fetal tissues.

In contrast to the placenta, DHA supplementation tended to increase expression of CD36 and FABPpm in fetal heart and skeletal muscle, which are thought to work together to mediate

sarcolemma fatty acid uptake into myocytes [62,63]. Expression of both proteins is promoted by PUFA-activated transcription factors such as peroxisome proliferator activate receptors (PPARs) [64], which also induce expression of CPT-1β [65]. Positive regulation of these enzymes by PUFA generally favors a partitioning of fatty acids towards mitochondrial oxidation pathways [66], which is consistent with the greater capacity of DHA-enriched fetal heart and muscle tissue to oxidize fatty acid over carbohydrate substrates in the present study. However, expression FATP1 and FATP4, which are also transcriptionally regulated by PPARs [67], were markedly lower in fetal skeletal muscle and unaffected (FATP1) or only marginally lower (FATP4) in heart. While the distinct functions of FATPs remain under investigation, both FATP1 [68] and FATP4 [69] possess long-chain (16-24 carbon) acyl-CoA transferase activity that is downregulated by the accumulation of long-chain acyl-CoAs [70]. Therefore, we speculate that the very low metabolic demand of fetal skeletal muscle *in utero* favors accumulation of acyl-CoA that downregulates FATP expression, thereby preventing excessive intracellular accumulation of lipids in the presence of higher CD36/FABPpm expression. The fetal heart has much higher metabolic demands at mid-gestation, which are met primarily by oxidation of glucose and lactate at mid-gestation [71]. However, the greater contribution of fatty acid oxidation in DHA-enriched cardiomyocytes may prevent excessive accumulation of acyl-CoAs, thereby attenuating the suppression of FATPs observed in fetal muscle. Whether such mechanisms explain this discrepancy and lack of effects of DHA feeding on fatty acid transporter expression on fetal kidney and brain despite similar tissue DHA enrichment merits further investigation.

An upregulation of fetal skeletal muscle fatty acid transport and oxidation observed following DHA supplementation herein has also been reported in response to maternal high-fat

feeding in sheep [41] and non-human primates [72]. This is not surprising given the overlapping mechanisms by which DHA and other long-chain fatty acids regulate lipid metabolism [73–75]. However, excessive fatty acid uptake by muscle and liver can contribute to the pathogenesis of insulin resistance and type 2 diabetes [76]. Indeed, the upregulation of fetal muscle fatty acid transport and oxidation by maternal high-fat feeding was associated with fetal insulin resistance and hepatic steatosis in sheep [41] and primates [77,78]. In the present study, we found no evidence for hepatic steatosis or $Ser^{307}$ phosphorylation of IRS-1 in fetal muscle or liver following prenatal DHA supplementation that was seen following maternal high-fat feeding in the same species [41]. The diverse effects of DHA feeding on fetal liver fatty acid transporters and OXPHOS proteins are difficult to interpret in this context, but demonstrate significant effects on hepatic nutrient metabolism that could impact handling of glucose and fatty acids. However, our observed reduction in muscle and liver IRS-1 protein expression in DHA group has been reported in primate fetal muscle following maternal high-fat feeding, which was associated with impaired insulin-stimulated muscle glucose uptake that persisted in offspring for at least 14 months [77]. While lower IRS-1 protein in fetal tissues may predispose offspring to insulin resistance in the offspring, the absence for $Ser^{307}$ phosphorylation status argues against the classic induction of this process in response to excessive exposure to lipids or inflammatory signaling [79]. Moreover, *n*3-PUFA supplementation has been shown to improve insulin sensitivity through actions on adult skeletal muscle [80], liver [81], and adipose tissue [82] in the presence of high-fat feeding, which extended to the offspring when fed in combination with a high-fat diet during pregnancy in rats [83]. However, such benefits have not been consistently demonstrated in humans [84], and there have been reports of greater adiposity and metabolic syndrome risk in children from DHA supplemented pregnancies [23,25,26]. Given the diverse

and complex interactions between DHA intake, fatty acid uptake, metabolism, and insulin signaling, future studies are clearly needed to better understand how of prenatal DHA supplementation impacts offspring metabolic risk trajectory.

In summary, the present study demonstrates the utility of an ovine model for investigating the biological effects of DHA supplementation on fetal development and metabolism. Reductions of maternal serum triglycerides and DHA enrichment of six fetal tissues were paralleled by tissue-specific impacts on fetal fatty acid transporters and metabolism that favor a greater capacity to oxidize lipids over carbohydrate substrates. These results are consistent with the diverse impacts of DHA on lipid metabolism observed in humans and other species and highlight the need for further study to understand its impacts on fetal and offspring metabolic health trajectory.

**Acknowledgments:** The authors wish to thank the maintenance crew at Animal Reproduction and Biotechnology Laboratory at CSU, especially Richard Brandes, Greg Harding, and Joel Artzer for their construction and maintenance of the sheep housing facility and assistance with animal care.

**Author contributions: Asma Omar**: Data curation, Formal analysis, Investigation, Validation, Supervision, Writing – original draft. **Lance Li Puma**: Formal analysis, Investigation. **Briana Risk**: Investigation. **Aria Witt**: Investigation. **Cheyanne Izon**: Investigation. **Luke Whitcomb**: Investigation. **Dorcas Kareng**: Investigation. **Quinton Winger**: Investigation, Methodology, Project administration, Resources. **Gerrit Bouma**: Investigation, Methodology, Project

administration, Resources. **Adam Chicco**: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Supervision, Methodology, Project administration, Resources, Funding acquisition, Writing – review and editing.

**Funding:** This work was supported by grants from the Colorado Agricultural Experiment Station supported by the United States Department of Agriculture (to AJC) and Agriculture and Food Research Initiative Competitive Grant No 2019-67015-29000 from the United States Department of Agriculture National Institute of Food and Agriculture (to GJB).

## References


[1] Bailey RL, Gahche JJ, Miller PE, Thomas PR, Dwyer JT. Why US adults use dietary supplements. JAMA Intern Med 2013;173:355–61. https://doi.org/10.1001/jamainternmed.2013.2299.

[2] Djaoudene O, Romano A, Bradai YD, Zebiri F, Ouchene A, Yousfi Y, et al. A Global Overview of Dietary Supplements: Regulation, Market Trends, Usage during the COVID-19 Pandemic, and Health Effects. Nutrients 2023;15:3320. https://doi.org/10.3390/nu15153320.

[3] Swanson D, Block R, Mousa SA. Omega-3 fatty acids EPA and DHA: health benefits throughout life. Adv Nutr 2012;3:1–7. https://doi.org/10.3945/an.111.000893.

[4] Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol 2018;9:345–81. https://doi.org/10.1146/annurev-food-111317-095850.

[5] Aung T, Halsey J, Kromhout D, Gerstein HC, Marchioli R, Tavazzi L, et al. Associations of Omega-3 Fatty Acid Supplement Use With Cardiovascular Disease Risks: Meta-analysis of 10 Trials Involving 77 917 Individuals. JAMA Cardiol 2018;3:225–34. https://doi.org/10.1001/jamacardio.2017.5205.

[6] Hu Y, Hu FB, Manson JE. Marine Omega-3 Supplementation and Cardiovascular Disease: An Updated Meta-Analysis of 13 Randomized Controlled Trials Involving 127 477 Participants. J Am Heart Assoc 2019;8:e013543. https://doi.org/10.1161/JAHA.119.013543.

[7] Skulas-Ray AC, Wilson PWF, Harris WS, Brinton EA, Kris-Etherton PM, Richter CK, et al. Omega-3 Fatty Acids for the Management of Hypertriglyceridemia: A Science Advisory From the American Heart Association. Circulation 2019;140:e673–91. https://doi.org/10.1161/CIR.0000000000000709.

[8] Wang T, Zhang X, Zhou N, Shen Y, Li B, Chen BE, et al. Association Between Omega-3 Fatty Acid Intake and Dyslipidemia: A Continuous Dose-Response Meta-Analysis of Randomized Controlled Trials. J Am Heart Assoc 2023;12:e029512. https://doi.org/10.1161/JAHA.123.029512.

[9] Harris WS, Bulchandani D. Why do omega-3 fatty acids lower serum triglycerides? Curr Opin Lipidol 2006;17:387–93. https://doi.org/10.1097/01.mol.0000236363.63840.16.

[10] Mozaffarian D, Wu JHY. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. J Am Coll Cardiol 2011;58:2047–67. https://doi.org/10.1016/j.jacc.2011.06.063.

[11] Cetin I, Carlson SE, Burden C, da Fonseca EB, di Renzo GC, Hadjipanayis A, et al. Omega-3 fatty acid supply in pregnancy for risk reduction of preterm and early preterm birth. Am J Obstet Gynecol MFM 2024;6:101251. https://doi.org/10.1016/j.ajogmf.2023.101251.

[12] Best KP, Gibson RA, Makrides M. ISSFAL statement number 7 - Omega-3 fatty acids during pregnancy to reduce preterm birth. Prostaglandins Leukot Essent Fatty Acids 2022;186:102495. https://doi.org/10.1016/j.plefa.2022.102495.

[13] Carlson SE, Gajewski BJ, Valentine CJ, Kerling EH, Weiner CP, Cackovic M, et al. Higher dose docosahexaenoic acid supplementation during pregnancy and early preterm birth: A randomised, double-blind, adaptive-design superiority trial. EClinicalMedicine 2021;36:100905. https://doi.org/10.1016/j.eclinm.2021.100905.

[14] Swinkels D, Baes M. The essential role of docosahexaenoic acid and its derivatives for retinal integrity. Pharmacol Ther 2023;247:108440. https://doi.org/10.1016/j.pharmthera.2023.108440.

[15] Mallick R, Basak S, Duttaroy AK. Docosahexaenoic acid,22:6n-3: Its roles in the structure and function of the brain. Int J Dev Neurosci 2019;79:21–31. https://doi.org/10.1016/j.ijdevneu.2019.10.004.

[16] Coletta JM, Bell SJ, Roman AS. Omega-3 Fatty acids and pregnancy. Rev Obstet Gynecol 2010;3:163–71.

[17] Basak S, Mallick R, Duttaroy AK. Maternal Docosahexaenoic Acid Status during Pregnancy and Its Impact on Infant Neurodevelopment. Nutrients 2020;12:3615. https://doi.org/10.3390/nu12123615.

[18] Gould JF, Smithers LG, Makrides M. The effect of maternal omega-3 (n-3) LCPUFA supplementation during pregnancy on early childhood cognitive and visual development: a systematic review and meta-analysis of randomized controlled trials. Am J Clin Nutr 2013;97:531–44. https://doi.org/10.3945/ajcn.112.045781.

[19] Dziechciarz P, Horvath A, Szajewska H. Effects of n-3 long-chain polyunsaturated fatty acid supplementation during pregnancy and/or lactation on neurodevelopment and visual function in children: a systematic review of randomized controlled trials. J Am Coll Nutr 2010;29:443–54. https://doi.org/10.1080/07315724.2010.10719881.

[20] Makrides M, Gibson RA, McPhee AJ, Yelland L, Quinlivan J, Ryan P, et al. Effect of DHA supplementation during pregnancy on maternal depression and neurodevelopment of young children: a randomized controlled trial. JAMA 2010;304:1675–83. https://doi.org/10.1001/jama.2010.1507.

[21] Carlson SE, Colombo J, Gajewski BJ, Gustafson KM, Mundy D, Yeast J, et al. DHA supplementation and pregnancy outcomes. Am J Clin Nutr 2013;97:808–15. https://doi.org/10.3945/ajcn.112.050021.

[22] Ramakrishnan U, Stein AD, Parra-Cabrera S, Wang M, Imhoff-Kunsch B, Juárez-Márquez S, et al. Effects of docosahexaenoic acid supplementation during pregnancy on gestational age and size at birth: randomized, double-blind, placebo-controlled trial in Mexico. Food Nutr Bull 2010;31:S108-116. https://doi.org/10.1177/15648265100312S203.

[23] Vinding RK, Stokholm J, Sevelsted A, Sejersen T, Chawes BL, Bønnelykke K, et al. Effect of fish oil supplementation in pregnancy on bone, lean, and fat mass at six years: randomised clinical trial. BMJ 2018;362:k3312. https://doi.org/10.1136/bmj.k3312.

[24] Wood K, Mantzioris E, Lingwood B, Couper J, Makrides M, Gibson RA, et al. The effect of maternal DHA supplementation on body fat mass in children at 7 years: follow-up of the DOMInO randomized controlled trial. Prostaglandins Leukot Essent Fatty Acids 2018;139:49–54. https://doi.org/10.1016/j.plefa.2017.09.013.

[25] Hull HR, Brown A, Gajewski B, Sullivan DK, Carlson SE. The Effect of Prenatal Docosahexaenoic Acid Supplementation on Offspring Fat Mass and Distribution at 24 Months Old. Curr Dev Nutr 2024;8:103771. https://doi.org/10.1016/j.cdnut.2024.103771.

[26] Vinding RK, Sevelsted A, Horner D, Vahman N, Lauritzen L, Hagen CP, et al. Fish oil supplementation during pregnancy, anthropometrics, and metabolic health at age ten: A randomized clinical trial. Am J Clin Nutr 2024;119:960–8. https://doi.org/10.1016/j.ajcnut.2023.12.015.

[27] Barry JS, Anthony RV. The pregnant sheep as a model for human pregnancy. Theriogenology 2008;69:55–67. https://doi.org/10.1016/j.theriogenology.2007.09.021.

[28] Baguma-Nibasheka M, Brenna JT, Nathanielsz PW. Delay of preterm delivery in sheep by omega-3 long-chain polyunsaturates. Biol Reprod 1999;60:698–701. https://doi.org/10.1095/biolreprod60.3.698.

[29] Roque-Jimenez JA, Oviedo-Ojeda MF, Whalin M, Lee-Rangel HA, Relling AE. Eicosapentaenoic and docosahexaenoic acid supplementation during early gestation modified relative abundance on placenta and fetal liver tissue mRNA and concentration pattern of fatty acids in fetal liver and fetal central nervous system of sheep. PLoS One 2020;15:e0235217. https://doi.org/10.1371/journal.pone.0235217.

[30] Roque-Jiménez JA, Oviedo-Ojeda MF, Whalin M, Lee-Rangel HA, Relling AE. Ewe early gestation supplementation with eicosapentaenoic and docosahexaenoic acids affects the liver, muscle, and adipose tissue fatty acid profile and liver mRNA expression in the offspring. J Anim Sci 2023;101:skad144. https://doi.org/10.1093/jas/skad144.

[31] Lane K, Derbyshire E, Li W, Brennan C. Bioavailability and potential uses of vegetarian sources of omega-3 fatty acids: a review of the literature. Crit Rev Food Sci Nutr 2014;54:572–9. https://doi.org/10.1080/10408398.2011.596292.

[32] Adarme-Vega TC, Lim DKY, Timmins M, Vernen F, Li Y, Schenk PM. Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. Microb Cell Fact 2012;11:96. https://doi.org/10.1186/1475-2859-11-96.

[33] Moran CA, Morlacchini M, Keegan JD, Fusconi G. The effect of dietary supplementation with Aurantiochytrium limacinum on lactating dairy cows in terms of animal health, productivity and milk composition. J Anim Physiol Anim Nutr (Berl) 2018;102:576–90. https://doi.org/10.1111/jpn.12827.

[34] Lourenço M, Ramos-Morales E, Wallace RJ. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. Animal 2010;4:1008–23. https://doi.org/10.1017/S175173111000042X.

[35] Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids. Washington, D.C.: National Academies Press; 2007. https://doi.org/10.17226/11654.

[36] Mulligan CM, Le CH, deMooy AB, Nelson CB, Chicco AJ. Inhibition of delta-6 desaturase reverses cardiolipin remodeling and prevents contractile dysfunction in the aged mouse heart without altering mitochondrial respiratory function. J Gerontol A Biol Sci Med Sci 2014;69:799–809. https://doi.org/10.1093/gerona/glt209.

[37] Zhao Z, Xu Y. An extremely simple method for extraction of lysophospholipids and phospholipids from blood samples. J Lipid Res 2010;51:652–9. https://doi.org/10.1194/jlr.D001503.

[38] Li Puma LC, Hedges M, Heckman JM, Mathias AB, Engstrom MR, Brown AB, et al. Experimental oxygen concentration influences rates of mitochondrial hydrogen peroxide release from cardiac and skeletal muscle preparations. Am J Physiol Regul Integr Comp Physiol 2020;318:R972–80. https://doi.org/10.1152/ajpregu.00227.2019.

[39] Pesta D, Gnaiger E. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. Methods Mol Biol 2012;810:25–58. https://doi.org/10.1007/978-1-61779-382-0_3.

[40] Chicco AJ, Le CH, Schlater A, Nguyen A, Kaye S, Beals JW, et al. High fatty acid oxidation capacity and phosphorylation control despite elevated leak and reduced respiratory capacity in northern elephant seal muscle mitochondria. J Exp Biol 2014;217:2947–55. https://doi.org/10.1242/jeb.105916.

[41] Omar AK, Li Puma LC, Whitcomb LA, Risk BD, Witt AC, Bruemmer JE, et al. High-fat diet during pregnancy promotes fetal skeletal muscle fatty acid oxidation and insulin resistance in an ovine model. Am J Physiol Regul Integr Comp Physiol 2023;325:R523–33. https://doi.org/10.1152/ajpregu.00059.2023.

[42] Curran-Everett D, Benos DJ, American Physiological Society. Guidelines for reporting statistics in journals published by the American Physiological Society. Am J Physiol Endocrinol Metab 2004;287:E189-191. https://doi.org/10.1152/ajpendo.00213.2004.

[43] Dyall SC, Balas L, Bazan NG, Brenna JT, Chiang N, da Costa Souza F, et al. Polyunsaturated fatty acids and fatty acid-derived lipid mediators: Recent advances in the understanding of their biosynthesis, structures, and functions. Prog Lipid Res 2022;86:101165. https://doi.org/10.1016/j.plipres.2022.101165.

[44] Campbell FM, Dutta-Roy AK. Plasma membrane fatty acid-binding protein (FABPpm) is exclusively located in the maternal facing membranes of the human placenta. FEBS Lett 1995;375:227–30. https://doi.org/10.1016/0014-5793(95)01216-2.

[45] Merz KE, Thurmond DC. Role of Skeletal Muscle in Insulin Resistance and Glucose Uptake. Compr Physiol 2020;10:785–809. https://doi.org/10.1002/cphy.c190029.

[46] Morrison JL, Berry MJ, Botting KJ, Darby JRT, Frasch MG, Gatford KL, et al. Improving pregnancy outcomes in humans through studies in sheep. Am J Physiol Regul Integr Comp Physiol 2018;315:R1123–53. https://doi.org/10.1152/ajpregu.00391.2017.

[47] Pankey CL, Odhiambo JF, Smith AM, Ford SP. Effects of maternal obesity in an ovine model on metabolic outcomes in F2 adults and F3 neonates. Domest Anim Endocrinol 2021;76:106628. https://doi.org/10.1016/j.domaniend.2021.106628.

[48] Ford SP, Long NM. Evidence for similar changes in offspring phenotype following either maternal undernutrition or overnutrition: potential impact on fetal epigenetic mechanisms. Reprod Fertil Dev 2011;24:105–11. https://doi.org/10.1071/RD11911.

[49] Banstola A, Reynolds JNJ. The Sheep as a Large Animal Model for the Investigation and Treatment of Human Disorders. Biology (Basel) 2022;11:1251. https://doi.org/10.3390/biology11091251.

[50] Pinnapureddy AR, Stayner C, McEwan J, Baddeley O, Forman J, Eccles MR. Large animal models of rare genetic disorders: sheep as phenotypically relevant models of human genetic disease. Orphanet J Rare Dis 2015;10:107. https://doi.org/10.1186/s13023-015-0327-5.

[51] Larqué E, Demmelmair H, Berger B, Hasbargen U, Koletzko B. In vivo investigation of the placental transfer of (13)C-labeled fatty acids in humans. J Lipid Res 2003;44:49–55. https://doi.org/10.1194/jlr.m200067-jlr200.

[52] Gil-Sánchez A, Larqué E, Demmelmair H, Acien MI, Faber FL, Parrilla JJ, et al. Maternal-fetal in vivo transfer of [13C]docosahexaenoic and other fatty acids across the human placenta 12 h after maternal oral intake. Am J Clin Nutr 2010;92:115–22. https://doi.org/10.3945/ajcn.2010.29589.

[53] Crawford MA, Hassam AG, Williams G. Essential fatty acids and fetal brain growth. Lancet 1976;1:452–3. https://doi.org/10.1016/s0140-6736(76)91476-8.

[54] Gil-Sánchez A, Demmelmair H, Parrilla JJ, Koletzko B, Larqué E. Mechanisms involved in the selective transfer of long chain polyunsaturated Fatty acids to the fetus. Front Genet 2011;2:57. https://doi.org/10.3389/fgene.2011.00057.

[55] Hanebutt FL, Demmelmair H, Schiessl B, Larqué E, Koletzko B. Long-chain polyunsaturated fatty acid (LC-PUFA) transfer across the placenta. Clin Nutr 2008;27:685–93. https://doi.org/10.1016/j.clnu.2008.05.010.

[56] Duttaroy AK, Basak S. Maternal Fatty Acid Metabolism in Pregnancy and Its Consequences in the Feto-Placental Development. Front Physiol 2021;12:787848. https://doi.org/10.3389/fphys.2021.787848.

[57] Duttaroy AK. Transport of fatty acids across the human placenta: a review. Prog Lipid Res 2009;48:52–61. https://doi.org/10.1016/j.plipres.2008.11.001.

[58] Larqué E, Krauss-Etschmann S, Campoy C, Hartl D, Linde J, Klingler M, et al. Docosahexaenoic acid supply in pregnancy affects placental expression of fatty acid transport proteins. Am J Clin Nutr 2006;84:853–61. https://doi.org/10.1093/ajcn/84.4.853.

[59] Schaiff WT, Barak Y, Sadovsky Y. The pleiotropic function of PPAR gamma in the placenta. Mol Cell Endocrinol 2006;249:10–5. https://doi.org/10.1016/j.mce.2006.02.009.

[60] Fonseca FCP da, Mucci D de B, Assumpção RP, Marcondes H, Sardinha FL de C, Silva SV, et al. Differential Long-Chain Polyunsaturated Fatty Acids Status and Placental

Transport in Adolescent Pregnancies. Nutrients 2018;10:220. https://doi.org/10.3390/nu10020220.

[61] Metherel AH, Irfan M, Klingel SL, Mutch DM, Bazinet RP. Compound-specific isotope analysis reveals no retroconversion of DHA to EPA but substantial conversion of EPA to DHA following supplementation: a randomized control trial. Am J Clin Nutr 2019;110:823–31. https://doi.org/10.1093/ajcn/nqz097.

[62] Chabowski A, Górski J, Luiken JJFP, Glatz JFC, Bonen A. Evidence for concerted action of FAT/CD36 and FABPpm to increase fatty acid transport across the plasma membrane. Prostaglandins Leukot Essent Fatty Acids 2007;77:345–53. https://doi.org/10.1016/j.plefa.2007.10.017.

[63] Koonen DPY, Glatz JFC, Bonen A, Luiken JJFP. Long-chain fatty acid uptake and FAT/CD36 translocation in heart and skeletal muscle. Biochim Biophys Acta 2005;1736:163–80. https://doi.org/10.1016/j.bbalip.2005.08.018.

[64] Sampath H, Ntambi JM. Polyunsaturated fatty acid regulation of gene expression. Nutr Rev 2004;62:333–9. https://doi.org/10.1111/j.1753-4887.2004.tb00058.x.

[65] Song S, Attia RR, Connaughton S, Niesen MI, Ness GC, Elam MB, et al. Peroxisome proliferator activated receptor alpha (PPARalpha) and PPAR gamma coactivator (PGC-1alpha) induce carnitine palmitoyltransferase IA (CPT-1A) via independent gene elements. Mol Cell Endocrinol 2010;325:54–63. https://doi.org/10.1016/j.mce.2010.05.019.

[66] Clarke SD. The multi-dimensional regulation of gene expression by fatty acids: polyunsaturated fats as nutrient sensors. Curr Opin Lipidol 2004;15:13–8. https://doi.org/10.1097/00041433-200402000-00004.

[67] Martin G, Schoonjans K, Lefebvre AM, Staels B, Auwerx J. Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. J Biol Chem 1997;272:28210–7. https://doi.org/10.1074/jbc.272.45.28210.

[68] Hall AM, Smith AJ, Bernlohr DA. Characterization of the Acyl-CoA synthetase activity of purified murine fatty acid transport protein 1. J Biol Chem 2003;278:43008–13. https://doi.org/10.1074/jbc.M306575200.

[69] Hall AM, Wiczer BM, Herrmann T, Stremmel W, Bernlohr DA. Enzymatic properties of purified murine fatty acid transport protein 4 and analysis of acyl-CoA synthetase activities in tissues from FATP4 null mice. J Biol Chem 2005;280:11948–54. https://doi.org/10.1074/jbc.M412629200.

[70] Li H, Herrmann T, Seeßle J, Liebisch G, Merle U, Stremmel W, et al. Role of fatty acid transport protein 4 in metabolic tissues: insights into obesity and fatty liver disease. Biosci Rep 2022;42:BSR20211854. https://doi.org/10.1042/BSR20211854.

[71] Fisher DJ, Heymann MA, Rudolph AM. Myocardial oxygen and carbohydrate consumption in fetal lambs in utero and in adult sheep. Am J Physiol 1980;238:H399-405. https://doi.org/10.1152/ajpheart.1980.238.3.H399.

[72] McCurdy CE, Schenk S, Hetrick B, Houck J, Drew BG, Kaye S, et al. Maternal obesity reduces oxidative capacity in fetal skeletal muscle of Japanese macaques. JCI Insight 2016;1:e86612. https://doi.org/10.1172/jci.insight.86612.

[73] Kersten S. Effects of fatty acids on gene expression: role of peroxisome proliferator-activated receptor alpha, liver X receptor alpha and sterol regulatory element-binding protein-1c. Proc Nutr Soc 2002;61:371–4. https://doi.org/10.1079/PNS2002169.

[74] Pégorier J-P, Le May C, Girard J. Control of gene expression by fatty acids. J Nutr 2004;134:2444S-2449S. https://doi.org/10.1093/jn/134.9.2444S.

[75] Sampath H, Ntambi JM. Polyunsaturated fatty acid regulation of genes of lipid metabolism. Annu Rev Nutr 2005;25:317–40. https://doi.org/10.1146/annurev.nutr.25.051804.101917.

[76] Glatz JFC, Luiken JJFP, Bonen A. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol Rev 2010;90:367–417. https://doi.org/10.1152/physrev.00003.2009.

[77] Campodonico-Burnett W, Hetrick B, Wesolowski SR, Schenk S, Takahashi DL, Dean TA, et al. Maternal Obesity and Western-Style Diet Impair Fetal and Juvenile Offspring Skeletal Muscle Insulin-Stimulated Glucose Transport in Nonhuman Primates. Diabetes 2020;69:1389–400. https://doi.org/10.2337/db19-1218.

[78] McCurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. J Clin Invest 2009;119:323–35. https://doi.org/10.1172/JCI32661.

[79] Schmitz-Peiffer C, Whitehead JP. IRS-1 regulation in health and disease. IUBMB Life 2003;55:367–74. https://doi.org/10.1080/1521654031000138569.

[80] Capel F, Acquaviva C, Pitois E, Laillet B, Rigaudière J-P, Jouve C, et al. DHA at nutritional doses restores insulin sensitivity in skeletal muscle by preventing lipotoxicity and inflammation. J Nutr Biochem 2015;26:949–59. https://doi.org/10.1016/j.jnutbio.2015.04.003.

[81] Rossmeisl M, Medrikova D, van Schothorst EM, Pavlisova J, Kuda O, Hensler M, et al. Omega-3 phospholipids from fish suppress hepatic steatosis by integrated inhibition of biosynthetic pathways in dietary obese mice. Biochim Biophys Acta 2014;1841:267–78. https://doi.org/10.1016/j.bbalip.2013.11.010.

[82] Zhang Y, He L, Chen X, Shentu P, Xu Y, Jiao J. Omega-3 polyunsaturated fatty acids promote SNAREs mediated GLUT4 vesicle docking and fusion. J Nutr Biochem 2022;101:108912. https://doi.org/10.1016/j.jnutbio.2021.108912.

[83] Guriec N, Le Foll C, Delarue J. Long-chain n-3 PUFA given before and throughout gestation and lactation in rats prevent high-fat diet-induced insulin resistance in male offspring in a tissue-specific manner. Br J Nutr 2023;130:1121–36. https://doi.org/10.1017/S000711452300017X.

[84] Lalia AZ, Lanza IR. Insulin-Sensitizing Effects of Omega-3 Fatty Acids: Lost in Translation? Nutrients 2016;8:329. https://doi.org/10.3390/nu8060329.

**Table 1:** Fetal characteristics

| | CON | DHA |
|---|---|---|
| Body Weight (g) | 225 ± 24.5 | 209 ± 12.2 |
| Body Length (cm) | 19.3 ± 0.4 | 20.1 ± 0.3 |
| Heart (g) | 1.9 ± 0.2 | 1.8 ± 0.1 |
| Liver (g) | 15.6 ± 1.1 | 14.6 ± 1.5 |
| Kidneys (g) | 2.5 ± 0.2 | 2.2 ± 0.1 |
| Brain (g) | 6.0 ± 0.3 | 6.4 ± 0.2 |
| Total Visceral Weight (g) | 24.2 ± 1.1 | 24.9 ± 1.9 |
| Visceral/Body Weight (%) | 12.0 ± 0.5 | 11.9 ± 0.2 |

Data are means ± SEM collected from GD75 fetuses obtained from pregnant ewes maintained on a control diet (CON; $N = 7$) or DHA-supplemented diet (DHA; $N = 6$). Total visceral weight is the sum mass of the four organs collected. There were no significant differences observed in any parameters between groups.

**Table 2. Fatty acid composition of phospholipids in the placental circulation**

| | Uterine artery | | Uterine vein | | Placental Tissue | | Umbilical Artery | | Umbilical Vein | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Fatty acid** | **CON** | **DHA** | **CON** | **DHA** | **CON** | **DHA** | **CON** | **DHA** | **CON** | **DHA** |
| **16:0** | 15.5 ± 0.3 | 15.4 ± 0.4 | 14.1 ± 1.7 | 14.5 ± 0.4 | ***13.5 ± 1.5*** | ***18.2 ± 0.7*** | ***21.1 ± 0.4*** | ***17.6 ± 1.0*** | ***20.8 ± 0.3*** | ***18.0 ± 0.6*** |
| **16:1** | 0.6 ± 0.1 | 0.5 ± 0.2 | 1.0 ± 0.5 | 0.9 ± 0.5 | 2.5 ± 0.3 | 2.5 ± 0.5 | 1.6 ± 0.1 | 1.8 ± 0.1 | 1.2 ± 0.3 | 1.7 ± 0.1 |
| **18:0** | 25.5 ± 0.6 | 23.9 ± 1.3 | 22.9 ± 3.1 | 22.2 ± 2.6 | 14.1 ± 1.7 | 15.7 ± 2.0 | ***19.1 ± 0.3*** | ***16.3 ± 0.9*** | *18.9 ± 0.3* | *16.9 ± 0.8* |
| **18:1*n*9** | 14.7 ± 2.9 | 13.8 ± 0.8 | 14.8 ± 1.9 | 13.5 ± 0.6 | 24.0 ± 3.4 | 30.3 ± 5.5 | 14.9 ± 0.8 | 14.6 ± 0.8 | *15.5 ± 0.9* | *13.4 ± 0.6* |
| **18:1*n*7** | 1.2 ± 0.6 | 0.3 ± 0.1 | 1.5 ± 0.7 | 0.9 ± 0.8 | 4.9 ± 0.4 | 4.9 ± 0.5 | 1.7 ± 0.7 | 1.4 ± 0.7 | 1.3 ± 0.7 | 1.5 ± 0.6 |
| **18:2*n*6** | 17.2 ± 1.7 | 15.8 ± 1.8 | 14.5 ± 2.1 | 13.5 ± 0.3 | 4.1 ± 1.0 | 3.8 ± 0.3 | ***1.0 ± 0.2*** | ***1.8 ± 0.3*** | ***1.1 ± 0.1*** | ***1.9 ± 0.2*** |
| **18:3*n*3** | *5.2 ± 1.5* | *1.3 ± 0.1* | 5.7 ± 1.4 | 1.5 ± 0.2 | 0.9 ± 0.5 | 0.4 ± 0.3 | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.1 ± 0.0 | 0.5 ± 0.0 |
| **20:0** | 0.3 ± 0.3 | 0.1 ± 0.1 | 0.8 ± 0.6 | 0.6 ± 0.3 | 1.0 ± 0.6 | 0.6 ± 0.5 | ***0.0 ± 0.0*** | ***0.6 ± 0.2*** | ***0.1 ± 0.1*** | ***0.9 ± 0.1*** |
| **20:1** | *0.2 ± 0.1* | *0.0 ± 0.0* | 0.8 ± 0.5 | 0.5 ± 0.4 | 1.5 ± 0.5 | 0.5 ± 0.4 | 0.3 ± 0.1 | 0.5 ± 0.2 | ***0.7 ± 0.1*** | ***0.1 ± 0.0*** |
| **20:2** | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.9 ± 0.5 | 0.4 ± 0.3 | 2.0 ± 0.5 | 0.9 ± 0.8 | 2.1 ± 0.6 | 1.5 ± 0.2 | ***2.9 ± 0.2*** | ***1.0 ± 0.1*** |
| **20:3*n*6** | 0.8 ± 0.3 | 0.4 ± 0.2 | 0.9 ± 0.5 | 1.0 ± 0.3 | 1.1 ± 0.5 | 0.6 ± 0.5 | ***0.3 ± 0.1*** | ***0.7 ± 0.2*** | ***0.3 ± 0.1*** | ***0.7 ± 0.0*** |
| **20:4*n*6** | 4.8 ± 0.6 | 4.6 ± 0.5 | 4.3 ± 0.4 | 4.2 ± 0.4 | 6.2 ± 0.5 | 6.4 ± 0.7 | 14.1 ± 1.3 | 13.0 ± 1.0 | 13.5 ± 1.2 | 11.9 ± 0.7 |
| **20:3*n*3** | 0.4 ± 0.3 | 0.2 ± 0.1 | 0.7 ± 0.5 | 0.1 ± 0.1 | 0.6 ± 0.5 | 0.0 ± 0.0 | *0.0 ± 0.0* | *0.2 ± 0.2* | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **20:5*n*3** | ***2.1 ± 0.7*** | ***4.4 ± 0.8*** | *2.6 ± 0.6* | *4.4 ± 0.6* | 2.3 ± 0.6 | 1.2 ± 1.0 | ***1.9 ± 0.8*** | ***5.5 ± 0.6*** | ***2.7 ± 0.7*** | ***5.3 ± 0.2*** |
| **22:5*n*3** | 3.6 ± 0.6 | 2.8 ± 0.7 | 3.8 ± 0.5 | 2.0 ± 0.2 | 2.7 ± 0.3 | 2.5 ± 0.2 | 6.8 ± 0.8 | 6.3 ± 0.4 | 6.9 ± 0.9 | 5.8 ± 0.4 |
| **22:6*n*3** | ***3.2 ± 0.2*** | ***12.0 ± 1.2*** | ***3.5 ± 0.3*** | ***11.5 ± 1.9*** | ***3.7 ± 0.2*** | ***4.8 ± 0.3*** | 11.8 ± 0.5 | 13.1 ± 1.2 | 12.1 ± 0.4 | 11.9 ± 0.5 |
| **SFA** | 41.4 ± 0.5 | 39.3 ± 1.5 | 38.4 ± 3.7 | 37.9 ± 2.2 | *29.6 ± 2.0* | *35.0 ± 2.2* | ***40.2 ± 0.7*** | ***34.8 ± 1.6*** | ***39.8 ± 0.6*** | ***36.3 ± 0.6*** |
| **MUFA** | 16.5 ± 3.0 | 14.6 ± 1.0 | 18.7 ± 0.6 | 16.2 ± 2.2 | 33.7 ± 2.8 | 38.6 ± 5.8 | 18.4 ± 0.5 | 18.5 ± 1.0 | *18.8 ± 0.5* | *17.1 ± 0.4* |
| **PUFA** | ***37.6 ± 1.2*** | ***42.6 ± 0.9*** | 39.0 ± 1.4 | 39.7 ± 1.4 | 26.0 ± 3.5 | 21.9 ± 3.3 | ***38.3 ± 1.5*** | ***43.0 ± 1.4*** | 39.8 ± 1.0 | 40.4 ± 0.6 |
| ***n*3 PUFA** | 14.5 ± 3.0 | 20.6 ± 2.2 | 16.2 ± 2.5 | 19.5 ± 2.1 | 10.1 ± 1.6 | 8.8 ± 1.7 | *20.6 ± 2.1* | *25.4 ± 1.8* | 21.8 ± 2.0 | 23.5 ± 0.8 |
| ***n*6 PUFA** | 22.8 ± 2.1 | 21.5 ± 2.6 | 20.5 ± 1.8 | 18.8 ± 0.3 | 12.1 ± 1.3 | 11.2 ± 1.2 | 15.3 ± 1.2 | 15.6 ± 0.9 | 14.8 ± 1.2 | 14.9 ± 0.7 |
| ***n*3/*n*6** | 0.7 ± 0.2 | 1.0 ± 0.2 | 0.9 ± 0.2 | 1.0 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 | ***0.9 ± 0.3*** | ***1.7 ± 0.2*** | 1.6 ± 0.3 | 1.6 ± 0.1 |

Values are mean ± SEM percentages of all fatty acids detected in the total phospholipid fraction extracted from serum or placental (cotyledon) tissue collected from CON (N = 5) and DHA (N = 4) ewes at GD75. Fatty acid nomenclature is C:B*n*X where C is the number of carbons, B is the number of double bonds present, and *n*X in the location of the first carbon in the double bond counting from the methyl (omega) end of unsaturated fatty acids. SFA, sum of all saturated fatty acids; MUFA, sum of all monounsaturated fatty acids; PUFA, sum of all polyunsaturated fatty acids. *n*3 and *n*6 represent the sum of all detected omega-3 and omega-6 PUFA in the sample, and the mean ratio of the totals for each group. ***Boldfaced italicized*** pairs of data are significantly different by independent samples *t*-test at $P < 0.05$, whereas *underlined font italicized* differ at $P < 0.10$.

**Table 3. Fatty acid composition of fetal tissue phospholipids**

| | Fetal Muscle | | Fetal Heart | | Fetal Liver | | Fetal Kidney | | Fetal Brain | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Fatty acid** | **CON** | **DHA** | **CON** | **DHA** | **CON** | **DHA** | **CON** | **DHA** | **CON** | **DHA** |
| **16:0** | 21.6 ± 1.3 | 23.5 ± 1.2 | ***16.9 ± 0.1*** | ***21.1 ± 1.1*** | *25.8 ± 0.4* | *24.3 ± 0.5* | ***27.9 ± 1.9*** | ***24.9 ± 0.9*** | 35.4 ± 0.7 | 35.8 ± 1.4 |
| **16:1** | *2.0 ± 0.1* | *1.7 ± 0.1* | *1.5 ± 0.1* | *1.2 ± 0.1* | 1.5 ± 0.1 | 1.5 ± 0.0 | 1.7 ± 0.2 | 1.4 ± 0.2 | 3.2 ± 0.2 | 2.9 ± 0.1 |
| **18:0** | 16.1 ± 0.4 | 16.7 ± 0.5 | 14.2 ± 2.2 | 17.5 ± 0.4 | ***20.1 ± 0.5*** | ***18.5 ± 0.6*** | 16.0 ± 0.7 | 17.0 ± 0.6 | 13.8 ± 0.2 | 13.8 ± 0.8 |
| **18:1*n*9** | ***35.7 ± 1.7*** | ***31.6 ± 0.5*** | 27.2 ± 2.2 | 23.7 ± 0.8 | 14.4 ± 0.3 | 13.9 ± 0.1 | ***25.8 ± 0.4*** | ***24.7 ± 0.2*** | ***16.1 ± 0.3*** | ***15.0 ± 0.5*** |
| **18:1*n*7** | 2.3 ± 1.0 | 1.8 ± 0.8 | 6.4 ± 3.3 | 1.5 ± 0.8 | 2.1 ± 0.6 | 2.2 ± 0.4 | ***4.6 ± 0.0*** | ***4.1 ± 0.1*** | ***3.7 ± 0.1*** | ***3.5 ± 0.1*** |
| **18:2*n*6** | ***0.9 ± 0.2*** | ***0.2 ± 0.2*** | ***2.1 ± 0.2*** | ***1.2 ± 0.3*** | ***0.0 ± 0.0*** | ***0.4 ± 0.2*** | ***1.1 ± 0.1*** | ***2.0 ± 0.2*** | 0.1 ± 0.1 | 0.0 ± 0.0 |
| **18:3*n*3** | 0.1 ± 0.0 | 0.0 ± 0.0 | ***0.2 ± 0.0*** | ***0.0 ± 0.0*** | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **20:0** | *0.2 ± 0.1* | *0.0 ± 0.0* | ***0.2 ± 0.1*** | ***0.0 ± 0.0*** | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **20:1** | *0.6 ± 0.2* | *0.2 ± 0.1* | ***1.5 ± 0.1*** | ***0.7 ± 0.2*** | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.0 ± 0.0 |
| **20:2** | ***3.6 ± 0.1*** | ***2.4 ± 0.0*** | ***3.6 ± 0.3*** | ***0.8 ± 0.3*** | ***1.5 ± 0.3*** | ***0.7 ± 0.2*** | 2.5 ± 0.6 | 2.0 ± 0.3 | 0.2 ± 0.2 | 0.0 ± 0.0 |
| **20:3*n*6** | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.5 ± 0.0 | 0.3 ± 0.2 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **20:4*n*6** | 7.0 ± 0.7 | 6.0 ± 0.4 | ***12.5 ± 1.0*** | ***9.5 ± 0.5*** | 13.6 ± 0.9 | 12.7 ± 0.4 | ***12.2 ± 0.9*** | ***9.8 ± 0.5*** | ***6.6 ± 0.3*** | ***5.5 ± 0.2*** |
| **20:3*n*3** | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **20:5*n*3** | ***0.4 ± 0.1*** | ***0.9 ± 0.3*** | ***2.4 ± 0.7*** | ***6.2 ± 0.7*** | ***0.7 ± 0.4*** | ***3.2 ± 0.4*** | ***1.6 ± 0.7*** | ***4.3 ± 0.3*** | 0.1 ± 0.1 | 0.0 ± 0.0 |
| **22:5*n*3** | ***2.1 ± 0.2*** | ***2.8 ± 0.1*** | 3.0 ± 0.2 | 2.6 ± 0.2 | 4.6 ± 0.4 | 4.7 ± 0.3 | 2.1 ± 0.1 | 2.2 ± 0.2 | 0.3 ± 0.3 | 0.0 ± 0.0 |
| **22:6*n*3** | ***2.1 ± 0.2*** | ***3.8 ± 0.2*** | ***3.0 ± 0.2*** | ***4.5 ± 0.3*** | ***11.7 ± 0.2*** | ***13.6 ± 0.3*** | ***3.7 ± 0.6*** | ***5.6 ± 0.2*** | ***11.1 ± 0.2*** | ***12.4 ± 0.5*** |
| **SFA** | 37.9 ± 1.4 | 40.2 ± 1.6 | ***31.3 ± 2.2*** | ***38.7 ± 1.3*** | ***45.9 ± 0.6*** | ***42.9 ± 0.8*** | 43.9 ± 2.6 | 42.0 ± 0.9 | ***49.2 ± 0.6*** | ***51.4 ± 0.2*** |
| **MUFA** | ***40.7 ± 1.3*** | **35.4 ± 1.0** | ***36.6 ± 1.8*** | ***27.2 ± 0.7*** | 18.0 ± 0.8 | 17.6 ± 0.3 | **32.1 ± 0.5** | **30.2 ± 0.5** | ***23.1 ± 0.3*** | ***21.4 ± 0.6*** |
| **PUFA** | 18.1 ± 1.3 | 16.6 ± 1.2 | ***28.5 ± 0.6*** | ***26.3 ± 1.0*** | ***32.1 ± 0.5*** | ***35.5 ± 0.5*** | ***22.3 ± 2.1*** | ***25.4 ± 0.6*** | 18.4 ± 0.7 | 17.9 ± 0.6 |
| ***n*3 PUFA** | ***4.6 ± 0.4*** | ***7.6 ± 0.4*** | ***8.6 ± 1.0*** | ***13.3 ± 0.8*** | ***17.0 ± 0.9*** | ***21.5 ± 0.7*** | ***7.1 ± 1.4*** | ***12.2 ± 0.6*** | 11.6 ± 0.5 | 12.4 ± 0.5 |
| ***n*6 PUFA** | 8.1 ± 0.9 | 6.4 ± 0.6 | ***15.2 ± 1.2*** | ***11.0 ± 0.8*** | 13.6 ± 0.9 | 13.2 ± 0.5 | ***12.7 ± 0.9*** | ***11.3 ± 0.3*** | ***6.7 ± 0.2*** | ***5.5 ± 0.2*** |
| ***n*3/*n*6** | ***0.6 ± 0.1*** | ***1.2 ± 0.1*** | ***0.6 ± 0.1*** | ***1.3 ± 0.1*** | 1.3 ± 0.2 | 1.6 ± 0.1 | ***0.7 ± 0.1*** | ***1.1 ± 0.1*** | ***1.7 ± 0.1*** | ***2.3 ± 0.1*** |

Values are mean ± SEM percentages of all fatty acids in the total phospholipid fraction extracted from GD75 fetal tissues of collected CON (N = 7) and DHA-supplemented pregnancies (N = 6). Fatty acid nomenclature is C:B*n*X where C is the number of carbons, B is the number of double bonds present, and *n*X in the location of the first carbon in the double bond counting from the methyl (omega) end of unsaturated fatty acids. SFA, sum of all saturated fatty acids; MUFA, sum of all monounsaturated fatty acids; PUFA, sum of all polyunsaturated fatty acids. *n*3 and *n*6 represent the sum of all detected omega-3 and omega-6 PUFA in the sample, and the mean ratio of the totals for each group. ***Boldfaced italicized*** pairs of data are significantly different by independent samples *t*-test at $P < 0.05$, whereas *underlined font italicized* differ at $P < 0.10$.

**Figure 1: Serum lipids in the placental circulation.**

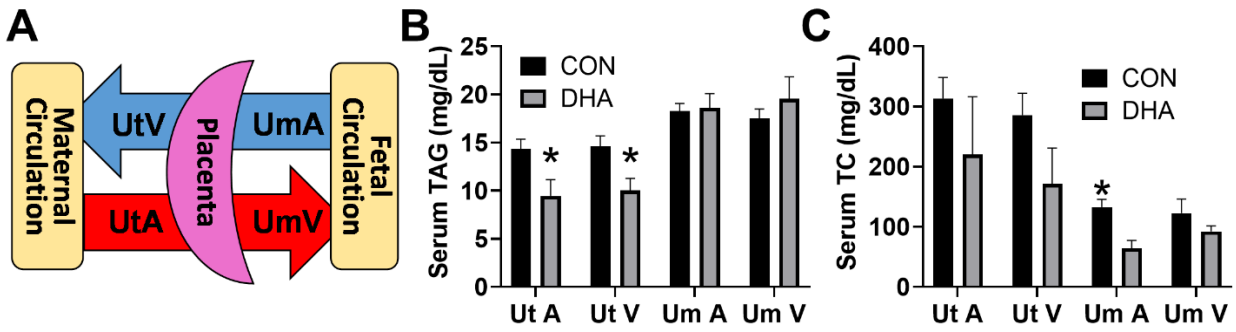



**Figure 1. Serum lipids in the placental circulation. A)** Schematic of placental circulation illustrating the relative positions and blood flow directions of the uterine artery (UtA), umbilical vein (UmV), umbilical artery (UmA), and uterine vein (UtV) from which sera were collected. Data are means ± SEM of total serum triglycerides (TAG) **(B)**, and total cholesterol (TC) **(C)** collected at GD75 from fasted anesthetized ewes maintained on a control (CON; N = 5) or DHA-supplemented diet (DHA; N = 4). * $P < 0.05$ vs. CON in the same vessel.

**Figure 2. Placental and fetal tissue fatty acid transporter expression**

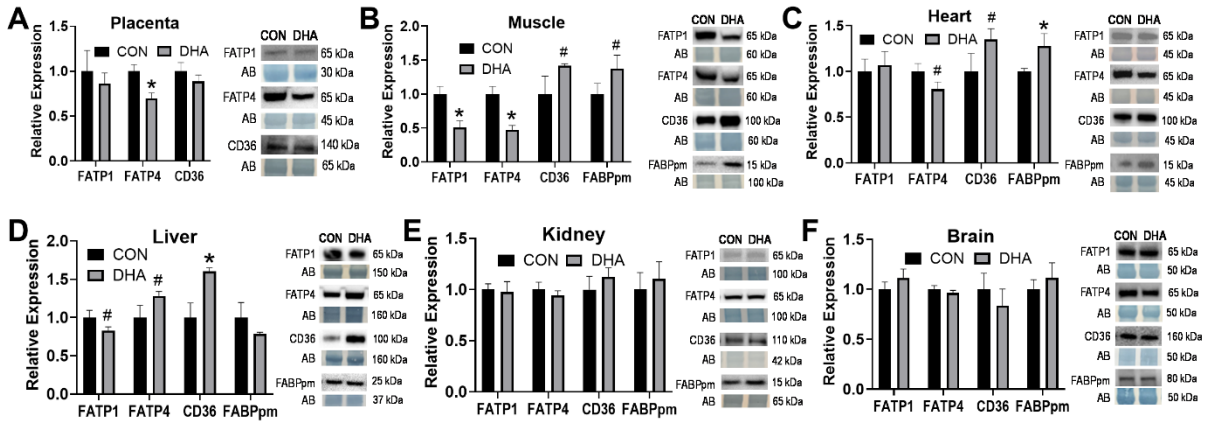



**Figure 2. Placental and fetal tissue fatty acid transporter expression.** DHA supplementation during pregnancy differentially affected the protein expression of the major fatty acid transport proteins in placental (colyedon) tissue (**A**), fetal skeletal muscle (*M. biceps femoris*) (**B**), fetal heart (left ventricular myocardium) (**C**), fetal liver (**D**), fetal kidney (**E**), and fetal brain (frontal cortex) tissue (**F**). FATP1, fatty acid transport protein 1; FATP4, fatty acid transport protein 4; CD36, fatty acid translocase; FABPpm, plasmalemmal fatty acid binding protein. Data are means ± SEM ($n$ = 5-7/group) of relative expression normalized to total sample protein by Amido Black (AB) staining with representative immunoblot scans and molecular weights from each experiment. $^{\#}P < 0.10$, $^{*}P < 0.05$ compared to CON of the same protein and tissue.

=

**Figure 3: Impacts on fetal cardiac metabolism**

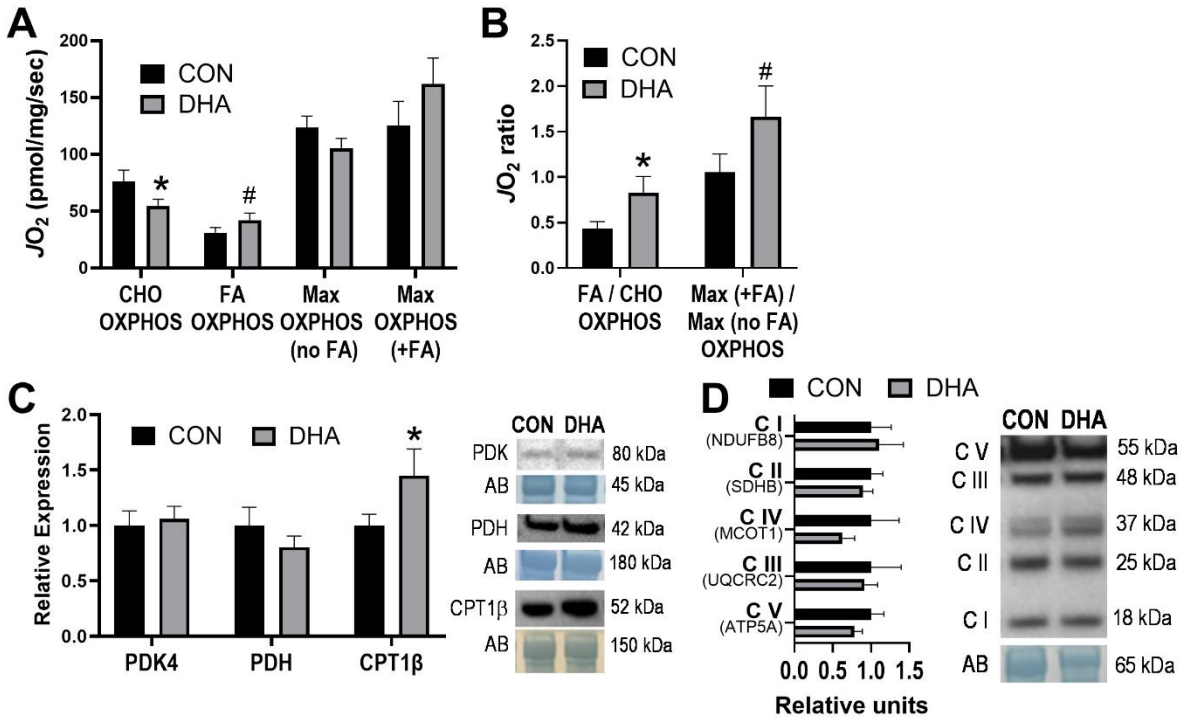



**Figure 3: Fetal cardiac metabolism. A)** Impacts of maternal DHA supplementation on the oxidative phosphorylation (OXPHOS)-linked respiratory capacities of permeabilized fetal cardiac muscle fibers supported by 5 mM pyruvate + 1 mM malate (CHO OXPHOS), 0.05 mM palmitoylcarnitine + 1 mM malate (FA OXPHOS), or mixed substrate combinations (Max OXPHOS; CHO + 10 mM glutamate and 10 mM succinate) in the presence (+FA) or absence (no FA) of 0.05 mM palmitoylcarnitine. **B)** Relative expressions of respiratory capacities presented in panel A, demonstrating higher OXPHOS capacities in DHA fetuses when fatty acid substrates are present. Tissue protein expression of enzymes known to regulate mitochondrial pyruvate and fatty acid oxidation **(C)** and each of the five mitochondrial OXPHOS complexes CI-V **(D)** normalized to total sample protein by Amido Black (AB) staining with representative immunoblots and molecular weights from each experiment. PDH, pyruvate dehydrogenase; PDK4, PDH kinase-4, CPT1β, carnitine palmitoyltransferase-1β; Data are means ± SEM (N = 5-7/group). $^{\#}P < 0.10$, $^{*}P < 0.05$ compared to CON of the same protein and tissue.

**Figure 4. Fetal skeletal muscle metabolism**

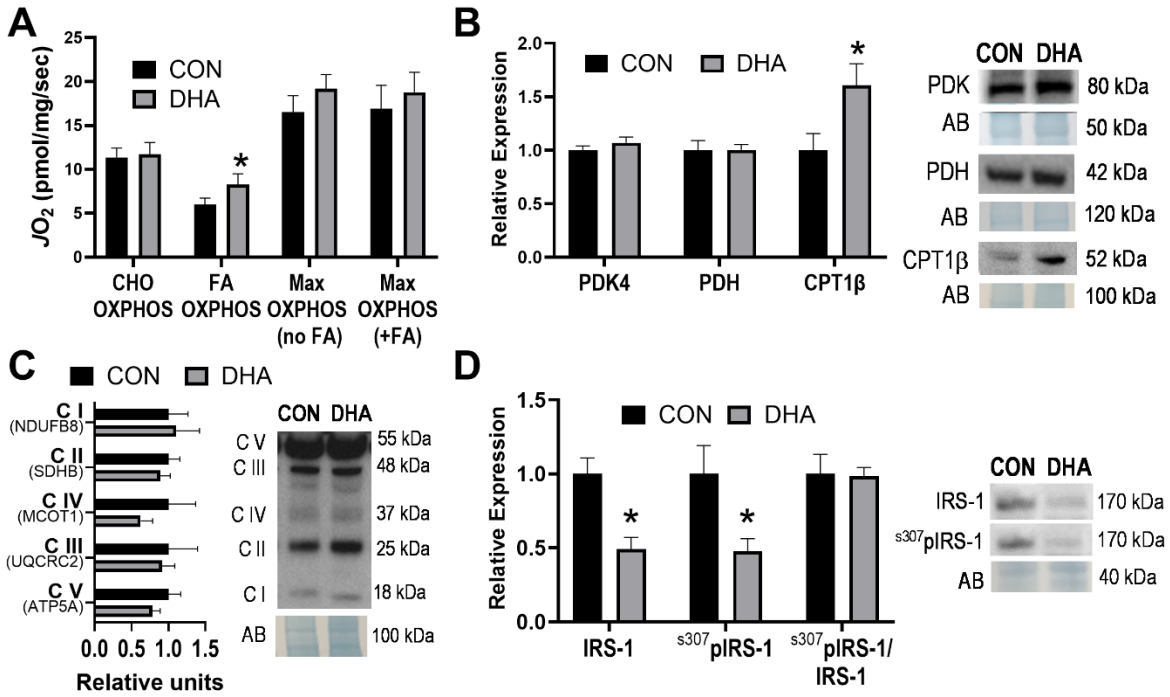



**Figure 4. Fetal skeletal muscle metabolism. A)** Impacts of maternal DHA supplementation on the oxidative phosphorylation (OXPHOS)-linked respiratory capacities of permeabilized fetal *M. biceps femoris* muscle fibers supported by 5 mM pyruvate + 1 mM malate (CHO OXPHOS), 0.05 mM palmitoylcarnitine + 1 mM malate (FA OXPHOS), or mixed substrate combinations (Max OXPHOS; CHO + 10 mM glutamate and 10 mM succinate) in the presence (+FA) or absence (no FA) of 0.05 mM palmitoylcarnitine. Tissue protein expression of enzymes known to regulate mitochondrial pyruvate and fatty acid oxidation **(B),** each of the five mitochondrial OXPHOS complexes CI-V **(C)**, and the total and ser-307 phosphorylated levels of insulin receptor substrate-1 (IRS-1) **(D)** normalized to total sample protein by Amido Black (AB) staining, with representative immunoblots and molecular weights from each experiment. PDH, pyruvate dehydrogenase, PDK4, PDH kinase-4, CPT1β, carnitine palmitoyltransferase-1β; Data are means ± SEM (N = 5-7/group). **P* < 0.05 compared to CON of the same protein and tissue.

**Figure 5. Fetal liver metabolism**

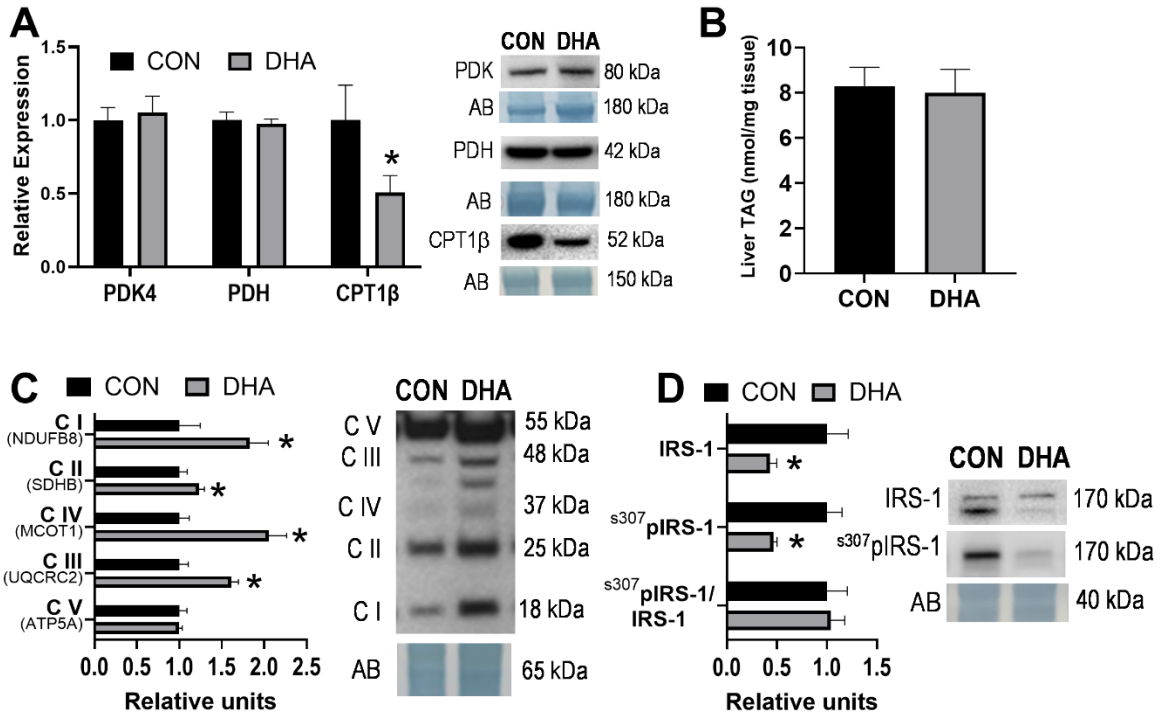



**Figure 5: Fetal liver metabolism.** Impacts of maternal DHA supplementation on fetal liver protein expression of **A)**enzymes known to regulate mitochondrial pyruvate and fatty acid oxidation**, B)** each of the five mitochondrial OXPHOS complexes CI-V, and **C)** the total and ser-307 phosphorylated levels of insulin receptor substrate-1 (IRS-1)**.** Immunoblot data are normalized to total sample protein by Amido Black (AB) staining, with representative blots and molecular weights from each experiment. PDH, pyruvate dehydrogenase, PDK4, PDH kinase-4, CPT1β, carnitine palmitoyltransferase-1β; Data are means ± SEM (N = 5-7/group). *$P < 0.05$ compared to CON of the same protein and tissue.

CHAPTER IV:

# IMPACTS OF PLACENTAL FATP4 GENE TARGETING ON FETOPLACENTAL SERUM FATTY ACID COMPOSITION AND FETAL TISSUE METABOLISM

Asma K. Omar, Ali Asghar, Quinton A. Winger, Gerrit J. Bouma, and Adam J. Chicco

Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1617

**Abstract**

Fetal development depends on regulated transfer of nutrients from the maternal to the fetal circulation by the placenta. In the previous two chapters, we examined the impacts of maternal high-fat feeding and docosahexaenoic acid (DHA) supplementation on placental and fetal fatty acid transport and metabolism. Among the several fatty acid transporters affected by these interventions, placental fatty acid transport protein 4 (FATP4) expression has been previously associated with the selective transfer of DHA to the umbilical circulation and increases in response to maternal overnutrition during pregnancy. Therefore, in the present study, we sought to investigate the influence of placental FATP4 on the fatty acid composition of serum phospholipids in the ovine fetoplacental circulation and fetal tissues, along with the fetal metabolic parameters evaluated in our previous studies. **Methods**: To this end, we employed a method for selective deletion of the FATP4 gene from the trophectoderm (pre-placental layer) of Day 9 ewe embryos (hatched blastocysts) via lentiviral transduction of a CRISPR-Cas9 construct targeting the ovine FATP4 gene *in vitro*. The transfected blastocysts were then implanted into recipient pseudo-pregnant ewes and allowed to develop until gestational day (GD) 75, after which the resulting placentomes and fetal tissues were collected for biochemical analyses. **Results:** As expected, transfection of D9 blastocysts with the lentiviral CRISPR construct successfully depleted FATP4 mRNA from the GD75 cotyledon (fetal side of the placenta).

However, this was not associated with a significant reduction in FATP4 protein in the cotyledon, perhaps due to migration of FATP4 transporters from the caruncle (maternal side of the placenta) where protein expression was higher compared to untreated animals. Despite this, FATP4 gene targeting decreased levels of DHA in the umbilical vein relative to the uterine artery, suggesting a possible disruption of DHA transfer from the maternal to fetal circulation. In contrast to the placenta, FATP4 gene targeting of blastocysts significantly decreased FATP4 protein expression in all five GD75 fetal tissues examined, with a >75% depletion in muscle, heart, liver and kidney compared to untreated animals. This was associated with an upregulation of FATP1 in fetal muscle and liver, and higher CD36 fatty acid transporter levels in fetal muscle, perhaps reflecting a compensatory response to maintain fetal uptake of long-chain fatty acids. The fatty acid composition of the fetal muscle and heart phospholipids was not significantly impacted by the loss of FATP4 proteins. However, fetal muscle capacity to oxidize pyruvate was significantly lower in transfected animals, which corresponded to >95% depletion of insulin receptor substrate-1 (IRS-1) and c-Jun N-terminal kinase (JNK) expression from both fetal muscle and liver. **Conclusions**: Taken together, these results highlight the potential pitfalls of CRISPR lentiviral transfection of D9 blastocysts as a method for selectively deleting a target protein from the GD75 placenta, both in terms of its placental selectivity and the possibility of protein migration from the caruncle or other tissues despite gene deletion from the cotyledon. Depletion of FATP4 protein in fetal tissues did not impact membrane fatty acid composition in heart or muscle, while its role in the placental fatty acid transfer remains inconclusive. The striking links between depletion of FATP4 with loss of IRS-1 and JNK protein from fetal muscle and liver are difficult to interpret, potentially reflecting co-regulation of these genes/protein, or more likely, off-target effects of the CRISPR-Cas9 constructs applied to D9 embryos and observed after two months of fetal development.

## 1. Introduction

Fetal development relies on the proper transfer of essential nutrients from the maternal circulation to the fetal circulation, which is the primary function of the placenta, an organ that develops during pregnancy. The placenta transfers nutrients such as glucose, fatty acids, amino acids and vitamins from the maternal circulation to the fetus, and transfers waste products from the fetus to the mother [1-3]. Any disruptions in maternal-fetal nutrient exchange, either due to maternal metabolic diseases or placental insufficiency, can lead to adverse outcomes in fetal growth and development [4].

Fatty acids play an important role in fetal growth and development through a variety of essential biological functions. Long-chain saturated fatty acids provide energy for cellular activities along with pyruvate derived from glucose catabolism [5]. Long-chain polyunsaturated fatty acids (PUFA) are primarily incorporated into cellular phospholipids where they contribute to membrane fluidity and structure [6] and may be mobilized to participate in cellular signaling [7], including regulation of nutrient uptake and metabolism [8]. As discussed in the previous chapter, long-chain omega-3 PUFA such as docosahexaenoic acid (DHA) also play a key role in retinal and nervous system development and may impact the metabolic risk trajectory of the developing fetus and offspring. Therefore, it is important to understand the mechanisms that regulate the transfer and utilization of fatty acids during pregnancy to optimize fetal growth and development [9].

Fatty acid transport proteins (FATPs) are membrane-bound proteins that transport long-chain fatty acids into cells [8, 10, 11]. Several transporters contribute to the effective transport of long-chain fatty acids across the placenta, including FATP4, FATP1, CD36 and fatty acid-binding proteins (FABPs) [10]. Among these, FATP1 and FATP4 are most highly expressed in the human

term placenta [12]. FATP4 transports long-chain fatty acids across the apical membrane of the trophoblast cells, then converts fatty acids into acyl-CoA derivatives [13], which is an important step in intracellular fatty acid utilization for energy production and lipid synthesis [5]. In addition, placental FATP4 is thought to regulate the transport of poly-unsaturated fatty acids to fetal circulation [12, 14] and is upregulated at mid-gestation in response to maternal overnutrition [15] and physical activity [16] during pregnancy. In our previous studies in the ewe model, we found that maternal high-fat feeding increases expression of FATP4 along with other fatty acid transporters in mid-gestation fetal tissues, but not placenta, with consequent upregulation of fatty acid oxidation capacity [17]. In contrast, maternal DHA supplementation decreased mid-gestation placental FATP4 expression and elicited diverse effects on the expression of FATP4 and other transporters in fetal tissues. Therefore, understanding the precise biological role and regulation of FATP4 during fetoplacental development merits further investigation.

The present study was the first to investigate the role of FATP4 in fetoplacental development using a loss-of-function approach in the pregnant ewe model. We utilized a method designed to selectively delete the *FATP4* gene from the trophectoderm in D9 blastocysts using CRIPSR-Cas9 lentiviral transfection in hopes of generating mid-term placenta that lacks FATP4 protein on the fetal side of the placenta (cotyledon). We predicted that placental *FATP4* deletion would impair the transport of long-chain PUFA (perhaps specifically DHA) to the offspring, which would perhaps have broader effect on fetal tissue membrane composition and nutrient metabolism.

## 2. Materials and Methods

*2.1 Experimental Model*. All animal procedures were approved by Colorado State University Animal Care and Use Committee in accordance with recommendations from the Declaration of Helsinki and the Guiding Principles on the Care and Use of Animals adopted by the American Physiological Society. White-faced ewes were purchased from a local vendor and housed in outdoor pens with free access to food and water. Heat cycles were determined using a vasectomized ram during the typical sheep breeding season (late October-November). Two weeks before pre-determined conception dates, ewes were started on standard control diet to support optimal pregnancy outcomes as used in our previous studies (Show-rite NewCo Lamb Feed; 17% protein, 5% Fat), and were maintained on this diet for the duration of the study. Ewes were randomly assigned to either control/natural pregnancy group (CON; N = 5), donor ewes from which blastocysts were collected for FATP4 gene targeting (N = 5), or recipient ewes within which the transfected blastocytes would develop with targeted deletion of *FATP4* (KO; N = 5) until gestational day 75 (GD75). Prior to the breeding season, three CRISPR guide RNAs targeting exons 2, 3 and 4 of the ovine FATP4 gene were designed and tested by Ali Asghar the Winger lab at CSU. The RNA targeted exon 2 below was ultimately selected and assembled into a LCV2 vector for *in vitro* lentiviral transduction of blastocysts obtained from donor ewes by Dr. Winger at the Animal Reproduction and Biotechnology Laboratory.

**CRISPR Guide RNA used in the present study targeting exon 2 of the ovine FATP4 gene:**

ATGCTGCTTGGGGCGTCTCTGTTGGGGGTGCTGCTGTTCTCCCGGCTGGTGCTGAAACTGCCCTGGACCC
AAGTGGGGTTCTCCCTGTTCTTCCTCTACCTGGGGTCTGGCGGCTGGCGCTTCATCCGAATCTTTATCAA
GACTGTCAGGCGTGATATCTT

**FW Oligo 5′ CACCGGGCTGGTGCTGAAACTGCCC 3′**

**REV Oligo 5′ AAACGGGCAGTTTCAGCACCAGCCC 3′**

GGCTGGTGCTGAAACTGCCCTGG Score (60.4 recommended is >66) +

Ewes on similar heat cycles were synchronized by two 2 mL doses of intramuscular Dinoprost Tromethamine (Lutalyse, Zoetis) two days prior to breeding with the same fertile ram to ensure efficient conception and minimize fetal genetic variations from paternal DNA. On gestational day 9 (D9), the uterus of donor ewes was removed and flushed to obtain the D9 hatched blastocyst, which was immediately exposed to the *FATP4* CRISPR-Cas9 lentivirus construct *in vitro* for 4-5 hours. The outermost single-cell layer of D9 blastocysts, the trophectoderm, will eventually develop into the fetal side of the placenta known as the cotyledon. In 2009, Georgiades et al. reported that short-term transduction of mouse blastocysts with a green fluorescent protein (GFP) transgene led to expression of GFP in the trophectoderm, but not in the inner cell mass/embryo, leading the authors to conclude that this lentivirus lineage-specific infection system facilitates early embryonic placental-specific gene targeting in the mouse and potentially other mammalian species [18]. This approach was subsequently adopted for use in D9 ovine blastocysts by research groups at CSU to target genes involved in placental development [19-23], which provided the basis for the methods used in the present study. Following 4-5 hours of incubation with the lentivirus, single transduced embryos were transferred to synchronized recipient ewes in the right or left horn ipsilateral to the corpus luteum on the ovary. The recipient ewes were maintained on antibiotics and were fed control diet until collection of placentomes and fetal tissues at mid-gestation as described below.

*2.2 Blood and Fetal Tissue Collection*. Seventy-five days following conception (GD75), pregnant ewes (confirmed by ultrasound) were fasted overnight and sedated by intravenous injection of diazepam (2 mL) and ketamine (10 mL) prior to establishing general anesthesia by 2% isoflurane inhalation. Following confirmation of deep anesthesia, the fetoplacental blood

vessels were surgically accessed for collection of blood from umbilical and uterine circulations, followed by removal of the placenta and fetus for morphological assessments and tissue collection. Sections of fetal heart, hindlimb skeletal muscle (*M. biceps femoris*), liver, kidney and brain (frontal cortex) were carefully dissected and snap frozen in liquid nitrogen prior to storage at -80°C for future biochemical experiments. In addition, 25-30 mg sections of skeletal muscle were placed in ice-cold BIOPS preservation medium containing (in mM) 10 Ca-EGTA (0.1 μM free calcium), 20 imidazole, 20 taurine, 50 K-MES, 0.5 DTT, 6.56 $MgCl_2$, 5.77 ATP, and 15 phosphocreatine, pH 7.1, for respirometry experiments on freshly prepared permeabilized fiber bundles on the same day. Following collection of the placental and fetal tissues, ewes were euthanized under general anesthesia.

*2.3 Serum Lipids and Glucose*. To determine if FATP4 gene targeting in D9 embryos impacted levels of major nutrients in the GD75 fetoplacental circulation, we used commercially available assay kits to measure serum levels of glucose (Invitrogen, Cat# EIAGLUC), triglycerides (Abcam, Cat# ab65336), total cholesterol, high-density lipoproteins (HDL), and low/very low-density lipoproteins (LDL+VLDL) (Abcam, Cat# ab65390) in venous and arterial blood from the uterine and umbilical circulations according to the manufacturer's instructions.

*2.4 Serum and Tissue Phospholipid Fatty Acid Composition*. The impacts of FATP4 gene targeting in D9 embryos on the fatty acid composition of phospholipids in the GD75 fetoplacental circulation, fetal muscle and heart were determined by gas chromatography of fatty acid methyl esters (FAMEs) hydrolyzed from the total phospholipid fraction as previously described [24]. Briefly, 10 mg of tissue was homogenized in 800 μL of precooled methanol in a

glass homogenizer to solubilize the total phospholipid fraction [25]. For serum, 50 µL was directly added to 600 µL of precooled methanol in a glass tube. After vortexing samples for 30 seconds, tubes were centrifuged at 900 × g for 5 min to pellet the non-phospholipid fraction. The supernatant (containing phospholipids) was transferred to a fresh glass tube, to which 25 µL of methoxide solution was added to synthesize methyl esters from hydrolyzed phospholipids. The reaction was stopped after 3 min by adding 75 µL of methanolic HCl, and FAMEs were extracted by adding 700 µL of hexane, transferring the upper hexane layer to 2 mL chromatography vials. FAMEs were dried under nitrogen flow and resuspended in hexane for gas chromatography (GC) analysis using an Agilent Technologies DB-225 30m x 0.250mm x 0.25µm column (model 122-2232, J&W Scientific) on an Agilent 6890 Series Gas Chromatograph with a flame ionization detector [24]. A fatty acid standard solution of 1 mg/mL was injected into the GC every 6-7 runs to recalibrate the fatty acid peaks on the chromatograph, enabling relative quantitation of up to 18 fatty acids commonly found in mammalian membranes expressed as a percentage of total phospholipid fatty acids. In addition, total levels of saturated fatty acid (SFA) monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) were calculated, along with total N3-PUFA, N6-PUFA and their ratio.

*2.5 Skeletal muscle oxidative metabolism*. The capacities of fetal skeletal muscle to oxidize fatty acids, pyruvate and mixed substrate combinations were assessed by high-resolution respirometry on permeabilized fiber bundles freshly prepared from hindlimb muscle sections as previously described [26]. Briefly, 10-15 mg of *M. biceps femoris* was trimmed free of connective tissue and gently teased with needle tip forceps in ice-cold BIOPS solution, then incubated with 50 µg/ml saponin for 20 min on ice with gentle rocking to permeabilize cell membranes while

leaving mitochondrial membranes intact [27]. Permeabilized fiber bundles were then rinsed in mitochondrial respiration medium (MiR05) containing (in mM) 0.5 EGTA, 3 $MgCl_2$ hexahydrate, 60 lactobionic acid, 20 taurine, 10 $KH_2PO_4$, 20 HEPES, 110 sucrose, and 0.1% BSA, pH 7.1 with KOH by rocking for 2 x 15 min on ice, then gently blotted dry on Whatman paper and weighed immediately before adding 5-6 mg of fiber bundles to the oxygraph chamber for respirometry experiments. Mass-corrected muscle oxidative capacities (per mg fiber bundle) were determined in MiR05 medium at 37°C using an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) by monitoring changes in the negative time derivative of the chamber oxygen concentration signal ($JO_2$) in response to carbohydrate (5 mM pyruvate + 1 mM malate), fatty acid (0.04 mM palmitoylcarnitine + 1 mM malate), or mixed substrate combinations (1 mM malate, 0.04 mM palmitoylcarnitine, 5 mM pyruvate, 10 mM glutamate, 10 mM succinate) following standardized instrumental and chemical background calibrations [28]. Following the addition of substrates, $JO_2$ was recorded in the absence of ADP to assess the capacity for non-phosphorylating "LEAK" $JO_2$ (facilitated by proton leak across the inner mitochondrial membrane), followed by the addition of 2.5 mM ADP to generate the maximal oxidative phosphorylation (OXPHOS)-linked $JO_2$. The extent of respiratory control by ADP was expressed as the OXPHOS coupling control factor [1-(LEAK/OXPHOS)] for carbohydrate and fatty acid oxidation, where a maximum value of 1.0 represents fully coupled mitochondria (100% control of respiration by ADP), and 0 represents fully uncoupled mitochondria (0% respiratory control). Respirometry chambers were maintained in a narrow hyperoxygenated environment (325–400 μM $O_2$) during experiments to avoid limitations of oxygen diffusion on *JO2* of permeabilized fiber bundles [29].

*2.6 Protein Immunoblotting*. Proteins were extracted from snap-frozen sections of placenta, fetal muscle and liver tissue by homogenization in lysis buffer containing (in mM): 150 NaCl, 1 EDTA, 1 EGTA, 5 sodium pyrophosphate, 1 sodium orthovanadate, 20 sodium fluoride added to Mammalian Protein Extraction Reagent (Pierce Cat# 78501) with supplemental Protease Inhibitor Cocktail (Sigma Aldrich Cat# P8340). Protein concentrations of 10,000 X g homogenate supernatants were detected by bicinchoninic acid assay (Thermo Scientific, #23225) using a colorimetric microplate reader (VersaMax, Molecular Devices). Proteins (30 µg) were electrophoresed on 4-12% Bis-Tris gels and transferred to PVDF membranes, then blocked for 1 h at room temperature with 5% non-fat milk or bovine serum albumin (for phosphorylated proteins) before incubating with the following primary antibodies (1:1000 dilution) overnight at 4C°: FATP1 (Abcam, ab81875), FATP4 (Abcam, ab200353), CD36 (Abcam, ab133625), FABPpm (Abcam, ab45966), IRS1 (Abcam, ab52167), pIRS1-Ser307 (Abcam, ab5599), pJNK (Abcam, ab47337), JNK (Abcam, ab199380). After three washes with Tris-buffered saline + 1% Tween-20 (TBST), the membrane was incubated in secondary antibodies (goat anti-rabbit-HRP (Abcam, ab6721) or goat anti-mouse-HRP (Abcam, ab6789) at 1:3000 dilution for one hour at room temperature. Blotted proteins were imaged using SuperSignal West Dura Extended Duration Substrate (Lot#VL314742) and a UVP ChemStudio blot imager (Analytik Jena, Germany), normalizing band densities to total protein staining of a 20-30 kDa range of bands near the target protein using AmidoBlack (Sigma A8181), quantified using ImageJ software (NIH).

*2.7 RNA isolation and quantitative PCR*. RNA was isolated from 50 mg fetal muscle using TRIZOL reagent (Sigma, T9424), with purity confirmed by a A260/230 of ≥ 1.8 using a

Nanodrop 1000. cDNA was made using Bio-Rad iScript cDNA synthesis kit (cat#1706891) on the same day as qPCR experiments performed on a Roche LightCycler 480. Primers against FATP1 (Forward: TGGTTCCTGTGGCTTCAACAG ; Reverse: TCTTCTTGTTCGTGGCGCTCT ), FATP4 (Forward: TTGGGGGTGCTGCTGTTCT ; Reverse: CATCTCGCTGCCAAAGACGA), MCAD (Forward: ACGGCTCCGTGAAGGACAA; Reverse: TTAAAGCCGCCTCCGACTTC), IRS-1 (Forward: GCTACGTGGACACCTCACCTGT; Reverse: GTTGCGGTTGGGACTGAGGT), SCHAD (Forward: GCATCCAAGGAGGACATCGAC ; Reverse: GGGCTGAAACAGGGGGTTCT), vLCAD (Forward: GGGCCTGGCAGACATCTTTA; Reverse: GTTCTCTGCTGGCACCCGTA) were synthesized by Fisher Scientific and tested by reverse transcriptase PCR (Invitrogen, Platinum PCR SuperMix, 12532-016). PCR amplification efficiencies were between 1.8 - 2.1, and qPCR was performed using with serial dilutions of cDNA to obtain a correlation between Log (concentration) of cDNA and Cp (crossing point) for each primer to confirm high primer efficiency, and relative expression levels of target mRNAs are presented by plotting mean $2^{-\Delta Cp}$ values using 18S ribosomal RNA as a reference gene.

*2.8 Statistical Analyses*. All data are presented as means ± SEM. Data from CON and DHA groups were compared using independent samples t-tests using GraphPad Prism 8 software, with statistically significant differences between groups being reported at both #$P < 0.10$ and *$P < 0.05$ to increase probability of capturing potentially important trends with modest numbers of animals per group, in accordance with guidelines from the American Physiological Society [30].

## 3. Results and Discussion

*3.1 Impacts on fetal characteristics*

This study compared the fetal morphology of CON and KO groups. The results are shown in **Table 1**, presented as mean ±SEM, demonstrated a trend toward reduced overall body and organ weights in the KO group compared to the CON group; however, none of these differences reached statistical significance.

**Table 1. Impact of placental FATP4 gene targeting on GD75 fetal morphology**

| | CON | KO |
|---|---|---|
| **Body weight, g** | 225.0 ± 24.5 | 181.3 ±8.0 |
| **Body length, cm** | 19.3 ±0.4 | 18.8 ±0.2 |
| **Heart, g** | 1.9±0.2 | 1.5±0.1 |
| **Liver, g** | 15.6±1.1 | 13.0±1.2 |
| **Kidneys, g** | 2.5±0.2 | 2.3±0.2 |
| **Brain, g** | 6.0±0.3 | 5.5±0.1 |
| **Total visceral weight, g** | 24.2±1.1 | 22.2±1.4 |
| **Visceral/ body weight, %** | 11.2±0.8 | 12.3±0.5 |

Data are means ± SEM collected from GD75 fetuses obtained from natural bred ewes (CON; N = 7) or ewes implanted with CRISPR transfected D9 blastocysts target FATP4 (KO; N = 4). Total visceral weight is the sum mass of the four organs collected.

*3.2 FATP4 gene targeting by CRISPR in D9 embryos selectively deleted FATP4 mRNA from the GD75 cotyledon*

**Figure 1** illustrates the mRNA expression of genes related to fatty acid transport and metabolism in the cotyletdon. FATP4 expression was essentially undetectable in the KO group compared to CON, confirming the successful knockout of this gene from the GD75 cotyledon following CRISPR targeting of D9 embryos. There was no effect of FATP4 gene targeting on FATP1 mRNA expression, indicating no compensatory upregulation in response to FATP4 KO. Similarly, other genes involved in fatty acid metabolism such as VLCAD (very long-chain acyl-CoA dehydrogenase), SCHAD (short-chain 3-hydroxyl-CoA dehydrogenase) and MCAD

(medium-chain acyl-CoA dehydrogenase) also showed no statistically significant difference between groups.

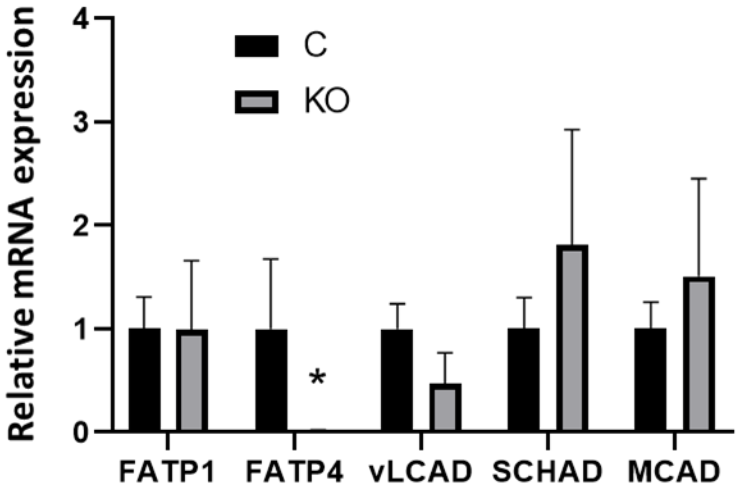



**Figure 1: Selective deletion of FATP4 mRNA in the GD75 placenta (cotyledon) following CRISPR gene targeting of D9 embryos**. Data are means ± SEM of mRNA expression by qPCR relative to 18S ribosomal RNA. Results demonstrate a complete lack of FATP4 mRNA in the FATP4-KO (KO) group compared to control (C), with no significant effect on mRNA expression of FATP1 or other fatty acid metabolism enzymes. FATP, fatty acid transport protein; vLCAD, very long-chain acyl-CoA dehydrogenase; SCHAD, short-chain acyl-CoA dehydrogenase, MCAD, medium-chain acyl-CoA dehydrogenase.

*3.3 FATP4 mRNA deletion from the GD75 cotyledon was not associated with lower FATP4 protein expression in the GD75 cotyledon or caruncle*

Despite successful deletion of FATP4 mRNA from the cotyledon, the protein expression of FATP4 was not significantly reduced in the same tissue (cotyledon) (**Fig 2A**) and was significantly greater in the KO compared to CON group on the maternal side of the placenta (caruncle) (**Fig 2B**; $P$=0.05). This may indicate a compensatory upregulation of FATP4 in the maternal side leading to trafficking of the protein to the fetal side of the placenta, but confirming such a hypothesis would require further study. Examination of the full immunoblots of FATP4 in

the cotyledon suggests partial disruption of the expected band in at least two KO sample, indicating that the CRISPR targeting might have at least partially disrupted protein expression as expected. Therefore, we decided to proceed with phenotypic analyses.

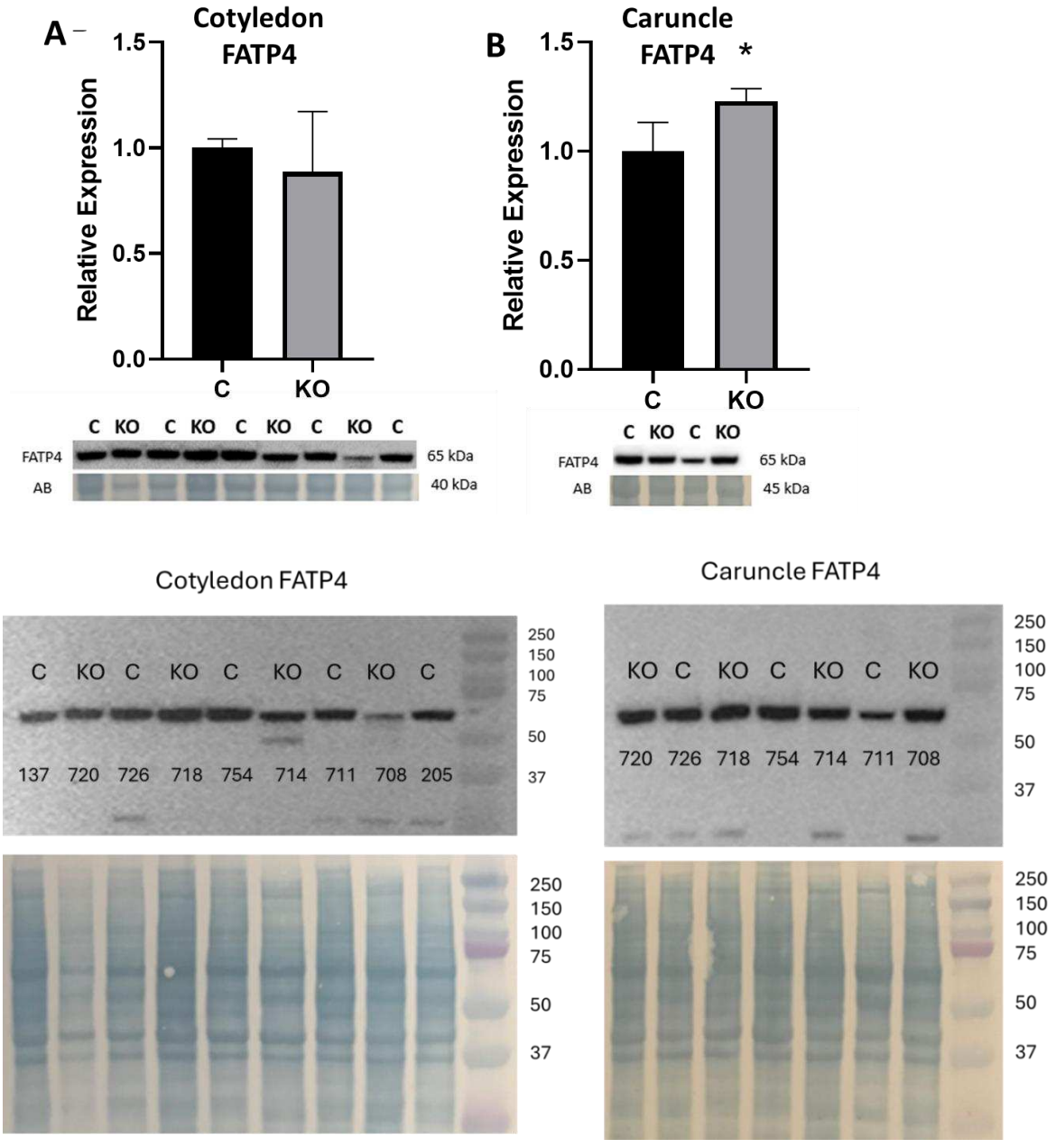



**Figure 2: Protein expression of FATP4 in the fetal side of the placenta (cotyledon; A) and maternal side of the placenta (caruncle; B) at GD75** following CRISPR gene targeting of D9 embryos (KO) or no intervention (CON) normalized to total sample protein by Amido Black (AB) staining, with representative immunoblots and molecular weights from each experiment. Data are means ± SEM of 5-7 blots per group, with representative full immunoblotted membranes and associated total protein (Amido Black)-

stained images demonstrating uniform sample loading across lanes, with corresponding molecular weight markers indicated on the right margins. *P = 0.05 vs CON.

*3.4 FATP4 mRNA deletion from the GD75 cotyledon was not associated with upregulation of FATP1 or CD36 in the GD75 cotyledon or caruncle tissue.*

To confirm that *FATP4* gene targeting by CRISPR in D9 embryos had no effect on the protein expression of other fatty acid transporters in the placenta at GD75, immunoblotting was performed and indicated no effect on the expression of FATP1 in the cotyledon (**Fig 3A)** or caruncle (**Fig 3B**), and no significant effect on expression of the fatty acid translocase CD36 in the cotyledon (**Fig 3C**) or caruncle (**Fig 3D**).

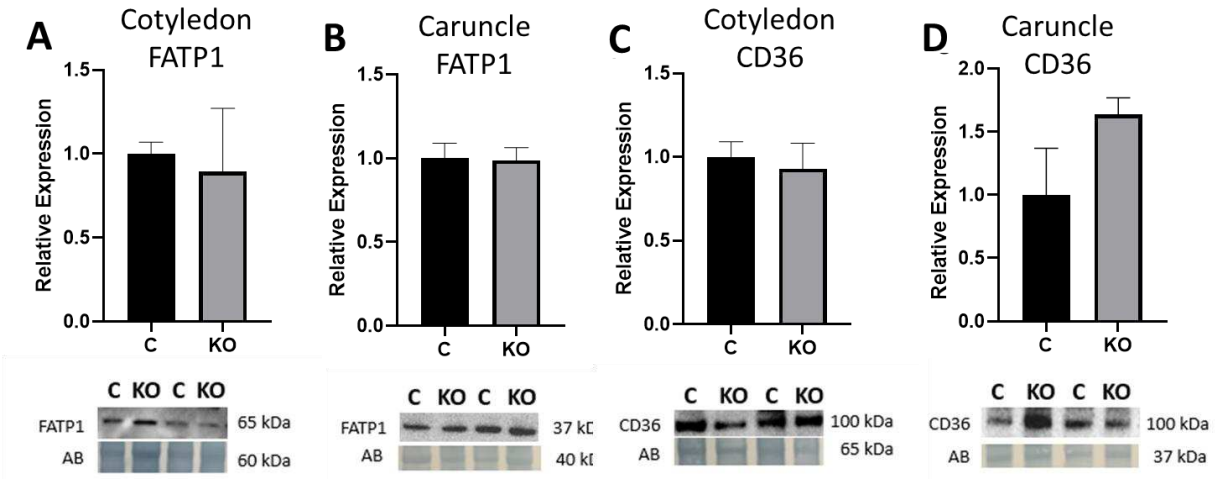



**Figure 3. Protein expression of FATP1 in the cotyledon (A) and caruncle (B), and fatty acid translocase CD36 in the cotyledon (C) or caruncle (D) at GD75** following CRISPR gene targeting of D9 embryos (KO) or no intervention (CON) normalized to total sample protein by Amido Black (AB) staining, with representative immunoblots and molecular weights from each experiment. Data are means ± SEM of 5-7 blots per group.

*3.5 FATP4 gene targeting by CRISPR in D9 embryos alters GD75 fetoplacental serum fatty acid composition.*

Given the evidence for potential selective disruption of cotyledonal FATP4 in the KO group and the putative effects of placental FATP4 on transfer of long-chain PUFA and DHA in particular

[31], we performed a detailed analysis of the phospholipid fatty acid composition of serum in the fetoplacental circulation (**Fig 4**). The schematic in Figure 4 illustrates the relative locations of the 4 distinct serum compartments of the maternal (uterine) and fetal (umbilical) circulations. The most notable effects of the KO treatment were a consistent trend for lower relative proportion of PUFAs and higher proportion of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in all four serum compartments, although this only reached statistical significance in the uterine artery (UtA; **Fig 4A**) uterine vein (UtV; **Fig 4B**), and umbilical artery (UmA; **Fig 4D**), with similar effect sizes in the umbilical vein (UmV; **Fig 4C**). As a rough estimate of the selective transfer of particular fatty acids from the maternal to fetal circulation, we expressed the proportion of major individual fatty acids (as % total within each compartment) in the UmV/UtA (**Fig 4E**). This ratio was significantly lower in the KO for serum DHA (22:6n3) and oleic acid (18:1n9), while 20:5n3 eicosapentaenoic acid (EPA) was greater ($P < 0.05$). These results are consistent with a potential role of placental FATP4 in regulating transfer of PUFA from the maternal to fetal circulation, with a potential preference for DHA as suggested by previous evidence for a correlational between placental FATP4 expression and DHA levels in the umbilical circulation [31]. However, the absence of clear FATP4 protein deficiency in the placenta weakens our ability to definitively conclude this connection.

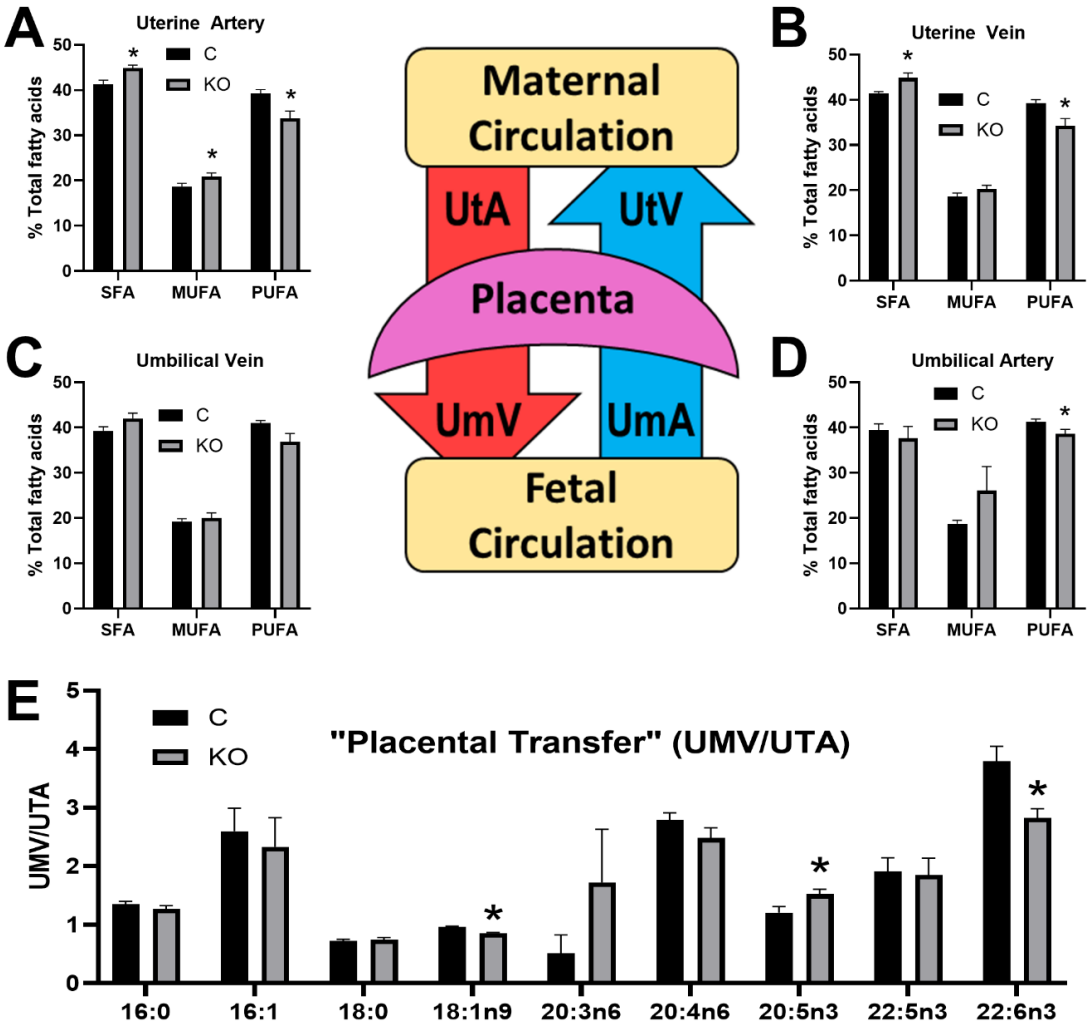



**Figure 4. Impacts of CRISPR targeting of the FATP4 gene in D9 embryos on the serum fatty acid composition of phospholipids in the fetoplacental circulation** from untreated (CON) and gene targeted embryos (KO) at GD75 (N = 4-5 samples/group). The central schematic illustrates the relative locations of the four distinct serum compartments of the maternal (uterine) and fetal (umbilical) circulations investigated, including the uterine artery (UtA; A) uterine vein (UtV; B), umbilical vein (UmV; C), and umbilical artery (UmA; D). Values are mean ± SEM percentages of all fatty acids detected for fatty acid classes including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). The relative proportion of major individual fatty acids in the UmV/UtA is presented as a rough estimate of the transfer of particular fatty acids from the maternal to fetal circulation (E). Fatty acid nomenclature is C:BnX where C is the number of carbons, B is the number of double bonds present, and nX in the location of the first carbon in the double bond counting from the methyl (omega) end of unsaturated fatty acids. Data are means ± SEM of 3-5 samples per group. *P < 0.05 compared to CON.

*3.6 Impacts of FATP4 gene targeting by CRISPR in D9 embryos on GD75 serum glucose, cholesterol and triglycerides in the fetoplacental circulation*.

To investigate the impact of *FATP4* gene targeting of D9 embryos on the levels of major fuel sources in the fetoplacental circulation, we also measured levels of serum glucose, lipoproteins/cholesterol, and triglycerides in uterine and umbilical veins and arteries (**Fig 5**). While there was no effect of *FATP4* gene targeting on total cholesterol lipoproteins (**Fig 5A**), glucose (**Fig 5E**), or low-density lipoproteins (LDL; **Fig 5B**), levels of high-density lipoproteins (HDL) trended lower in all four serum compartments (**Fig 5C**; P = 0.01 – 0.10), while levels of triglycerides tended to be higher in both the uterine and umbilical circulations (**Fig 5D**). Fig 5F presents a schematic of the fetoplacental circulation for reference. This suggests a broader impact of the CRISPR *FATP4* gene targeting intervention in D9 embryos on GD75 fetoplacental lipid metabolism and/or transfer that is difficult to interpret, but consistent with potential importance of FATP4 in regulating lipid homeostasis during pregnancy.

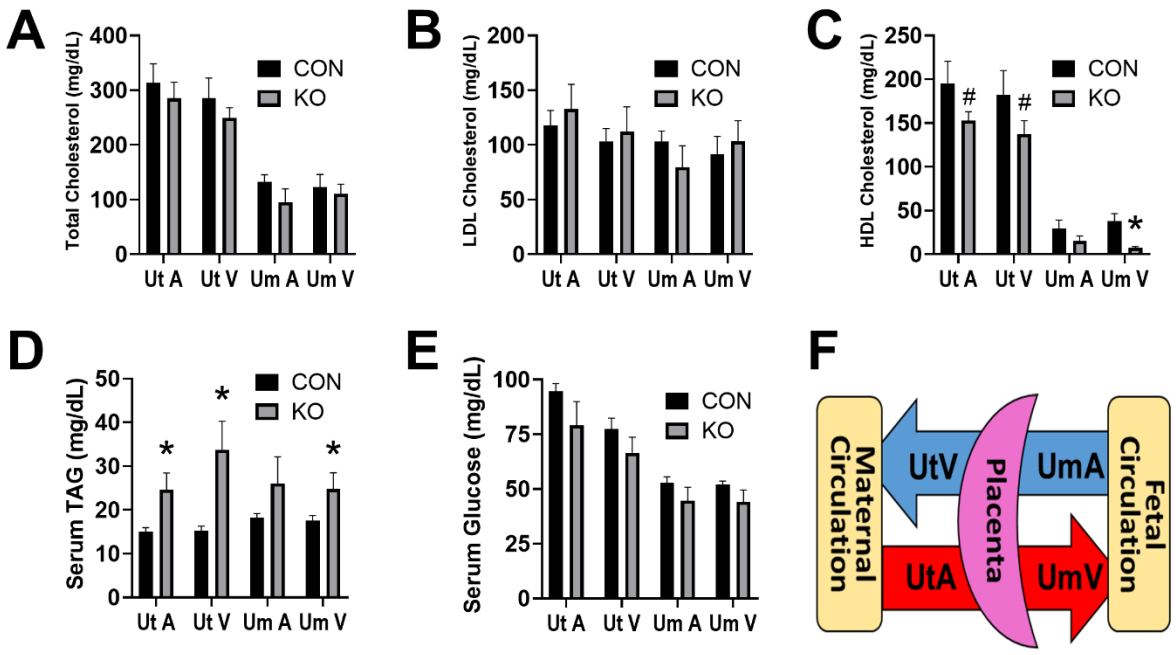


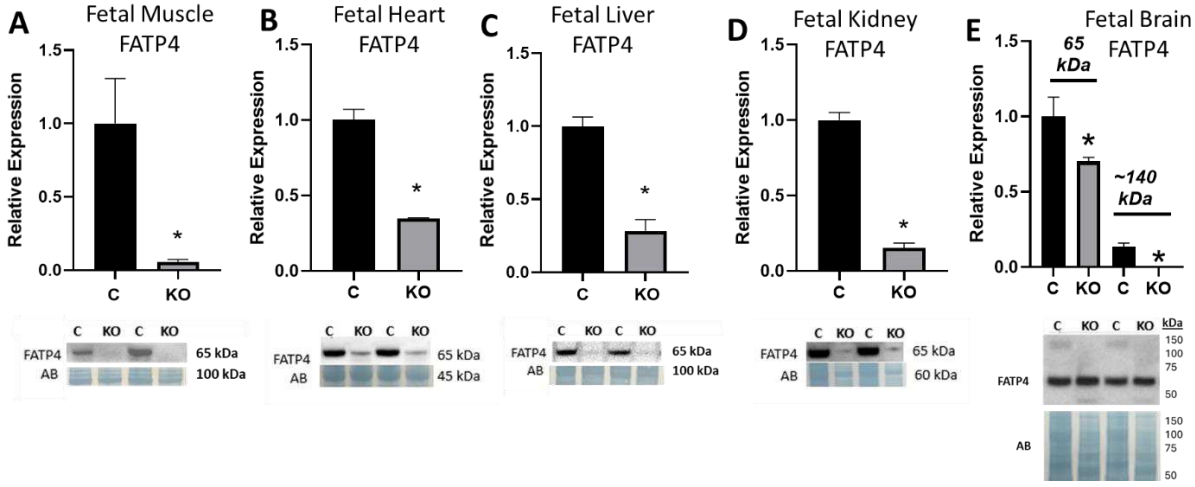
**Figure 5. Impacts of CRISPR targeting of the FATP4 gene in D9 embryos on the serum levels of glucose, cholesterol and triglycerides in the fetoplacental circulation** from untreated (CON) and gene targeted embryos (KO) at GD75. Values are mean ± SEM of the units indicated for total cholesterol lipoproteins (A), low-density lipoproteins (LDL; B), high-density lipoproteins (HDL; C), triglycerides (D), and glucose (E), with panel F presenting a schematic of the fetoplacental circulation for reference. Values of each metabolite are provided for the maternal (uterine) and fetal (umbilical) circulations investigated, including the uterine artery (UtA) uterine vein (UtV), umbilical vein (UmV), and umbilical artery (UmA). Data are means ±SEM of 3-5 samples per group. *P < 0.05, #P = 0.05- 0.10 compared to CON.

*3.7 FATP4 gene targeting by CRISPR in D9 embryos depletes FATP4 protein from GD75 fetal tissues.*

To determine of CRISPR FATP4 gene targeting of D9 embryos impacted expression of FATP4 protein in the GD75 fetus, we immunoblotted five fetal tissues for FATP4 protein (**Fig 6**). Strikingly, we found that FATP4 gene targeting was associated with a >75% depletion of FATP4 protein in the fetal skeletal muscle **(Fig 6A**), heart **(Fig 6B**), liver (**Fig 6C**), and kidney (**Fig 6D**), with comparatively minor effects on the fetal brain (**Fig 6E**). Inspection of full immunoblots from each tissue revealed highly consistent evidence of protein depletion in all KO samples of the muscle, heart, liver and kidney, with highly specific antibody binding and good resolution. Interestingly, the comparatively minor effects in the brain on protein bands at the same molecular weight seen in other tissues (~65 kDa) was associated with faint expression of a band at ~ 140 kDA that was notably depleted in the KO samples. We speculate that this could represent FATP4 protein dimers or perhaps a larger molecular weight FATP4 enzyme complex in brain that was deleted by the D9 embryonic gene targeting; however, this would require further investigation to confirm. An alternative explanation for the comparatively minor effect of *FATP4* gene targeting on the fetal brain despite its marked depletion in other fetal tissues is that the fetal brain arises from the ectoderm of the D9 blastocyst inner-cell mass, which is likely to be the least affected by lentiviral transduction. However, we have not demonstrated lentiviral transfection of the inner-cell mass in these experiments, as such would require direct evidence of Cas9-mediated excision

of the CRISPR-targeted nucleotide sequence within the FATP4 gene in fetal tissues, which we have not provided.

In their study, Georgiades et al. found that short-term lentiviral transduction of mouse blastocysts results in placental-specific gene targeting based on evidence that detectable GFP protein was only observed in the trophectoderm and lack of observed GFP expression in the inner-cell mass or later-stage embryo [18]. Importantly, subsequent studies using this technique in ovine D9 blastocysts at CSU have primarily evaluated impacts of gene deletion on early placental development [19-23]. To our knowledge, this is the first study to examine multiple GD75 ovine fetal tissues for expression of the protein encoded by the gene targeted in D9 blastocysts. While the widespread deletion of FATP4 in fetal tissues is consistent with some CRISPR-Cas9 construct reaching the D9 blastocyst inner cell mass during lentiviral transfection, we have not demonstrated this directly and so cannot rule out explanations for the depletion of FATP4 protein in fetal tissues by other mechanisms.



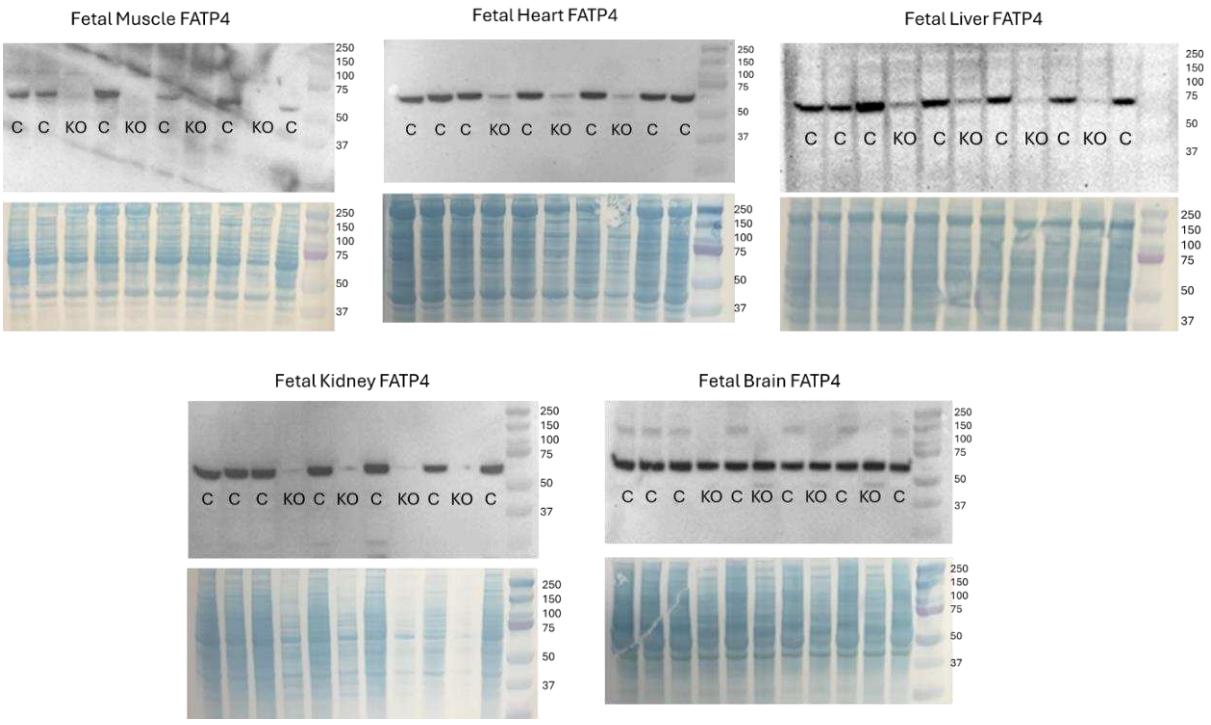



**Figure 6. Protein expression of FATP4 in GD75 fetal tissues** following CRISPR gene targeting of D9 embryos (KO) or no intervention (CON). FATP4 gene targeting was associated with a marked (>75%) depletion of FATP4 protein in the fetal skeletal muscle (A), heart (B), liver (C), and kidney (D), with comparatively minor effects on the fetal brain (E) normalized to total sample protein by Amido Black (AB) staining, with representative immunoblots and molecular weights from each experiment. Data are mean ±SEM of 5-7 blots per group, with representative full immunoblotted membranes and associated total protein (Amido Black)-stained images demonstrating uniform sample loading across lanes, with corresponding molecular weight markers indicated on the right margins. Data are means ±SEM of 5-7 blots per group.*P < 0.05 vs CON.

*3.8 Cas9 protein is undetectable in KO placental and fetal tissue at GD75.*

In an effort to specifically demonstrate that the CRISPR-Cas9 viral construct transfected in blastocyst inner cell mass that ultimately developed into GD75 fetal tissues, we performed immunoblotting of fetal liver samples from control and KO for Cas9 protein. Despite trying three separate Cas9 antibodies (Abcam ab203933; Active Motif cat#61577; and Invitrogen prod#MA5-23519, Lot#TK26656643I), we were not able to detect any Cas9 protein in fetal liver. However, we were also unable to detect any Cas9 protein in either cotyledon or caruncle

placental tissue of any KO animals. Therefore, based on the findings in **Fig 1** and **Fig 6**, we question the reliability of blotting for Cas9 protein in mid-term placenta or fetal tissues as a means of confirming transfection of a CRISPR-Cas9 construct to D9 blastocysts following over two months of cell differentiation, proliferation, and fetal development.

*3.9 FATP4 deletion from fetal muscle and heart has minimal effects on membrane fatty acid composition*.

Given the marked effects of CRISPR *FATP4* gene targeting of D9 embryos to deplete FATP4 protein from fetal muscle (**Fig 6A**) and heart (**Fig 6B**), we decided to investigate impacts on the fatty acid composition of membrane phospholipid extracted from these tissues (**Fig 7**). As noted in earlier chapters, phospholipids were evaluated as the primary locus of PUFA incorporation in biological tissues given the putative role of FATP4 in PUFA transport [31]. Somewhat surprisingly, there was no significant effect of tissue FATP4 depletion on the relative proportions of membrane SFAs, MUFAs or PUFAs in the fetal muscle (**Fig 7A**) or heart (**Fig 7B**). To determine if FATP4 depletion impacted the class of PUFA incorporated into tissue phospholipids, we calculated the total proportion of *n*3-PUFA and *n*6-PUFA in each tissue and found no effect of FATP4 KO in fetal muscle (**Fig 7C**), with a trend for lower levels of both classes in fetal heart (**Fig 7D**; P = 0.05-0.10 for *n*3-PUFA). A more detailed evaluation of specific fatty acid levels revealed mildly significant impacts of FATP4 KO on three fatty acids in fetal muscle (**Fig 7E**; P = 0.05-0.10) and lower 22:5n3 (DPA) in fetal heart (**Fig 7F**; P = 0.05-0.10), but no other notable trends. Taken together, we conclude that protein expression of FATP4 does not play a major role in regulating the proportions of fatty acid classes or individual species incorporated into fetal membranes during development – at least in muscle and heart.

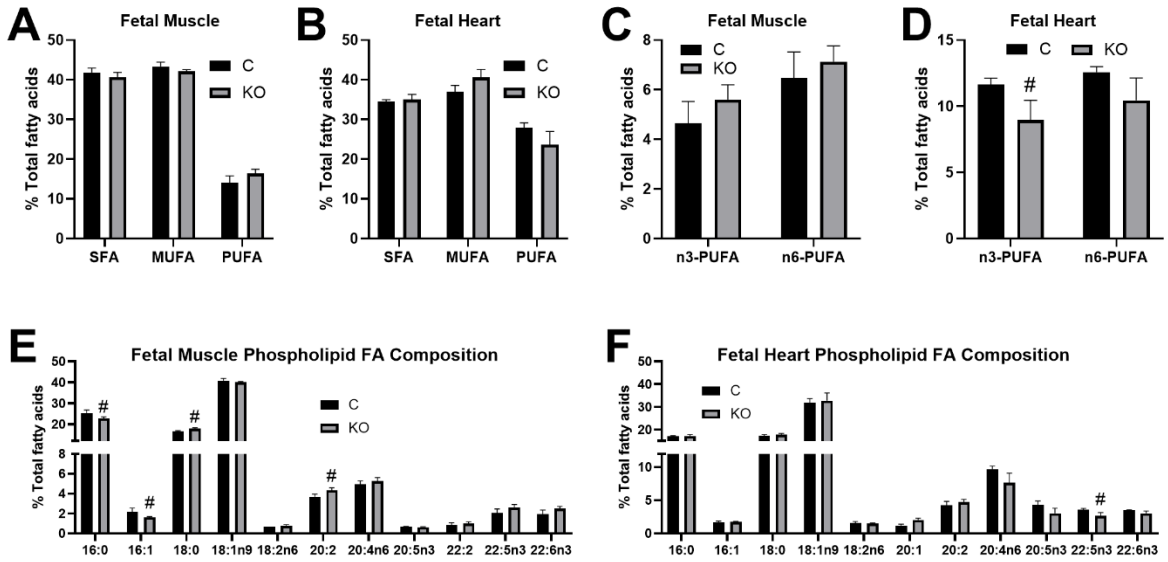



**Figure 7. Impacts of CRISPR targeting of the FATP4 gene in D9 embryos on the fatty acid composition of phospholipids in the fetal muscle and heart**. Values are mean ± SEM percentages of all fatty acids detected for fatty acid classes including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) detected in the fetal muscle (A) and heart (B) of untreated (CON) and gene targeted (KO) at GD75 (N = 4-5 samples/group). The total proportion of all n3 and n6-PUFA detected in muscle (C) and heart (D), along with proportions of all major individual fatty acids detected in muscle (E) and heart (F) are also presented. Fatty acid nomenclature is C:B*n*X where C is the number of carbons, B is the number of double bonds present, and *n*X in the location of the first carbon in the double bond counting from the methyl (omega) end of unsaturated fatty acids. Data are means ±SEM of 4-5 samples per group. $^{\#}$P = 0.05 - 0.10 compared to CON.

*3.10 Deletion of FATP4 triggers upregulation of FATP1 and CD36, but not FABPpm, in fetal muscle.*

To determine if the depletion of FATP4 protein from fetal muscle was associated with compensatory changes in the expression of other fatty acid transport proteins, we immunoblotted fetal muscle homogenates expression of FATP1 (**Fig 8A**), CD36 (**Fig 8B**) and the plasmalemmal fatty acid binding protein (FABPpm; **Fig 8C**). Not surprisingly, we found significantly higher levels of FATP1 and CD36 in the FATP4 KO muscle, consistent with a compensatory

upregulation of these transporters in response to FATP4 depletion. Interestingly, levels of FABPpm were lower in KO compared to CON, perhaps indicating an interaction of these proteins in coordinated uptake/transfer of fatty acids into fetal muscle tissue [32].

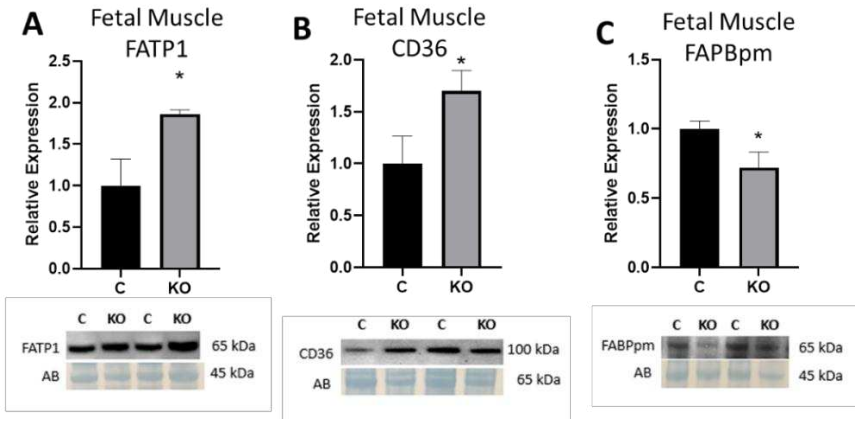



**Figure 8. Protein expression of fatty acid transporters other than FAP4 in GD75 fetal muscle.** Protein levels of FATP1 (A), fatty acid translocase CD36 (B) and plasmalemmal fatty acid binding protein (C) at GD75 following CRISPR *FATP4* gene targeting of D9 embryos (KO) or no intervention (CON) normalized to total sample protein by Amido Black (AB) staining, with representative immunoblots and molecular weights from each experiment. Data are means ±SEM of 5-7 blots per group. *P < 0.05 vs CON.

*3.11 FATP4 deletion is associated with loss of IRS-1 and impaired carbohydrate oxidation in fetal muscle.*

Given our previous studies demonstrating impacts of higher fetal muscle fatty acid transporter expression on metabolic parameters [17], we investigated the effect of FATP4 depletion with FATP1 and CD36 upregulation on fetal muscle metabolism (**Fig 9**). Consistent with the favoring of fatty acid of carbohydrate oxidation capacity observed in the presence of higher FATP1 and CD36 in our previous study [17], we observed a non-significant trend for higher palmitoylcarnitine oxidation capacity in permeabilized muscle fibers from KO compared to CON fetuses (**Fig 9A**) that paralleled a significantly lower capacity to oxidize pyruvate (**Fig 9B**; $P < 0.05$). Unexpectedly, this was associated with complete deficiency in insulin receptor substrate-1 (IRS-1) protein expression in the KO muscle (**Fig 9C**), as well post-translational regulator kinase

c-Jun N-terminal kinase (JNK) expression (**Fig 9D**). The depletion of these proteins is difficult to interpret in the context of FATP4 deletion, suggesting that expression of IRS-1 and JNK may be linked to that of FATP4 in some way not previously described. Along these lines, it is interesting to note that ~50% lower FATP4 levels in GD75 fetal muscle following maternal DHA supplementation in our previous study (**Chapter 3; Fig 2B**) was associated with 50% lower IRS-1 protein levels as well (**Chapter 3; Fig 4D**). While there is no literature precedence for such a relationship, there is evidence that FATP4 regulates glucose handling and insulin sensitivity [33, 34]. Alternatively, while there is no sequence homology between FATP4 with either IRS-1 or JNK, we cannot definitively rule out off-target effects of the CRISPR-Cas9 transfection of Day 9 blastocysts on the proteins in developing embryo and fetal tissues 2 months later.

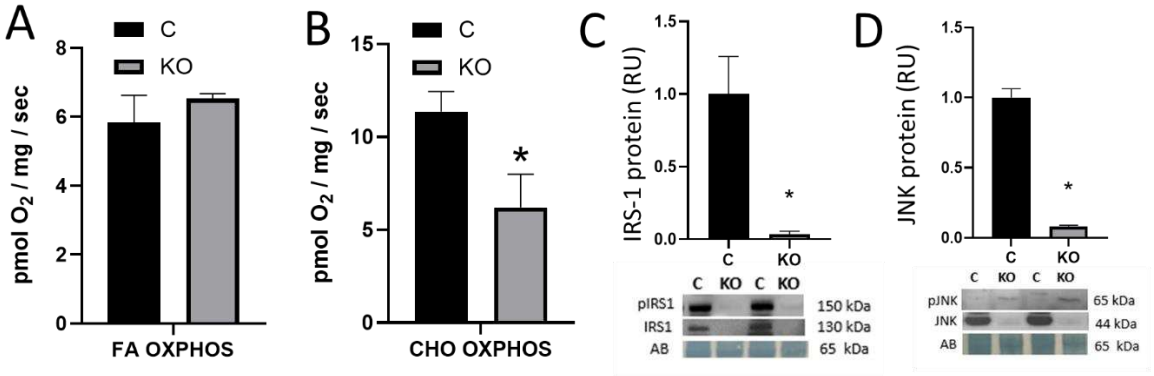



**Figure 9. FATP4 gene targeting on D9 embryos impacts GD75 fetal muscle metabolism**. A) Oxidative phosphorylation (OXPHOS)-linked respiratory capacities of permeabilized fetal *M. biceps femoris* muscle fibers supported by 0.05 mM palmitoylcarnitine + 1 mM malate (FA OXPHOS) (A), and 5 mM pyruvate + 1 mM malate (CHO OXPHOS) (B). Tissue protein expression of the total and ser-307 phosphorylated levels of insulin receptor substrate-1 (IRS-1) (C) and c-Jun N-terminal Kinase (JNK; D) normalized to total sample protein by Amido Black (AB) staining, with representative immunoblots and molecular weights from each experiment. Data are means± SEM (N = 5-7/group). *P < 0.05 compared to CON of the same protein and tissue.

*3.12 FATP4 depletion is associated with higher FATP1, loss of IRS-1 and JNK, and triglyceride accumulation in GD75 fetal liver*.

Given the findings in fetal muscle above and the dual roles of muscle and liver in regulating systemic glucose homeostasis, we decided to also investigate impacts of FATP4 depletion in fetal liver on expression of other fatty acid transporters, levels of tissue triglycerides, and protein expression of IRS-1 and JNK (**Fig 10**). Consistent with findings in fetal muscle, loss of FATP4 protein in the fetal liver was associated with an increase in FATP1 protein expression (**Fig 10A**), with no effect on CD36 (**Fig 10B**) or FABPpm (**Fig 10C**). Interestingly, this was associated with significantly higher levels of triglycerides (TAGs) in liver tissue (**Fig 10D**) and a similar depletion of IRS-1 (**Fig 10E**) and JNK protein (**Fig 10F**).

Taken together, these effects strongly favor disruption of glucose and fatty acid handling by hepatocytes in a manner consistent with the development of severe insulin resistance [35, 36], although the interconnected relationships of these pathways are complex and still a matter of debate [37]. Development of fetal hepatic steatosis is consistent with elevated levels of triglycerides observed in the maternal and fetal circulation (**Fig 5D**) and has been reported previously in the context of maternal overnutrition [38], suggesting that disruption of fetal FATP4 expression favors a pathologic metabolic health trajectory. Indeed, FATP4 deficiency and inhibition has been shown to shift hepatic fatty acid toward greater triglyceride synthesis and export to the circulation [39]. However, drawing concrete biological insights from these results is difficult given the unclear effects of *FATP4* gene targeting on the placenta and our inability to rule out potential off-target effects of CRISPR gene targeting in early embryos on GD75 fetal tissue [40].

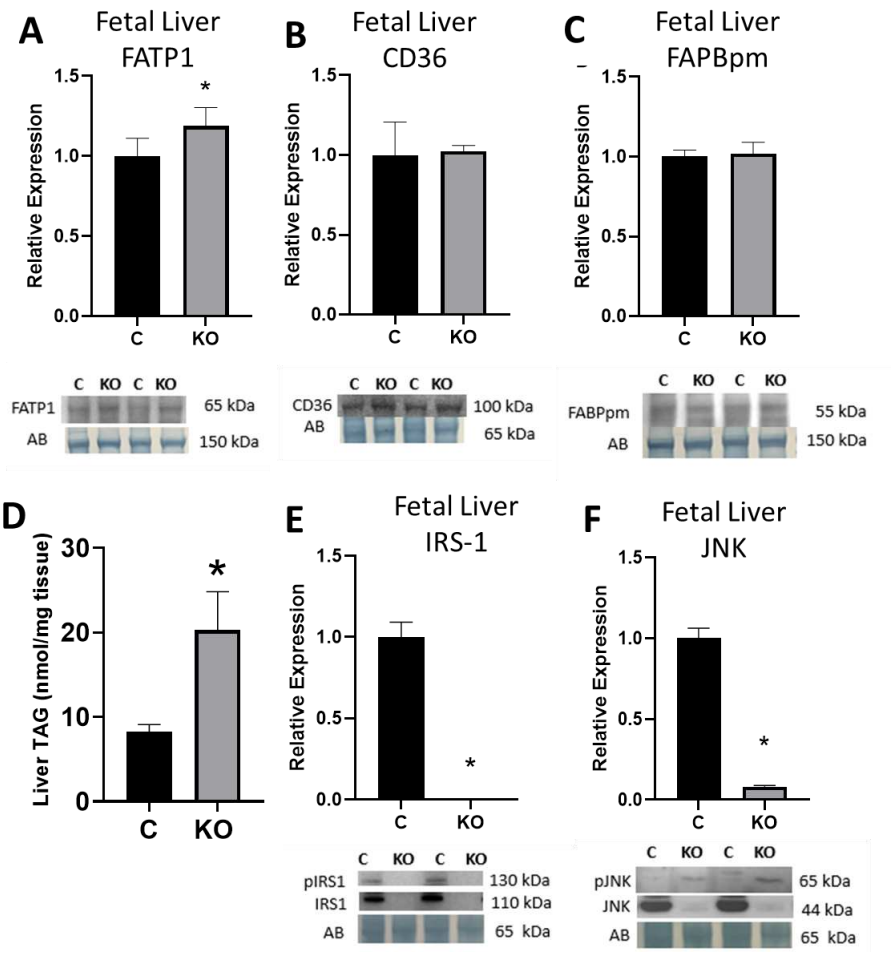



**Figure 10. FATP4 gene targeting on D9 embryos impacts GD75 fetal liver metabolism**. A) Fetal liver tissue protein expression of fatty acid transfer protein 1 (FATP1; A), fatty acid translocase (CD36; B), plasmalemmal fatty acid binding protein (FABPpm; C), levels of triglycerides in liver tissues (D), and the total and ser-307 phosphorylated levels of insulin receptor substrate-1 (IRS-1; E) and c-Jun N-terminal Kinase (JNK; F). All blots were normalized to total sample protein by Amido Black (AB) staining, with representative immunoblots and molecular weights from each experiment. Data are means ± SEM (N = 5-7/group). *P < 0.05 compared to CON of the same protein and tissue.

### *3.13 Summary and Conclusions*

This study represents the first attempt to specifically examine the role of FATP4 in fetoplacental biology using a loss-of-function gene targeting approach during early embryonic development. Our results confirm that lentiviral transfection of the CRISPR-Cas9 construct targeting the *FATP4* gene in D9 hatched blastocyst successfully deleted *FATP* mRNA on fetal side of the

placenta (cotyledon) as expected. However, persistent FATP4 protein expression in both the fetal and maternal sides of the placenta at GD75 suggests that trafficking of FATP4 protein might have occurred from the maternal side of the placenta where expression was higher in KO animals, or perhaps supply from the maternal circulation [41]. Despite this, *FATP4* gene targeting increased serum and fetal hepatic triglycerides and altered phospholipid fatty acid composition in the fetoplacental circulation, consistent with a disruption of placental DHA transfer and overall handling of serum lipids during pregnancy. However, marked depletion of FATP4 protein from several fetal tissues could also have impacted serum lipids in the fetoplacental circulation. As discussed above, as we have not provided conclusive evidence for lentiviral transfection of the inner cell mass during D9 blastocyst incubation. we cannot rule out alternative explanation for the depletion of FATP4 in fetal tissues in this study.

Depletion of FATP4 protein from fetal tissues presented an opportunity to examine its role on fetal metabolism, which tended to favor a compensatory increase in FATP1 expression and an impairment of carbohydrate utilization in fetal muscle and liver, including a loss of IRS-1 and its regulator kinase JNK by mechanisms that require further investigation. While we cannot rule out off-target effects of CRISPR-Cas9 transfection in D9 blastocysts on GD75 fetal tissues, these results are consistent with induction of a pathologic metabolic risk trajectory when FATP4 expression is deficient. Such findings have potential relevance to human pregnancy complications, including gestational diabetes [42] and intrauterine growth restriction [43, 44] when placental lipid transport and metabolism are disrupted [45]. Therefore, further investigation of fetoplacental FATP4 expression and its dysregulation during fetoplacental development could reveal insights relevant to placental insufficiency and pathologic fetal metabolic programming.

**References**


1. Gude, N.M., et al., Growth and function of the normal human placenta. Thrombosis research, 2004. 114(5-6): p. 397-407.
2. Islam, A., et al., Omega-3 fatty acids transport through the placenta. Asian Journal of Medical and Biological Research, 2016. 2(1): p. 1-8.
3. James-Allan, L.B., et al., Changes in placental nutrient transporter protein expression and activity across gestation in normal and obese women. Reproductive Sciences, 2020. 27(9): p. 1758-1769.
4. Bowman, C.E., Z. Arany, and M.J. Wolfgang, Regulation of maternal–fetal metabolic communication. Cellular and molecular life sciences, 2021. 78: p. 1455-1486.
5. Stremmel, W., et al., A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. Lipids, 2001. 36: p. 981-989.
6. De Carvalho, C.C. and M.J. Caramujo, The various roles of fatty acids. Molecules, 2018. 23(10): p. 2583.
7. Innis, S.M., Fatty acids and early human development. Early human development, 2007. 83(12): p. 761-766.
8. He, Q., et al., Cellular uptake, metabolism and sensing of long-chain fatty acids. Frontiers in Bioscience-Landmark, 2023. 28(1): p. 10.
9. Uauy, R. and A.D. Dangour, Nutrition in brain development and aging: role of essential fatty acids. Nutrition reviews, 2006. 64(suppl_2): p. S24-S33.
10. Zhang, S., et al., Placental adaptations in growth restriction. Nutrients, 2015. 7(1): p. 360-389.
11. Herrmann, T., et al., Mouse fatty acid transport protein 4 (FATP4): characterization of the gene and functional assessment as a very long chain acyl-CoA synthetase. Gene, 2001. 270(1-2): p. 31-40.
12. Larqué, E., et al., Expression pattern of fatty acid transport protein-1 (FATP-1), FATP-4 and heart-fatty acid binding protein (H-FABP) genes in human term placenta. Early human development, 2006. 82(10): p. 697-701.
13. Mishima, T., et al., The expression and function of fatty acid transport protein-2 and-4 in the murine placenta. PloS one, 2011. 6(10): p. e25865.
14. Brett, K.E., et al., Maternal–fetal nutrient transport in pregnancy pathologies: the role of the placenta. International journal of molecular sciences, 2014. 15(9): p. 16153-16185.
15. Zhu, M.J., et al., Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2010. 299(5): p. R1224-R1231.
16. Hutchinson, K.A., et al., Physical activity during pregnancy is associated with increased placental FATP4 protein expression. Reproductive Sciences, 2020. 27(10): p. 1909-1919.

17. Omar, A.K., et al., High-fat diet during pregnancy promotes fetal skeletal muscle fatty acid oxidation and insulin resistance in an ovine model. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2023. 325(5): p. R523-R533.

18. Georgiades, P., et al., Trophoblast-specific gene manipulation using lentivirus-based vectors. Biotechniques, 2007. 42(3): p. 317-325.

19. Purcell, S.H., et al., The involvement of proline-rich 15 in early conceptus development in sheep. Biology of reproduction, 2009. 81(6): p. 1112-1121.

20. Ali, A., et al., The role of LIN28-let-7-ARID3B pathway in placental development. International Journal of Molecular Sciences, 2020. 21(10): p. 3637.

21. Baker, C.M., et al., Development of ovine chorionic somatomammotropin hormone-deficient pregnancies. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2016. 310(9): p. R837-R846.

22. Hord, T.K., et al., Placenta specific gene targeting to study histone lysine demethylase and androgen signaling in ruminant placenta. Animal Reproduction, 2020. 17(3): p. e20200069.

23. Ali, A., et al., Trophectoderm-specific knockdown of LIN28 decreases expression of genes necessary for cell proliferation and reduces elongation of sheep conceptus. International journal of molecular sciences, 2020. 21(7): p. 2549.

24. Mulligan, C.M., et al., Inhibition of delta-6 desaturase reverses cardiolipin remodeling and prevents contractile dysfunction in the aged mouse heart without altering mitochondrial respiratory function. Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences, 2014. 69(7): p. 799-809.

25. Zhao, Z. and Y. Xu, An extremely simple method for extraction of lysophospholipids and phospholipids from blood samples. Journal of lipid research, 2010. 51(3): p. 652-659.

26. Li Puma, L.C., et al., Experimental oxygen concentration influences rates of mitochondrial hydrogen peroxide release from cardiac and skeletal muscle preparations. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2020. 318(5): p. R972-R980.

27. Pesta, D. and E. Gnaiger, High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. Methods Mol Biol, 2012. 810: p. 25-58.

28. Chicco, A.J., et al., Adaptive remodeling of skeletal muscle energy metabolism in high-altitude hypoxia: lessons from AltitudeOmics. Journal of Biological Chemistry, 2018. 293(18): p. 6659-6671.

29. Li Puma, L.C., et al., Experimental oxygen concentration influences rates of mitochondrial hydrogen peroxide release from cardiac and skeletal muscle preparations. Am J Physiol Regul Integr Comp Physiol, 2020. 318(5): p. R972-R980.

30. Curran-Everett, D., D.J. Benos, and S. American Physiological, Guidelines for reporting statistics in journals published by the American Physiological Society. Am J Physiol Endocrinol Metab, 2004. 287(2): p. E189-91.

31. Larqué, E., et al., Docosahexaenoic acid supply in pregnancy affects placental expression of fatty acid transport proteins. The American journal of clinical nutrition, 2006. 84(4): p. 853-861.

32. Schwenk, R.W., et al., Fatty acid transport across the cell membrane: regulation by fatty acid transporters. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 2010. 82(4-6): p. 149-154.

33. Okura, T., et al., Postprandial fatty acid-binding protein 4 is associated with muscle insulin resistance. Diabetologia, 2024. 67(10): p. 2304-2315.

34. Stahl, A., et al., Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. Developmental cell, 2002. 2(4): p. 477-488.

35. Toyoshima, Y., et al., Deletion of IRS-1 leads to growth failure and insulin resistance with downregulation of liver and muscle insulin signaling in rats. Scientific Reports, 2025. 15(1): p. 649.

36. Matsuzaka, T. and H. Shimano, Molecular mechanisms involved in hepatic steatosis and insulin resistance. Journal of diabetes investigation, 2011. 2(3): p. 170-175.

37. Farese, R.V., et al., The problem of establishing relationships between hepatic steatosis and hepatic insulin resistance. Cell metabolism, 2012. 15(5): p. 570-573.

38. Brumbaugh, D.E. and J.E. Friedman, Developmental origins of nonalcoholic fatty liver disease. Pediatric research, 2014. 75(1): p. 140-147.

39. Döring, S., et al., Elevation of blood lipids in hepatocyte-specific fatty acid transport 4-deficient mice fed with high glucose diets. Molecular Genetics and Metabolism, 2019. 126(1): p. 30-38.

40. Jeon, J., et al., Retroelement insertion in a CRISPR/Cas9 editing site in the early embryo intensifies genetic mosaicism. Frontiers in Cell and Developmental Biology, 2019. 7: p. 273.

41. Li, H., et al., Role of fatty acid transport protein 4 in metabolic tissues: insights into obesity and fatty liver disease. Bioscience reports, 2022. 42(6): p. BSR20211854.

42. Wijendran, V., et al., Maternal plasma phospholipid polyunsaturated fatty acids in pregnancy with and without gestational diabetes mellitus: relations with maternal factors. The American journal of clinical nutrition, 1999. 70(1): p. 53-61.

43. Cetin, I., et al., Intrauterine growth restriction is associated with changes in polyunsaturated fatty acid fetal-maternal relationships. Pediatric Research, 2002. 52(5): p. 750-755.

44. Felton, C., et al., Umbilical vessel wall fatty acids after normal and retarded fetal growth. Archives of Disease in Childhood-Fetal and Neonatal Edition, 1994. 70(1): p. F36-F39.

45. von Beckerath, A.-K., et al., Perinatal complications and long-term neurodevelopmental outcome of infants with intrauterine growth restriction. American journal of obstetrics and gynecology, 2013. 208(2): p. 130. e1-130. e6.

# CHAPTER V:
# CONCLUDING REMARKS AND FUTURE DIRECTIONS

Maternal diet during pregnancy has been associated with offspring metabolic health later in life; however, the specific biological links between diet, the intrauterine environment, and fetal metabolism that provide the bases for this relationship are not well understood. The studies in this dissertation established the feasibility of using an ovine model to study these interactions, specifically demonstrating impacts of maternal high-fat feeding on circulating fetoplacental nutrient supply and fetal muscle metabolism in Chapter 2 that parallel recent evidence from non-human primates. In particular, results of this study corroborated evidence for impaired insulin signaling and a shift in utilization of fat over carbohydrates for energy production, which may predispose offspring to develop metabolic disease later in life. Future studies are recommended to investigate the longer-term consequences of these effects during pregnancy in this model, along with potential mechanisms responsible for fetal "programming" of offspring metabolic status *in utero* (e.g., epigenetic modifications such as DNA methylation and histone acetylation impacting nutrient metabolism or insulin action). Such studies are also recommended to elucidate the longer-term impacts of prenatal DHA supplementation on offspring metabolic phenotype given the diverse tissue-specific effects on fetal fatty acid transporter expression and metabolism revealed by our studies in Chapter 3. This study also revealed parallels between maternal DHA supplementation and high fat feeding that merit further study, particularly in light of emerging evidence from human observation studies suggesting potential adverse effects of prenatal DHA supplementation on offspring metabolic risk.

There are several limitations of these studies that are worth noting, including the generally small sample size that reduced statistical power and prevented our ability to detect fetal sex differences in responses to the high-fat or DHA supplemented diets. We also acknowledge that only a single dose/level of supplemental fat and DHA were studied in Chapters 2 and 3, so we cannot rule out different response to higher or lower doses than those used in our studies. This being said, we did select levels of excess fatty acid and DHA supplementation that are well within the range of doses used in previous experimental studies in both ruminants and monogastric mammals, which reasonably parallel achievable intake levels in humans. It is also important to note that our static measurements of fetal tissue and serum fatty acids and metabolism in the fetoplacental circulation are insufficient for determining actual rates placental transfer or fetal uptake or *in vivo* metabolic flux. Perhaps most importantly, we reveal the potential pitfalls of using CRISPR-Cas9 targeting genes of D9 blastocysts via lentiviral transfection for selectively deleting a target protein from the GD75 placenta in Chapter 4, both in terms of placental selectivity and the possibility of protein migration from the caruncle or maternal circulation despite mRNA depletion from the cotyledon. In addition, the striking links between depletion of FATP4 with loss of IRS-1 and JNK protein from fetal muscle and liver in this study may reflect either co-regulation of these genes/proteins during development or off-target effects of the CRISPR-Cas9 constructs applied during early embryo development. Clearly, additional studies are needed to resolve true biological effects from experimental artifacts regarding the impacts of FATP4 on fetoplacental metabolism, as well as to further establish the feasibility of selective gene targeting in the trophectoderm of D9 ovine blastocysts as means of studying impacts of placental gene expression on fetal development – particularly when the target gene is highly expressed in fetal tissues.