High fructose intake during pregnancy adversely affects insulin resistance, lipid profiles, and affects placental gene expression of nutrient transporters and angiogenesis in rat dams and their pregnant offspring

by

Aleida Song

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

Doctor of Philosophy

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

University of Alberta

ABSTRACT

Fructose intake has increased over the past several decades due to high consumption of sugary foods and drinks. In non-pregnant humans and animals, high fructose intake results in hyperlipidemia, insulin resistance and weight gain, although its effects on metabolic adaptations to pregnancy and on offspring have not been well-defined thus far. Three studies were conducted for this thesis with the overall purpose of assessing the effects of fructose intake on physiological changes related to pregnancy in dams and their pregnant offspring.

In each study, female rats received a 10% fructose solution (FR) or distilled water (CONT) prior to and during pregnancy. Offspring remained on the same diet treatment as their mothers (Dams) prior to and during pregnancy. Glycemic control was assessed by measuring glucose and insulin concentrations throughout the study and an oral glucose tolerance test toward the end of pregnancy. Body weights and body composition were also measured. Gene expression for nutrient transporters and vascular development were measured in the placenta of Dams and Offspring.

The first study compared the effects of fructose intake between pregnant and age-matched, non-pregnant rats (Dam-FR, n=6; NP-FR, n=5; Dam-CONT, n=4; NP-CONT, n=6). Dams and NP rats given fructose solution had increased concentrations of insulin (P=0.0031) and triglyceride (P=0.0476) compared with CONT (Dams and NP). Pregnancy did not accentuate the effects of FR on these outcomes.

The second study compared the metabolic effects of intake of the fructose solution vs. distilled

water on pregnant rats (Dams) and their female offspring (Offspring) when pregnant (Dam-FR, n=16; Dam-CONT, n=14) and female offspring when pregnant (Offspring-FR, n=10; Offspring-CONT, n=10). Fructose intake increased body weight (P=0.0003), plasma glucose, insulin and triglyceride concentrations (all P<0.0001) and % fat mass (P<0.0001) in Dams and Offspring. Offspring-FR had higher plasma triglyceride concentrations than Dams-FR (P=0.0099) and both CONT groups. Placenta from Dams-FR and Offspring-FR had increased mRNA expression of FABP1 (P=0.0004) but not other nutrient transporters (GLUT1 and SNAT2). Markers of angiogenesis (VEGF-A, P=0.0015; VEGFR-2, P=0.0014) were altered and CD31 expression was reduced (marker of endothelial cells; P=0.0129) with FR. Placentae and fetuses from Dams and Offspring given FR weighed less than those from CONT groups (P=0.0002 and P=0.0004, respectively).

The third study compared the effect of intake of fructose (Dam-FR, n=14) vs. distilled water (Dam-CONT, n=12) on body weight and metabolic characteristics in two sequential pregnancies. Body weights were higher in the second than the first pregnancy (P<0.0001). Plasma glucose concentrations were higher in the second pregnancy at the mid-way point (P=0.0341) compared with the first pregnancy. Insulin concentrations were higher throughout the second vs. the first pregnancy (P=0.0182). Fructose intake did not exacerbate these effects.

Overall, intake of a 10% fructose solution induces insulin resistance and hypertriglyceridemia in pregnant rats and the adverse effects of fructose intake during pregnancy were similar in the first and second pregnancy. Hypertriglyceridemia was more pronounced in pregnant Offspring consuming FR. This suggests that the effect of dietary programming on lipid metabolism occur in conjunction with a physiological challenge and points to the potential of dietary intake of

fructose to adversely influence insulin resistance and lipid metabolism in mothers and their offspring when pregnant. Alterations in maternal metabolites and body composition were correlated with changes in placental development, particularly nutrient transporters, angiogenesis and vascular development. Overall changes in maternal metabolic profiles and placental development may be related to adverse health outcomes (such as hyperlipidemia) in the mother and offspring in later life.

PREFACE

This thesis is an original work by Aleida Song. The study was developed by Dr. Rhonda Bell, and funded by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Faculty of Medicine & Dentistry at the University of Alberta. The study protocol (Protocol # AUP191) was approved by the Research Ethics Office of the University of Alberta, in accordance with regulations set by the Canadian Council on Animal Care (CCAC).

This thesis has six chapters: Chapter 1 provides a general introduction and a review of previous studies. I was responsible for a literature search to understand the potential molecular links between excess fructose intake and health consequences by searching the literature regarding the effects of maternal nutrition on health and feto-placental development. Chapter 2 provides the background, objectives and hypothesis of three studies (Study A, Study B and Study C). Chapter 3 describes experimental designs, protocols, methods and statistical analysis that were used in three studies. Mrs. Abha Dunichand-Hoedl and Mrs. Nicole Coursen provided assistance with animal care as well as tissue collection and tissue processing following termination. I and Dr. Stuart Astbury were responsible for animal monitoring, tissue collection and performing laboratory experiments. Mr. Brent Nielsen provided technical assistance in animal monitoring, tissue collection, primer design and performing RT-polymerase chain reaction (PCR). *In situ* hybridization protocol was provided by Dr. David Simmons (University of Queensland) and Dr. David Natale (University of California). Dr. David Natale provided a primer design protocol, and Dr. Kunimasa Suzuki helped with the primer design and provided guidance and assistance for

RNA extraction and gene expression analysis using RT-PCR. Mr. Gary Sedgwick guided the analysis of α-tocopherol in the liver and the placenta by HPLC. Mr. Sedgwick and I optimized existing protocols of α-tocopherol analysis for placenta samples. Mrs. Kristina MacNaughton helped with immunohistochemistry using markers for endothelial cells and oxidative stress in the placentae. Dr. Caroline Richard helped with statistical analysis using repeated measures in SAS. Chapter 5 provides an overview and synthesis of the results from three studies and describes limitations. Chapter 6 describes general conclusion of the studies and future directions.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Rhonda Bell for the opportunity to study in her research group, her support and encouragement throughout the course of my Ph.D. I would also like to thank the members of my supervisory committee, Dr. Denise Hemmings and Dr. Walter Dixon for the guidance, support and advice.

Financial support for this work was provided by the Natural Sciences and Engineering Research Council of Canada and the Faculty of Medicine & Dentistry. I am grateful to the University of Alberta and the Alberta Diabetes Institute for supporting me through the Ph.D. program.

Many people encouraged and supported me on various occasions during the study. I would like to thank Mrs. Abha Dunichand-Hoedl for teaching me many laboratory skills and supporting me with all the experiments that I conducted. A special thank goes to Dr. David Natale for guidance in *in situ* hybridization, and Mr. Gary Sedgwick for guidance in the HPLC analysis. I am grateful for the technical support on mRNA analysis and *in situ* hybridization that I received from Dr. Kunimasa Suzuki. The work would not have been possible without his help. A special thank goes to Mrs. Nicole Coursen for her supports with animal care and sample collections, and Mrs. Kristina MacNaughton for assistance with lab experiments. I would also like to thank Ms. Susan Goruk and Mrs. Marnie Newell for their encouragement and support. Many thanks to Mrs. Jody Forslund, the graduate program administrator of the Department of Agricultural, Food and Nutritional Sciences, and Mrs. Tracy Vetsch, the HR/finance administrator of Human Nutrition Research Division.

I would like to thank my fellow graduate students, both past and present, for their friendship and

help: Dr. Fatheema Begum Subhan, Jocelyn Graham, Doaa Dahlawi, Yuzhu Liang, Hara Nikolopoulos, Jessica Thompson, Dr. Megan Jarman, Dr. Sarah Elliott, Laura Adam, Jill Morris, Maira Quintanilha, Leticia Cristina Radin Pereira, Stephanie Ann Babwik, Janis Baarda. A special thank you goes to Dr. Stuart Astbury for his great help with lab experiments and academic writing and for being a companion for the trip to the Starbucks every day. Thank you to Brent Nielsen and Rachel Jihyon Son for their help in the experiments. I would also like to thank Dr. Caroline Richard for her help with statistical analysis of the study. I will not forget the great memories and support from my friends and colleagues: Dr. Hyunju Chung, Erin Lewis, Dr. Wenlin Yu, Lira Yun, Bomi Kim, Euna Oh.

I would also like to express my special thanks to Dr. John Kennelly who was my father's Ph.D. supervisor and became my great mentor and supporter. A special thank you goes to my Canadian mother, Louise Kennelly. I will not forget her great support and the memories she gave me in my life. I would like to express my gratitude for the support and encouragement from my Canadian family, Mr. John Collier, Mrs. Judy Collier, my sisters Jennifer Michalycia and Jacquelyn Collier, and all members of Collier family. I would not have been able to complete the Ph.D. program without their support.

Finally, I would like to thank my Korean family. I am grateful for the support and love I received from my parents, Man-Kang Song and Hyang Sook Lee, and my sister, Helene Song. I would like to thank my father especially for being a great mentor throughout the course of my Ph.D. I would also like to thank my parents-in-law, Won-Jae Lee and In-Hyun Song, and brother-in-law, James Jung-Jun Lee. Lastly, I would like to thank my husband, John Jungjin Lee, for being by my side. I would not have been able to accomplish this without his support and love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	acetyl-coenzyme A carboxylase 1
Acetyl-CoA	acetyl-coenzyme A
ACTH	adrenocorticotrophin hormone
Ang-1	angiopoietin-1
AOPP	advanced oxidation protein products
AUC	area under the curve
BCA	bicinchoninic acid assay
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BSA	bovine serum albumin
CAT	catalase
CD36	cluster of differentiation 36
CNS	central nervous system
CONT	control group
ChREBP	carbohydrate-responsive element-binding protein
CRH	corticotrophin-releasing hormone
Ct	threshold cycle
CuZnSOD	copper/zinc superoxide dismutase
DAPI	4,6-diamidino- 2-phenylindole
DBP	diastolic blood pressure
DHE	dihydroethidium
DHR	dihydrorhodamine 123
DIG	digoxigenin
DOHaD	Developmental Origins of Health and Disease
EB	ethidium bromide
ECSOD	extracellular superoxide dismutase
11β-HSD	11β-hydroxysteroid dehydrogenase
eNOS	endothelial nitric oxide synthase
FABP	fatty acid binding protein

FABPpm	plasma membrane fatty acid binding protein
FAS	fatty acid synthase
FAT	fatty acid translocase
FATP	fatty acid transport protein
FFA	free fatty acid
FLK-1	fetal liver kinase-1
FLT	fms-like tyrosine kinase receptor
FOXO1	forkhead box protein O1
FR	fructose group
FSH	follicle-stimulating hormone
GD	gestational day
GDM	gestational diabetes mellitus
GLUT	glucose transporter
GnRH	gonadotropin-releasing hormone
GPX	glutathione peroxidase
GR	glucocorticoid receptor
GSH	glutathione
GSSG	glutathione disulfide
H&E	hematoxylin and eosin
H_2O_2	hydrogen peroxide
HBSS	hanks balanced salt solution
hCG	human chorionic gonadotropin
HDL	high-density lipoprotein
HFCS	high fructose corn syrup
HIF-1	hypoxia inducible factor-1
HOMA-IR	homeostasis model assessment- insulin resistance
HPA	hypothalamic-pituitary-adrenal
HPFS	Health Professionals Follow-Up Study
hPL	human placental lactogen
HPLC	high-performance liquid chromatography

IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding proteins
ISH	*in situ* hybridization
IUGR	intrauterine growth restriction
JZ	junctional zone
KDR	kinase insert domain receptor
LAT	large neutral amino acid transport
LBW	low birth weight
LDL	low-density lipoprotein
LH	luteinizing hormone
LPL	lipoprotein lipase
LZ	labyrinth zone
MABT	maleic acid buffer containing tween 20
MDA	malondialdehyde
MeAIB	methylaminoisobutyrate
MMP-9	metalloproteinase 9
MnSOD	manganese superoxide dismutase
NHANES	National Health and Nutrition Examination Survey
NHS	National Health Service
NBT	nitro-blue tetrazolium chloride
Nox	nicotinamide adenine dinucleotide phosphate oxidase
OCT	optimal cutting temperature compound
OGTT	oral glucose tolerance test
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECAM	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
PlGF	placental growth factor
pO_2	oxygen partial pressure

QMR	quantitative magnetic resonance analysis
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
SBP	systolic blood pressure
SDS	sodium dodecyl sulfate
sFLT	soluble fms-like tyrosine kinase receptor
SGA	small for gestational age
SGLT	sodium/glucose co-transporter 1
SMA	smooth muscle actin
SNAT	sodium-coupled neutral amino acid transporter
SOD	superoxide dismutase
SREBP1	sterol regulatory element-binding protein 1
SSB	sugar-sweetened beverages
STZ	streptozotocin
TBARS	thiobarbituric acid reactive substances
TCA cycle	tricarboxylic acid cycle
TG	triglycerides
TMB	tetramethylbenzidine
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor 1
VEGFR-2	vascular endothelial growth factor receptor 2
VLDL	very low-density lipoprotein
WHO	World Health Organization
w/v	weight:volume
w/w	weight/weight

CHAPTER 1: LITERATURE REVIEW

1. Fructose

1.1 Fructose properties

Fructose ($C_6H_{12}O_6$) is a ketohexose monosaccharide arranged in a five-carbon furanose ring structure (Figure 1-1) and forms a common disaccharide, sucrose, when bound to glucose (1). Purified fructose is white and crystalline in solid form and is known to be the sweetest of all carbohydrates (1). It has a synergistic effect on sweetness which means that it intensifies the sweet taste of other nutritive and non-nutritive sweeteners such as sucrose and aspartame (2). D-fructose is the form found commonly in nature and, therefore, is the most important form from the perspective of human nutrition and physiology (2).

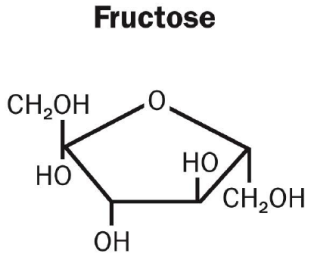


Figure 1-1: The chemical structure of D-fructose

1.2 Food sources of fructose, fructose consumption and recommendation for sugar

Fructose occurs naturally in high concentrations in some foods such as honey and is present in many fruits either as sucrose or fructose (3). It is also derived from corn syrup. Purified fructose

is used as a sweetener in many processed foods and beverages (3). Sucrose was the main source of dietary fructose until the development of the industrial process that has allowed for the production of high fructose corn syrup (HFCS) in the early 1970's (4). Today, three types of HFCS are used commonly in foods and drinks: HFCS-42, HFCS-55, and HFCS-90 (4). The number indicates the percentage of fructose present (4). HFCS-42 is used mostly in processed foods and baked goods, whereas HFCS-55 is added to soft drinks (4). Since the United States Department of Agriculture allowed the replacement of more expensive sucrose with HFCS in processed foods, HFCS is now one of the most common sources of dietary fructose among North Americans (4).

Fructose consumption has increased largely through sugar-sweetened beverages (SSB) and sweet desserts (5). Elliott et al. (2002) reported that fructose intake from sucrose and HFCS increased from 64 g/day in 1970 to 81 g/day in 1997 (6). Bray et al. (2004) estimated total fructose intake to increase from 8.8 % of total calories in 1977 – 1978 to 11.5 % of total calories in 1994 – 1998 among Americans greater than two years of age, according to data collected by the United States Department of Agriculture (7). Miriam et al. (2008) estimated that fructose consumption in the U.S. was 54.7 g/day (10.2% of total calorie intake) in 1988 – 1994 (3), based on data collected in the large, nationally representative sample of U.S. adults and children called the National Health and Nutrition Examination Survey (NHANES) III. Miriam et al. (2008) found that the main contributor of dietary fructose was SSB (30.1 %), grains (cakes, pies, and cereals; 21.5 %), and fruit juices (19.4 %) (3). Fructose intake was the highest among adolescents 12 – 18 years of age (22.4 %) compared with all other age groups. In general, those in the younger age groups (2 – 5, 6 – 11, 12 – 18, and 19 – 30 years) had significantly higher fructose intake than those in the 31 –

50-year age group (3).

There is little information available about fructose intake from added sugar[1] and HFCS among Canadians (8). In a recently published paper, Brisbois et al. (2014) estimated consumption of added sugars and the main food sources of sugar in the diets of Canadians using data from the 2004 Canadian Community Health Survey (8). Total sugar[2] consumption was 116 g/day, which is equivalent to 21 % of total calorie intake of Canadians. Added sugar accounted for 11 – 13 % of total energy intake (8). Adolescents (9 – 18 years) consumed total sugars mostly from soft drinks, and flavoured milk and fruits, whereas adults (19^{+} years) consumed total sugars largely from soft drinks, table sugar and fruits (8). Although the consumption of HFCS may be overestimated (i.e. all soft drinks were considered to be caloric) or underestimated (category "sugar and syrups" did not include corn sweeteners), these data were best estimates (8).

In March in 2015, a new World Health Organization (WHO) guideline recommends both adults and children reduce their intake of added sugars[1] to <10 % of total energy intake/day. For additional health benefits, the guideline recommends to reduce daily consumption of added sugars to <5 % (25 g, 6 teaspoons)/day. In Canada, only a Tolerable Upper Intake level is set for added sugars[1] by Canada's Food Guide. A maximal intake of added sugars is ≤25 % of total energy intake for both adults and children.

[1] Added sugar: sugars added to foods (8)

[2] Total sugar: all sugars, both naturally occurring and added (8)

1.3 Digestion, absorption and metabolism of fructose

1.3.1 Digestion and absorption

Following ingestion, free fructose is primarily absorbed in the jejunum, whereas sucrose needs to be first broken down into its two monosaccharides, glucose and fructose, by sucrase (9). After fructose reaches the intestine, it is transported across the brush border membrane of the enterocyte by glucose transporter 5 (GLUT5) or glucose transporter 2 (GLUT2) (9). GLUT5 is a facilitated fructose transporter that has a low affinity but high capacity for fructose (9). GLUT2 has a low affinity and high capacity for glucose, but it also has the ability to transport three monosaccharides including glucose, galactose, and fructose (9). A diet high in sucrose can result in saturation of sodium/glucose co-transporter 1 (SGLT1), which induces translocation of GLUT2 to the apical membranes of enterocytes, and saturation of GLUT5 (10). This change can facilitate additional uptake of glucose and fructose by enterocytes. After absorption is complete, GLUT2 is re-localized to the basolateral membrane (9).

The capacity and tolerance for fructose absorption in healthy individuals varies from less than 5g of fructose to more than 50 g of fructose for three to four hours after ingestion (11). However, high-fructose intake or fructose intolerance as a result of a defect in hepatic aldolase B function can cause incomplete absorption of fructose, resulting in adverse gastrointestinal symptoms including bloating, flatulence, loose stools, and diarrhea (12). The mechanisms contributing to these symptoms are not clearly understood, but might be due to the osmotic effects of fructose in the intestine (13) and/or incomplete bacterial fermentation of absorbed fructose in the colon to short-chain fatty acids (14). The consumption of fructose with glucose and galactose, particularly

if equal amounts of fructose and glucose can be consumed, may help to prevent fructose malabsorption (11, 15). Thus, fruits (ex. banana, strawberries) that provide equal amounts of glucose and fructose may be readily tolerated and reduce the symptoms of fructose intolerance (11). On the other hand, consumption of processed foods containing a high amount of free fructose or excess fructose vs. glucose may aggravate gastrointestinal symptoms in people with fructose intolerance, or other bowel-related disorders and may increase these adverse symptoms in healthy populations (11).

1.3.2 Liver metabolism of fructose

Once absorbed, fructose is transported to the liver through the hepatic portal vein (6). In the liver, two GLUT transporter isoforms, GLUT2 and GLUT5, have the capacity to take up fructose (6). Once in the liver, fructose is phosphorylated to fructose-1-phosphate by fructokinase and fructose-1-phosphate is then split by aldolase B into glyceraldehyde and dihydroxyacetone phosphate (Figure 1-2, 6). Triose kinase converts glyceraldehyde to glyceraldehyde 3-phosphate while triose-phosphate isomerase converts dihydroxyacetone-phosphate to glyceraldehyde 3-phosphate (6). Both glyceraldehyde and dihydroxyacetone phosphate bypass the rate-limiting step of phosphofructokinase-1 in the glycolytic pathway, thus ingestion of fructose can rapidly increase the substrates for glycogenesis and lipogenesis (6).

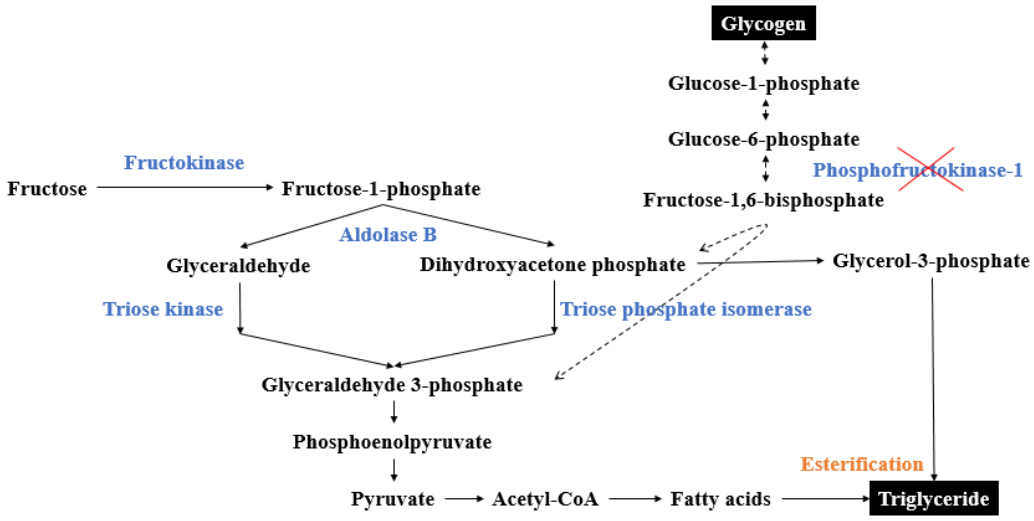


Figure 1-2: Fructose metabolism in the liver

De novo lipogenesis Fructose in the liver generates pyruvate which is transformed to acetyl-coenzyme A (acetyl-CoA) by pyruvate dehydrogenase in the mitochondria (Figure 1-3, 16). Acetyl-CoA is oxidized to generate ATP, H_2O and CO_2 through the tricarboxylic acid cycle (TCA cycle) (16). In the cytosol, acetyl-CoA is catalyzed to malonyl-CoA by acetyl-CoA carboxylase 1 (ACC1) and utilized to form palmitate by fatty acid synthase (FAS). Palmitate is then available to synthesize triglycerides (TG) and very low-density lipoprotein (VLDL) (16). After the liver releases VLDL into circulation, TG are removed from VLDL by lipoprotein lipase (LPL) for energy storage and utilization (16).

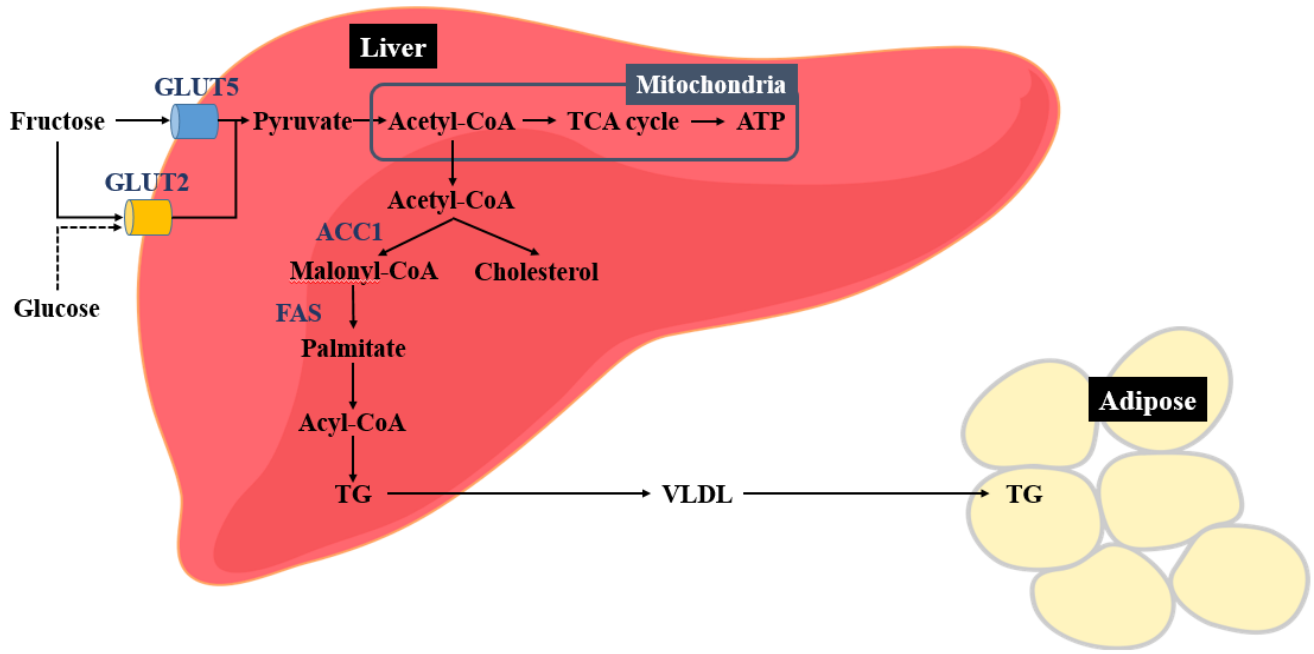


Figure 1-3: Fructose-induced de novo lipogenesis

Comparison of De novo lipogenesis following consumption of sucrose, fructose or HFCS

There is some suggestion that fructose in its different forms may have different metabolic effects since a recent study found that sucrose, purified fructose and HFCS appear to have different effects on hepatic lipogenesis (349). In Mock's study (2017), 4-week-old female Sprague-Dawley rats were provided with water containing 13 % (w/v) of fructose, sucrose or HFCS-55 for eight weeks (349). The mRNA expression of enzymes involved in hepatic lipid synthesis was measured by qPCR, and TG content in the liver was determined using a colorimetric assay kit and histology (349). In this study, rats fed the fructose solution consumed higher amount of food than rats fed the sucrose while those given the HFCS-55 solution had the highest total caloric intake than the other groups. This indicates that the HFCS-55 group

consumed a larger number of calories from their fluid compared to other rats (349).

In the liver, all solutions increased hepatic gene expression of FAS and stearoyl-CoA desaturase-1 which are both involved in lipid metabolism (349). However, differences were found in β-oxidation since there was decreased gene expression of peroxisome proliferator-activated receptor-α in the liver of rats fed HFCS-55 and sucrose solutions but not those fed fructose solution. Furthermore, fatty acid esterification processes could have been affected since there was (increased exression of diglyceride acyltransferase 2 in the liver of rats given sucrose, but not HFCS-55 and fructose solutions (349). The authors note that HFCS-55 consumption lead to the most extensive *de novo* lipogenesis as suggested by the increased hepatic lipid accumulation that was accompanied by down-regulation of peroxisome proliferator-activated receptor-α and no change in outflow (349). The authors suggested that based on these results it appears that consumption of beverages sweetend with HFCS could be more lipogenic than intake of sucrose and fructose, and thus, intake of HFCS should be limited (349). Further study in humans is needed to corroborate these findings.

1.4 The health implications of high fructose consumption

As the prevalence of obesity and metabolic disorders has been increasing globally over the past several decades, high fructose consumption has been considered as a key determinant in developing these disorders. Both animal and human studies suggest that high fructose intake (>10 % of total calorie intake) may lead to adverse health outcomes; these are described in detail below.

1.4.1 Weight gain and obesity

HFCS consumption, weight gain and development of obesity in humans High fructose intake has attracted public health concerns since HFCS began to replace sucrose in the food supply in the 1970's (5, 6). Several investigators have noted that fructose consumption from HFCS or free fructose is associated with weight gain or the prevalence of obesity (17, 18, 19). For example, Malik et al. (2013) conducted a meta-analysis of prospective cohort studies and randomized controlled trials to examine the relationship between the consumption of SSB and prevalence of childhood obesity (20). Total 22 cohort studies and two randomized controlled trials showed the positive association between the consumption of SSB and weight gain in both adults and children (20). In children, the relationship between SSB intake and childhood obesity was described by Ludwig and colleagues in 2001 by studying 548 children, beginning at grades six and seven in 1995 (baseline) to 1997 for 19 months (18). Children's BMI and the incidence of obesity increased with each additional serving of SSB after adjustment for baseline anthropometrics and demographics (18). Bray et al. (2004) examined the relationship related to fructose production and the prevalence of obesity in the U.S. (7) by combining studies done by Vuilleumier (1993) examining HFCS and crystalline fructose production (19) and by Flegal et al. (2002) that examined the prevalence of overweight (BMI≥25 kg/m^2) and obesity (BMI≥30 kg/m^2) using NHANES data (21). Bray's analyses noted that increased HFCS availability is associated with increases in obesity rates over several decades (7). However, a number of studies found no association between HFCS intake and the prevalence of obesity (22, 23, 24), and, Klurfeld et al. (2013) suggested that it was difficult to imagine, from a physiological perspective, that the prevalence of obesity was significantly increased by HFCS consumption but not sucrose. This is

important since there are relatively small differences in the proportions of glucose and fructose in the different types of HFCS compared with sucrose (23). In addition, sucrose and HFCS are absorbed at the same rates through the gastrointestinal tract. Clearly the link between HFCS, weight and the development of obesity deserves further attention.

Fructose intake and body weights in rats There is a considerable body of work in animal models that indicate that high fructose intake causes increased body weight (15, 25). In Bocarsly's study (2010), an 8 % (weight:volume, w/v) HFCS solution, a 10 % (w/v) sucrose solution or water was given to male rats in their drinking water along with *ad libitum* chow for short (8 weeks) and long-term (6 – 7 months) (25). In the short-term, rats provided with HFCS gained significantly more weight than rats fed the sucrose solution (25). During the long-term experiment, rats with 12-hour access to HFCS experienced a 234 % increase in their body weight, while those with 24-hour access to HFCS had a 257 % increase in body weight, above baseline (25). Rats fed HFCS had increased body adiposity at six months of age compared to rats fed *ad libitum* chow with water and this was most pronounced in the abdominal region (25). Light et al. (2009) also reported that 5-week-old female Sprague-Dawley rats fed 13 % (w/v) HFCS solution with *ad libitum* chow for 8 weeks had the highest increase in body weight and adiposity compared to rats that consumed a 13 % (w/v) glucose solution or tap water (26). In this study, the rats consuming fructose solution had significantly higher amounts of retroperitoneal and gonadal adipose tissue than rats fed the glucose solution or tap water (26).

Not all studies have observed higher body weights in rats given high amounts of fructose (27, 28). Hwang et al. (1987) indicated that the body weights of young male Sprague-Dawley rats, weighing 160 – 180 g at the start of the study and fed a diet containing 66 % of calorie from

fructose for two weeks, did not differ from those of male rats fed a chow diet (27). Bell et al. (1996) also reported that consumption of a fructose diet did not alter the body weights of male rats (28). The authors examined the effects of diet-induced insulin resistance on glucose and lipid metabolism as well as β-cell mass (28). In this study, male Wistar rats at nine – ten weeks of age underwent islet transplantation and were assigned to receive a 40 % (weight/weight, w/w) fructose, a 70 % (w/w) fructose, or a control diet (28). The body weights of male rats in both the 40 % (w/w) and 70 % (w/w) fructose diet groups did not differ from those of rats fed a control diet (28). Thus, fructose intake does not always alter body weights of growing male rats. Moreover, most of previous studies have focused on the effects of fructose intake on body weights of male rats only. Therefore, information on the relationship between fructose intake and body weights in female rats is still limited.

Differences in the effects of fructose intake on body weights between studies could be dependent on the form of fructose delivery (liquid vs. solid) There was a suggestion that the increases in body weight may be largely promoted by fructose in the liquid rather than a solid form (29, 30). DiMeglio and Mattes (2000) conducted a cross-over study to examine the effects of liquid vs. solid forms of fructose intake on total calorie intake and body weight (29). Seven men and eight women were provided with 450 kcal/day of HFCS in liquid (soft drinks) vs. solid form (jelly beans) for four weeks, followed by a four-week washout and another four weeks of dietary treatment. Energy intake during the liquid-fructose-intake period was not changed from baseline but was significantly reduced during the solid-fructose-intake period (29). Body weight and BMI were significantly increased by the end of the period when participants consumed liquid fructose compared to baseline (body weight: After soft drinks=68.2 ± 14.5 kg vs.

baseline=67.7 ± 14.7 kg; BMI=21.9 ± 2.1 vs. 21.8 ± 2.2 kg/m^2) (29). Therefore, fructose in liquid form may promote body weight gain in people more than fructose in a solid form.

Mechanisms Although the effects of high fructose ingestion on body weight are somewhat inconsistent, several investigators have proposed potential mechanisms that could underlie the resulting weight gain when observed (31, 32, 33, 34, 35, 36). One mechanism could be through an acute reduction in the concentrations of insulin and leptin following consumption of foods or beverages containing significant amounts of fructose. Insulin and leptin are key hormones in glucose metabolism, and both have a critical role in the central nervous system (CNS) for long-term energy metabolism (31). Insulin is produced from pancreatic β-cells after glucose administration and regulates glucose uptake into cells as well as leptin production from adipocytes (31, 32). Following glucose ingestion, insulin increases the circulating leptin concentration, and this has a role in the inhibitory control of hunger (31, 32). Thus, reductions in both insulin and leptin production resulting from acute fructose intake (relative to acute glucose intake) may increase one's desire for food intake, and the increased caloric intake then contributes to weight gain.

Additional evidence for involvement of insulin and leptin in these responses comes from studies that confirm that fructose, unlike glucose, does not acutely stimulate insulin production (33, 34). However, evidence from a study using rhesus monkeys indicated that acute ingestion of fructose resulted in lower insulin levels and did not change circulating leptin levels compared to that observed following ingestion of the same amount of glucose (35). Glucose infusion increased glucose and insulin concentrations in plasma and subsequently increased plasma leptin levels, whereas fructose infusion did not stimulate insulin and circulating leptin (35). Teff et al. (2004)

conducted a randomized cross-over study to investigate whether high fructose intake reduces leptin concentrations compared with high glucose intake (36). Twelve normal-weight women (BMI=19.8 – 26.7 kg/m^2) consumed a diet containing 55 % carbohydrate, 30 % fat and 15 % protein as total calorie intake with additional 30% of the carbohydrate intake from either a fructose or glucose-sweetened beverage (36). Blood was collected every 30 – 60 minutes for 24 hours on two days during which subjects consumed meals with the macronutrient distribution described above, and postprandial leptin concentrations were measured (36). The area under the curve for leptin was reduced during the entire period (24 hours) in the fructose group compared to the glucose group (Fructose: 218.5 ± 22.8, glucose: 300.8 ± 30.7 ng/ml/24hour) (36). Thus, long-term fructose intake may decrease satiety and increase energy intake due to dysregulated secretion of insulin and leptin.

In summary, it is clear that the response of insulin and leptin after fructose intake is different from the response of those hormones after glucose consumption. Fructose intake may also promote weight gain and be associated with the prevalence of obesity, but this relationship is not consistent in human and animal studies.

1.4.2 Hyperlipidemia

Fructose intake and lipid synthesis in humans There are a number of studies reporting that regular ingestion of high quantities of fructose increases the levels of low-density lipoprotein (LDL), VLDL cholesterol and TG in animals and humans (37, 38, 39). For example, in humans, fasting TG in plasma were increased by 2-fold and fractional *de novo* lipogenesis was elevated by 5-fold in healthy males who were provided 3 g of fructose/kg body weight as 20 % (w/v)

fructose solution for six days (37). Le et al. (2006) presented similar results in which intake of 1.5 g fructose/kg body weight for four weeks increased TG and glucose levels in plasma of healthy male subjects (38). Intra-hepatocellular and intra-myocellular lipids were also measured, but these concentrations were not changed after four weeks of fructose consumption (38). Le suggested that the formation of ectopic lipids could be prevented by increased formation of VLDL in plasma (38). Hallfrisch et al. (1983) proposed that an elevation of the TG level may be accelerated in people with metabolic disorders (39). In Hallfrisch's study, an increased rate of TG synthesis was observed in participants with hyperinsulinemia but was not observed in healthy individuals after five-weeks of a high fructose diet (39). For example, a fructose diet (15 % of calorie intake as fructose) increased TG levels by 1.07-fold in healthy subjects, whereas the same diet increased TG levels by 1.6-fold in hyperinsulinemic participants (39). In summary, high fructose intake increases lipogenesis in the liver and circulating LDL, VLDL and TG concentrations in blood, and this is accentuated among those who have concomitant hyperinsulinemia. Based on these findings, it appears that intake of high amounts of free or purified fructose is associated with the development of hyperlipidemia.

Fructose intake and lipid synthesis in rats Similar to humans, rats fed a fructose-rich diet (63 % of total calories from fructose) for two weeks had significantly higher fasting hepatic TG and plasma VLDL concentration than did glucose-fed rats (40). Rats that consumed moderate amounts of fructose (18 % fructose and 11 % saturated fatty acids as total calorie intake) had elevated plasma insulin, VLDL, LDL and TG concentrations compared to a control group consuming glucose as the carbohydrate source (41). Intake of high amounts of free or purified fructose significantly increase expression of fructokinase, aldolase B, pyruvate kinase, FAS and

glycerol-3-phosphate acyltransferase, which contribute to the observed increases in circulating lipids and *de novo* lipogenesis (41).

1.4.3 Insulin resistance

Fructose intake and insulin resistance in humans Several investigators have suggested that high fructose intake contributes to the development of insulin resistance in humans, although the data for this is inconsistent. Healthy male subjects were provided with a diet (55 % carbohydrates, 30 % fat and 15 % protein as total calorie intake) containing 1.5 g purified fructose/kg body weight/day as part of the source of carbohydrates for four weeks (38). Plasma lipids, free fatty acid (FFA) and glucose disposal rates were measured before and after the treatment (38). Although the fasting plasma concentrations of TG and FFA were significantly increased after providing the fructose diet, the fasting insulin concentration was not changed compared to before consuming a fructose diet (38). Moreover, the fructose diet did not alter the hepatic glucose output or the glucose disposal rate, suggesting that insulin resistance was not altered (38). Another study, this time in healthy individuals and participants with type 2 diabetes , were given a high fructose diet (55 % carbohydrate, of which 35 % was purified fructose, 30 % fat and 15 % protein as total calorie intake) for seven days (42). All participants had significantly increased fasting VLDL concentrations and lipid accumulation in the liver and muscle, but the diet did not increase plasma glucose or insulin concentrations (42). On the other hand, a study done by Stanhope et al. (2009) showed that fructose consumption decreased insulin sensitivity in overweight and obese subjects (43). Overweight and obese men and women (BMI=25 – 35 kg/m^2) received three servings of glucose- or fructose-sweetened beverages/day (25 % of energy requirements) with a diet containing 15 % protein, 30 % fat and 55 % carbohydrates for ten

weeks (43). Insulin concentrations during an oral glucose tolerance test (OGTT) were significantly higher after consuming fructose beverages, and the insulin sensitivity was decreased by 23.6 % in women and 11.7 % in men consuming fructose (43). Tappy and Kim noted that evidence for an association between high-fructose intake and insulin resistance in humans may be inconsistent since fructose does not invoke acute insulin secretion (22).

Fructose intake and insulin resistance in animals Although it is still unclear whether fructose directly or indirectly reduces insulin sensitivity in humans, a number of studies using animal models support the suggestion that fructose intake is positively associated with insulin resistance (44, 45, 46). One of the potential mechanisms for this could relate to the observation that fructose appears to induce a down-regulation of insulin receptors, thereby leading to insulin resistance (44). Gatena et al. (2003) reported that the mRNA expression of insulin receptors was decreased in skeletal muscle and liver of rats fed a 66 % (of calories) fructose diet compared to that of rats fed a standard chow diet (44). These authors suggested that expression of insulin receptors was lower as a consequence of a chronic increase in plasma insulin concentrations resulting from high fructose intake (44). A second potential mechanism may relate to increased levels of FFA following high fructose intake, which could then alter glucose transport and glucose metabolism in muscle, adipose and hepatic cells (45). Insulin resistance, induced by a high concentration of FFA, is caused by an inhibition of insulin access to the liver and skeletal muscle by changing the expression of the insulin receptor (45). Insulin resistance and increases in circulating FFA following a high fructose diet also alters insulin receptor substrate-1 (IRS-1) levels (45). For example, tyrosine-phosphorylation of insulin receptor substrate-1 was decreased in the liver of rats fed a fructose diet (62 % fructose as a total calories) compared with rats fed a

control diet containing cornstarch as the carbohydrate source (45). A third potential mechanism by which fructose intake may reduce insulin sensitivity involves changes in adiponectin secretion (46). These authors examined this possibility using experiments in 3T3-L1 cells, an adipocyte cell line (46). Expression of adiponectin was increased when 5, 10, and 20 mM fructose was added compared with that observed for the non-treated cells (46). However, the increases in adiponectin expression in cells that were treated with 10 and 20 mM fructose were lower than that seen in cells treated with 5 mM fructose (46). Although the concentrations of fructose used in this experiment are all supraphysiological, they do suggest that exposure to high concentrations of fructose could induce insulin resistance and thereby reduce production and secretion of adiponectin (46).

1.4.4 Hypertension

Fructose intake and hypertension in humans Hypertension is another chronic condition thought to be induced by regular consumption of high amounts of fructose. Experimental evidence in humans suggests that fructose may negatively impact blood pressure (55, 56). For example, healthy adults in a randomized control study who consumed 200 g of 10 % (w/v) fructose solution/day (750 kcal) for two weeks had increased 24-hour SBP and diastolic blood pressure (DBP) (by 7 ± 2 and 5 ± 2 mmHg, respectively) (55). Similar results were found in the study done by Brown et al. (2008) in which the blood pressure of healthy normal-weight participants (nine men and six women) was increased two hours after consuming 500 ml of drinks containing 60 g of fructose (56). Blood pressure was measured continuously from the baseline (30 minutes before drinking fructose solution) to 2 hours after consuming the fructose drink (56). After fructose ingestion, SBP and DBP increased significantly (changes in SBP from

baseline: fructose=4.3 mmHg, control=1.2 mmHg; DBP: fructose=3.5, control=1.3 mmHg) (56).

Some epidemiological data suggests that high fructose intake has a negative impact on blood pressure although not all the studies conducted concur (57, 58). Analysis of data collected from the NHANES (2003 – 2006, USA), examined the relationship between fructose intake from added sugars and blood pressure in adults without a history of hypertension (57). After adjustment for demographics and other dietary factors that have been reported to influence blood pressure such as total calorie intake, total carbohydrate, sodium, potassium, alcohol, and vitamin C intake, higher fructose consumption was significantly associated with elevated blood pressure (57). However, a recent study done by Jayalath et al. (2014) did not support this association (58). Jayalath and colleagues conducted a meta-analysis of three prospective cohort studies: The Health Professionals Follow-Up Study (HPFS), the Nurses' Health Study Ⅰ (NHS-Ⅰ), and the Nurses' Health Study Ⅱ (NHS-Ⅱ); all in U.S. health professionals (58). These three cohorts included 223,230 participants, with the HPFS following men and the other two studies following women (58). Total fructose intake was calculated from food frequency questionnaires, and hypertension (SBP≥140, DBP≥90 mmHg) was either diagnosed by physicians or self-reported on questionnaires (58). The relationship between fructose intake and the prevalence of hypertension was analyzed after adjustments for ages, BMI, physical activity, smoking status, and the following dietary factors: total calorie intake, alcohol, caffeine, folate, and vitamin C consumption (58). Median fructose intake was 5.7 – 6.0 % of total energy intake in each study, and fructose consumption from SSB and fruit juice was 35.2 % (HPFS), 35.6 % (NHS-Ⅰ) and 44.0 % (NHS-Ⅱ) respectively (58). The incidence of hypertension was not associated with fructose intake in these three cohorts (risk ratios=1.02, 95 % CI, 0.99 – 1.04) (58).

Fructose intake and hypertension in rats Studies by Hwang et al. were among the first to describe fructose as a dietary ingredient that induced hypertension and insulin resistance in rats (27). Subsequent studies by Reaven and his lab group suggested that changes in the renin-angiotensin-aldosterone system and lipid metabolism induced by dietary fructose could contribute to hypertension in rats (47, 48). Extensive work in this area has continued and has been the subject of several recent reviews and debate (50, 51). Many of the recent animal studies have identified possible mechanisms by which fructose could cause hypertension (52, 53, 54). For example, rats fed a 5, 10 or 20 % (w/v) fructose solution for 12 weeks, had significantly higher systolic blood pressure (SBP) than that of rats consuming tap water (52). There was no significant difference in blood pressure between rats fed 10 % (w/v) and 20 % (w/v) fructose solution, but blood pressure in rats fed those two solutions was significantly higher than that in rats fed 5 % (w/v) fructose solution, suggesting that a dose-response to fructose intake may occur (52). Kamide et al. (2002) noted that male Sprague-Dawley rats provided with a diet containing 66 % of total calories from fructose (Fr) had a significant elevation in mean SBP as well as in urinary excretion of epinephrine and norepinephrine compared with control rats fed chow *ad libitum* (CONT) for four weeks, (SBP: Fr=131, CONT=117 mmHg; epinephrine: Fr=70.9 ± 9.8, CONT=18.8 ± 4.9 ng/day; norepinephrine: Fr=232.7 ± 43.4, CONT=113.3 ± 33.4 ng/day) (53). Their findings suggest that fructose induces changes in these hormones and may thereby contribute to hypertension (53). Sanchez-Lozada et al. (2008) suggested that fructose-induced hypertension may be induced by hyperuricemia and renal damage (54). Therefore, regular consumption of high amounts of fructose appears to activate mechanisms that could contribute to the development of hypertension (54).

In summary, both human and animal studies suggest the possibility that fructose has a role in increasing blood pressure. The animal studies suggest a number of mechanisms could be affected, including the renin-angiotensin system, through angiotensin 2 receptors type 1 and 2, as well as production or excretion of epinephrine and norepinephrine. Epidemiological studies may continue to shed light on the long-term effects of fructose intake and blood pressure control in humans. Some authors have suggested a need for randomized and double-blind controlled clinical trials to provide more solid evidence to support or refute these findings (58), but these are clearly not ethical or practical for studying the relationship between regular intake of fructose from added sugars and any of the health effects described in this section.

1.4.5 Oxidative stress

Oxidative damage to DNA, proteins and lipids has been known to contribute to many chronic diseases such as diabetes, metabolic syndrome and cancer (61, 62). Oxidative damage is induced by increased oxidative stress which denotes an imbalance between free radical production and antioxidant capacity (Figure 1-4). Although only a few studies have focused on the association between high fructose intake and oxidative stress in humans, a number of studies using animal models have found relationships between increased fructose intake, increased free radical production, reduced antioxidant nutrient status and reduced antioxidant enzyme capacity.

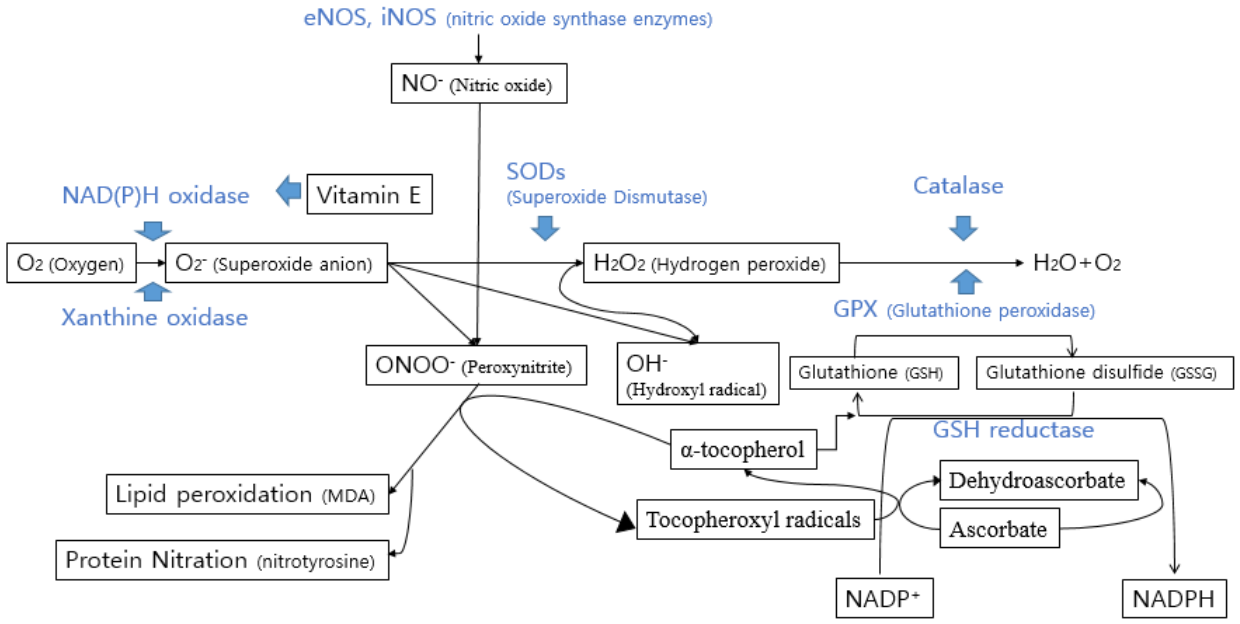


Figure 1-4: The pathway of oxidative stress. Superoxide anion is generated by the reduction of triplet-state molecular oxygen ($^3O_2$); Superoxide dismutase (SODs) converts superoxide into hydrogen peroxide (H_2O_2) by an enzymatic reaction; H_2O_2 is converted into a highly reactive hydroxyl radical (OH-). H_2O_2 is also converted into water by the enzymes, catalase and glutathione peroxidase (GPX); Glutathione disulfide (GSSG) is produced from the oxidation of glutathione (GSH) by GPX, which can be converted back to GSH by GSH reductase in an NADPH-dependent process. Nitric oxide (NO) is produced by the oxidation of the terminal guanidino nitrogen atoms of L-arginine by nitric oxide synthase enzymes, NOS; NO can be converted into nitrogen species such as peroxynitrite ($ONOO^-$) and nitrotyrosine. The figure is Adapted from Droge (2001) (60), Djamali (2007) (63) and Irshad and Chaudhuri (2002) (64)

Fructose intake and oxidative stress in humans Only a few studies have focused on whether high fructose intake has a direct effect on the level of oxidative stress and antioxidant enzymes in humans (65). Fisher-Wellman et al. (2013) examined acute changes in the levels of pro-oxidants and antioxidants in 10 healthy males (BMI=24.9 ± 4.0 kg/m^2) after consuming a high-fat (whipping cream), high carbohydrate (100 % of total calorie as carbohydrate from refined sugar),

high protein meal (100 % of total calorie as protein from protein powder) or a combination of the three (33 % of each based on kilocalories), in a randomized cross-over study with one week separating each treatment (65). Blood samples were collected at one, two, four and six hours postprandially, and Malondialdehyde (MDA), hydrogen peroxide, a product that stimulates the formation of peroxynitrite, and nitrite, a breakdown product of nitric oxide, concentrations were measured (65). One hour after the meal, only the high-fat diet significantly increased the concentrations of MDA, hydrogen peroxide and nitrite in blood compared to the high-carbohydrate, high-protein and mixed meals (65). Fisher-Wellman's study was limited in that it only investigated the direct effects of fructose intake on the levels of oxidative stress detectable in blood, and used unrealistic proportions of macronutrients (65). Further research is needed to determine the association between fructose intake and oxidative stress in humans.

Fructose intake, production of oxidants and activities of antioxidants A number of studies in rat models suggest that high fructose feeding increases production of oxidative products and reduces the activity of antioxidant enzymes (59, 65, 66, 67, 68, 69; studies are summarized in Appendix 1).

Delbosc et al. (2005) conducted a study of oxidative stress using 8-week-old Sprague-Dawley rats fed a regular chow diet with 60% (w/v) fructose solution for 42 days (66). The plasma concentrations of thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, was measured by fluorimetry and the plasma concentrations of advanced oxidation protein products (AOPP), an indicator of protein oxidation, was estimated by spectrophotometry (66). Superoxide anion production was measured in tissues and polynuclear cells on a luminometer (66). In rats fed the fructose solution, there were increases in the concentrations of

TBARS (control=0.594 ± 0.039, fructose=0.724 ± 0.033 moles/L) and AOPP (control=8.69 ± 0.50, fructose=18.59 ± 0.91) (66). The production of superoxide anions in the ventricle and aorta was also increased in rats fed the fructose solution from day 14, 21, 28 and 42 to the end of the treatment (66). Bagul et al. (2012) also fed male Sprague-Dawley rats a control diet containing 65 % (w/w) cornstarch or a 65 % (w/w) fructose diet for eight weeks (67). After eight weeks of feeding, total nitrate/nitrite were determined in serum while TBARS, glutathione (GSH), SOD, catalase (CAT), glutathione peroxidase (GPX) and vitamin C concentrations were determined in liver tissue (67). Fructose intake significantly increased the levels of serum nitrate/nitrite (control=30, fructose=130 mM) and TBARS (control=0.15, fructose=0.27 nmol/g tissue) (67), decreased SOD activity (SOD activity: control=24, fructose=15 % inhibition) and reduced vitamin C concentrations (control=90, fructose=20 mg/dl) (67). Zhou et al. (2012) found a similar result that plasma nitrite was increased in male Wistar rats fed a 60 % (w/w) fructose diet for >4weeks (control=2.5, fructose=15 mmol/L) (67). More recently, Lozano et al. (2016) reported that the intake of a 25 % (w/v) fructose solution for two months was associated with increased levels of TBARS in the liver (control=20.4 ± 1.1, fructose=23.9 ± 0.1 μmol/L), but not in plasma, in male rats (59). After eight months of fructose feeding, the activity of CAT was significantly reduced (control=0.246 ± 0.03, fructose=0.120 ± 0.03 μmol/L), but there were no changes in the activity of SOD and the level of TBARS in plasma and the liver (59). Together these studies suggest that fructose intake is positively associated with increases in oxidative stress (59). It is unclear why lipid peroxidation was more pronounced in fructose-fed rats after two months compared with eight months (59), but these results should be investigated further.

Alterations in antioxidant activity, however, were not always induced by high fructose intake

(68). Evidence for this comes from a study by Zagrodzki et al (2007), in which young male Wistar rats were fed a fructose diet (31 % fructose as total calorie) for nine weeks (68). After nine weeks of the treatment, blood and tissues (liver, kidneys and heart) were collected to analyze enzyme and antioxidant status (68). Four enzymes were chosen to evaluate antioxidant status: the activity of SOD in red blood cells; ferric reducing ability of plasma; and activity of GPX and thioredoxin reductase in tissues (68). Lipid peroxidation in plasma was measured by MDA assay (68). In rats fed the fructose diet, the activity of GPX in the liver was significantly decreased (control=0.98 ± 0.09, fructose=0.88 ± 0.18 U/mg protein), but the activity of the remaining enzymes and the concentration of MDA were not significantly different compared to those in rats fed a control diet (68).

Fructose intake and antioxidant micronutrients Antioxidant micronutrients such as vitamin A, vitamin C and vitamin E have been widely studied for their physiological characteristics and beneficial effects on health (69, 70). Vitamin E has been studied as an important lipid-soluble antioxidant. It prevents oxidation by reacting with lipid radicals in the lipid peroxidation chain reaction (70). Faure et al. (2007) examined the effects of antioxidants (vitamin E, zinc and selenium) on oxidative stress in rats fed a fructose diet (69). Rats were divided into three groups: a control group=rats fed a control diet containing 61 % (w/w) carbohydrate (without fructose) with 50 IU/kg of vitamin E, 29 mg/kg of zinc and 0.05 mg/kg of selenium; a fructose group=rats fed a 58 % (w/w) fructose diet without vitamin E, zinc and selenium; and a supplementation group=rats fed the fructose diet with vitamin E, zinc and selenium supplementation (vitamin E=500 IU/kg, zinc=50 mg/kg, selenium=0.15 mg/kg) (69). After six weeks of feeding, rats were euthanized and blood collected to examine GSH and GSSG activity along with TBARS

concentrations (69). As a result, plasma TBARS was increased (control=2.31 ± 0.12, fructose 3.19 ± 0.15 μmol/L) and GSH/GSSG ratio in blood was reduced by fructose consumption (control=110 ± 31, fructose=61 ± 11) (69). Although, the rats fed the fructose diet with supplementary antioxidants had a significantly higher GSH/GSSG ratio (891 ± 91) compared to the rats fed the control diet, plasma TBARS was not decreased by supplementation (69). Therefore, results from this study suggest that dietary antioxidants may help to reduce oxidative stress in rats fed a high fructose diet (69).

Taken together, it appears that high fructose intake induces oxidative stress in rats by increasing the level of oxidants and decreasing the activity of antioxidant enzymes. In addition, dietary intake of antioxidants may have a beneficial effect on reducing the level of oxidative stress associated with high fructose feeding. However, the association between high fructose intake and oxidative stress has not been well established in humans. Moreover, most of the previous studies have studied fructose intake and oxidation in male subjects, not in female subjects. Thus, the effects of fructose intake on oxidative stress still need to be examined in humans and particularly in females.

2. Pregnancy

2.1 Physiological and anatomical changes during pregnancy

During pregnancy, women undergo a coordinated set of physiological and functional changes to support fetal development. Tan and Tan (2013) recently reviewed many of the physiological and anatomical changes that are observed in pregnant women (71). These authors highlight the importance of changes to the cardiovascular, respiratory, hematological, renal, gastrointestinal

and endocrine systems that are required to allow optimal fetal development and for the survival of the mother and the baby into the postpartum period (71). Several of these important changes are highlighted below.

2.1.1 Haematological and cardiovascular changes

Plasma volume increases by 30 – 50 % during pregnancy. The increases in plasma volume are progressive, beginning in the first six to eight weeks of pregnancy and leveling off in the second trimester (72). This increase in plasma volume is due to an increase in oestradiol levels, which activate the renin-angiotensin system to reabsorb sodium and water (73). The elevated plasma volume is important to support increased blood flow to the uterus and maternal organs, and may also be important in preparation for blood loss during and after delivery (75). The formation of red blood cells increases by 18 – 25 % (71) due to an increase in circulating erythropoietin (72) and is needed to promote iron transfer between the mother and the fetus. The elevated plasma volume leads to a dilution of the concentration of red blood cells in plasma and decreased blood viscosity, resulting in elevated blood flow to maternal organs and the fetus (72).

The heart also undergoes anatomical changes during pregnancy (71, 72, 77). Firstly, Tan and Tan (2013) describe that the heart is "pushed upwards, rotated and its left border is laterally displaced" during pregnancy (71). Pregnant women also experience increases in cardiac output, reduced blood pressure and heart rate. Cardiac output, the product of stroke volume (L/beat) and heart rate (beats/min), increases by 30 – 50 % over the course of pregnancy and is characterized by rapid increases over the first eight weeks of gestation and then slower rates of increase for the rest of pregnancy (71, 72). Maternal heart rate is elevated by 15 – 20 beats/minute by 32 weeks

gestation, thus increased heart rate is the primary contributor to increased cardiac output as pregnancy advances (72). Stroke volume, the total blood volume pumped from the heart per contraction, increases 25 – 30 % in the first and second trimesters compared with the non-pregnant state as the volume of blood returning to the heart is elevated (71, 77). Although both blood volume and heart rate increase, blood pressure does not increase during pregnancy due to a systemic decrease in vascular resistance (331). It is known that increased progesterone levels and decreased vascular responses to vasopressors reduce vascular resistance by increasing the productions of vasodilators (i.e. nitric oxide) (331). A larger drop in vascular resistance occurs in the uterus than in the rest of the body, thus a large proportion of cardiac output reaches the growing fetus via uterus and the placenta (77, 331). Insufficiency in vascular and hematological adaptations to pregnancy may result in pregnancy complications such as hypoxia or pre-eclampsia along with intrauterine growth restriction and miscarriage (71).

2.1.2 Hormonal changes

Multiple hormones, including 1) Gonadotropins, 2) Steroid hormones, 3) Human placental lactogen, 4) Prolactin, 5) Hypothalamic-pituitary hormones and 6) Insulin, all have a critical role in the progression of pregnancy, and are discussed below.

1) Gonadotropins Gonadotropin-releasing hormone (GnRH) is released from the hypothalamus, and stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary to trigger ovulation (71). In a normal menstrual cycle, the levels of FSH and LH decrease but, if the egg is fertilized, the hormone, human chorionic gonadotropin (hCG) supports the development of the corpus luteum that produces progesterone

and estrogen at the beginning of gestation (78). hCG is released from the syncytiotrophoblast after fertilization (79). The secretion of hCG rapidly decreases as the corpus luteum degenerates between the first seven to ten weeks of gestation (78). ***2) Steroid hormones*** After ovulation, the corpus luteum produces progesterone and estrogen and the levels of the two hormones are increased as pregnancy progresses (78). During pregnancy, increased progesterone levels have a critical role in implantation, whereas an increased level of estrogen has a major role in enhancing endothelial functions (80, 81, 82). ***3) Human placental lactogen (hPL)*** Once the level of hCG decreases, the syncytiotrophoblast in the placenta then releases hPL which has similar actions to growth hormone, which stimulates the formation of tissues (78). ***4) Prolactin*** The release of prolactin increases as pregnancy progresses for the preparation of breastfeeding after delivery (78). ***5) Hypothalamic-pituitary hormones*** During pregnancy, the hypothalamic-pituitary-adrenal (HPA) axis is important for regulating several metabolic activities (71, 72). The HPA axis regulates the secretion of many pregnancy-related hormones including corticotrophin-releasing hormone (CRH) and adrenocorticotrophin hormone (ACTH) (83). CRH is synthesized by the placenta and the level of CRH increases as pregnancy progresses (83). The major functions of CRH are in a) promoting the development of fetal organs such as the lung, liver, and gut, b) maintaining blood glucose levels, c) modulating immune functions, and d) maintaining cardiovascular tone (83). CRH stimulates ACTH secretion from the pituitary gland, which has a role in the secretion of cortisol in the bloodstream (84). In the second and third trimesters, the placenta also secretes CRH to the maternal bloodstream (84). Cortisol secretion is stimulated by CRH and ACTH until the second trimester, but it starts to be attenuated in the third trimester (84).

Insulin responses to glucose in normal pregnancy Pregnancy is characterized by insulin resistance (85). In early pregnancy, insulin sensitivity remains unchanged, although insulin secretion is elevated (85). However, as pregnancy progress, insulin resistance increases (or insulin sensitivity decreases), as evidenced by the fact that insulin responses to a glucose infusion during late pregnancy is increased by 200 – 250 % to maintain euglycemia in the mother (86). It was found that the plasma insulin response to glucose during the OGTT in normal pregnant women was 3.7 times greater than that in normal non-pregnant women (85). During late pregnancy, insulin action that normally decreases glucose disposal and lipolysis is suppressed (85), leading to significant increases in glucose and FFA in pregnant women compared to those in non-pregnant women. Severe insulin resistance in pregnant women may be present among those with pre-diabetes prior to pregnancy or gestational diabetes mellitus (GDM), and these conditions can accelerate the production of FFA and glucose (85).

The reason for increased insulin secretion is uncertain (85, 87), but is clearly part of a normal pregnancy. Seely and Solomon (2003) note that insulin resistance is mediated by several hormones including hPL, which is present in all pregnancies (87). hPL stimulates insulin secretion in β-cells (291) and increases glucose uptake and oxidation in adipocytes (87). In addition, an increase in LDL, FFA, leptin and other markers (vascular cell adhesion molecule, tumor necrosis factor) during pregnancy are considered to promote insulin secretion (87).

2.1.3 Adaptations in maternal organs during pregnancy

Kidney Pregnancy results in significant changes in the structure and functions of organs such as the kidney, lung, and gut (88, 89). The kidney undergoes alterations in hemodynamics,

glomerular filtration and tubular reabsorption. Kidney size increases by approximately one centimeter due to an expansion of vasculature and interstitial volume (88, 89). The urinary system is promoted through the dilation of the renal tubules during the first seven weeks of gestation (71). This results in hydroureteronephrosis, which becomes more critical as pregnancy progresses, showing as frequent urination in the third trimester (90). Alterations in glomerular filtration rate and charge selectivity of the basal lamina layer of the glomerulus increases the clearance of protein, albumin, and glucose (71). Thus, the excretion of creatinin, protein, albumin and glucose also rises throughout pregnancy (71). However, the level of plasma sodium concentrations is not decreased in the body since the absorption of sodium is increased through the changes in the kidney (91).

Lungs and blood vessels Pregnancy increases the volume of gas exchange between the mother and fetus through the activity of hormones; an increase in progesterone concentrations in the mother acts as a respiratory stimulant by decreasing airway resistance (72). The muscle relaxation in the thoracic region also causes increased air volume/minute by 150 %, resulting in a "mild respiratory alkalosis" in pregnant women (72). The condition of mild alkalosis promotes gas exchange between the mother and fetus through the placenta. Neck circumference and blood vessels in the nose also expand (92). Thus, pregnant women sometimes experience nosebleeds and nasal congestion (93).

Gastrointestinal tract Alterations in the gastrointestinal system are necessary to meet nutritional needs for both the mother and fetus and this topic has been recently reviewed (94). Animal models show that the surface area and weight of the gut increases during pregnancy. Villous height increases in the duodenum by mid-pregnancy (95) and it increases in the jejunum

by later pregnancy in rodents (96). Progesterone's action on smooth muscles decreases intestinal movement, which results in a delay in intestinal transit time (97). The expansion of the uterus also inhibits gastric emptying by pushing the small intestine upward. These alterations may trigger the symptoms such as bloating, nausea and vomiting in pregnant women (98).

2.1.4 Gestational weight gain and body composition

Physiological changes Weight gain during pregnancy reflects the significant body composition changes in the mother along with increased volume of maternal organs and development of the fetus and the placenta (99). In humans, pregnancy-associated weight gain is expected to be about 4 kg fat, 1 kg protein, and 7 – 8 liters of water (99). Approximately 25 % of the total maternal weight gain in pregnancy is from the fetus, 11 % is from the placenta and amniotic fluid, and the remaining weight is tissue growth and deposition within maternal depots (72). Changes in plasma volume, fat mass and protein accumulation take place throughout pregnancy and follow a particular pattern (72). Plasma volume increases by 50 ml at ten weeks of gestation and by ~1500 ml at 40 weeks of gestation (recently reviewed by King, 100). The circulating plasma concentration of many micronutrients decreases, but the reduction is less than the alterations in plasma volume suggesting that their absolute amount in plasma actually increases (100). Total fat accumulation is approximately 328 g at ten weeks of gestation and continuously increases to about 3,825 g by 40 weeks of gestation (100). Protein accretion in maternal tissues at ten weeks of gestation is approximately 36 g, and it increases to 925 g at 40 weeks of gestation (100). These physiological changes in pregnancy induce a minimal increase in weight in the first trimester, and higher amounts of weight gain in the second and third trimesters (101).

The Institute of Medicine Committee (IOM) on Gestational Weight Gain (2009) recommends

that women with different pre-pregnancy BMIs gain different amounts of weight during pregnancy (101). The updated IOM guidelines were adopted by Health Canada in 2010. Women with a pre-pregnancy BMI of <18.5, 18.5 – 24.9, 25.0 – 29.9 or ≥30.0 kg/m^2 are recommended to gain 12.5 – 18, 11.5 – 16, 7 – 11.5, and 5 – 9 kg respectively (101). Maternal weight gain is a general predictor of pregnancy outcomes (102, 103, 104). For example, low weight gain is associated with preterm birth and increased risk for an infant to be born small for gestational age (SGA), gaining recommended amounts of weight is associated with optimal pregnancy outcomes, whereas high weight gain is related to an increased risk of macrosomia and higher postpartum weight retention (102, 103, 104).

2.2 Maternal metabolism of nutrients

Pregnancy induces marked changes in carbohydrate, lipid and protein metabolism. These changes are aimed at ensuring the most efficient metabolic utilization of substrates for the mother and fetus.

2.2.1 Carbohydrates

Carbohydrate metabolism changes during pregnancy to meet the increased energy needs of both the mother and fetus. Fasting glucose concentrations are reduced in pregnant (vs. non-pregnant) women by 10 – 20 % due to increased glucose utilization (105). Maternal glucose utilization is elevated by the prevalence of increased number of red blood cells and enlarged maternal organs such as heart, lung, uterus, and breast (105). At term pregnancy, the fetus requires 40 g of glucose/day and the mother needs 55 – 60 g of additional glucose/day, relative to the non-pregnant state, resulting in an additional 100 g of glucose/day being required for maintaining

pregnancy (105). Thus, glucose levels in the blood are low during pregnancy, especially in the third trimester (105). In addition, hyperplasia of pancreatic β-cells leads to increased insulin secretion and further contributes to low glucose concentrations at this time prior to the development of significant insulin resistance (105).

2.2.2 Lipids

Plasma levels of lipid fractions such as FFA, TG, cholesterol and phospholipids increase during pregnancy (106). Moderate postprandial hyperinsulinemia creates an optimal condition for lipid synthesis and fat storage during pregnancy (107). The increase in cholesterol and TG synthesis stimulates maternal fat accumulation in the second trimester (107). In the third trimester, accumulated fat declines corresponding to a phase of rapid lipolytic activity of adipose tissue (107). The plasma concentrations of TG and lipoprotein complexes such as VLDL, LDL and high-density lipoprotein (HDL) increase in the early stages of pregnancy (107). The level of VLDL is elevated by an increased level of both fatty acids and glycerol through up-regulated *de novo* lipogenesis and endogenous and exogenous fatty acids (107). Estrogen increases the production of VLDL and inhibits the activity of lipoprotein lipase in adipose tissue (107). In addition, a reduction in progesterone secretion in late pregnancy decreases the activity of lipase in adipose tissue by 60 %, resulting in a low removal rate (107). The increases in the circulating FFA pool help to meet the maternal needs for increased basal energy expenditure (107). In addition, elevated lipolysis increases the amount of glycerol for gluconeogenesis (107).

2.2.3 Protein

The anabolic processes needed to support tissue growth and accumulation by the mother and

fetus suggests a need for increased protein intake throughout pregnancy (106). Thus, protein metabolism increases during pregnancy; the rate of protein synthesis is highest at 12 weeks of gestation and an additional 4.7 g/day of protein is needed to maintain the additional tissue and the fetus in the last four months of gestation (106). To meet the needs of protein during pregnancy, maternal body changes the metabolism to prevent a net loss in body protein (106). During the postprandial period, plasma concentrations of serine, isoleucine, alanine, and leucine are lower in pregnant women than those in non-pregnant women (106). These reductions are accompanied by greater hepatic retention during pregnancy (106). In normal pregnant women, the plasma alanine pool at term increases about 35 % (106). Although the total amount of amino acids does not change during pregnancy, their plasma concentrations are decreased as a result of hemodilution (106). Urinary nitrogen excretion does not increase during pregnancy, thereby supporting increased amino acid availability in plasma to support tissue growth (106).

In summary, macronutrient metabolism changes during the course of normal pregnancy to meet the increased metabolic needs of the mother and feto-placental unit. Glucose utilization in the fetus and placenta increases and there is increased fatty acid oxidation. Amino acids are transported to the liver to generate glucose, and they are also used for the synthesis of maternal, placental and fetal tissues.

2.2.4 Vitamins and minerals

Vitamins and minerals are essential for metabolism since they are involved in processes such as cell signaling, tissue growth and homeostasis (108). In pregnancy, the roles of micronutrients are required to support healthy placental and fetal development. The importance of a few

micronutrients during pregnancy has been elucidated in previous studies as deficiencies of micronutrients such as folate, vitamin B_6, vitamin B_{12} and iron in the mother are known to induce anemia, infant morbidity, mortality and other birth defects. A summary of functions of micronutrients and deficiency-associated risks is presented in Table 1-1. Since the study design of this thesis included measurement of vitamin E status, more details of vitamin E during pregnancy are described in this section.

Table 1-1: Summary of functions of a few micronutrients and deficiency-related risks during pregnancy

Micronutrients	Functions	Deficiency-related outcomes
Folate, vitamin B_6, B_{12} (109, 110)	Have a role in gametogenesis, fertilization and implantation which may affect DNA and histone methylation of the oocyte	Neural tube defects
Iodine (108, 111, 112).	Utilized for fetal brain development in the first and second trimesters	Severe iodine deficiency induces cretinism (severe stunting and reduced mental capacity) in humans, neurological damage to the fetus in animal models
Iron (108, 113, 114, 115)	Required for fetal brain development by enzymes involved in neurotransmitters serotomin, dopamine and myelination	The most prevalent micronutrient deficiency globally Adversely affects the infant's developing hippocampus, and compromises energy metabolism in the brain and myelination

Vitamin E During pregnancy, vitamin E is considered to be one of the most essential nutrients for the physiology of reproduction (116), although maternal-fetal vitamin E transfer is limited (117). Vitamin E is a lipid-soluble, essential nutrient that comprises eight compounds: α, β, γ and δ-tocopherols and α, β, γ and δ-tocotrienols (118). These compounds have both antioxidant and non-antioxidant activities (118). The most well-known capacity of α-tocopherol is to protect lipids and lipoproteins against oxidative damage (119). However, recent studies have found that the function of α-tocopherol is not limited to antioxidant roles, but that it also modulates cell signaling and proliferation (116). In particular, α-tocopherol together with other antioxidants including ascorbate (vitamin C) has a critical role in reducing lipid peroxidation in LDL (116). Similar to the functions of α-tocopherol, γ-tocopherol has a beneficial effect such as reducing the level of superoxide anion, lipid peroxidation and LDL oxidation (120). Other compounds such as β and δ-tocopherols and tocotrienols are also known to have a similar function to that of the antioxidant activities of α-tocopherol (120).

The role of vitamin E in gestation is still unclear, but a number of studies showed the association between maternal vitamin E status and reproductive success (121, 122). Studies using mouse placentae inferred that α-tocopherol may have a critical role in proliferation of syncytiotrophoblast cell (121). In pregnant women, when the concentrations of α-tocopherols in plasma were lower, newborns had significantly lower volume of vitamin E in their plasma (122). Moreover, the risk of miscarriage was increased by 2-fold in women with vitamin E deficiency (123).

2.3 Maternal nutrition and fetal programming

Significant changes in maternal physiology during pregnancy are required to provide adequate supplies of nutrients to the fetus. Mothers who experience abnormal physiological adaptations to pregnancy due to poor nutritional intakes or underlying disease processes have an increased risk of maternal and fetal morbidity and mortality. In addition, recent studies strongly suggest that the health of women of childbearing age has influences on the long-term health of their offspring. Studies done with animals and in humans support the hypothesis linking maternal health and nutrition to fetal development. This section will review the literature on the relationship between maternal nutrition and short and long-term effects on offspring health.

2.3.1 The developmental origins of health and disease (DOHaD)

The "Developmental Origins of Health and Disease (DOHaD)" hypothesis describes how environmental factors such as maternal lifestyle, disease, smoking and diet during pregnancy influence fetal development (International Society for Developmental Origins of Health and Disease). The DOHaD, previously called the "fetal origins hypothesis" was put forward by Sir David Barker in collaboration with Dr. Nick Hales (1992). The theory is therefore, often called the "Barker hypothesis" or, sometimes, "fetal programming". In 1986, Barker et al. investigated the relationship between birth weight and the mortality rate due to ischemic heart disease in men who were born in England (124). In this study, Barker found that babies who were born with a lower birth weight were at higher risk of ischemic heart disease in later life than babies who were born with a normal weight (124). Data from the Dutch famine birth cohort study was analyzed to examine the effects of acute famine (1944 – 1945) on birth weight, length and ponderal index of

babies (125). Babies whose mothers were exposed to famine while they were *in utero* were more susceptible to chronic diseases such as heart disease, diabetes, obesity and renal disease on adults (125). These two studies suggested that fetal development is sensitive to environmental exposures, and that lasting changes that are "programmed" *in utero* can result in adverse health outcomes in the offspring at birth and their health in later life. Moreover, several studies indicate that exposure to famine in early, mid or late pregnancy has different health outcomes on the fetus (126). Babies whose mothers experienced famine in early pregnancy were heavier and longer at birth and had more "atherogenic lipid profile" than those babies whose mothers were not exposed to the famine (126). However, babies whose mothers were exposed to the famine during mid and late pregnancy were lighter, shorter and had smaller heads and placentae than those whose mothers were not exposed to the famine (126). The babies whose mother experienced famine, which occurred while they were in mid and late gestation, also had reduced glucose tolerance in adult life (125). These studies indicate that fetal development during critical periods of gestation can be impaired by environmental factors, and health outcomes in the offspring differ depending on the stage of pregnancy (125, 126). More studies have highlighted the importance of maternal nutrition on fetal development during pregnancy and birth outcomes in animal models and human studies.

2.3.2 The relationship between birth outcomes and adult disease in later life

A number of epidemiological studies have demonstrated that low birth weight (<2,500 g or 5.5 lbs.) and high birth weight (>4,000 g or 8.8 lbs.) are associated with an increased risk of adult diseases in later life (127, 128, 129). These conditions include coronary heart disease, stroke, diabetes, and obesity.

Birth weight, body size and coronary heart disease in later life Osmond et al. (1993) followed 10,141 men and 5,585 women who were born in Hertfordshire, England from 1911 – 1930 and analyzed the relationship between their birth weight and their prevalence of coronary heart disease in later life (127). Mortality from coronary heart disease was least likely in both males and females whose weight at birth was higher than 5.5 lbs. and lower than 9.5 lb compared with those whose birthweights were outside of this range (127). Similarly, Frankel et al. (1996) examined birth weight and the prevalence of coronary heart disease in 1,258 men from South Wales who were born from 1979 to 1983 (129). Birth weights of these men were divided into quartiles: Quartile 1 indicates low birth weight, Quartile 2 and 3 indicate normal weight at birth, Quartile 4 indicates high birth weight (129). The study found that the incidence of coronary heart disease was significantly increased in men who were light at birth (Quartile 1) compared to those whose birthweight were in the highest quartile (129). Thus, the study concluded that low birth weight is highly associated with an increased risk for developing coronary heart disease (129). However, a major limitation of this study was that birth weight was obtained by participant recall, not from birth records (129). Thus, Frankel may not have defined birth weight categories with the same degree of accuracy as other studies (129). In 1997, Rich-Edwards and his colleagues collected birth weight data from 121,700 women in a cohort study, the NHS-I (128). Birth weight of women was divided into six categories; birth weight < 2.3, 2.3 – 2.5, 2.5 – 3.2, 3.2 – 3.9, 3.9 – 4.5, or >4.5 kg (128). The authors found a negative association between birth weight and the development of stroke in women (128). Relative to women whose birth weight was 3,175 – 3,856 g (the reference group), the relative risk of experiencing cardiovascular disease was 1.49 for women whose birth weight was <2,268 g, 1.25 for women whose birth weight was 2,268 – 2,495 g, 1.12 for women whose birth weight was >2,495 – 3,175 g, 0.96 for women whose birth

weight was > 3,856 – 4,536 g, and 0.68 for women whose birth weight was >4,536 g (128). The relationship was still significant after adjusting for childhood socioeconomic factors (128).

Like body weight, body size at birth is related to the onset of chronic disease in adult life (130). Eriksson et al. (2001) performed a longitudinal study in Finland to determine the association between body size at birth and the risk of coronary heart disease in 4,630 men who were born in 1934 – 44 (130). The authors analyzed birth outcomes (birth weight, length, ponderal index) and infant growth of men who developed coronary heart disease and who died of that disease later in life (130). A low ponderal index (≤25 kg/m^3) at birth in men was correlated with an increased risk for developing coronary heart disease (Hazard ratios: 1.66) compared with men who had ponderal index >29 kg/m^3 (referent ponderal index) (130). In addition, anthropometric characteristics at 1 year of age were also related to men's risk of coronary heart disease in adulthood. Infants who were light (≤9 kg, hazard ratio: 1.82), short (≤73 cm, hazard ratio: 1.55) and had a low BMI (≤16 kg/m^2, hazard ratio: 1.83) relative to the referent groups at one year of age had higher risk of developing coronary heart disease (130).

In the study done by Stein et al. (1996), information about birth weight and size of men and women who were born between 1934 and 1954 in South India was used to examine the association between these variables and the prevalence of heart disease (131). The development of coronary heart disease was significantly higher in both men and women who had a low birth weight (≤5.5 lbs.), short birth length (≤18 inches), and small head circumference (<13 inches) (131). In addition, the highest risk of disease was in people who were light (≤5.5 lbs.) and whose mothers weighed less than 45 kg during pregnancy (131).

Birth weight, non-insulin dependent diabetes and glucose intolerance in later life Low and high birthweights were also associated with increased risk of Type 2 diabetes and obesity in the offspring, as several studies showed (132, 133, 134, 135). One example is a longitudinal study of diabetes that was conducted among the Pima Indians (132). The prevalence of non-insulin dependent diabetes was high in the lowest (<2,500 g) and highest (≥4,500 g) birth weight groups (132). In addition, Robinson et al. (1992) reported that men who were lighter at birth in the UK, had higher plasma glucose levels during the OGTT (134). In 40 men aged 18 – 25, plasma glucose concentration was 8.8mmol/L in men whose birth weight was ≤ 3,204 g, 8.2 mmol/L in men whose birth weight was between 3,204 and 3,572 g, and 7.3 mmol/L in men whose birth weight was >3,572 g after glucose administration (134). In the Health Professionals Follow-Up Study (HPFS) conducted in the U.S., low birth weight (<5.5 lbs.) was associated with a high risk of adult diseases such as diabetes and hypertension, whereas a high birth weight (≥10 lbs.) was related to an increased risk of obesity (135). The men who were lighter than 5.5 lbs. at birth had a 1.8-fold odds of developing diabetes and a 1.3 increased odds of developing hypertension compared to the men who weighed 7.0 – 8.5 lbs. (referent weight) (135). The odds ratio of the highest BMI quintile (>28.2 kg/m^2) was 2.08 in men whose birth weight was ≥10 lbs. compared to the reference group (birth weight 7.0 – 8.5 lbs.) (135). In another epidemiological study in England, men aged 58 – 70 (mean 64) who had records of weight at birth and one year of age had plasma glucose and insulin levels measured during a two-hour OGTT in adulthood (133). Low weight at both birth (≤5.5 lbs.; ≤2,495 g) and one year of age (≤18 lbs.; ≤8.16 kg) was associated with an increased risk of having impaired glucose tolerance and non-insulin dependent diabetes in later life (133).

Body proportions at birth, placental weight and development of adult disease in later life

Body proportions such as head and abdominal circumferences at birth and placental weights may also be related to the development of adult disease in later life. Using the data from the NHS Central Register, Barker et al. (1992) found that the blood pressure in adult men and women aged 46 to 54 years was associated with head circumference of babies at birth and with placental weight (136). Subjects who had lower birth and heavier placental weights tended to have higher blood pressure in adult life (136). SBP increased as the placental:birth weight ratio increased (136). Moreover, the mean SBP of subjects increased as head circumference to length ratio increased at birth (136). Martyn et al. (1996) analyzed the relationships between death rates of stroke and coronary heart disease and birth size characteristics in men who were born between 1911 and 1930 by using two cohort studies, one from Hertfordshire and the other from Sheffield, UK (137). Information about individuals' birth characteristics, specifically body size (weight, length), head circumference, and placental weight were examined relative to information on these same individuals' death certificates regarding the cause of death from either of these diseases (137). The death rates from stroke and coronary heart disease were highest in men whose birthweight was low (≤5.5 lbs.) (137). In particular, mortality from coronary heart disease was increased when head circumference was small (<14 inches), and the placental weight was light (≤1.25 lbs.) at birth (137). Martyn suggested that adaptations to maternal undernutrition during pregnancy may induce alterations in fetal physiology such as reducing the growth of the head, tissues and the placenta (137).

2.3.3 The effects of maternal nutrient intake on fetal development

Maternal nutrition during pregnancy plays a critical role in fetal development. Undernourished

and overweight women are known to be more susceptible to adverse birth outcomes and their offspring are at higher risk of long-term chronic health conditions than well-nourished women of normal weight. This section provides an overview of current knowledge on the effects of maternal nutritional status on fetal development. This section also focuses on potential mechanisms of fetal programming of chronic disease through maternal under and overconsumption of energy, protein, fat, vitamin E and fructose (including consumption of SSB and added sugars) during pregnancy.

2.3.4 Undernutrition

It has been shown in both animals and humans that maternal undernutrition during pregnancy is positively associated with fetal growth restriction (138). Maternal undernutrition during pregnancy can result from any number of situations including a limited supply of food, for example during famine, or not meeting the increased nutritional requirements of pregnancy in adolescence or severe hyperemesis (139, 140). For example, young girls may enter pregnancy with increased nutritional needs since these are required for both maternal growth and the growth of the fetus (139). Severe nausea and vomiting can limit nutrient intake by the mother and may place the fetus at risk for low nutrient availability, particularly those nutrients that are not extensively stored or mobilized from maternal tissue (140). Thus, energy and nutrient restriction are considered as a major nutritional problem in the world. This section reviews studies on the effects of maternal undernutrition on fetal growth and the risk of developing disease in offspring in later life.

Energy restriction Previous studies in an animal models have shown that reductions in

maternal energy intake on the order of <30 % of requirements leads to fetal growth restriction and alterations in glucose regulation in the offspring in later life (140). Offspring body weights from severely energy-restricted mothers (i.e. receiving 30 % of *ad libitum* calorie intake), were significantly reduced at birth (Fetal weight: 3.45 ± 0.24 g vs. *ad libitum* group=5.15 ± 0.11 g) (141). These effects align with observations from studies in humans that showed a positive association between maternal caloric restriction and low birth weight.

The timing of caloric restriction during pregnancy may also affect glucose homeostasis and insulin resistance in adult offspring (125, 142). Gardner et al. (2005) showed that offspring of ewes who received 50 % of the normal caloric intake in late pregnancy (from gestational day 110 – term) had increased glucose and insulin responses to a glucose bolus compared to offspring of ewes fed 50 % calories in early pregnancy. Offspring of ewes exposed to caloric restriction in early pregnancy had responses that were similar to those whose mothers met caloric requirements during the entire pregnancy (142). This observation is consistent with reports about offspring conceived during the Dutch Famine Winter in which adult offspring of mothers exposed to famine during late gestation had higher glucose concentrations during and after the OGTT than those whose mothers were exposed to famine either in early or mid-gestation (125).

Protein Restriction Proteins and their component amino acids are essential nutrients, and they play a critical role in all cellular processes including anabolism, catabolism, DNA replication and responses to stimuli; thus, the protein supply to the fetus during pregnancy is critical for optimal fetal development (106). Similar to energy restriction, maternal protein restriction during pregnancy is associated with low birth weight and impairments in organ development in the fetus, and these effects are consistent across mammalian species (143, 144). Pregnant rats fed a

protein-restricted diet (8% of total calories from protein) throughout pregnancy had significantly lower litter weights at birth (protein-restricted dams=77 ± 25 g, control dams=100 ± 30 g). These fetuses also had lower organ weights (including pancreas, muscle, liver spleen, heart, kidney, thymus, lung, brain) compared to the fetuses of dams fed a diet containing 20 % protein diet (143). At weaning (21 days of age), weights of the pancreas, spleen, muscle, liver, lung, and brain were still all lower in the offspring from the protein-restricted group compared with those from the control group (143). Similarly, in a pig model, Pond et al. (1991) found that sows fed a low protein diet (0.7 % of total calories from protein) gave birth to piglets with low birth weights and with reduced liver, kidney, and gastrointestinal tract weights compared to sows fed a control diet (13 % of total calories from protein) (144).

Possible Mechanisms A number of mechanisms underlying the effects of maternal undernutrition on fetal programming have been suggested (141, 145). Holmes (2006) suggested that a reduction in placental 11β-hydroxysteroid dehydrogenase (11β-HSD) activity occurs in protein-restricted female rats, and this could inhibit fetal growth (141). Since placental 11β-HSD prevents an excess amount of glucocorticoids from crossing the placenta, a reduction in placental 11β-HSD activity could allow an excess amount of glucocorticoids to affect the fetus, resulting in intrauterine growth restriction (IUGR) (141). Lower concentrations of Insulin-like growth factor-1 (IGF-1) in animals maintained on a low protein or a low-calorie diet may also contribute to IUGR (146, 147). Ewes were fed *ad libitum* or a calorie-restricted diet prior to, and during, pregnancy and plasma IGF-1 was measured between gestational day 105 and 115 (147). The reduction in IGF-1 levels in response to maternal undernutrition was significant in both the mother and the fetuses (147). The authors also suggested that IGF-1 has a critical role in the

promotion of cell proliferation and inhibition of apoptosis, and both of these processes are critical determinants of appropriate fetal growth (147). Thus, the reductions in IGF-1 as a result of low maternal protein intake may play a role in inducing fetal growth restriction (147).

Several mechanisms have been proposed as the means by which protein restriction during pregnancy may alter insulin responses to a glucose challenge in offspring later in life (125, 148, 149, 150). Firstly, Ravelli et al noted that maternal undernutrition leads to impaired glucose sensing in β-cells of offspring (125). Rasschaert et al. corroborated these finding and noted that the activity of glucokinase and glycerolphosphate dehydrogenase was reduced in β-cells of offspring from protein-restricted dams (150). Snoeck observed lower β-cell number and reduced vascularity of islets in offspring of protein-restricted mothers (149). In addition, blood glucose levels in the offspring of dams fed a protein-restricted diet may reflect permanently altered activity of hepatic enzymes of glycolysis and gluconeogenesis such as glucokinase and phosphoenolpyruvate carboxykinase (148). The alterations in hepatic enzyme activities influence glucose levels in offspring in both fasted and normal states, and if they are dysregulated, impaired glucose metabolism may ensue (148). Together, these studies suggest that glucose regulation may be affected by a number of different mechanisms, all contributing to hyperglycemia in the offspring.

More recent studies suggest that maternal protein deficiency may adversely affect the fetal genome by influencing DNA and protein methylation within the fetus. Wu et al. (2004) suggested that energy deficiency may inhibit DNA methylation and histone modification (151) as a result of reductions in the activity of DNA methyltransferases that catalyze DNA methylation with S-adenosylmethionine as a methyl donor (152). Within this process, amino acids such as

glycine, histidine, methionine, and serine as well as B vitamins, provide methyl donors for both DNA and protein methylation (153). Epigenetic changes, a result of an insufficient amino acid supply, and resulting inhibition of DNA methylation could contribute to lasting impairment of metabolic pathways in the offspring (152).

In conclusion, low energy intake or low protein in "critical periods" *in utero* can lead to alterations in body weight, organ development, and insulin secretion in the offspring through changes in metabolism, hormonal secretion and DNA methylation into their adult life. These alterations may induce irreversible changes in the physiology of the offspring, which may significantly increase the risk of chronic disease in later life.

2.3.5 Overnutrition

Findings from the studies discussed above suggest that many chronic diseases that are manifested in adult life may be driven by processes that are programmed by the *in utero* environment, which in turn is influenced by factors such as maternal nutritional status, physical activity, and other lifestyle habits prior to, and during, pregnancy. Although the literature exploring the effects of maternal undernutrition is extensive, there has been a growing interest in understanding the potential for the effects of maternal overnutrition on offspring weight, particularly the risk of obesity, through modifying energy metabolism, endocrine regulation and appetite control of the offspring. Such alterations may also contribute to the risk and onset for weight-related health conditions such as increased BMI (154), hyperphagia (155), and insulin resistance (156). Studies that examined the effects of maternal overnutrition on fetal development have been conducted in both animal and human subjects. Animal experiments are

well controlled, and randomized, but animals have different reproductive characteristics than humans, such as shorter gestation and larger litter size. Most human studies, on the other hand, rely on longitudinal cohort studies to examine these effects. A longitudinal cohort study is an observational study that does not include any intervention with participants. Thus, such a study has the advantages that researchers can observe the patterns of variables over time and analyze the associations between factors such as the relationship between maternal dietary intake and birth weight of the fetus. However, this type of study may be limited in external validity, since they are often conducted with well-educated and healthy women, and the numerous confounding factors may be imperfectly controlled for during analyses. Therefore, it is critical to take results from both animal and human studies into account to fully understand the relationship between maternal overconsumption and fetal development.

This section reviews studies on the effects of maternal overnutrition on fetal growth and the risk of developing disease in offspring in later life. Since overnutrition has been examined in different ways, overnutrition includes following categories; **a) high energy intake:** "junk food" with an *ad libitum* chow diet, 154; "obesogenic diet (≥11 % fat, 33 % sugar as total energy intake)", 155, 156; "Western diet", 157; receiving 190 % of energy requirements, `58, **b) high fat intake:** ≥40 % fat as total energy intake, 159, 160, **c) high fructose intake:** 10 % (w/v) fructose or 33 % (w/v) sucrose solution with an *ad libitum* diet; 60 % fructose as total energy intake; diets containing ≥55 % (w/w) simple sugars, 161, 162, 163, 164, 165.

Maternal high energy intake Recent studies show that diet-induced obesity during pregnancy may affect the long-term health of the offspring (154, 155, 156). Bayol et al. (2007) showed that offspring of rats fed "junk food" have increased body weight in post-weaning (154). Pregnant

rats were fed "junk food" (including biscuits, marshmallows, cheese, jam donuts, chocolate chip muffins, butter flapjacks, potato crisps, caramel/chocolate bars) with *ad libitum* chow or only a chow diet (control group) (154). Rat dams that were fed the "junk food" during pregnancy were divided into those either a chow diet or "junk food" in lactation and their offspring similarly followed the diets of their mother until postnatal week 10 (154). Rat dams fed "junk food" consumed significantly higher proportions of calories from fat, carbohydrates, and sucrose than the control group during pregnancy and lactation (154). At post-natal week 10, the BMI (body lengths and body weights of offspring were used to calculate BMI, i.e. kg/m^2) of "junk food" fed offspring whose dams were fed "junk food" both during pregnancy and lactation was higher than the BMI of the offspring of control dams and of "junk food" fed offspring whose dams were fed "junk food" during either pregnancy or lactation (154). Samuelsson et al. (2008) also examined the effects of maternal diet-induced obesity on metabolism and cardiac functions in the offspring in mice (155). Female mice were fed either an obesogenic diet (16 % fat, 33 % sugar as total energy intake) or a control diet (3 % fat, 7 % sugar as total energy intake) before and during pregnancy for six weeks (155). The offspring of dams that consumed the obesogenic diet were hyperphagic from four to six weeks of age compared to those of dams fed a control diet (155). Body weight was significantly increased in male offspring at 12 weeks of age and in female offspring from five weeks of age (155). Both male and female offspring of dams fed the obesogenic diet had reduced skeletal muscle mass and increased body adiposity at three and six months of age (155). Moreover, glucose and insulin secretion during the 2-hour OGTT was higher in pups of the obese dams (155). Similarly, Nivoit et al. (2009) investigated the relationship between diet-induced obesity in dams and glucose homeostasis in the offspring (156). Female rats were fed either an obesogenic diet (11 % kcal as fat, 43 % sugar) or a control

diet (3 % kcal as fat, 7 % sugar) for eight weeks before and during pregnancy and lactation (156). Offspring were weaned onto the control diet and assessed until they were twelve months of age (156). Insulin and glucose responses to the OGTT were determined in three-month-old male and female offspring (156). The pups from dams fed an obesogenic diet had higher body weight and were hyperphagic, as shown by higher weekly food intake, than those from dams fed a control diet from weaning (156). The concentration of plasma insulin during the glucose tolerance test was also high in three-month-old offspring of obese dams, especially in male offspring (156). Body weight and white adipose tissues (mesenteric, perigonadal, perirenal, subcutaneous white adipose tissues) weight of male and female offspring from dams fed the obesogenic diet was significantly increased at 12 months of age compared to male and female offspring from control dams (156). Increases in organ weights (i.e. adrenals, heart, kidneys) were found in female offspring from dams fed the obesogenic diet at 12 months of age only (156).

In humans, there appears to be an inverse relationship between maternal consumption of obesogenic diets during pregnancy and infant birth weight (157). For example, Knudsen et al. (2008) investigated the relationship between maternal dietary patterns, including energy intake, and SGA using data from the Danish National Birth Cohort study (157). Pregnant women were classified as having one of the following three dietary patterns based on a food frequency questionnaire and a 7-day weighed food record in mid-pregnancy: the “western diet” (a diet high in red and processed meat, high-fat dairy), a “health conscious diet” (a diet high in vegetables, fruits, poultry, and fish), or an “intermediate” dietary pattern (combination of “Western diet” and “health conscious patterns”) (157). Mothers with the “Western diet” had a significantly increased risk of having a SGA baby compared to women in either of the other groups (157). Results

remained significant after adjusting for the potential confounders such as maternal age, smoking in pregnancy (never vs. ever), mother's height and weight and father's height (157).

Studies in animals have noted that the inverse relationship between maternal overconsumption and fetal growth in humans may be a result of alterations in oocyte maturation and implantation, which may affect placental growth, impair placental transfer efficiency, and reduce the rate of fetal development (158, 166). Rattanatray et al. (2010) developed a sheep model in which non-pregnant ewes were either overnourished (received 190 % of energy requirements) or not (received 100 % of energy requirements, control) for four months prior to insemination (158). Donor embryos were implanted in non-obese recipients and fat mass in female lambs at four months of age was measured (158). Total fat mass was higher in female lambs of overnourished dams than in female lambs of control dams (158). The authors noted that exposure of the oocyte to maternal overnutrition may result in a greater synthesis of TG (158). This suggests that maternal nutrition during the periconceptional period can influence fetal programming in pregnancy (158). Igosheva et al. (2010) also examined the oocytes and zygotes of mice exposed to a diet containing high saturated fat and sugar (10 % kcal as sucrose, 20 % kcal as animal lard) for 6 weeks prior to pregnancy (166). Oocytes from mouse dams exposed to the experimental diet had higher amounts of mitochondrial DNA and higher rates of reactive oxygen species (ROS) generation compared to control dams (166). Oxidative stress was also increased in oocytes from dams fed the high fat/sugar diet as evidenced by an intracellular redox state favouring oxidation (166). Thus, Igosheva's study showed that regular consumption of a diet high in saturated fat and sugar before pregnancy may compromise oocyte quality and could contribute to poor rates of reproductive success in obese women (166).

Maternal high-fat intake A number of studies have shown that maternal high-fat diets during pregnancy result in abnormal glucose homeostasis (167), elevated blood pressure (168) and increased body adiposity (169) in the offspring. Srinivasan et al. (2006) observed that dams fed a high-fat diet (59.5 % fat of the total calories) prior to, and during, pregnancy had significantly lower birth weight offspring compared to the offspring of normal dams on gestational day (GD) 21 (159). The concentrations of insulin, glucose, TG and FFA in plasma were higher, and body weights were heavier in adult offspring of high-fat fed dams than those in the offspring of normal dams (159). In addition, Cerf et al. (2005) showed that the pups exposed to a maternal high-fat diet (40 % kcal as fat) during pregnancy had significantly increased plasma glucose levels and altered α, β cell development (160). The pups from dams exposed to a high-fat diet in the first or second weeks of pregnancy only were hypoglycemic on postnatal day one, whereas the pups from dams exposed to the same high-fat diet for the entire pregnancy were hyperglycemic on postnatal day one (160). In addition, the number and volume of pancreatic β-cells were reduced while the α-cell number and volume were increased in neonates from rats fed a high-fat diet during the whole gestation (160). From this point of the view, it appears that a maternal high-fat diet during pregnancy is associated with alterations in the development of pancreatic cells, and the specific effects depend on the timing of exposure.

Maternal vitamin E intake Vitamin E in deficiency has long been known to cause embryonic failure and resorption of fetuses in female rats (344), thus suggesting that it could have an important role in maintaining a healthy pregnancy. Vitamin E status has not been frequently measured in human pregnancies, thus it is difficult to know how it relates to pregnancy outcomes. In situations where vitamin E status has been documented to be low, vitamin E supplementation

has had beneficial effects, for example in the case of HIV-positive women in Tanzania (345, 346). In contrast, the effects of vitamin E supplementation in adequately nourished women appears to be minor (reviewed in Traber, 2014; 347). For example, Rumbold et al. (2015) recently conducted a meta-analysis of 21 randomized controlled trials to examine the effects of vitamin E supplementation and health outcomes in pregnant women and their babies (329). Vitamin E supplementation occurred in combination with vitamin C and other agents in many of the studies. There was no clear difference between women supplemented with vitamin E in combination with vitamin C or other supplements compared with placebo for pre-eclampsia, IUGR, neonatal death, or preterm birth (329). However, vitamin E in combination with vitamin C supplementation decreased a risk of having placental abruption compared to a placebo group (risk ratios=0.64, 95 % CI, 0.44 – 0.93) (329). They also noted that vitamin E supplementation was not associated with any harmful effects in these studies. Thus, this author highlighted the importance of further investigation on the relation between vitamin E intake and placental abruption. Clearly better documentation of vitamin E status in pregnancy in humans is also needed.

Although most of previous studies did not find any benefitial effects of vitamin E supplementation on reducing adverse birth outcomes in humans, studies using animals have shown that vitamin E has a beneficial effect on pregnancy by preventing oxidative damage to cells and scavenging reactive oxygen and reactive nitrogen species (118, 119). Cederberg et al. (2001) provided vitamin C and E supplementation to normal or streptozotocin (STZ)-induced diabetic female Sprague-Dawley rats (weighing 250 g) (171). Normal (N) and diabetic pregnant rats (D) were randomized to 1 of 3 groups: No supplementation during pregnancy (control); Low Vit E and C (0.5 % vitamin E and 1 % (w/w) vitamin C; N 0.5+1 or D 0.5+1) or High Vit E and

C (2 % vitamin E and 4% (w/w) vitamin C; N 2+4 or D 2+4) (171). On GD20, pregnant rats were euthanized, and maternal blood, maternal liver and fetal liver were collected (171). The concentration of α-tocopherol was measured in maternal plasma and the liver using high-performance liquid chromatography (HPLC), the maternal plasma levels of ascorbic acid were determined by a spectrophotometric method and the degree of lipid peroxidation (TBARS) in fetal liver was analyzed using commercial kits (171). Low and high vitamin supplementation reduced the rates of fetal resorptions in diabetic dams, and did not adversely affect N dams (171). The plasma and liver concentrations of α-tocopherol and vitamin C were significantly increased in supplemented diabetic dams, but not in supplemented normal dams compared to normal and diabetic dams without the supplementation (171). The level of lipid peroxidation was higher in the livers of fetuses from diabetic dams (TBARS=200 nmol/g) than those from diabetic dams given vitamin supplements (TBARS in supplemented groups=60 – 70 nmol/g) (171). Vitamin supplementation did not impact TBARS levels in N rats (171). Cederberg's study (2001) indicated that the combination of vitamin E and C supplementation may help to reduce oxidative stress in diabetic rats but has little benefit in normal healthy rats (171). Similarly, Viana et al. (2000) showed that female STZ-diabetic rats, supplemented with vitamin E (150 mg/day of vitamin E, administered by gavage) had decreased rates of malformations, reabsorptions and TBARS compared to diabetic dams not provided with vitamin E (172).

Although the beneficial effects of vitamin E supplementation are more pronounced in diabetic rats than in normal rats, vitamin E in combination with vitamin C supplementation also improved endothelial dysfunction in normal rats (173). For example, Franco et al. (2003) examined whether vitamin E and C improve vascular functions of male offspring of nutritionally restricted

or control dams (173). Female Wistar rats at nine to eleven weeks of age were given either a control diet (22 % (w/w) protein, 43.5 % carbohydrates, 4.2 % fat) or a restricted diet (50 % of nutrients of the control diet) during pregnancy (173). Offspring of both groups received the control diet until 14 weeks of age (173). At 14 weeks of age, offspring of dams fed the restricted diet were treated with vehicle, vitamin C (ascorbic acid 150 mg/kg/day) or vitamin E (350 mg/kg alpha tocopherol/day) for 15 days (173). At 16 weeks of age, SBP and the load of ROS was measured *in vivo* (173). SBP was significantly higher in vehicle-treated compared to Vitamin C or E-treated groups (vehicle-treated=140, vitamin C-treated=123, vitamin E-treated=120, control=110mmHg) (173). Other studies conducted by Viana in 1999 and 2003 also showed that vitamin E supplementation in female rats increased vitamin E concentrations in plasma and the liver of the mother, fetal plasma and the placenta (174, 175). Viana et al. (1999) suggested that HDL transfers α-tocopherol to the placenta by passive transfer, resulting in higher concentrations of α-tocopherol in the placenta and fetus (174). Thus, these studies support the idea that maternal vitamin E supplementation in pregnancy may reduce oxidative stress in the mother, placenta and fetus, resulting in increasing reproductive success.

2.3.6 High fructose intake

There has been growing interest in metabolic, physiological and clinical consequences of fructose intake in pregnant women and their babies because of its potential to undermine maternal and infant health. Previous studies have found that fructose intake during pregnancy increases *de novo* lipogenesis, insulin responses to a glucose challenge and liver glycogen in rodent models (161, 162, 163, 164, 165, 178, 179, 180). Although the effects of fructose intake on offspring weight vary, two studies showed that the fetal:placental weight ratios and the

weights of neonates at weaning were reduced in dams given fructose during pregnancy (162, 178). Some rodent studies also indicated that the effects of maternal fructose consumption during pregnancy on metabolites (i.e. insulin, leptin concentrations) and physiology (i.e. body weight, the weight of fat mass, liver and muscle) of offspring were more pronounced in female than male offspring in a rodent model (182, 183). In humans, studies have found that consumption of SSB and added sugar increases a risk of developing preeclampsia (176, 177), infant body fat mass and neural tube defects (332, 334). This section summarizes the outcomes of the effects of fructose intake on the mother and offspring in both humans and rodents.

In humans Two studies have examined the relation between intake of SSB and a risk of preeclampsia in pregnant women (176, 177), building on the animal model work in male or non-pregnant females suggesting that high fructose intake negatively impacts blood pressure. In a study of 60,761 pregnant women in the Norwegian Mother and Child Cohort Study, drinking >1 serving size (> 250 mL) of SSB per day was positively associated with the risk of preterm delivery (adjusted OR: 1.25; 95 % CI: 1.08, 1.45) (176) while, Borgen et al. (2012) consumption of added sugars and SSB by pregnant women was positively associated with the risk of preeclampsia (OR: 1.27; 95 % CIs: 1.05, 1.54) (177). Elevated risks of neural tube defects were also observed in mothers who had high sucrose intake during the periconceptional period (three months before and three months after conception) (332). In a population-based case-control study (332), intakes of glucose, fructose and sucrose were measured by food frequency questionnaires in 454 mothers whose infants had neural tube defects cases and 462 control mothers whose infants did not have neural tube defects cases (control) (332). In this study, higher intake of sucrose during the periconceptional was associated with a 2-fold increase in risk of

neural tube defects in the infant, while higher intakes of glucose or fructose were not (332).

Grundt et al. (2012) noted an interesting association between sugar intake at a national level and birth weights in Norway between 1989 – 2010 (333). In this ecological study, intake of SSB was that reported by industrial beverage producers in Norway (333) and infant birth weights were from national data. The pattern of birth weight increase from 3,580 g to 3,633 g from 1989 to 2001, and subsequent decreased to 3,583 g in 2009 was mirrored by the increase and decrease in SSB consumption during this same time period (333). Although national data of SSB consumption may not reflect intake by individuals these trends are interesting and deserve further study (333).

In rodents The effects of high fructose consumption on pregnancy have been primarily examined in rodent models. It is well known that fructose intake increases *de novo* lipogenesis and total fat mass relative to body weight in pregnant rats and mice (161, 164, 165, 178, 179, 180). In a rat model, consumption of a 10 % (w/v) fructose solution with *ad libitum* standard chow during pregnancy increased mRNA expression of ACC, FAS and sterol regulatory element-binding protein 1 (SREBP1) in the liver on GD21 (ACC: control=1.23 ± 0.28, fructose=2.46 ± 0.2; FAS: control=3.80 ± 1.12, fructose: 9.93 ± 1.19) (161). Moreover, plasma concentrations of TG and liver TG contents were raised in pregnant rats fed a fructose solution (plasma TG: control=65.9 ± 14.9, fructose=117.9 ± 12.6 mg/dL; liver TG: control=11.1 ± 0.7, fructose=17.0 ± 1.6 mg/g tissue) (161). In other studies, pregnant rats consuming a 10 % (w/v) fructose solution had increased energy intake on GD14 and GD20 (162, 163), increased insulin responses to a glucose challenge on on GD14 (162, 163), raised plasma glucose and insulin concentrations on GD19 (178), higher liver glycogen content and increased proportions of fat on

GD19 (178). There is emerging evidence to suggest that high fructose intake in pregnancy is linked to inappropriate vascularity in the placenta (lower vessel area (%)) in pregnant rats (Alzamendi et al., 2012). Tain et al. (2015) reported that intake of a 60 % (w/w) fructose diet during pregnancy increased both systolic and diastolic blood pressure, suggesting that high fructose intake in pregnancy may have detrimental effects in several aspects of vascular growth (181).

The effects of feeding high fructose throughout gestation on fetal and placental weights vary among studies (163, 179, 162). Lineker et al. (2015) and Rodriguez et al. (2016) found no changes in litter size, fetal weight, placental weight or the placental:fetal weight ratio on GD20 (163, 179). By ~9 months of age, offspring body weights were higher among those whose mothers consumed a 10 % (w/v) fructose solution vs. those whose mothers consumed water (179). In Rawana's study (1994), body weights of individual neonates and total litter weights at weaning were lower in rats fed a 10 % (w/v) fructose solution than those of offspring born to rats fed tap water prior to, and during, pregnancy (178). However, Alzamendi et al. (2012) showed that the fetal:placental weight ratio was higher in pregnant rats fed a 10 % (w/v) fructose solution at GD20 compared with controls, suggesting that fetal weight was higher, but there was no change in placental weight in the fructose group (162).

In part, the differences in fetal weights may indicate sex-specific responses to maternal fructose intake during gestation. Two studies showed that body weights of female but not male fetuses were higher than controls at GD18 – 20 (182, 183). For example, Samuelsson et al. (2013) followed offspring of (mouse) dams fed a sucrose-rich diet (26 % of calorie intake) prior to, and throughout pregnancy, and reported increased body weight of female offspring, but not male

offspring, at three months of age (182). Fat mass relative to body weight was increased and liver, heart and muscle weighed more in females, born to dams fed the sucrose-rich diet vs. female offspring of dams fed a standard chow diet at three months of age (182). These differences were not observed in male offspring.

Some studies have examined serum metabolites and blood pressure in male and female offspring of sucrose-fed mice. Results indicate that insulin and leptin concentrations were increased in female but not male offspring compared to offspring of control dams, while systolic and diastolic blood pressure, heart rate and physical activity were increased in male but not female offspring of sucrose-fed vs. control dams (182).

Although a few studies reported sex-specific effects of prenatal fructose exposure, most of the previous studies did not detect significant different outcomes of consuming fructose on males and females. A number of experimental studies provided evidence that concentrations of insulin and TG, *de novo* lipogenesis and fat mass are significantly increased in both female and male offspring born to fructose-fed dams (179, 163, 165, 178, 183). In Rawana's study (1994), the plasma concentration of insulin at weaning was higher in offspring of rats fed a 10 % (w/v) fructose solution than that in rats fed tap water and a 10 % (w/v) glucose solution (178). Lineker et al. (2015) also showed that both plasma glucose and insulin concentrations were higher in offspring of rats fed a 10 % (w/v) fructose solution than those in offspring of control dams (163). Moreover, it has been clearly shown that hepatic lipid synthesis was significantly increased in offspring born to fructose-fed dams (179, 184). Clayton et al. (2015) found that offspring of dams fed fructose (20 % of calorie intake) had an increase in mRNA expression of GLUT5, SREBP1c and peroxisome proliferator-activated receptor-α on postnatal day 10 (184). In

Rodriguez's study (2016) female rats were provided either tap water (C), a 10 % (w/v) fructose solution (F) or a 10 % (w/v) glucose solution (G) during pregnancy (179). Rats were allowed to litter out and offspring were kept on a standard chow diet with 10 % (w/v) fructose solution, resulting in C/F, F/F and G/F groups (179). Offspring were euthanized at 240 days of age (179). Plasma was collected to determine TG and FFA, the liver was used to examine TG content, and mRNA expression of hepatic genes as measured by qPCR (179). The mRNA expression of ACC was higher, but that of phosphoenolpyruvate carboxykinase and uncoupling protein was low in F/F at 240 days of age (179). In addition, F/F had increases in plasma TG, FFA and TG contents in the liver compared to C/F and G/F (179). Therefore, Rodriguez's study indicated that maternal fructose intake during pregnancy significantly alters lipid metabolism, of offspring in later life, specifically by increasing *de novo* lipogenesis.

Together these studies suggest that a number of physiological processes may be adversely affected by maternal intake of fructose or sucrose (main source of fructose intake), and in some cases the changes observed may be sex-specific. The mechanisms and importance of these findings require further study.

Li et al. (2015) is one of the few studies that has combined dietary treatments to study the effects of high fructose and fat intake during pregnancy on offspring, and extended the experiments until offspring were 13 weeks of age (185). In Li's study, mice fed either a standard-chow diet (NC), high-fat diet (HFFD, 60 % of calorie intake as fat) with a 10 % (w/v) fructose solution for ten weeks before mating, during pregnancy and lactation (185). Female offspring were fostered to dams fed either NC or HFFD until weaning. After weaning, half of the female offspring were provided with NC and the remaining female offspring were provided with the HFFD, resulting in

four groups, NC/NC, NC/HFFD, HFFD/NC, HFFD/HFFD (185). Dams were euthanized at 17 weeks of age (at weaning) and offspring were euthanized at 13 weeks of age (185). The concentrations of blood glucose, insulin, FFA and TG were assessed using commercial kits, and mRNA expression of hepatic genes was analyzed by PCR (185). In dams, those in the HFFD group had increased blood glucose and plasma insulin concentrations during the OGTT, and had increased body weights and fat mass relative to body weight at 17 weeks of age (185). NC/HFFD and HFFD/HFFD offspring had elevated plasma concentrations of FFA and TG and increased liver weights and beta cell mass compared to NC/NC and HFFD/NC offspring at 13 weeks of age (185). In offspring, hepatic expression of PGC-1α was higher and GLUT2 and glucokinase were lower in NC/HFFD and HFFD/HFFD (185). Although Li's study (2015) designed "obesogenic diets (186)" using both high fat and high fructose for the pregnant female mice, this study also showed long-term effects of the maternal "obesogenic diet" on metabolites and physiology of offspring in adult life. This may indicate that both prenatal and postnatal fructose exposures are both key determinants that contribute to metabolic disorders in offspring in later life.

Although a number of recent works have focused on the effects of "prolonged fructose exposure" (187), it is still unclear how maternal fructose intake during gestation can influence metabolic and physiological aspects of offspring in adult life. Regnault et al. (2013) suggested that early life adaptations may be regulated or modified by maternal metabolic and placental adaptations, and this may permanently change metabolism in offspring in later life (187). Therefore, further studies are required to examine whether maternal fructose intake has influences on early life adaptations by inducing morphological and functional changes in the placenta during fetal development.

3. Placenta

The placenta is a critical organ during pregnancy since it plays a central role in mediating fetal growth and development through hormone production, nutrient transfer, exchange of gases and removal of waste products and by acting as a physical barrier between the mother and fetus (188). This section describes developmental stages and the functions of the placenta in humans and rats. In addition, this section outlines some of the important similarities and differences that should be considered in generalizing between humans and rat models. Lastly, this section considers the role that maternal nutritional intake may play in affecting placental development.

3.1 Placental development

In humans, the placenta is formed from the chorion and allantois and parts of the blastocyst in early pregnancy; thus, it is a "chorioallantoic placenta" (189). The fetal side of the placenta has an umbilical cord that is attached to the chorionic plate (Figure 1-5; 189). The opposite side of the placenta is the basal plate, deciduas basal, or maternal surface. At term, the basal plate has between 10 – 40 cotyledons that consist of a number of villous trees which includes the intermediate and terminal villi (189).

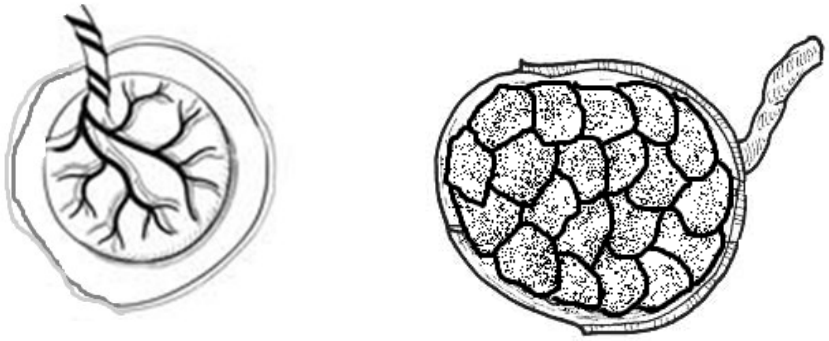


Figure 1-5: The fetal side (left) and maternal side (right) of human placenta at term. The

figure is adapted from Huppertz (190)

The placenta develops in parallel with the fetus during pregnancy and each trimester is marked by specific features within the placenta (188). The following paragraphs describe the stage of placental development during **a) first trimester, b) second trimester** and **c) third trimester** in humans (188).

a) During the first trimester (week one to week twelve in humans, early pregnancy), a fertilized egg grows into a blastocyst and implants in the uterus. In the embryonic stage (~week eight), the outer layer of the embryo is subdivided into two layers: the cytotrophoblast (internal site) and the syncytiotrophoblast (external site) and together these develop into the placenta (188). As pregnancy progresses, the cytotrophoblasts continue to proliferate and fuse to form the syncytiotrophoblast, and the syncytiotrophoblast covers the surface area of the villi (Figure 1-6; 191, 192). This process further contributes to the villous structure and vessel branches by trophoblast proliferation, vasculogenesis, and angiogenesis (193). Villous formation is most active in the first and second trimesters (190). At approximately six to eight weeks of gestation, the development of the villous system begins (194). At about ten weeks of gestation, cytotrophoblast from placental villi invades the space around the spiral artery (330). This process causes the loss of muscle layer and the endothelium is replaced by extravillous cytotrophoblast (330). This remodeling of the spiral arteries increases the diameter of the vessels about 10-fold, which results in increased blood volume to the intervillous space (330). This system ensures that adequate nutrients and respiratory gases are provided for fetal growth (330). The process is completed by the end of the first trimester (194). More details on villous formation are described in Figure 1-6 (330). Failure of spiral artery remodeling as can happen when there is reduced

expansion of the vascular lumen and fibrinoid necrosis of the artery is known to cause IUGR and pre-eclampsia (330). **b) During the second trimester** (week 13 – week 27 in humans, mid-pregnancy), the placenta takes over the regulation of maternal-fetal interactions from the corpus luteum (190). These functions include the production of hormones (hCG, estrogen, progesterone, and gonadotropin releasing hormone) and nutrient transport including glucose, amino acids and fatty acids to the fetus by transporter proteins (190; more details will be described in section 3.4, page 70 – 72). **c) During the third trimester** (week 28 – end of pregnancy in humans, late pregnancy), nutrient transfer efficiency in the placenta increases, which helps to accelerate fetal growth (162).

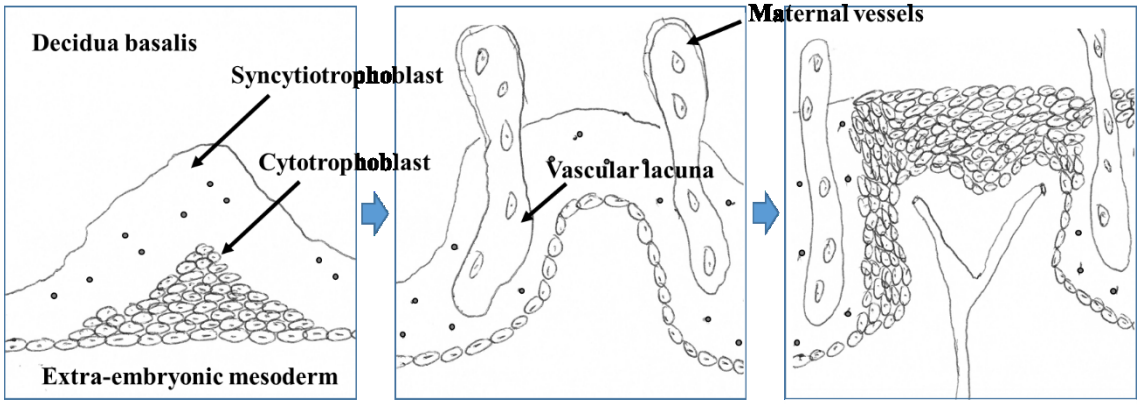


Figure 1-6: The development of placental villi. Villous structures develop starting from appoximately 8 – 10 weeks gestation onward. Formation of the secondary villi occurs as the trophoblastic trabeculae penetrate the central core of the cytotrophoblasts more deeply than do the primary villi. The tertiary villi are developed from the secondary villi and occur as fetal vessels become surrounded by trophoblasts. These tertiary villi have complete layers of both cytotrophoblasts and syncytiotrophoblast. The villous core in the tertiary villi later divides into four types: immature intermediate villi, stem villi, terminal villi and syncytial sprouts. The tertiary villi can efficiently diffuse gas and nutrients between the maternal-fetal circulation. The tertiary villi become prominent in the last trimester. *The figure is adapted from Huppertz (2011) (190) and an electronic source from Placenta and Extraembryonic Membranes at University of Michigan Medical School (link below: **http://www.med.umich.edu/lrc/coursepages/m1/embryology/embryo/06placenta.htm**).

3.2 Vasculogenesis and angiogenesis

Development of the villous tree is necessary to ensure adequate nutrient transfer and gas exchange between the mother and the fetus (190). Thus, appropriate branching of placental vasculature is critical to adequate fetal growth (190). Vasculogenesis and angiogenensis are the processes needed to support vessel growth in the placenta (190).

Vasculogenesis, or the process of new vessel formation, begins with villi formation (190). At approximately day 21 post conception, endothelial cells begin invading secondary villi (190). Hemangiogenic progenitor cells (from mesenchymal cells) and macrophages (derived from progenitor cells) differentiate within the core of the placental villi to connect between the embryo and the placenta (190). After this process, new cells differentiate within the mesenchymal stroma of the placental villi, and are referred to as tertiary villi (190). Further cell recruitment leads to hematopoietic cells and angioblastic cells; hematopoietic cells develop into blood cells, and

angioblastic cells differentiate into endothelial precursor cells and form the first vessels (190). Vasculogenesis eventually evolves into angiogenesis (190). Angiogenesis is the formation of new blood vessels from pre-existing vessels and occurs through elongation, intercalation, sprouting and intussusception as follows: 1) Elongation is the growth of a vessel from the ends of pre-existing vessels; 2) Intercalation is the growth of a vessel from pre-existing vessels, but excluding the ends; 3) Sprouting occurs as new branches extend from the pre-existing vessel; and 4) Intussusception forms a new vessel wall in the center of a pre-existing vessel (190). Through these processes, the placenta is able to create a complete vascular tree.

Vasculogenesis and angiogenesis require the secretion of growth factors to recruit trophoblast cells within the villous core (190). Hematopoietic cells, angioblastic cells and macrophages are firstly derived from the placenta (macrophages are derived from progenitor cells before villous formation) (190). These cells, called Hofbauer cells, are widely found in villi and secrete growth factors, primarily vascular endothelial growth factor (VEGF) (190). Other types of cells like smooth muscle cells also secrete VEGF, fibroblast growth factor and angiopoietin (190). The secretion of growth factors leads to the formations of villous trees. Details on growth factors are described below:

The vascular endothelial growth factor (VEGF) family of molecules VEGF is a family of pro-angiogenic cytokines that is produced by macrophages, T cells and cytotrophoblasts (195) that regulate angiogenesis and vasculogenesis by affecting vascular permeability (195, 196). The VEGF family consists of **a) VEGF-A and VEGF-B, b) placental growth factor (PlGF), c) VEGF receptor 1** (VEGFR-1, also known as fms-like tyrosine kinase receptor-1 (FLT-1)), **and, d) VEGF receptor 2** (VEGFR-2, also known as kinase insert domain receptor (KDR) and fetal

liver kinase 1 (Flk-1) in humans) (197, 198).

a) VEGF-A, and -B VEGF-A promotes angiogenesis by stimulating vascular endothelial cell proliferation, reducing apoptosis, increasing vascular permeability and promoting vasodilation through the nitric oxide pathway (199, 200). VEGF-B plays an indirect role in angiogenesis in the placenta since it acts to recruit PlGF (201).

b) placental growth factor (PlGF) PlGFs are known to stimulate vessel growth by directly stimulating vascular endothelial cell proliferation and indirectly recruiting pro-angiogenic cells (201). In addition, PlGF increases proliferation of cells such as fibroblasts and smooth muscle cells (201, 202) In the placenta, PlGF is abundantly expressed by the villous syncytiotrophoblast and stem vessels (203). The functions of PlGF in VEGF-dependent angiogenesis are still unclear (200), but PlGF is considered to enhance VEGF signaling, which results in promoting angiogenesis (201). Excess production of PIGF appears to inhibit angiogenesis since overexpression of PlGF results in the formation of VEGFA-PlGF heterodimers, which ultimately reduces the free VEGF-A pool (201). This suggests that there may be an optimal range for VEGF-A concentration to support angiogenesis (201, 204).

c) VEGF receptor 1 and 2 VEGFR-1 is expressed in trophoblasts in the placenta and binds to VEGF-A, VEGF-B and PlGF with high affinity (197, 205). It is a primary receptor of VEGF-A in endothelial cells (200). Both the expression of VEGF and VEGFR-1 is highest at early gestation and declines as pregnancy progresses (206). Ahmed et al. (2000) also described that binding of VEGF to VEGFR-2 has a major role in invasion of immature intermediate villi during the first trimester, and binding of PlGF and VEGFR-1 help to form the long terminal capillary

loops in the last trimester (199).

3.3 Oxidative stress: Factors that regulate vasculogenesis and angiogenesis

Pregnancy is a state of oxidative stress, primarily due to increased production of ROS and the additional mitochondrial activity in the placenta (207). ROS production includes formation of nitric oxide, carbon monoxide and peroxynitrite (207). Superoxide is essential for placental vascular development (350), but excess levels of superoxide increase the production of peroxynitrite which is known as a powerful oxidant induced by the reaction between superoxide and nitric oxide (207). An imbalance between ROS and antioxidant potential in cells and tissues may contribute to pathologic conditions of pregnancy such as preeclampsia, diabetes and obesity (207). This section will discuss the effects of oxidative stress on placental vasculogenesis and angiogenesis, and the types of pro-oxidants and antioxidants found in the placenta. In addition, this section will review oxidative stress in pathologic conditions of pregnancy.

3.3.1 Oxidative stress and the development of placental villi

For the first ten weeks of pregnancy in humans, the process of placentation and embryogenesis takes place in a hypoxic environment (207, 208). Extravillous trophoblasts block the maternal spiral arteries, which results in no blood flow through these vessels and a low degree of oxidative stress (209). During this period, low oxygen concentrations and a low degree of ROS increase the expression of VEGF by hypoxia inducible factor-1 (HIF-1) (210). From eight to 12 weeks of gestation, extravillous trophoblasts collapse and penetrate maternal spiral arteries which triggers blood flow into the placenta (209). This process is accompanied by significantly increased levels of oxidative stress and increases in the expression of PlGF, and decreases in the expression of

VEGF (209). Therefore, poor perfusion in the placenta during the first trimester leads to the inhibition of VEGF expression and changes the timing of PlGF growth, resulting in immature vessel development (211). The placenta also begins to synthesize antioxidant enzymes such as copper/zinc superoxide dismutase and mitochondrial superoxide dismutase starting at approximately 11 weeks of gestation (207, 212, 213). Thus, the placenta may be protected from excessive oxidative stress. From 12 weeks of gestation, blood vessels maintain optimal blood flow between maternal and fetal circulation (209). Fetal capillaries are expanded by angiogenesis between nine to 23 weeks of gestation and reach blood vessel maturation at 23 to 24 weeks of gestation (207, 209, 211, 213, 214).

Oxidative stress is defined as an imbalance between a number of antioxidants and pro-oxidants (Details are described in Figure 1-4). These pro-oxidants and antioxidants play a critical role in vascular development in the placenta. This section focuses on a few pro-oxidants and one antioxidant (among a number of factors involved in oxidative stress in the placenta) which are important to my study.

Oxidants and vascular development in the placenta A normal level of oxidative stress is well-controlled by appropriate levels of pro-oxidants and antioxidants (207, 215). For example, under normal conditions, superoxide itself is limited by a reduction of membrane transport, lipid solubility and SOD activity in the placenta (207). However, when tissues produce high levels of superoxide and nitric oxide, the reaction between these two pro-oxidants generates peroxynitrite anion ($ONOO^-$) which is a strong oxidant (207). Peroxynitrite inhibits cellular respiration, stimulating lipid peroxidation and oxidizing sulfhydryl groups in proteins by reducing mitochondrial electron transport (216). Peroxynitrite also reacts with proteins and produces a

stable product, nitrotyrosine (217). An increased formation of both peroxynitrite and nitrotyrosine has been associated with vascular damage in many pathologic conditions such as atherosclerosis (217) and lung injury (218). In the placenta from women with preeclampsia and diabetes, the formation of nitrotyrosine residues was increased in the vascular endothelium in the placenta compared to those in the placenta of healthy pregnant women (219). An increased formation of peroxinitrite and nitrotyrosine residues and lipid peroxidation were also found in the placenta in preeclampsia, diabetes and intrauterine growth restriction (IUGR) (207, 220, 221).

An antioxidant (vitamin E) and vascular development in the placenta Recently, antioxidants, particularly vitamin E which is known to prevent oxidation by reacting with lipid radicals in the lipid peroxidation chain reaction (70), have been found to play a critical role in placental vascular development. The role of vitamin E in vascular development was determined through *in vitro* studies using human umbilical vein endothelial cell culture (222, 223). These *in vitro* studies demonstrated that treating human umbilical vein endothelial with α-tocopherol stimulates angiogenesis and vasculogenesis by phosphorylation of α-tocopherol to α-tocopheryl phosphate (222, 223). α-tocopheryl phosphate stimulates the Pl3K/Akt signaling pathway that increases HIF-1α/PGC-1α, leading to an increased expression of VEGF (224). The second role of vitamin E has been suggested to protect the fetus and the placenta from oxidative stress, although this function is less clear in humans (225). Vitamin E is known as one of the major antioxidants that inhibits lipid peroxidation in lipid membranes and lipoproteins (226). α-tocopherol is converted into tocopheroxyl radical by reacting with lipid peroxyl radicals that are generated from polyunsaturated fatty acids (225). After the first reaction, tocopheroxyl radical is reduced to

tocopherol by reacting with ascorbate. α-tocopherol also regenerates GSH from glutathione disulfide (GSSG), an oxidized dimer of two GSH molecules (225). Therefore, an increased level of GPX is able to catalyze decomposition of hydrogen peroxide and reduce oxidative stress (225).

Lipid peroxidation and vitamin E A few studies have examined oxidative stress in the placenta by analyzing the levels of lipid peroxidation and vitamin E in the placenta of women with preeclampsia (227, 228, 229). Poranen et al. (1996) examined the level of lipid peroxidation and antioxidants in the placenta from 15 normal and 15 preeclamptic women (229). TBARS was measured to determine the level of lipid peroxidation, and the concentration of vitamin E and activities of SOD, GPX, glucose-6-phosphate-dehydrogenase, glutathione transferase were measured to determine the level of antioxidants in the placenta (229). Placental lipid peroxidation was significantly elevated in preeclampsia while the activity of placental SOD and glucose-6-phosphate-dehydrogenase was lower in preeclampsia compared to normal pregnancy (229). However, this study did not find any alterations in the activity of GPX, glutathione transferase, or the concentration of vitamin E in the placenta from women with preeclampsia (229). In their later study, Porane et al. (1998) confirmed that the concentration of vitamin E in the placenta is unchanged in preeclampsia (230). A more recent meta-analysis done by Rumbold et al. showed that vitamin E in combination with vitamin C supplementation can prevent placental abruption that is known to be caused by increased oxidative stress in the placenta (323). However, the author mentioned that the role of vitamin E on preventing placental abruption was not clear since the results observed in the study could be from vitamin E or vitamin C or both (323). It is hard to determine the mechanisms by which vitamin E may affect oxidative stress in

the placenta since oxidative stress is a complicated process. Further studies are required to understand the undelying mechanisms relating vitamin E intake and status with, oxidative stress and adverse birth outcomes, particularly placental abruption.

3.4 Nutrient transporters in the placenta

The placenta transports nutrients from maternal circulation to the fetus (231). Layers of the placental villi establish the interface between maternal and fetal circulation. These structures regulate the exchange of nutrients and waste products between mother and fetus (231). The syncytiotrophoblast that faces the maternal circulation, consists of the microvillus membrane and the basal plasma membrane (231). The microvillus membrane is closest to the maternal circulation, whereas the basal plasma membrane faces toward the fetal capillaries (231). After nutrients cross syncytiotrophoblast and cytotrophoblast, they reach the fetal capillary epithelium, cross this tissue's cytoplasm and intermembranous space, and eventually enter fetal circulation (231). Nutrients are transported across the syncytiotrophoblast through a combination of active and passive transport mechanisms, including facilitated diffusion and bi-directional transport processes (232). Many of the nutrient transport proteins are embedded in the microvillus and/or and basal plasma membranes. Membrane-associated transporters for glucose, amino acids and fatty acids are discussed below; micronutrient transporters are also present in the syncytiotrophoblastic membrane but are beyond the scope of this thesis.

3.4.1 Glucose transporters

Glucose is a major energy substrate supporting growth of the fetus and placenta. There is almost no gluconeogenesis within either the fetus or the placenta, thus glucose is available to the fetus

and placenta from maternal circulation and moves across the syncytiotrophoblast using facilitated diffusion that occurs through the GLUT transport molecules (190, 231). GLUT1 is known as the basal glucose transporter and is widely expressed in almost all tissues (231). It is the most abundant glucose transporter in the placenta in early and late pregnancy (231). GLUT1 is expressed in both the microvillus and basal plasma membranes, but has lower expression in the basal plasma membrane than in the microvillus membrane, suggesting that the rate-limiting step of glucose transfer may exist in the basal plasma membrane (231). GLUT3, a placenta-specific glucose transporter, and is considered to play a role in glucose transport during early pregnancy since its expression decreases gradually as pregnancy progresses (231). GLUT3 is embedded in the microvillus membrane of the syncytiotrophoblast, as well as in cytotrophoblasts and endothelium (233). GLUT4 is an insulin-sensitive glucose transporter that is expressed primarily in muscle and adipose tissue but is also localized in the cytosol of the syncytiotrophoblast and intervillous stromal cells. GLUT4 expression in the placenta decreases substantially at term (234). Thus, GLUT1, 3 and 4 seem to be specifically localized in different parts of the placenta at different times during pregnancy. Although most older studies have failed to detect other members of the GLUT family including GLUT 2, GLUT5, GLUT9 and GLUT10, emerging evidence suggests that they may actually be present (235). A recent study by Novakovic et al. (2013) identified temporal changes in DNA methylation of GLUT2, GLUT3, GLUT5, GLUT9 and GLUT10 in term placentae (236), however, their functions in the placenta remain to be determined.

3.4.2 Amino acid transporters

The concentration of amino acids is higher in fetal than maternal circulation, suggesting an

active transport of amino acids across the syncytiotrophoblast into the fetal circulation (237). A number of amino acid transporters are embedded in the syncytiotrophoblast's plasma membrane (237). System A and System L are the most well characterized of the amino acid transporters in the placenta (237). System A is a sodium-dependent accumulative transport system that transfers small neutral amino acids such as alanine, serine, and glycine (231, 237). System A has three main isoforms in the placenta, namely, sodium-coupled neutral amino acid transporter (SNAT) 1 (also known as SLC38A1), SNAT2 (SLC38A2) and SNAT4 (SLC38A4) (238). These transporters are expressed in the microvillus membrane during the third trimester mostly (238). SNAT2, in particular, has been suggested to play a critical role in the regulation of fetal growth since lower expression of SNAT2 was reported in the placentae of pups that experienced IUGR compared to placentae of control rats (239). It was suggested that the reduction of placental SNAT2 may be a cause, rather than a consequence, of IUGR (239). System L is a sodium-independent transporter for non-essential amino acids such as leucine (240). The isoforms of these transporters are large neutral amino acid transport (LAT) 1, LAT2, LAT3, and LAT4 (240). LAT1 exists on the microvillus membrane, whereas LAT2, LAT3 and LAT4 exist on the basal plasma membrane of the syncytiotrophoblast (241). SNAT and LAT transporters work together to provide adequate substrate for protein synthesis and signaling pathways required for normal fetal development.

3.4.3 Fatty acid transporters

Placental TG lipase breaks TG into FFA and glycerol since TG cannot cross the syncytiotrophoblast due to its large molecular size (242). Lipoprotein lipase and endothelial lipase hydrolyze TG at the microvillus membrane and FFA are then transported to fetal

capillaries through fatty acid transporters such as FATP, fatty acid translocase (FAT/CD36), plasma membrane FABP (FABPpm) and FABP (231). FATPs are located on both the microvillus and basal plasma membranes of the syncytiotrophoblast to transfer long-chain fatty acids (231). The FABP family of transporters is important for the uptake of long-chain polyunsaturated fatty acids (231). Five members of FABP (FABP1, 2, 3, 4, 5) are the most abundant fatty acid transporters in all cell types (231). However, the functions of the FABP family in the placenta are not well known.

3.5 The rat as an animal model for studying human placental function

Animal models, especially rats, are useful models to investigate some aspects of placental and fetal development due to their short gestational period, the potential for obtaining relatively large numbers of samples and the fact that their placenta share many physical similarities with the human placenta (243). Results from studies in rodent models also allow for studying *in vivo* effects on placental development since these processes take place under physiological conditions, in contrast to studies in cell culture in which cells are removed from their natural environment, and in which there are limited interactions and mechanisms that may exist between tissues.

The rat placenta has been used as a model of human placental development in a number of types of studies since they share several structural features (244). For example, both human and rat placentae are classified as chorioallantoic placenta, “a placenta in which the chorion is formed by the fusion of the allantoic mesoderm and vessels to the inner face of the serosa” (medical dictionary) (244). In addition, both the rat and human have a hemochorial type of placenta and share similar milestones in placental development described above (244).

However, there are also several important differences that exist between the structure and physiology of the placentae of rats and humans. These include differences in the timing of feto-placental development, the number of layers (human, hemomonochorial with one layer while the rat has a hemochorial structure with three layers) and the villous structure (human=villous structure; rat=labyrinthine structure). Knowledge of the similarities and differences between placentae in rats and humans helps to ensure that results are interpreted appropriately.

3.5.1 The morphology of rat placenta

In rats, embryo implantation occurs at the anti-mesometrial side while the placenta forms at the mesometrial side (244). The placenta develops different layers and structures from GD6 to GD16 (244). From mid- to late pregnancy, the rat placenta has a discoid shape and a hemochorial type of placentation like humans (244). Structurally, the fetal part of the placenta consists of labyrinth and junctional zone (JZ), and the maternal part of the placenta consists of deciduas and metrial gland (244).

Fetal parts of the placenta The labyrinth zone (LZ) of the rat placenta is composed of maternal sinusoids and trophoblast epithelium. The maternal sinusoids are an open pore capillary, through which maternal blood passes freely between trophoblastic septa (244). The trophoblast epithelium, also known as cytotrophoblast, is directly exposed to the maternal blood space and contains a number of microvilli on its surface area (244). Syncytiotrophoblast-1 and -2 and gap junctions form a placental barrier under the trophoblast layer (Figure 1-7, 244). Gap junctions are protein channels that are composed of connexins (245). They provide channels for the diffusion of small molecules (cAMP, cGMP, inositol triphosphate and calcium) and ions (245). In rats,

connexin-26 forms a number of gap junctions which connect syncytiotrophoblast-1 and -2, and transfers glucose along with GLUT1 (247). The LZ becomes the major part of the placenta to exchange gas and nutrients since maternal and fetal blood are very close to each other in this area (244).

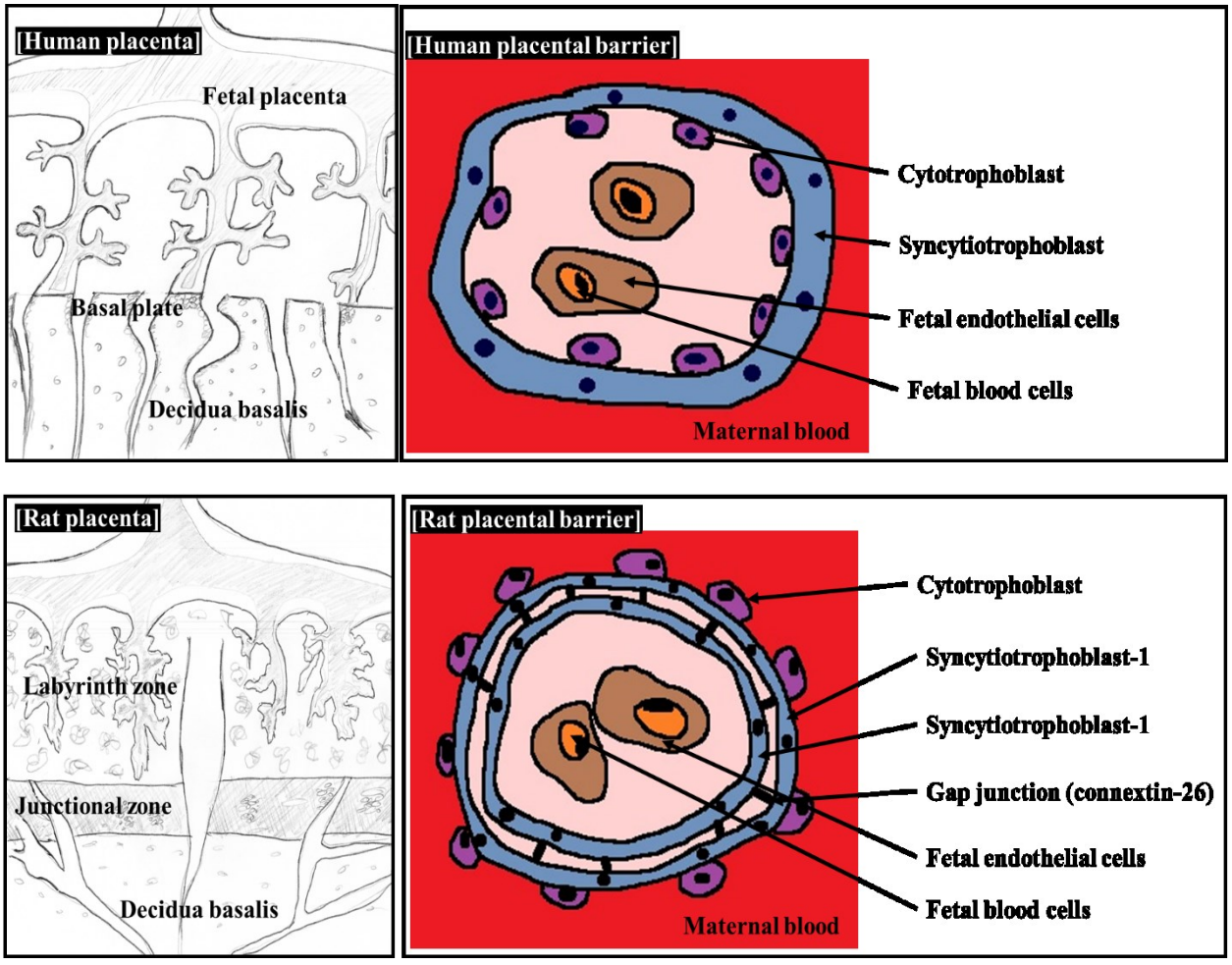


Figure 1-7: Comparison between rat placenta and human placenta. The figure is adapted from Furukawa (244)

Maternal blood traverses the labyrinth through maternal sinusoids and reaches the JZ (also

known as the basal zone) (244). The JZ contains three types of the cells: Spongiotrophoblast cells, trophoblastic giant cells, and glycogen cells (244). The spongiotrophoblast cells are the main components of the JZ (244). These cells are located directly next to the trophoblastic giant cells and some glycogen cells. The trophoblastic giant cells have a critical role in the endocrine functions of the placenta such as synthesizing and secreting prolactin (244). Glycogen cells are known to be derived from the spongiotrophoblasts during mid-pregnancy, but most of them disappear at the end of pregnancy (244). The role of glycogen cells is not clearly understood (244).

Maternal part of the placenta The maternal portion of the placenta is called the deciduas basalis and is the main area in which angiogenesis is observed (246). The deciduas basalis also produces a number of hormones, cytokines and other growth factors, which together have a critical role in growth and development of the placenta (246). The metrial gland is located in the mesometrial triangle in rat placenta and is composed of various cell types including decidualized endometrial stromal cells, uterine natural killer cells, spiral shaped artery, and fibroblasts (248).

3.5.2 Nutrient transporters in rat placenta

Similar to humans, a number of nutrient transporters are found in the rat placenta. GLUT1 is expressed in both the JZ and LZ of the placenta, but it is significantly highly expressed on syncytiotrophoblast layer 1 and 2 during mid-pregnancy (149). GLUT3 is primarily expressed in the LZ, and is embedded in the membranes facing the maternal circulation (249). Functionally, GLUT3 is thought to protect the fetus from maternal hyperglycemia and hypoglycemia by regulating glucose transfer across the placental barrier (249). Placentae of diabetic rats have

increased GLUT3 expression, while their GLUT1 expression remains unchanged in this condition (249). This suggests that changing the expression of GLUT3 helps to regulate glucose transfer between maternal-fetal circulations while the expression of GLUT1 is not altered (249). Amino acid transporters and fatty acid transporters have also been identified in the rat placenta.

In summary, the placenta has a critical role in fetal development by regulating nutrient transfer, hormone secretion and protecting the fetus from external stimuli. In each trimester of pregnancy, placental development is marked by specific features such as the development of placental villi by angiogenesis and expression of nutrient transporters. The rat has been used as a model for the study of the human placenta because of structural similarities between the two. However, there are still several differences in the structure of the trophoblast layers and the timing of the processes of placental development between those two species. Thus, it is important to consider the differences in the characteristics of human and rat placentae and then analyze outcomes from a rat model in order to understand how it applies to the human system.

3.6 Maternal dietary intake and its effects on placental development

Variation in maternal nutrient intake has been known to influence fetal development and have long-term effects on the individual offspring in later life. Although it is still unclear how maternal diets specifically influence fetal development, scientists have reported that under and overconsumption of calories and other nutrients by the mother affects the birth weight of the fetuses (as described previously). Moreover, a correlation between weight at birth and placental weight, an important variable in fetal development, was shown in a number of studies. Under conditions of maternal energy restriction or overconsumption during pregnancy, the placenta

performs compensatory functions for optimal fetal growth, and these lead to clinical consequences for the fetus, such as intrauterine growth restriction. Therefore, it is important to understand how maternal nutrition during pregnancy can affect placental development and function, with a specific focus on maternal under and overnutrition. The goal of this section is to outline understanding of how maternal nutrition, particularly overnutrition with respect to energy, protein, fat and fructose (including sucrose), can have a lasting impact on fetal health via changes in the placenta. This is because more and more studies are now focusing on the adverse effects of overnutrition, due to westernized diets, on fetal development. The various studies about describing the adverse effects of maternal undernutrition (low protein, low energy intake) on placental development and fetal growth are summarized in Appendix 4.

Overconsumption of a diet high in fat leads to excess calorie intake and increases the risks for developing chronic diseases. Previous studies have shown that early programming of the fetus can be influenced by maternal undernutrition, and that this may be associated with poor placental development. Similar to maternal malnutrition, maternal high energy, and particularly high fat intake during pregnancy, can alter the functions and the morphology of the placenta, resulting in fetal growth restriction and significant health problems for the offspring in adult life. More researchers are also moving their focuses on the adverse effects of fructose intake on the fetus and the placenta. This section mainly focuses on the reported effects of maternal overconsumption of calories, fat and fructose on placental development. Studies are summarized in Appendix 5.

The development of placental cells It has been found that maternal high-fat consumption inhibits the proliferation rate of placental cells during pregnancy (250, 251). Kim et al. (2014)

examined the rate of cell proliferation and apoptosis in the placentae of mice fed a high-fat diet (251). Four-week-old mice were fed either a control diet (10 % of total energy intake as fat) or a high-fat diet (45 % of total energy intake as fat) for eight weeks (251). Tissues were collected on GD15.5 or 17.5. In the placentae, proliferation was examined by staining with an antibody against phosphohistone H3, and apoptosis was analyzed by staining with an antibody against activated caspase-3 (251). The data showed that the proliferation rate, but not the apoptosis rate, of cytotrophoblasts appeared to be reduced in the LZ of the placenta of high-fat fed mice at both GD15.5 and 17.5 (251). The authors suggested that a reduction in proliferation in cytotrophoblasts may inhibit syncytiotrophoblast formation since the syncytiotrophoblast is maintained through the fusion of underlying cytotrophoblast cells (251). Liang et al. (2010) also observed that maternal high-fat intake (60 % of total energy intake as fat from one month before conception and during pregnancy) significantly decreased the type of trophoblast cells in LZ of the placenta of mice on GD19 (250). In addition, the results of the placental morphological analysis indicated that the placentae from high-fat fed mice had areas with a high density of fibrinonecrosis, a form of necrosis, and cellular fragmentations in endothelium in the LZ (250). These two studies suggest that cell proliferation in the placenta is inhibited by maternal high-fat consumption.

Placental blood vessels Maternal high energy intake, particularly high fat intake, appears to adversely affect the vascular development of the placenta in a number of different ways, including by increasing angiogenesis and possibly by affecting the dimensions of the fetal capillaries and maternal spaces in term placenta (252, 253). For example, Hayes (2012) analyzed the effects of maternal high-fat intake on the growth of placental endothelial cells in rats fed a

diet containing 45 % energy from fat (252). Placentae were collected at GD15 and formalin-fixed (252). Then, placentae were sectioned, and the expression of cluster of differentiation 31 (CD31; an endothelial cell marker) and smooth muscle actin (SMA; a marker of blood vessel maturity) were measured by immunohistochemistry (252). Mature blood vessels were determined by SMA positive cells/one tissue section (total four sections) (252). A high-fat diet (45 % total kcal from fat) increased the level of CD31, which indicates a greater number of blood vessels had formed (252). This result suggested that maternal high-fat intake promotes the development of placental vascularization (252). However, interestingly, the expression of SMA was decreased by 30 % in the placenta of high-fat fed rats, which suggests immature vessel formation (252). A reduction in SMA expression is commonly shown in mouse models of preeclampsia, and it is associated with increased blood pressure and reduced fetal growth (253). Similarly, in Hayes's study, the proportion of SGA birth was significantly higher in high-fat fed dams than in dams in the control group (16 % of total energy intake as fat) (252). Thus, Hayes's study indicated that a maternal high-fat diet inhibits mature blood vessel formation, and this change was associated with fetal growth restriction.

It is currently unclear how maternal energy/fat intake can affect vessel formation and maturity, but a possible mechanism has been suggested. Li (2013) suggested that upregulation of HIF-1α and VEGF-A expression is associated with abnormal vascular development (254). A diet-induced GDM model was developed using female mice that were fed either a high-fat diet (40 % of total energy intake as fat) or a control diet (14 % of total energy intake as fat) for 14 weeks before and during pregnancy (254). Placentae were collected at GD18.5, and the mRNA and protein expression of HIF-1α, VEGF-A and CD31 were determined using real-time PCR and

immunohistochemistry (254). mRNA expression of both HIF-1α and VEGF-A was increased by 4.7-fold and 7.4-fold respectively in the placentae of high-fat fed dams (254). However, the mRNA levels of CD31 was increased by 1.3-fold only in the placentae of high-fat fed dams (254), which means the increased level of CD31 was not parallel with the increased level of HIF-1α and VEGF-A. Thus, placental angiogenesis may be insufficient in the placentae of rats fed a high-fat diet compared to that of rats fed a diet containing a regular amount of fat (14 % of total energy intake as fat) (254).

Oxidative stress Only a few studies have focused on the effects of maternal overnutrition on the level of oxidative stress in the placenta. In humans, however, there are growing interests in the association between maternal obesity and oxidative stress in the placenta. We may gain insight into the relationship between overnutrition and placental oxidative stress by examining the placenta of obese women since overnutrition is one of the factors that contributes to increased risk of developing obesity. Roberts et al. (2009) found that maternal BMI was positively associated with placental oxidative stress in humans (255). In Roberts' study (2008), placentae were collected from lean (BMI 18.5 – 24.9 kg/m^2 at delivery), overweight (BMI 25 – 29.9 kg/m^2 at delivery) and obese (BMI 30 – 40 kg/m^2 at delivery) women within 39 minutes of delivery (255). In the placenta, nitrotyrosine, manganese SOD (MnSOD), copper/zinc SOD (CuZnSOD) and extracellular SOD (ECSOD) were measured by western blots, and total nitrotyrosine and activities of SOD and GPX were analyzed by ELISA (255). The results showed that measurement of band intensity of MnSOD, CuZnSOD, ECSOD by western blots did not reveal differences among the BMI groups (255). The activities of SOD and GPX were not different among the placentae of lean, overweight and obese women, but the concentration of

nitrotyrosine was significantly increased in the placentae from obese women compared to lean and overweight women (obese=0.35, lean=0.19, overweight=0.17 ug/mg protein) (255).

In obese rats, alterations in activities of certain antioxidant enzymes were found in the placenta (256). Female Wistar rats were divided into a control (220 g/kg diet of protein, 631 g/kg diet of carbohydrates, 43 g/kg diet of fat), an obese (300 g/kg diet of protein, 351 g/kg diet of carbohydrates, 195 g/kg diet of fat) or a malnourished (90 g/kg diet of protein, 761 g/kg diet of carbohydrates, 43 g/kg diet of fat) group (256). Oxidative stress markers (GPX, glutathione reductase, Glutathione S transferase, SOD, GSH, GSSG, GSH/GSSG ratio) were measured in the placentae on GD17 (256). The study found no difference in these oxidative stress markers in the placentae between control and malnourished rats, but the placentae from obese rats had an increased level of GPX (obese=57.4 ± 1.2, control=52.4 ± 1.6 mU/mg protein) and a decrease in glutathione S transferase (obese=3.5 ± 0.05, control=3.8 ± 0.1 mU/mg protein) (256). However, it is still hard to draw a conclusion as to whether maternal overconsumption affects the level of oxidative stress from results of only two studies (255, 256). There is a need for future studies to investigate the extent to which overnutrition during pregnancy has influences on placental oxidative stress by measuring numerous oxidant and antioxidant markers.

Fructose intake is known to increase free radical production and reduce antioxidant capacity (pages 19 – 23), but little is known about how maternal fructose intake during pregnancy influences oxidative stress in the placenta. Recently, Rodrigo et al. (2016) examined the association between maternal fructose intake and oxidative stress in the mother, the placenta and the fetus using a rat model (334). The study also determined whether the effect of high fructose intake on oxidative stress is more pronounced compared to those effects from high glucose

intake (334). Female Sprague-Dawley rats (weighing 200 – 220 g) were assigned to receive a 10 % (w/v) fructose solution, a 10% (w/v) glucose solution or distilled water (control group) throughout gestation (334). On GD 21, blood, tissues (liver, placentae) and the fetuses were weighed and lipid peroxidation (MDA) levels in these tissues was measured (334). In rats, plasma MDA values were reduced in both fructose and glucose groups compared to the control group, but the liver MDA levels were significantly higher in dams fed the fructose solution than dams fed glucose or distilled water (334). Plasma and liver MDA values were higher in the fetuses of fructose-fed rats, but no difference was found in the fetuses of glucose-fed rats compared to the controls (334). Placenta MDA values were higher in the fructose group, but not in the glucose group, than controls (334). Rodrigo also found that increased MDA levels in the placenta in the fructose group were accompanied by a reduction in nuclear factor-erythroid 2-related factor-2 and its target gene, heme oxygenase-1 in the placenta; these molecules are antioxidants (334). Thus, this model clearly showed that maternal fructose intake, but not glucose intake, increases placental oxidative stress (334).

Similar results were found by Asghar et al. (2016) since maternal fructose consumption (66.8 % of total calorie intake as fructose) for six weeks led to increased lipid peroxidation in the placentae of C57BL6/J female mice at GD 18.5 (335). To further characterize the oxidative state in the placentae of those fructose-fed mice, SOD1, SOD2, glutathione peroxidase and catalase, were measured by immunoblot analysis (335). Mice given fructose had increased levels of SOD2 and decreased amounts of glutathione peroxidase and catalase in the placentae (335). Asghar also found reductions in forkhead box protein O1 (FOXO1) and SOD2 in the uterus of the fructose-fed mice compared to the control group (58 % (w/w) complex carbohydrate) (336).

FOXO1 activates genes involved in apoptosis, DNA repair and antioxidant defense system by inducing SOD2 (336). Thus, this study showed that fructose intake is able to impair antioxidant capacity, resulting in increased oxidative stress (336). These adverse effects were associated with a 25% increase in resorption rates in fructose-fed mice (336). In addition, Asghar found increased uric acid levels in the placentae of fructose-fed rats, and these observerations were consistent in a substudy using human placentae (335). A positive relation between maternal serum fructose concentrations and placental uric acid levels in humans was also found in this substudy (335). Asghar suggested that excess uric acid levels may elevate *de novo* lipogenesis in fructose-fed rats and humans, resulting in increased oxidative stress and inflammation (335). Together these studies suggest that fructose intake is associated with increases in pro-oxidants and decreases in antioxidants, possibily due to increased uric acid levels and lipotoxicity.

Placental nutrient transporters Gaccioli et al. (2013) recently reviewed studies that examine the effects of matenal nutrient intake on placental transporters in animal models and humans (338). However, information in Gaccioli's review paper was limited in scope since it summarized previous studies that showed changes in nutrient transporters in the placenta of the mothers and dams who have gestational diabetes or obesity, but not mothers who had overnutrition (338).

This section reviews a number of studies that examined the expression of nutrient transporters in the placenta such as those for glucose, amino acids, and fatty acids in several different models of excessive maternal calorie intake during pregnancy. The results from these studies are inconsistent, suggesting that nutrient transporter expression could be sensitive to maternal dietary intake as well as a host of factors that are not well characterized. The studies reviewed below provide examples of the findings in this area.

A few studies have shown that maternal high-fat intake increased the levels of glucose transporters in the placenta, whereas other studies did not observe any differences in these transporters between dams fed a high-fat diet and a control diet (186, 257, 258, 337). Jones et al. (2009) fed female mice with a high-fat diet (32 % of total energy intake as fat) or a control diet (11 % of total energy intake as fat) for 8 weeks from eight weeks of age until GD18.5 (258). On GD18.5, placentae in each dam were pooled, and protein expression of GLUT1 and GLUT3 was analyzed by western blotting (258). The protein expression of GLUT1 was increased 5-fold in the microvillus membrane, whereas that of GLUT3 was not changed by maternal high-fat intake (258). Another study done by Sferruzzi-Perri et al. (2013) investigated the expression of GLUT3 in the placenta of rats fed either an obesogenic diet (containing 30 % of total energy intake as fat and 36 % of total energy intake as simple sugar) or a control diet (containing 11 % of total energy intake as fat and 7 % of total energy intake as simple sugar) from GD1 to the end of the experiment (until GD16 or 19) (186). Placentae were collected on either GD16 or 19, and the mRNA expression of GLUT1 and GLUT3 was measured by RT-PCR (186). Rat dams fed an obesogenic diet had no changes in GLUT1 expression at either GD16 or 19, and although the expression of GLUT3 in placentae was not altered by an obesogenic diet at GD19, it increased at GD16 compared to that in placentae of control dams (186). Lin et al. (2012) also observed changes in GLUT3 expression in the placentae of high-fat fed dams (257). In particular, Lin investigated the effects of the level of both fat and fiber in a diet on GLUT1 and GLUT3 expression in the placenta (257). Rats were fed a diet containing high fat/low fiber (HL; 25 % (w/w) fat/2.29 % (w/w) fiber), high fat/high fiber (HH; 25 % (w/w) fat/10.94 % (w/w) fiber), low fat/low fiber (LL; 5 % (w/w) fat/2.29 % (w/w) fiber), or low fat/high fiber (LH; 5 % (w/w) fat/10.94 % (w/w) fiber) four weeks before pregnancy until the placentae were collected on

GD13.5 and 17.5 (257). GLUT1 and GLUT3 expression in the placentae were examined by real-time PCR and the data were analyzed by multiple comparisons for a 2x2 factorial design (257). The mRNA expression of GLUT1 was significantly decreased in the placentae by maternal high fat intake at both GD13.5 and 17.5 (257). However, GLUT3 expression was increased by a high-fat diet at GD17.5 only, but not at GD13.5 (257). More recently, Reynolds et al. (2015) fed Sprague-Dawley rats with a control diet (1 % (w/w) salt and 10 % kcal from fat), a high salt diet (4 % (w/w) salt and 10 % kcal from fat), a high fat diet (1 % (w/w) salt and 45 % kcal from fat) or a high fat and high salt diet (4 % (w/w) salt and 45 % kcal from fat) for three weeks prior to, and during, gestation (337). In this study, diets high in salts, fat or both represent Western diets (337). At GD18, the fetuses and the placentae were weighed and mRNA expression of GLUT1 and GLUT4 in the placentae was analyzed by RT-PCR (337). In rats, the weights of male placentae were lower, but not female placentae, in high-fat fed dams, high-salt fed dams and both high-fat/high-salt fed dams than the control dams (337). There was no difference in both GLUT1 and GLUT4 expression between groups with female placentae, but those were increased in male placentae of high-fat fed dams and high-fat/high-salt fed dams (337). Reynolds suggested that fetal growth may be supported by potential compensatory mechanisms by increased nutrient availability, although increased nutrient availability (increased GLUT1 and 4 expression in the placenta) did not support the growth of male fetuses (337). The authors explained that differences in sex chromosome number may induce sex-specific responses to maternal modified diets, thus only male fetuses, but not female fetuses, had responses to maternal high fat and high salt diets (337).

The results of changes in the expression of GLUT1 and 3 in the placenta from these four studies

(Jones et al., 2009; Sferruzzi-Perri et al., 2013; Lin et al. 2012; Reynold et al. 2015) were inconsistent. Two major reasons have been proposed for why these studies reached different conclusions. Firstly, the diet manipulations were different among those studies. Mice in the high-fat group were fed 32 % of total energy intake as fat in Jones's study, whereas rats were fed a diet containing 30 % of total energy intake as fat and 36 % of total energy intake as simple sugar in Sferruzzi-Perri's study. Lin also provided diets containing yet another different nutrient composition (HH, HL, LH, LL) to the rat and Reynold provided a high fat diet (45 % kcal from fat) or a high fat/high salt diet (45 % kcal from fat, 4 % (w/w) salt). Secondly, GLUT1 and 3 may have different roles in glucose transport at different times during pregnancy, by increasing or decreasing their expression (231). Thus, the timing of sample collection may contribute to apparently contradictory results (259). Based on this information, it is still unclear whether maternal high fat and obesogenic diets alter GLUT1 and 3 in the placenta.

The timing of expression and expression levels of placental amino acid transporters were also evaluated in the Jones (2009), Sferruzzi-Perri (2013), Lin (2012) and Reynolds (2015) studies (186, 258, 257, 337) mentioned above. In the Jones' study (2009), protein expression of SNAT2 and SNAT4 was measured by western blotting in the placentae from rats fed a high-fat diet (258). The protein expression of SNAT2 was significantly elevated 9-fold, but that of SNAT4 was not changed in the placentae of high-fat fed dams compared to the placentae from control dams at GD18.5 (258). Sferruzzi-Perri et al. (2013) examined the mRNA expression of SNAT1, 2 and 4 in the placentae of rats fed an obesogenic diet by real-time PCR (186). In this study, the mRNA expression of SNAT2 in the placentae at GD16 was increased, but that of SNAT1 and SNAT4 was not changed (186). On GD19, none of the amino acid transporters were altered in their

expression in the placentae of rats fed an obesogenic diet (186). In Lin's study (2012), the mRNA expression of placental SNAT4 was significantly reduced, while SNAT2 expression was unchanged by high-fat intake (186) on GD13.5 and 17.5. Thus, similar to the observations with glucose transporters, the alterations in placental amino acid transporters in response to maternal high-fat and obesogenic diets were inconsistent among these three studies. Differences among these results may be due to the same reasons as mentioned previously when different diet composition and timing of sample collections may have contributed to the different outcomes. In addition, Sferruzzi-Perri (2013) suggested that changes in transporter abundance seems to be a common response to changes in nutrient availability, whatever its cause (186). For example, an increase in placental SNAT2 was detected in both a high-fat diet model (258) and a low-protein diet model (260). Thus, inconsistency in the patterns of regulation of the alterations in nutrient transporters may indicate temporal changes in response to the timing of nutrient transfer from maternal-fetal circulations at the time when samples were collected. Finally, Reynolds et al. found that mRNA expression of SNAT2 at GD18 was increased in placentae from male but not female fetuses from dams fed a high-salt diet, a high-fat diet and a high-fat/high-salt diet (337). The expression of SNAT4 was increased in placentae from male fetuses from high-fat/high-salt fed dams, but no differences were observed in any other groups (337). These observations lead the authors to conclude that there by sex-specific responses to maternal high fat and high salt intake.

Abundance of mRNA for fatty acid transporters such as FATP and FAS were changed by high-fat and high-energy intake of dams (186, 261), although not many studies have examined the alterations in these transporters. Zhu et al. (2010) showed that mRNA expression of FATP1 and

FATP4 was enhanced in the placenta of obese ewes (fed 150 % of nutrient requirement prior to and during pregnancy) at GD 75 (341). Sferruzzi-Perri (2013) reported that the level of FATP mRNA expression was significantly elevated in the placenta of mice fed an obesogenic diet at GD19 (186). The mRNA expression of FAS was reduced in the placentae of obesogenic-diet fed dams at GD16, but it was not changed at GD19 (186). The mRNA expression of fatty acid transporters in response to maternal high-fat intake was also measured in the placentae of Japanese macaques (261). In this study, Japanese macaques were fed either a control-chow diet (15 % of total energy intake as fat) or a high-fat diet (35 % of total energy intake as fat) for four to seven years before and during pregnancy (261). On GD130 (term=173 days), placentae were collected, and the mRNA expression of placental transporters (FATP1, FATP2, FATP4, FATP6, FABP3, FABP4, FABP5, FABPpm) were measured in the tissues by real-time PCR (261). Plasma membranes were isolated from placental villous tissues and used to measure protein expression of FABPpm and FATP4 by immunoblotting (261). mRNA expression of FATP1, FATP2, FATP6, FABP3, FABP4, FABP5 was not affected by maternal high-fat intake whereas mRNA expression of FABPpm and FATP4 was significantly decreased (261). However, protein expression of FABPpm and FATP4 were not altered in the placentae of dams fed the high-fat diet (261). Thus, the authors suggested that a maternal high-fat diet does not affect fatty acid uptake by the placenta (261).

In humans, fatty acid transporters in the placentae of the mothers who consume diets high in fat are not well characterized. Considerable more information is available regarding placentae from women who had gestational diabetes or who are obese. For example, the protein expression of liver-type FABP was increased by 112 % in term placentae (37 – 41 weeks) from pregnancies

complicated with gestational diabetes compared to pregnancies where the mother has not had gestational diabetes (339). Placental FABP4 expression was increased primary cultures of human trophoblasts from the placenta of obese diabetic women (BMI of > 30 kg/m^2) (340). Brett et al. (2014) conducted a study on women to determine whether prenatal physical activity and diet composition affected the expression of nutrient transporters in the placenta (262). Six women (BMI of 18.5 – 24.9 kg/m^2) were classified as "active" or "non-active" women based on their physical activity levels (262). Physical activity was measured with accelerometer and adherence to the Canadian Physical Activity Guidelines for Adults was used to determine "active" or "non-active" women (262). Diet composition of women was assessed by 24-hour dietary recalls and placental gene expression was analyzed using PCR (262). Women who met the guidelines had lower gene expression of placental FATP4 (262). However, there was no difference in dietary fat intake between "active" and "non-active" women (262). Moreover, % daily calorie intake from fat was not associated with the expression of placental FABP3, FABP4, FABP5, FATP2 and FATP4 (262). In summary, the expression of fatty acid transporters in the placentae of non-human primate and humans seems to be unaffected by maternal high-fat intake based on the cited studies, although the expression of FATP and FAS was altered in the placentae of mice fed an obesogenic diet. It is still not clear whether the expression of placental fatty acid transporters is altered by maternal high fat intake, and whether the responses of transporters vary among species since only a few studies have approached that question. Further investigations are required to determine the effects of a high-fat diet on fatty acid transporters in the placenta.

The morphology and weight of the placenta A few studies have shown that a maternal high fat, high sugar and high fructose diet induces changes in the area of the zones and sometimes it

also alters placental weight (186, 251, 303, 335, 336). However, results are inconsistent. Kim et al. (2014) measured area of placental layers in mice fed the high-fat diet (described above) (251). Placentae of mice were taken at either GD15.5 or 17.5, and the overall size of the placenta and the area of placental layers were assessed by histology (251). Maternal high-fat intake did not alter the overall size of the placenta, but it did induce a reduction in the area of the LZ at both GD15.5 and 17.5 (251). The JZ in the placenta was not affected by high-fat intake at either day (251). In Sferruzzi-Perri's study, the placentae of mice fed the obesogenic diet were stained with hematoxylin and eosin to determine total LZ and JZ surface area (186). On GD16, both total JZ and LZ surface area were not changed by maternal obesogenic-diet intake (186). However, on GD19, dams fed the obesogenic diet had reduced LZ surface area, but no effects on the JZ compared to those of control dams (186). In this study, placental weight was reduced in dams fed the obesogenic diet at both GD16 and 19 (186). Sferruzzi-Perri suggested that the reduced LZ surface area in response to the obesogenic diet may have limited transfer ability by reducing diffusion rate of nutrients (186).

The effects of maternal high-fat intake or high-energy intake on placental weight have been inconsistent in previous studies (186, 251, 263, 264). In Sferruzzi-Perri's study, placental weight was lower in rats fed the obesogenic diet compared to that in mice fed the control diet at both GD16 and 19 (186). However, in Kim's study, placental weight was significantly increased in mice dams fed a high-fat diet compared to that in mice dams fed a control diet at GD15.5 (251). In O'Tierney-Ginn's study, the placental weight of Japanese macaques was not affected by maternal high-fat consumption at GD130 (264). Gadd et al. (2000) also showed that placentome mass was significantly reduced in ewes that had a diet switched from 50 g/day to 300 g/day on

GD52 and a diet switched a diet from 300 g/day to 50 g/day on GD52 groups at GD104 (263). This result indicates that placentome mass is reduced by maternal calorie overconsumption during mid and late pregnancy, regardless of the amount of a diet that ewes consumed during early pregnancy (263).

Maternal fructose intake also induces changes in the morphology and the weight of the placenta. In Asghar's study, intake of a high-fructose diet (66.8 % of total energy as fructose) induced a larger decidua area and smaller labyrinth zone (335), and placental weights were significantly heavier, resulting in a significant decrease in the fetal:placental weight ratios compared to the control group (335). In Saben's study, consumption of a fructose diet (also 66.8 % of total energy as fructose) resulted in placentae that weighed significantly less than placentae from control dams (336). In Vickers's study, female Wistar rats received either fructose solution (20 % of caloric intake) during pregnancy or regular tap water (303). The placentae and the fetuses were dissected and weighed at GD21 (303). Vickers found that intake of the fructose solution did not change fetal weight, but reduced placental weight of female fetuses, not male fetuses, which resulted in an increase in the fetal:placental weight ratios in female fetuses (303). Vickers suggested that the placentae of female fetuses may be more sensitive to fructose transfer, growth factors (i.e. IGF) or other placental transporters than the placentae of male fetuses (303).

As shown in previous pharagraphs, in most studies, placental weight seems to be affected by maternal high fat intake, high energy intake and high fructose intake, but the results are inconsistent. The changes in placental weight in response to maternal diets may vary between genders, durations and amount of fructose in the diet. Although placental weight seems to be associated with alterations in trophoblast development and growth hormone signaling, it is still

unclear why previous findings regarding placental weight of dams fed a high-energy diet are inconsistent.

Summary Maternal overnutrition during pregnancy appears to induce placental perturbations and may be associated with deleterious effects in the offspring in later life. Many studies over the last several decades have focused on the effects of high-calorie intakes and their effects on offspring. These studies have shown that high-calorie intake during pregnancy appears to be associated with increases or decreases in birth weight, but similarly increases the risk of developing chronic diseases in the offspring in later life (154, 155). However, a western diet not only means high-calorie foods, but it means high-calorie foods from an excess amount of nutrients like fat and sugar including a high amount of glucose and fructose. Previous studies showed that a maternal diet that is high in either calories or nutrients has adverse effects on placental morphology and function. Alterations in the vascular network were associated with changes in nutrient signaling pathways, which influence placental layers and morphology and weight of the placenta. Moreover, alterations in the level of oxidative stress may be associated with changes in vascularization via angiogenic growth factors. However, a number of important caveats should be noted. Firstly, placental structure may be not only altered by energy intake, but also may be additionally affected by the relative proportion of nutrients above nutrient requirements since mechanisms of alterations in placental development were not fully explained in previous studies. Future studies should provide consistent details about dietary composition being used in the specific experimental model. Second, it is hard to apply the results from animal studies to humans due to the difference among these species. Use of *in vitro* models using different placental cell types may give some indication of the direct effects of nutrients on cell

development, but this does not give us observations needed to understand effects on the entire placenta as an organ system. Results from this review of relevant literature highlight the need for additional studies that examine the molecular basis of structural and functional changes in the placenta using animal models with well-defined dietary treatments. Information arising from these studies could then be applied to examine effects in humans and will give us a better understanding of the effects of maternal dietary intake on placental development.

CHAPTER 2: RATIONALE

This thesis was conducted with the overall purpose of assessing the physiological effects of high intakes of purified (or free) dietary fructose on physiologic profiles of pregnant rats and was divided into three sub-studies. The first study, Study A, focused on the effects of fructose intake in pregnant rats compared with non-pregnant rats. The second study, Study B, focused on the effects of fructose intake in pregnant rats and their pregnant female offspring. The third study, Study C, focused on the effects of fructose intake on rat dams in two sequential pregnancies.

Study A. The effects of high fructose intake on the body weight, metabolic profiles and fat mass of non-pregnant and pregnant rats

For several decades, most studies have focused on whether fructose intake is associated with an increased risk of developing metabolic syndrome and related conditions such as insulin resistance, glucose intolerance, weight gain, hypertension and oxidative stress. These studies have generally been carried out in male subjects in both humans and rodents (pages 8 – 23). Few of these studies included females, thus it is less clear whether fructose has similar effects in females. Recently, a few studies have started to provide evidence that fructose intake during pregnancy alters the metabolic and physiologic profiles of pregnant rats (pages 54 – 59). However, none of these studies have compared the metabolic profiles of pregnant and age-matched, non-pregnant rats consuming fructose.

Thus, the research question of Study A was:

Does dietary fructose intake affect the body weights, metabolic profiles and body composition of

pregnant rats and non-pregnant rats compared to rats not consuming fructose?

The research objective of Study A pursued in order to answer the research questions were:

Objective A1.

To compare the effects of dietary fructose intake on body weights, metabolic profiles (glucose, insulin, TG concentrations) and body composition (proportion of fat mass and lean mass at GD21) between pregnant and non-pregnant rats consuming and not consuming fructose.

Hypothesis A1.

Regular intake of dietary fructose will result in higher body weights, higher concentrations of glucose, insulin and TG (i.e. a worse metabolic profile) and higher proportions of body fat in pregnant vs. non-pregnant rats.

Study B. The effects of high fructose intake on metabolic and physiologic characteristics of rat dams and placentae across two generations

The DOHaD hypothesis suggests that alterations in fetal development during pregnancy may influence the lifelong health of the offspring (page 36 − 37). It highlights the key roles of maternal nutrition on the developing fetus. A number of studies have shown that poor maternal nutrition during pregnancy is associated with lower birth weights and this is independently associated with an increased risk of developing non-communicable diseases in later life (pages 37 – 40).

Several recent studies using rodent models have provided insight into the effects of maternal fructose intake during pregnancy on later health of her offspring (161 – 165, 178 – 180, 183). These studies found that offspring of dams fed high fructose had increased plasma concentrations

of leptin, fructose, glucose FFA and TG (163, 165, 178, 179, 183). However, most of the studies have not examine the effects of exposure to a high-fructose maternal diet during pregnancy on metabolic regulation in the offspring in adult life, and no other studies have examined metabolism in the female offspring when when they are pregnant. Moreover, previous studies have not compared the effects of fructose intake between dams and offspring. This is important to examine since it is possible that the effects of fructose intake in the offspring may be exaggerated compared to their mothers.

A few studies in the area of developmental origins of health and disease have highlighted that the placenta plays a critical role in fetal development since the placenta performs compensatory functions for optimal fetal growth. This is supported by a number of studies that showed a positive association between poor fetal development and compromised placental development. Changes in the placenta that have previously been noted include abnormalities of the placenta vasculature, maternal-fetal nutrient transport and excessive oxidative stress in the placenta (pages 66 − 69). However, the question remains as to whether maternal fructose intake may influence fetal development through changes in the placenta. Thus, investigations involving the placenta are an obvious starting place.

Study B used a rat model to understand the effects of consuming fructose on the physiology of pregnancy and placental development, and its long-term effects on pregnancy in female offspring. Responses to a long-term high-fructose diet may differ between humans and rats, but animal models could shed light on the extent to which dietary fructose contributes to programming physiological characteristics in offspring. Understanding the so-called “transgenerational effects” of maternal dietary intake in pregnancy are important to advance our understanding of the

underpinnings of increased risk of chronic diseases across generations. At this point, the long-term effects of maternal fructose intake during pregnancy on the health of adult offspring has not been examined in detail, and no other studies have examined the consequences of fructose intake during pregnancy on female offspring whose dams have also consumed fructose.

Thus, the research questions of Study B were:

What are the effects of dietary fructose on metabolic and physiologic profiles of dams and their female offspring prior to and during pregnancy?

How are the placentae from rat dams and their female offspring affected by fructose feeding?

The research objectives of Study B pursued in order to answer the research questions were:

Objective B1. To characterize the effects of fructose feeding on body weight, metabolic profiles, and body composition of rat dams and their female offspring prior to and during pregnancy.

Hypothesis B1. Body weights will be higher, metabolic profiles will be worse (i.e. glucose, insulin, triglyceride concentrations will be higher), and the proportion of body fat will be higher among offspring vs. dams given fructose.

Objective B2. To examine the effects of dietary fructose intake on the expression of genes involved in glucose and fat metabolism (FAS, GLUT2, GLUT5) and the degree of oxidative stress (α-tocopherol concentrations and lipid peroxidation) in the livers of dams and female offspring in late gestation.

Hypothesis B2. Hepatic expression of FAS, GLUT2 and GLUT5 in late gestation will be higher in offspring compared to rat dams given fructose and compared with controls receiving water.

Female offspring given fructose will have lower concentrations of α-tocopherol and higher levels of lipid peroxidation in their livers compared with dams given fructose and compared with controls receiving water.

Objective B3. To characterize the effects of dietary fructose intake on gene expression (FABP1, SNAT2, GLUT1, VEGF-A, PlGF, VEGFR-1, VEGFR-2), oxidative stress (α-tocopherol concentrations, lipid peroxidation, superoxide expression, and peroxynitrite expression) and morphological aspects (ratio of LZ:JZ) in the placentae from dams and female offspring in late gestation.

Hypothesis B3. Placenta from pregnant offspring given fructose intake will have altered expression of genes related to nutrient transporters (FABP1, SNAT2, GLUT1), angiogenesis (VEGF-A, PlGF, VEGFR-1, VEGFR-2), higher levels of oxidative stress (lower α-tocopherol concentrations and higher amounts of lipid peroxidation, superoxide expression, and peroxynitrite expression) and a reduction in the area (LZ, JZ) of the placenta compared to placenta from dams given fructose and from controls receiving water.

Study C. The effects of high fructose intake on metabolic and physiologic profiles of female rats during two sequential pregnancies

Studies in humans suggest that women who experience GDM in one pregnancy are at high risk for developing it in subsequent pregnancies (265, 266). This may indicate that physiological impairments that are seen in one pregnancy are also likely to appear again in subsequent

pregnancies. No studies in animal models have investigated whether alterations in maternal physiology induced by inappropriate nutrition in one pregnancy, are aggravated in subsequent pregnancies.

Thus, the research question of Study C was:

What are the effects of dietary fructose on body weights, metabolic profiles, and body composition of female rats during two sequential pregnancies?

The research objectives of Study C pursued in order to answer the research questions were:

Objective C1. To examine the effects of dietary fructose intake on body weights, metabolic profiles (glucose, insulin, TG), body composition (fat mass, lean mass) of pregnant rats during two sequential pregnancies.

Hypothesis C1. Body weight and metabolic profiles (glucose, insulin and TG concentrations) will be higher in a second pregnancy than in a first pregnancy in rat dams consuming fructose. Moreover, body fat will be higher and lean mass will be lower in a second pregnancy compared to a first pregnancy in rat dams fed fructose.

CHAPTER 3: MATERIALS AND METHODS

Experimental Designs and General Protocols for All Studies

Animals, Diets and Fluids For each of the studies described in this thesis (Study A, Study B and Study C), 7-week-old female and male Wistar rats (Charles River Canada, Montreal, Quebec) were pair-housed in shoebox cages (female and male rats were separated) in a temperature-controlled room (18 – 23 ℃; 40 – 70 % humidity) with a 12hour light:dark cycle. They received food *ad libitum* (Purina 5001, Table 3-1, see Appendix 6 for more details; Purina Mills, St. Louis, MO) throughout the study and distilled water was available to all rats during the one-week acclimation period that followed their arrival at the University of Alberta Health Sciences Lab Animal Unit. During each of the studies described below, rats that were randomized to the fructose (FR) group consumed a 10% fructose solution (w/v; Amresco, Solon, OH, USA) during the time indicated. Rats in the control group (CONT) continued to consume distilled water throughout the entirety of all studies. All procedures used in these studies were approved by the University of Alberta Animal Ethics Committee in accordance with the Canadian Committee on Animal Care Guidelines.

Table 3-1: Diet composition of Purina 5001

	Protein	**Fat**	**Carbohydrates**
% of calories	29.829	13.427	56.744

Study A. The effects of high fructose intake on the body weight, metabolic profiles and fat mass of non-pregnant and pregnant rats

Study A-1. Experimental Design and General Protocol

Study A was part of a larger project designed to examine insulin secretory responses from pancreatic islets isolated from pregnant and non-pregnant rats given distilled water or 10 % (w/v) fructose solution. Results from the isolated islets are not part of this thesis. Twenty-one 7-week-old female Wistar rats were purchased from a commercial breeder as described above. Following one week of acclimation, and at 8 weeks of age, animals were randomly assigned to receive either distilled water (CONT; control group) or a 10 % (w/v) fructose solution (FR; fructose group) for three weeks. At 11 weeks of age, 10 rats were co-housed overnight with male rats that had been maintained on distilled water with food *ad libitum*. Pregnancy was confirmed by vaginal lavage the following morning, and a positive sperm test was considered to represent gestational day (GD) 0. Female rats remained on either CONT (Dam-CONT; n=4) or FR (Dam-FR; n=6) treatment for the three weeks of pregnancy. Eleven female rats were not mated, and therefore not pregnant (NP) and remained on either CONT (NP-CONT; n=6) or FR (NP-FR; n=5) treatment for three weeks. At GD21 (14 weeks of age for NP), all female and male rats were euthanized. Body weights were not recorded, plasma was not collected, body composition was not analyzed in male rats.

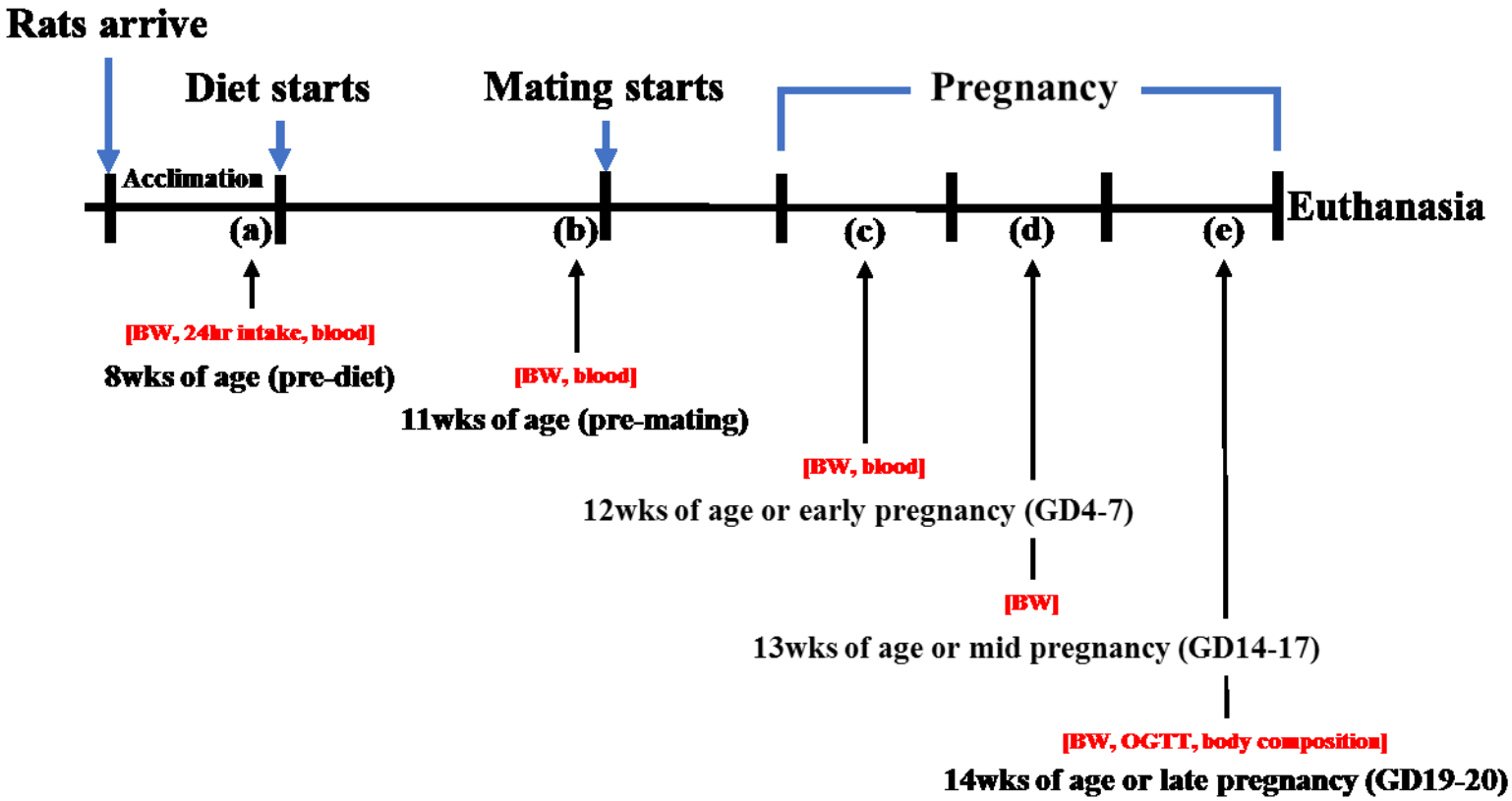


Figure 3-1: Timelines for Study A. Measurements were made as indicated, in pregnant and non-pregnant female rats. BW=body weight

Study A-2. Regular Monitoring of Body Weight and Metabolic Profiles

Body weights of NP-CONT and NP-FR were recorded during the study, beginning at (a) 8 weeks of age (pre-diet, the day before starting the fructose treatment), (b) 11 weeks of age, (c) 12 weeks of age, (d) 13 weeks of age and (e) 14 weeks of age.

Body weights of Dam-CONT and Dam-FR were recorded on the same schedule as described for NP rats, i.e. (a) 8 weeks of age (pre-diet, the day before starting the fructose treatment), (b) 11 weeks of age (pre-mating, the day before mating), (c) early pregnancy (GD 4 – 7, 12 weeks of age), (d) mid pregnancy (GD14 – 17, 13 weeks of age) and (e) late pregnancy (GD19 – 20, 14 weeks of age) (Figure 3-1).

At each time point, morning whole blood samples were collected from the tail vein of non-fasting rats into micro-container tubes containing K_2 EDTA (BD, Franklin Lakes, NJ, USA) and kept on ice until separated by centrifugation (Eppendorf Centrifuge 5415, Germany, 16,000 x g; 8 minutes). Plasma was removed, aliquoted, and stored in microfuge tubes (Fisher Scientific Ltd, Ottawa, ON, Canada) at -20 ℃ until analysis of glucose, insulin and TG.

Study A-3. Analyses in Plasma

Glucose assay Plasma glucose concentration was determined spectrophotometrically by Trinder's glucose oxidase assay (Figure 3-2, 267) using a commercially available kit (Trinder assay kit, Genzyme Diagnostics, Charlottetown, PE, Canada). Plasma (2.5 µL) was incubated at room temperature in 250 µL of a working reagent (A solution containing (after reconstitution) 0.25 mmol/L 4-aminophenazone, 20 mmol/L p-hydroxybenzoate, >40,000 U/L glucose oxidase, >2,000 U/L peroxidase, and preservatives) for 15 minutes. After

incubation, the increase in absorbance was measured at 505 nm with a recording spectrophotometer.

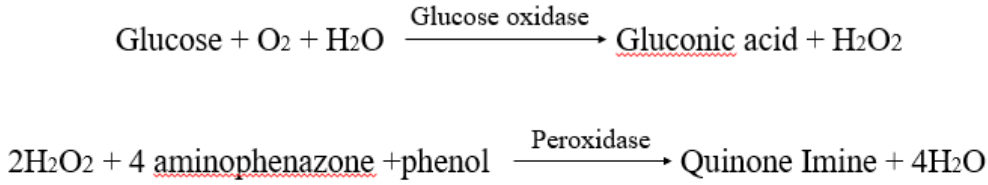


Figure 3-2: Glucose Trinder assay. Glucose in plasma is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling of 4-aminophenazone with phenol to yield a purple coloured product, quinoneimine, whose formation can be measured using a spectrophotometer. A more intense colour at A_{505} indicates higher glucose concentration in plasma.

Insulin assay Plasma insulin concentration was measured spectrophotometrically using a sandwich type immunoassay kit (rat Ultrasensitive ELISA Immunoassay kit, ALPCO Diagnostics, Salem, NH, USA). Plasma (25 µL) was incubated with 100 µL of conjugate (Detection Antibody) in a 96-well microplate coated with a monoclonal antibody specific for rat insulin at room temperature for one hour. After incubation, plate was washed with 350 µL of wash buffer and then incubated with 100 µL of tetramethylbenzidine (TMB) substrate (supplied) for 30 minutes at room temperature. After a second incubation, the increase in absorbance was measured at 562 nm with a recording spectrophotometer. Figure 3-3 shows the antibody binding procedure.

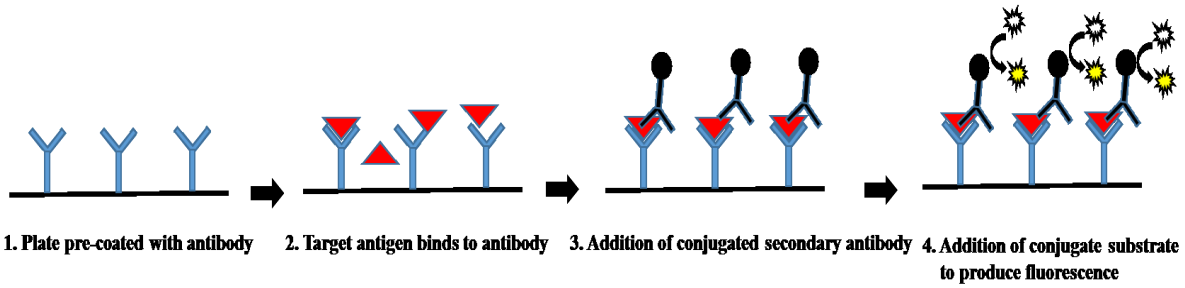


Figure 3-3: Example of antibody binding procedure in Sandwich type ELISA. Secondary antibody is conjugated to an enzyme (i.e. HRP), which will generate a coloured product on addition of a conjugate substrate (i.e. TMB). Incubation of TMB with HRP produces a blue clour, and then produces yellow colour when reaction is stopped by addition of stop solution (i. e. sulphuric acid).

Triglyceride assay Plasma triglyceride concentration was measured using the Trinder method (Triglyceride-SL assay kit, Genzyme Diagnostics, Cambridge, Massachusetts, USA; Figure 3-4). Plasma (2.0 µL) was incubated at room temperature in 150 µL of a working reagent (0.4 mmol/L 4-aminophenazone, 2.6 mmol/L adenosine triphosphate, 3.0 mmol/L p-chlorophenol, >2,400 U/L glycerol phosphate oxidase, >1,000 U/L lipoprotein lipase, >540 U/L peroxidase, >400 U/L glycerol kinase, stabilizers and preservatives) for 15 minutes. After incubation, the increase in absorbance was measured at 505 nm with a recording spectrophotometer.

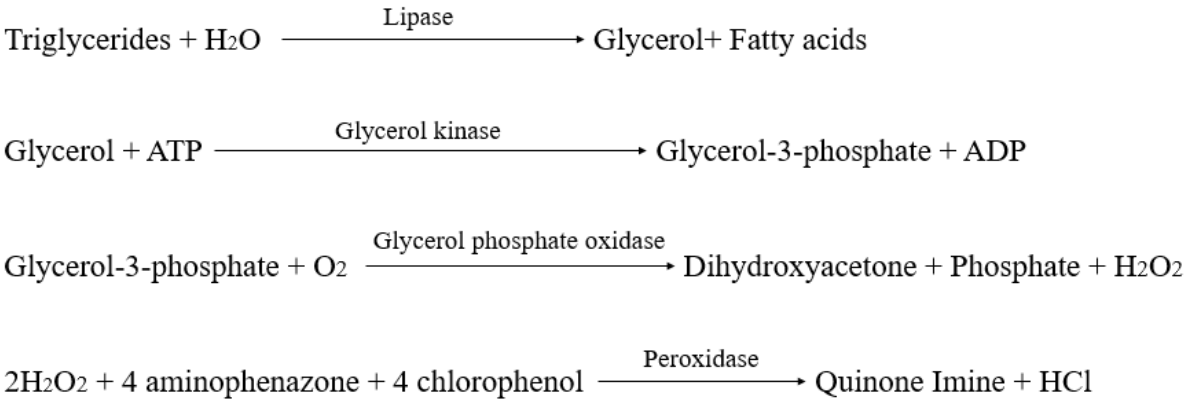


Figure 3-4: TG Trinder assay. TG in plasma is hydrolyzed to glycerol and fatty acids by lipase. Glycerol from the first reaction is converted to glycerol-3-phosphate through the action of glycerol kinase, and this, in turn, is converted to dihydroxyacetone, phosphate and hydrogen peroxide by glycerol phosphate oxidase. The hydrogen peroxide produced by this reaction is used in a subsequent oxidative coupling reaction of 4-aminophenazone with 4-chlorophenol in the presence of peroxidase to generate quinoneimine, a coloured product that can be measured spectrophotometrically at A_{505}.

Oral glucose tolerance test (OGTT) OGTTs were carried out at time point (e) in both non-pregnant rats (when they were 14 weeks of age) and pregnant rats (during late pregnancy). Rats were fasted for ~4 hours, beginning at ~8 am. Prior to administration of the oral glucose bolus, a blood sample was collected from the tail vein and placed on ice. At 0 min, 3 g/kg glucose solution was administered by gavage. Blood samples were collected from the tail vein into micro-container tubes containing K_2 EDTA (BD, Franklin Lakes, NJ, USA) at 0, 15, 30, 45, 60 and 90 minutes after glucose administration and placed on ice. Blood samples were centrifuged, and plasma was removed, aliquoted, and stored (-80 ℃) until analyzed for glucose, insulin and TG. Area under the response curve (AUC) for glucose and insulin was calculated with the following formula (Figure 3-5).

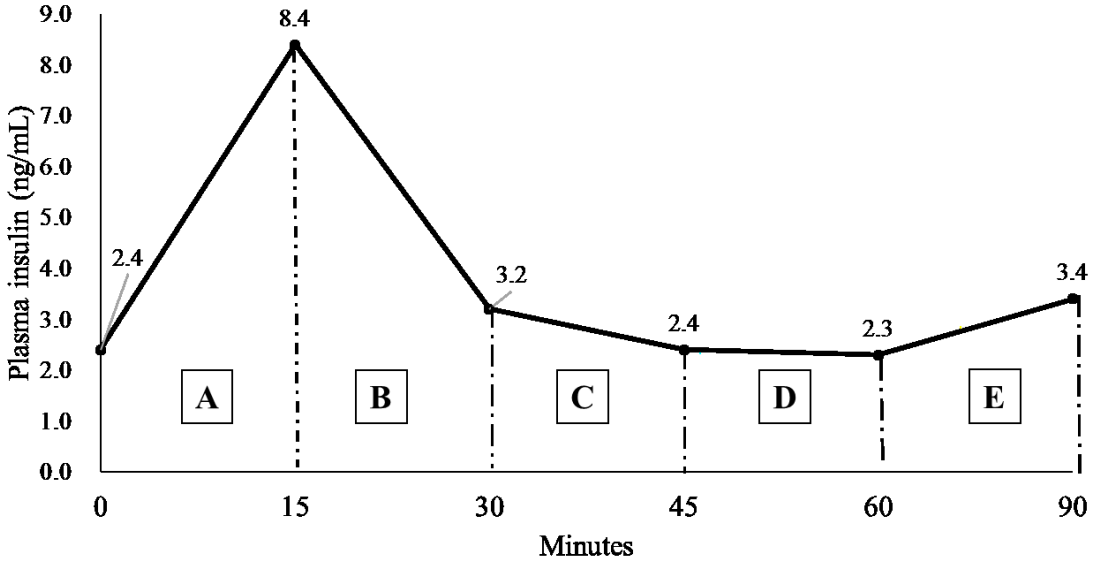


Figure 3-5: The Area Under the Curve (AUC). This figure is adapted from Arvidsson-Lenner (268)

AUC = A+B+C+D+E

$$= \frac{(ins.con.@T0 + ins.con.@T15)}{\frac{15}{2}(min)} + \frac{(ins.con.@T15 + ins.con.@T30)}{\frac{15}{2}(min)} + \frac{(ins.con.@T30 + ins.con.@T45)}{\frac{15}{2}(min)} +$$

$$\frac{(ins.con.@T45 + ins.con.@T60)}{\frac{15}{2}(min)} + \frac{(ins.con.@T60 + ins.con.@T90)}{\frac{30}{2}(min)}$$ **ins.con.* : insulin concentration

Example)

A= (2.4+8.4)*7.5 = 81

B= (8.4+3.2)*7.5 = 87

C=(3.2+2.4)*7.5 = 42

D=(2.4+3.4)*7.5=43.5

E=(2.3+3.4)*15 = 85.5

Total AUC for 1.5hour = 81 + 87 + 42 + 43.5 + 85.5 = 339 ng*min/mL

Homeostasis model assessment-insulin resistance (HOMA-IR) Fasting plasma glucose and insulin concentrations (at t=0) during OGTT were used to calculate the homeostasis model assessment-insulin resistance (HOMA-IR) (351) as an indicator of insulin resistance with following equation (352):

$$\mathbf{HOMA-IR} = \frac{\text{Fasting plasma insulin (pmol/L) x fasting glucose (mmol/L)}}{135}$$

* Insulin unit conversion = ng/mL (μg/L) to pmol/L

= 1 ng/ml (μg/L) x 172.1 = 172.1 pmol/L

Example) Fasting plasma insulin = 1 ng/mL, fasting glucose = 5 mmol/L

$$\mathbf{HOMA-IR} = \frac{\text{Fasting insulin (1 x 172.1) x fasting glucose (5)}}{135}$$

= 6.3

Study A-4. Determination of body composition

At 14 weeks of age, body composition (% fat and % lean of body weight) of NP rats and pregnant Dams was measured using quantitative magnetic resonance analysis (QMR; EchoMRI LLC 4-in-1 whole-body composition analyzer; Echo MRI; Echo Medical Systems, Houston, TX, USA). Shortly thereafter, rats were euthanized using CO_2 and final body weights were recorded.

Study A-5. Statistical Analyses

Values are presented as mean ± standard error of the mean (S.E.M) and all statistical analyses

were performed using SAS 9.2 (SAS Institute, Cary, NC, USA). The normality of each variable distribution was verified prior to statistical analysis, and those that were not normally distributed were log-transformed before analysis. Data that were not normally distributed were $AUC_{glucose}$ and $AUC_{insulin}$ during OGTT and body composition.

Group differences were assessed by two-way ANOVA and included repeated measures where appropriate such as body weight from pre-diet to late pregnancy, and plasma glucose, insulin and TG concentrations in monitoring samples taken from pre-diet to late pregnancy. Pair-wise post hoc comparisons using a Tukey correction were used to identify individual group differences in analyses using repeated measures. A *p*-value of 0.05 was accepted as statistically significant.

Study B. The effects of high fructose intake on metabolic and physiologic characteristics of rat dams and placentae across two generations

Study B-1. Experimental Design

Twenty 7-week-old female Wistar rats were purchased and acclimated for one week as described above. At 8 weeks of age, animals were randomly assigned to receive either distilled water (Dam-CONT; n=10) or a 10 % (w/v) fructose solution (Dam-FR; n=10) for three weeks. At 11 weeks of age, all Dams were co-housed with male rats that had been maintained on distilled water with food *ad libitum*. Pregnancy was confirmed by vaginal lavage, and a positive sperm test was considered to represent GD0. Five Dam-CONT and five Dam-FR were euthanized at GD21. Remaining Dam-CONT (n=5) and Dam-FR (n=5) were left to litter out, and all litters were culled to achieve 10 – 12 pups/litter. Dams continued on their assigned treatment during lactation (finishing at 21 days after delivery), at which point

two female pups from each Dam were randomly selected to remain in the study as Offspring. Male offspring were euthanized at this time, without recording any measurements or collecting any tissues. Female Offspring received distilled water with food *ad libitum* until eight weeks of age. At that point, they were placed on the same treatment as their Dams and they received either distilled water (Offspring-CONT, n=10) or a 10 % (w/v) fructose solution (Offspring-FR, n=10). These treatments continued through mating and pregnancy. Pregnant Offspring were euthanized at GD21 (Figure 3-6). All male rats that were co-housed with female rats were euthanized at the end of the study. Samples were not collected from male rats.

Since Dam-CONT (n=4) and Dam-FR (n=6) in Study A followed the same protocol (i.e. diet, timing) as Dams in Study B, data for the variables that were common to both studies were combined. Body weight, 24-hour food and water intake, metabolic profiles (glucose, insulin, TG), OGTT and body composition for dams in Study A and Study B were combined.

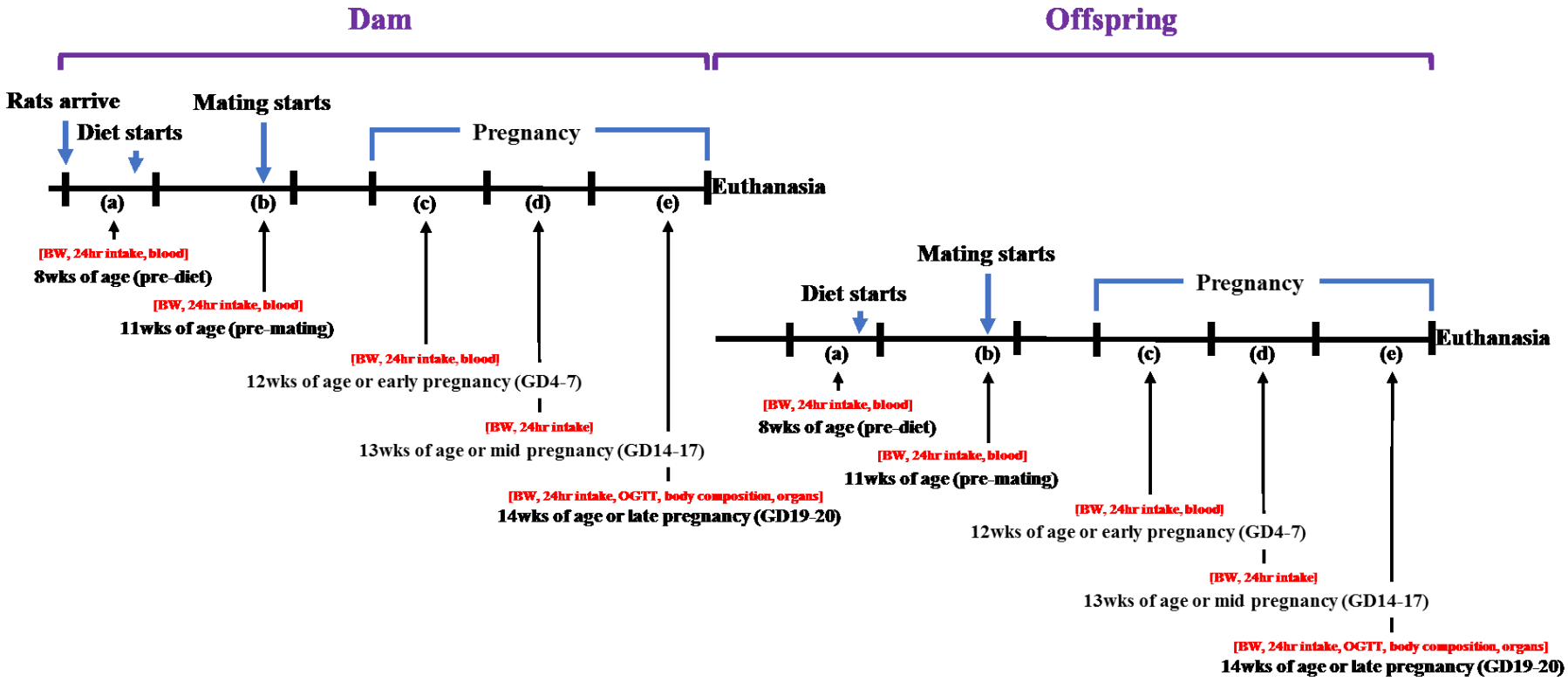


Figure 3-6: Timelines for Study B. Measurements were made as indicated, in Dams and Offspring. BW=body weight, 24hr intake=food and water intake during 24 hours, organs: pancreas, liver, adipose tissues (uterine, retroperitoneal and brown/interscapular), fetuses and placentae were collected.

Study B-2. Regular Monitoring of Body Weight, 24-hour Food and Fluid Intake and Metabolic Profiles

Body weights and 24-hour food and water intake of Dams and Offspring in Study B were recorded on the same schedule as described for Dams in Study A (Figure 3-1, page 102). Total calorie intake from food and the 10 % (w/v) fructose solution was calculated based on the diet composition information provided for Purina 5001 (Table 3-1, page 100). Blood samples from Study B Dams and Offspring were also collected during the study as described for Study A-2 (page 103). Plasma was separated, stored frozen, and used for analysis of glucose, insulin, TG and protein concentrations.

Study B-3. The Calculation of Caloric Intake from Food and Fluid Intake

Purina 5001 pellets contain 29.8 % of energy as protein, 13.4 % of energy as fat, 56.7% of energy as carbohydrates and has an energy density of 4.09 kcal/g (Purina 5001, Table 3-1). Caloric intake from food was calculated with the following formula.

Example)

A rat consumed 100 g of pellets and 50 g of 10 % (w/v) fructose solution

Energy intake from pellets = 100 g of pellets x 4.09 kcal/g = 409 kcal

- Energy intake from protein = 409 kcal x 0.29829 = 122 kcal
- Energy intake from fat = 409 kcal x 0.13427 = 55 kcal
- Energy intake from carbohydrates = 409 kcal x 0.56744 = 232 kcal

Energy intake from 10 % (w/v) fructose solution = 50 g of the fructose solution x 0.1 x 4 kcal = 20 kcal

Total energy consumption of a rat

= 409 kcal from pellets + 20 kcal from the fructose solution = 429 kcal

---

Study B-4. Analyses in Plasma

Plasma glucose, insulin and TG concentrations were measured by the assays described above (page 103 – 108).

Protein assay Total plasma protein concentrations were measured from rats in Study B only and was used as a measure of protein status. Plasma protein concentration was measured by the bicinchoninic acid assay (BCA assay) (Pierce BCA protein assay, Pierce Biotechnology, Rockford, IL) which is a calorimetric method that measures the reduction of Cu^{2+} to Cu^{1+} (biuret reaction) and the subsequent formation of a Cu^{1+} complex in an alkaline medium (269). Ten (10) µL of plasma was incubated at 37 ℃ in 200 µL of a BCA reagent for 30 minutes. After incubation, absorbance was measured at 562 nm with a spectrophotometer.

Study B-5. Determination of Body Composition

At GD21, body composition (% fat and % lean of body weight) and body weight of Dams and Offspring was measured using quantitative magnetic resonance analysis (details described above, page 108).

Study B-6. Tissue Dissection and Preparation

After measurements of body composition and body weight at GD21, Dams and Offspring were euthanized using CO_2. The whole liver, pancreas and adipose tissues (uterine, retroperitoneal and brown/interscapular) were excised and weighed. The whole pancreas and adipose tissues were snap-frozen in liquid nitrogen immediately. Approximately 400 mg of the liver was collected, snap-frozen and stored in microfuge tubes (Fisher Scientific Ltd, Ottawa, ON, Canada) at 80 ℃. Placentae and fetuses were dissected free from the uterus, counted, individually weighed, and the placental:fetal weight ratio was calculated. The gender of the fetuses was not determined. Three placentae on each end of the uterine horn were excluded from sample collection, and six randomly selected placentae/rat (Dams and Offspring) were collected from the middle of the uterine horns. Three placentae were snap-frozen at -80ºC for later determination of gene expression, and another three placentae/rat (Dams and Offspring) were embedded in paraffin for histological and in-situ hybridization analyses (detailed below).

Study B-7. Analyses of the Rat Dam and Offspring Liver Tissue

In frozen liver of Dams and Offspring, gene expression of FAS, GLUT2, GLUT5 were measured using qPCR (Qiagen RNeasy mini kit; Qiagen N.V., Hilden, Germany). The concentrations of α, β, γ, δ tocopherols were measured using HPLC (Supelguard Diol guard column; Supelco, Bellefonte, PA, USA). Hepatic TG concentrations were measured to aid in interpretation of the results from the analyses of tocopherols. Lipid peroxidation in liver tissue was measured as an indicator of oxidative stress using methods described below.

Study B-7.1. RNA extraction and determination of gene expression in the livers using

quantitative polymerase chain reaction (qPCR)

RNA extraction was carried out by following the commercial kit instruction. Approximately 20 – 25 mg of frozen liver was ground in liquid nitrogen and homogenized in 600 µL of Buffer RLT (containing a guanidine salt) with β-mercaptoethanol (10 µL of β-mercaptoethanol in 1mL Buffer RLT). The supernatant was collected by centrifugation at 8,200 x g for two minutes and mixed with one volume of 70 % (v/v) ethanol. The mixture (up to 700 µL) was transferred to an RNeasy spin column followed by centrifugation (8,200 x g, 15 second). The RNeasy spin column was worked by adding 500 µL of buffer RPE followed by centrifugation (8,200 x g, 15 second). Thereafter, 30 µL of RNase free water was added to the RNeasy spin column and collected by centrifugation into a new tube (8,200 x g, 2 minutes).

The RNA concentration and purity were confirmed using a Nanodrop instrument (Thermo Scientific, Waltham, MA, USA), and reverse transcription PCR was carried out using the High Capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). qPCR was carried out using SYBR Green dye and the StepOne Plus PCR machine (Applied Biosystems). The target genes and their primers are shown in Table 3-2. Product sizes and primer specificity were confirmed using classical PCR and gel electrophoresis before and melting-curves following qPCR.

The results were presented as fold changes relative to the control group, according to the $2^{-\Delta\Delta CT}$ method. The $2^{-\Delta\Delta CT}$ method is a mathematical model that uses a calibrator sample for comparison of gene expression of unknown samples (270). This method uses a ratio of change in threshold cycle (Ct) between a target gene and a reference gene by the formula, $\Delta\Delta Ct=\Delta Ct$ (Ct (target gene

in treatment group) – Ct (reference gene in treatment group)) – ΔCt (Ct (target gene in control group) – Ct (reference gene in control group)). Ct is "the cycle number at which the fluorescence signal crosses threshold (271)", the point where amplification of the fluorescence signal starts (270). This method assumes that cDNA strands are replicated entirely in each PCR cycle (270).

All results from the analyses of gene expression in the liver were normalized to two reference genes, GAPDH and RPLP0. The expression of a reference gene should remain constant in any condition for normalization purposes (272). However, it is always important to ensure the consistency of the reference genes since the expression of some commonly used reference genes vary in certain environments and tissues (272, 273). Any inconsistency of the expression of reference genes may lead to misinterpretation of results for the genes of interest (273). To solve this problem, a number of studies have recommended using multiple reference genes for obtaining reliable data (273, 275). In this study, two reference genes were chosen by the tool, RefFinder (Web-site: http://fulxie.0fees.us/?type=reference). The web-program RefFinder chooses the most stable reference gene based on the major computational programs (Bestkeeper, 276; NormFinder, 277; Genorm, 278; the comparative ΔCt method, 279) (280). Based on these results, the geometric mean of GAPDH and RPLP0 was used as the reference value for liver samples.

Table 3-2: Primers used for analysis of gene expression in the liver

Gene	5′ Forward primer 3′ Reverse primer	Reference
FAS	AAGCGGTCTGGAAAGCTGAA ACCAGTGTTTGTTCCTCGGA	*
GLUT2	AAAGCCCCAGATACCTTTACCT TGCCCTTAGTCTTTTCAAGC	*
GLUT5	CTTCGGAGTGTCTTGGAAGC GGCAGGGACTCCAGTCAG	*
GAPDH	TGGAGTCTACTGGCGTCTT TGTCATATTTCTCGTGGTTCA	247
RPLP0	GATGCCCAGGGAAGACAG CACAATGAAGCATTTTGGGTAG	282

*Synthetic forward and reverse primers were designed in house using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were designed to be intron-spanning to avoid amplification of genomic DNA.

Study B-7.2. Biochemical analysis of α-tocopherol and triglyceride concentrations in the liver

The concentrations of tocopherols in tissues were measured as potential anti-oxidant agents. Tocopherols were extracted from the livers according to the methods described by Kasimanickam et al. (282). Tocopherols (α, β, γ, δ) and α-tocopherol acetate were analyzed by HPLC using the procedure of Kramer et al. (283). Approximately one gram of frozen tissue was ground in liquid nitrogen, homogenized in 400 µL of 0.1 M tris buffer (pH 7.3) and then

transferred to a screw capped glass tube. Four-hundred (400) µL of 100 % ethanol, 40 µL of 15 % (w/v) of ascorbic acid and 400 µL of 0.9 % (w/v) NaCl were added to the tissue homogenates. Without saponification, tocopherols were extracted by adding 400 µL of hexane followed by centrifugation (3,000 x g, 10 minutes). Upper organic layer was collected for HPLC.

The Varian 9010 HPLC system was equipped with a Varian Prostar 235 pump with a Varian Prostar 410 auto sampler (Varian, Walnut Creek, CA, USA). The column used was a Supelcosil LC-Diol (4.6 mm x 250 mm, 5 micron) equipped with a Supelguard Diol guard column (4.0 mm x 20 mm). Compounds were detected using a Shimadzu (Shimadzu, Kyoto, Japan) RF-535 Fluorescence detector (Ex 295 nm, EM 330 nm). Chromatograms were acquired and integrated using Varian galaxie software. Standards were prepared as 5, 10, 20 40 and 60 nmol/mL of α-tocopherol acetate and α, β, γ, δ-tocopherols (Sigma-Aldrich, St. Louis, MO, USA; Figure 3-7).

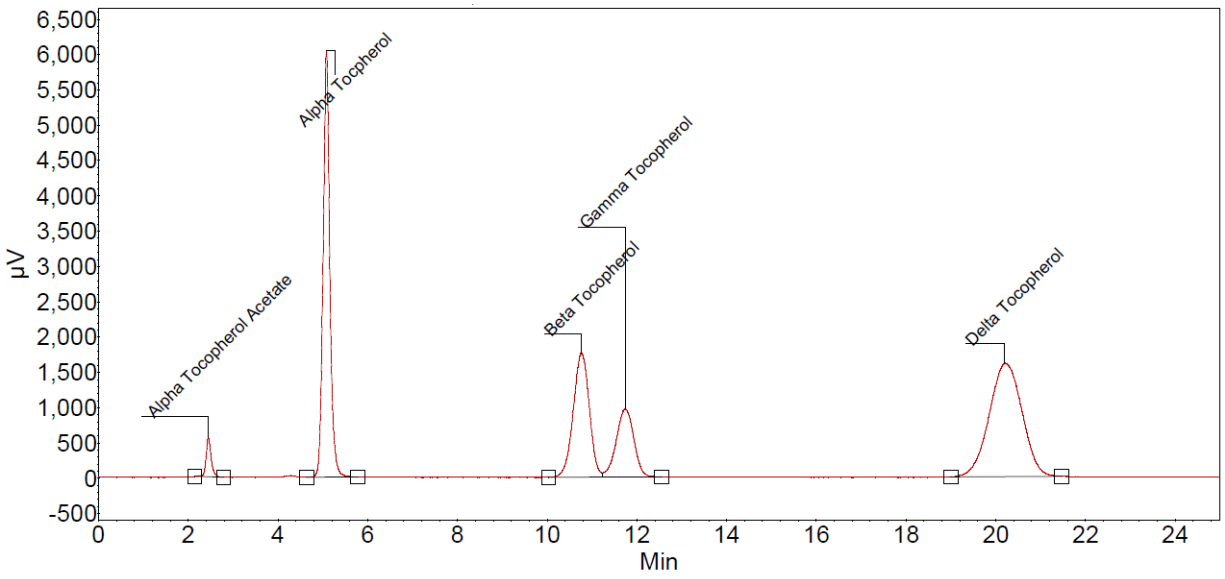


Figure 3-7: Sample chromatogram of HPLC separation of α-tocopherol acetate and α, β, γ, δ-tocopherols

This protocol was also tested for any benefits to carrying out saponification of tocopherols. Saponification has been commonly applied to liberate α, β, γ, δ-tocopherols from tissues in many studies since the high fat content of the liver may pose a problem in the extraction of tocopherols (284). Saponification in this protocol involved the heating of the samples in alkaline medium (adding 200 µL of 10 (w/v) % KOH to tissue homogenates and boiling for 15 to 30 minutes at 60 °C or 80 °C), using the procedure of Kramer et al. (283). As a result of the extraction, there was no difference seen between sample preparation with and without saponification. Therefore, saponification of tocopherols was not applied to liberate tocopherols in the liver in this study.

Hepatic TG content was used to interpret α-tocopherol concentrations in the liver (mmol/g tissue) as suggested by Kasimanickam et al. (282). Hepatic TG concentration was determined spectrophotometrically using a commercially available kit (Triglyceride Quantification Kit, ab5336, Abcam, Cambridge, UK). Briefly, approximately 100 mg of frozen tissue was ground in liquid nitrogen and homogenized in 1 mL of 5 % (v/v) NP-40/ddH2O. Lipid was extracted by heating (80 °C, 3 minutes) followed by centrifugation (20,000 x g, 2 minutes), and the supernatants were collected and diluted 1:10 with ddH_2O for analysis of TG. Twenty-five (25) µL of supernatant was incubated with 2 µL of lipase at room temperature for 20 minutes and then incubated with 50 µL of TG reaction mix (46 µL of Triglyceride Assay buffer, 2 µL of Triglyceride Probe and 2 µL of Triglyceride Enzyme Mix) at room temperature for 60 minutes. After incubation, absorbance was measured at 562 nm with a spectrophotometer.

Study B-7.3. Determination of lipid peroxidation in liver as an indicator of oxidative stress

Lipid peroxidation was assessed in the liver using a commercial kit (TBARS assay kit, Abnova, Walnut, CA, USA). Approximately 50 mg of frozen liver tissue was ground in liquid nitrogen and homogenized in 500 µL of radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors and then centrifuged at 1,600 x g for 10 minutes at 4 ℃. Supernatants were collected and diluted 1:1 with RIPA buffer. Supernatants (100 µL) were boiled with sodium dodecyl sulfate (100 µL SDS) solution and 4 mL of color reagent for 1 hour, placed in an ice bath for 10 minutes, and centrifuged at 1,600 x g for 10 minutes at 4 ℃. Absorbance was measured at 530 nm.

Total hepatic protein concentration was used to interpret the concentrations of TBARS in the liver. Approximately 50 mg of frozen liver was ground in liquid nitrogen and homogenized in 500 µL of radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. Tissue homogenates were incubated at 37 ℃ in 200 µL of a BCA reagent for 30 minutes. After incubation, absorbance was measured at 562 nm with a spectrophotometer.

Study B-8. Analyses of the Dam and Offspring Placentae

One of the frozen placentae per rat pregnancy (Dams and Offspring) was used to determine the levels of mRNA expression of nutrient transporters (SNAT2, FABP1) and angiogenic factors (VEGF-A, PlGF, VEGFR-1, VEGFR-2) by qPCR as described below. A second frozen placenta per rat pregnancy was used to analyze the concentrations of α, β, γ, δ tocopherols as a marker of antioxidant status. A third frozen placenta per rat pregnancy was embedded in optimal cutting

temperature compound (OCT) and sectioned at 5 µm as described below. Slides were stained with Dihydroethidium (DHE) to determine superoxide, or Dihydrorhodamine 123 (DHR) to examine peroxynitrite and Cluster of differentiation 31 (CD31) to determine vascular development by immunohistochemistry.

Three freshly dissected placentae/each rat (Dams and Offspring) were immediately placed in ice-cold phosphate buffered saline (PBS) and fixed overnight in 4 % (v/v) paraformaldehyde (PFA) in PBS. Thereafter, tissues were alcohol-dehydrated, xylene-cleared and paraffin-embedded in an automatic tissue processor (Leica TP1020, Germany). Paraffin-embedded placentae were sectioned at 5µm and mounted on Super Frost Plus slides (VWR); placentae were stained with hematoxylin and eosin (H&E) and the remaining slides were used for determination of GLUT1 using *in situ* hybridization (details described below).

Study B-8.1. RNA extraction and determination of gene expression in the placentae using quantitative polymerase chain reaction (qPCR)

RNA extraction and qPCR were carried out in the placenta by the methods described above (pages 114 – 116). The target genes and their primers are shown in Table 3-3. To ensure consistency of expression of reference genes, all results from the placentae were adjusted to the reference genes, GAPDH and TBP. These reference genes were chosen by RefFinder as described above (pages 115 – 116).

Table 3-3: Primers used for analysis of expression of placental genes

Gene	5′ Forward primer 3′ Reverse primer	Reference
SNAT2	AGTTGGGACATAAGGCATACGG GGACACCAGAAGGACCAGATAG	*
FABP1	AAGTACCAAGTGCAGAGCCAAG GACCCATAGGTGATGGTGAGTT	*
VEGF-A	ATCATGCGGATCAAACCTCACC GGTCTGCATTCACATCTGCTAT	285
PlGF	CCGGCCCTGGCTGCATTGAA CAGGCAAAGCCCACAGGCGA	286
VEGFR-1	CGACACTCTTTTGGCTCCTTCTAAC TGACAGGTAGTCCGTCTTTACTTCG	288
VEGFR-2	GTCCGCCGACACTGCTGCAA CTCGCGCTGGCACAGATGCT	286
GAPDH	TGGAGTCTACTGGCGTCTT TGTCATATTTCTCGTGGTTCA	288
TBP	GCCTGCGGCTGCTCGTTTTG TGGGGAGGCCAAGCCCTGAG	289

*Synthetic forward and reverse primers were designed in house using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were designed to be intron-spanning to avoid amplification of genomic DNA.

Study B-8.2. Biochemical analysis of α-tocopherol in the placentae

The concentrations of tocopherols in the placentae were measured as potential anti-oxidant agents. Tocopherols were extracted from the placentae and were analyzed by HPLC according to the methods described above (pages 117 – 118).

The protocol was tested for benefits of saponification of tocopherols. There was no difference observed between sample preparation with and without saponification. Therefore, saponification of tocopherols was not applied to liberate tocopherols in the placentae.

Study B-8.3. Determination of lipid peroxidation as an indicator of oxidative stress in the placenta

Oxidative stress was assessed in the frozen placentae by determining the TBARS concentration using the methods described above (page 119). TBARS concentrations were corrected for total tissue protein concentration, determined using the BCA method, described above.

Histology in paraffin-embedded placentae The area of LZ and JZ of the placentae was measured by histology. Sections of paraffin-embedded placentae from rats (Dams-CONT=5, Dams-FR=5, Offspring-CONT=9, Offspring-FR=7) were stained with H&E according to standard procedures and visualized with a microscope (AX10 Imager.M1, Carl Zeiss Microimaging, Thornwood, NY, USA) using AxioVision software (Zeiss, Version 4.5, Germany). To determine the areas of the LZ and JZ, placenta sections (magnification, x50) were photographed and approximately 60 images were merged to visualize a whole section of the placenta, and then the image was converted to an 8-bit format. Threshold values of the LZ and JZ were adjusted in Image J software (National Institutes of Health,

Bethesda MD). The LZ and JZ of the placentae were circled by freehand selection (a tool used to create an irregular shaped selection area) and the ratio of LZ:JZ was calculated (Figure 3-8). The average of the ratio between LZ:JZ in the placentae of rats in each group was calculated. Measurement was done by one technician and one student who were blinded to each other's observations.

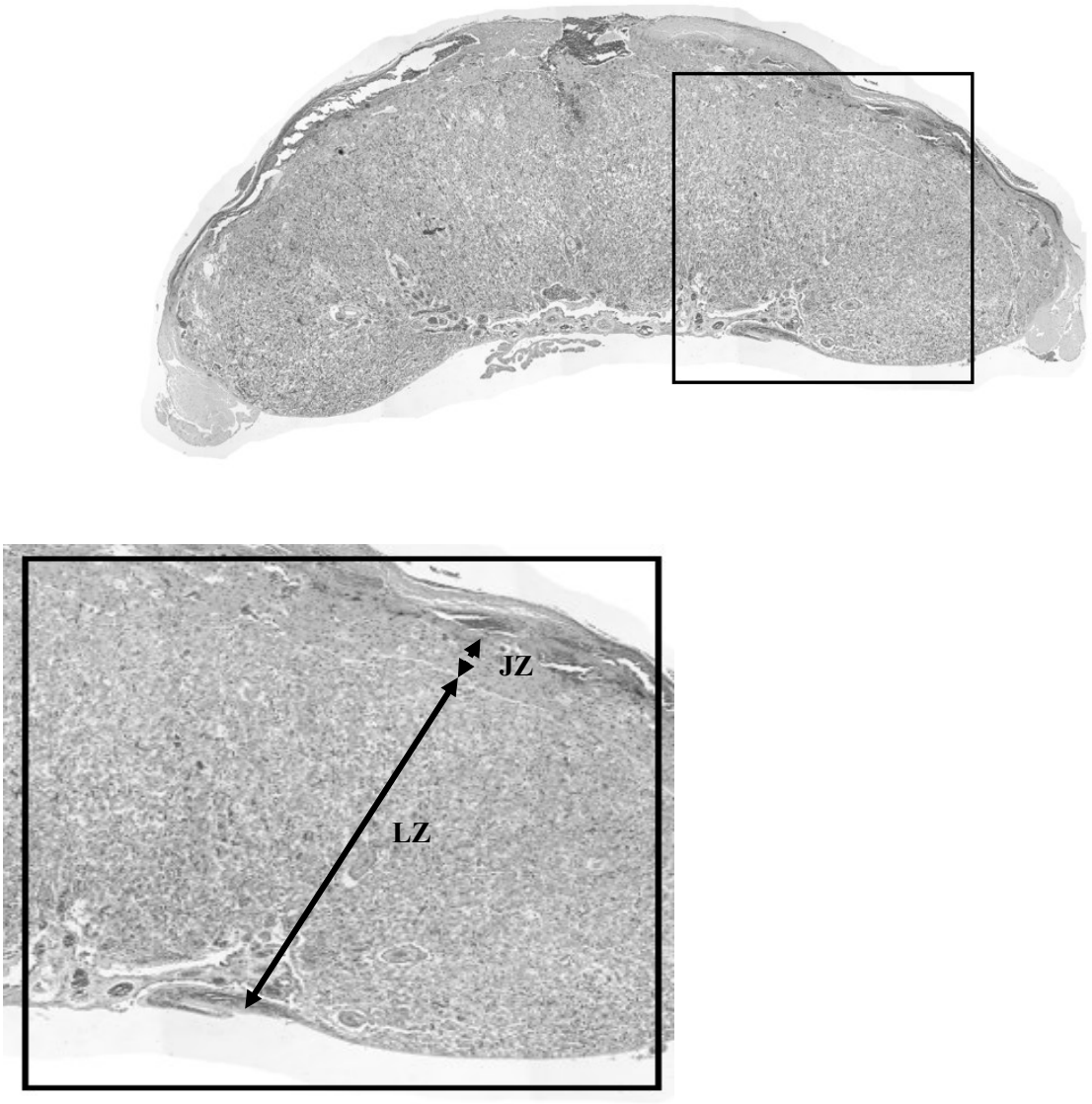


Figure 3-8: The identification and measurement of the labyrinth zone (LZ) and junctional

zone (JZ) in a vertically-sectioned placenta

GLUT1 expression and distribution measured in placental sections *In situ* hybridization (ISH) was used to examine GLUT1 expression and distribution within the placentae. This measurement builds on previous work in our lab indicating that mRNA expression of GLUT1 was reduced 2-fold in the placentae of rats fed a 10 % (w/v) fructose solution compared with rats fed distilled water at GD20 (163).

ISH was performed as described by Simmons et al. (290). Riboprobe for GLUT1 was synthesized with T7 RNA polymerase (Sense probe) and T3 RNA polymerase (Antisense probe) (final products: forward primer, 5'- AATTAACCCTCACTAAAGGG-3' and reverse primer, TAATACGACTCACTATAGGG) and labeled with DIG-UTPs (Roche). Synthetic forward and reverse primers were designed using Primer3 software (developed at the Whitehead Institute for Biomedical Research, by Steve Rozen and Helen J. Skaletsky; available at the Primer3 Software Distribution Web site; http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and product sizes and primer specificity were confirmed using PCR and gel electrophoresis. Slides cut from paraffin-embedded placentae from rats (Dams-CONT=5, Dams-FR=5, Offspring-CONT=9, Offspring-FR=7) were fixed in 4 % (v/v) PFA for ten minutes followed by rehydration in PBS for ten minutes. Slides were treated with proteinase K (0.3 % (v/v) of proteinase K (10 mg/mL) in proteinase K buffer; Roche) for 20 minutes at room temperature, and acetylated (0.25 % (v/v) acetic anhydride in DEPC-treated water; Sigma) for 10 minutes. After acetylation, DIG-labeled probes (final dilution of 1:2000) were heated in hybridization buffer (1x salts (containing 1.14 g of NaCl, 0.1 g of Tris HCl, 1.34 g of Trise Base, 0.078 g of $NaH_2PO_4 \bullet 2H_2O$, 0.071 g of Na_2HPO_4, 1 ml of 0.5 M EDTA pH8), 50 % (v/v) deionized formamide, 10 % (w/v) dextran sulfate, 0.1 %

(v/v) yeast tRNA, 1x Denhardt's solution (1 % (w/v) BSA, 1 % (w/v) Ficoll, 1 % (w/v) polyvinylpyrrolidone in DEPC-treated water) for 10 minutes at 70 °C, and then 1 µL of denatured probe was diluted in 200 µL of hybridization buffer. Slides were incubated overnight in hybridization buffer containing digoxigenin (DIG)-labeled probes overnight at 65 °C in a sealed humidified box. Following hybridization, slides were washed twice in post-hybridization wash solution (containing 1xSSC, 50 % (v/v) formamide, 0.1 % (v/v) Tween-20; 1x SSC containing 0.15 M sodium chloride and 15 mM trisodium citrate in Milli-Q water) for 30 minutes at 65 °C. This was followed by washing in MABT (150 mM sodium chloride, 100 mM maleic acid 0.1 % (v/v) Tween 20 in Milli-Q water filtered through a one-micron filter) for 30 minutes at room temperature and washing in 1x RNA wash (0.5 M sodium chloride, 0.1 M Tris (pH 8), 0.05 M EDTA) for ten minutes at 37 °C. Slides were next washed in 1x RNA wash containing 20 µg/mL RNase A for 30 minutes at 37 °C, then washed in 1xRNA wash for 5 minutes at 37 °C and finally washed in 1x MABT for 5 minutes at room temperature. Next, slides were blocked in 1x maleic acid buffer containing tween 20 (MABT) (2 % (v/v) blocking reagent; 20 % (v/v) goat serum) for 1 hour, and incubated with anti-DIG antibody (1:2500) overnight at 4 °C in a humidified chamber. After incubation with anti-DIG antibody, slides were washed in 1x MABT for 15 minutes at room temperature followed by washing in NTMT (containing 5 M sodium chloride, 1M magnesium chloride, 1M tris and 10 % (v/v) Tween-20) for 10 minutes at room temperature and washing in NTMT containing levamisole (100 mg levamisole in 200 mL of NTMT) for 10 minutes at room temperature. Probes on the slides were detected by incubation with colour substrate (4.5 µL of nitro-blue tetrazolium chloride (NBT) and 2.5 µL of 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP); Promega) for 12 hours at 37 °C. Staining was stopped by adding 1xPBS, and the slides were mounted with Permount (Fisher

Scientific Company, Pittsburgh, PA, USA) and analyzed using Image J software (source or reference) as described above for H & E analyses.

Study B-8.4. Immunohistochemistry in OCT-embedded placentae

Measurement of oxidative stress Oxidative stress was identified by visualization of DHE and DHR as markers of superoxide and peroxynitrite respectively. Placentae were cut into 10 µm-thick sections using a cryostat and washed with Hanks Balanced Salt Solution (HBSS). The samples were incubated with either 20 mM DHE solution (1:100, Biotium, Hayward, CA, USA) or 5 mM DHR (1:50, Molecular Probes, Eugene, OR, USA) for 30 minutes at 37 ℃. After incubation, samples were washed with HBSS. Fluorescent images were obtained with an Olympus microscope (Olympus, Tokyo, Japan) using cellSens Dimension software (Olympus). Nine (9) to 10 regions (Figure 3-9) of the placenta were photographed into and saved as TIFF images at a final magnification of 20x. The intensity of positive staining was quantified using Image J software (National Institutes of Health, Bethesda MD).

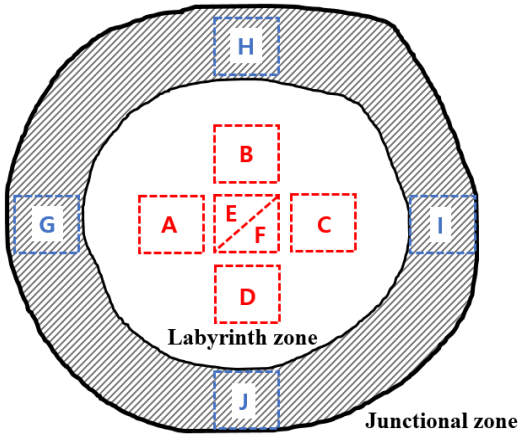


Figure 3-9: Horizontal cross-section of the placenta and identification of the areas used to visualize DHE and DHR. 9 to 10 regions in the placenta were randomly selected: A, B, C, D, E

and F represent random regions within the Labyrinth zone, G, H, I and J represent random regions within the Junctional zone.

Measurement of vascularity Cryosectioned placentae were fixed with cold acetone for 10 minutes and air-dried for 5 minutes. The slides were washed with 1xPBS (pH 7.5) and then incubated with blocking solution (2 % bovine serum albumin; BSA) for 60 minutes at room temperature. After blocking, the slides were incubated with mouse anti-rat CD31 (BD BioSciences, San Jose, CA, USA) diluted 1:10 with 2 % (w/v) BSA overnight at 4 ℃ and this was followed by 60-minute incubation with Goat anti-mouse (Alexa Fluor 546, BD BioSciences, San Jose, CA, USA; diluted 1:250 in 2 % (w/v) BSA; Molecular Probes, Eugene, OR, USA) at room temperature. After incubation, slides were washed and stained with 1µL of mounting medium with 4,6-diamidino-2-phenylindole (DAPI). Each slide was viewed by fluorescence microscopy (Olympus, Tokyo, Japan) using cellSens Dimension software (Olympus). Nine to ten regions (Figure 3-10) of the placenta were photographed and saved as TIFF images at a final magnification of 20x. The intensity of positive staining was quantified using Image J software (National Institutes of Health, Bethesda MD). There was no positive expression in JZ, thus the intensity was measured only in LZ of the placenta.

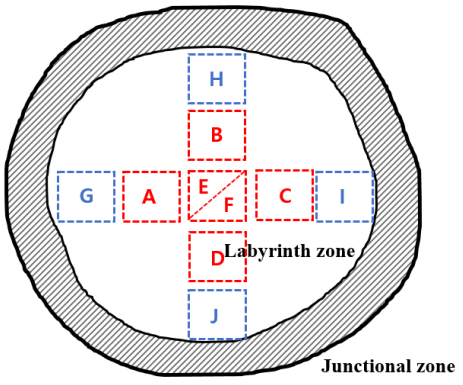


Figure 3-10: Horizontal cross-section of the placenta and identification of the areas used to

visualize CD31. **9 to 10 regions were randomly selected: A, B, C, D, E, F, G, H, I and J represent random regions within the labyrinth zone.**

Study B-9. Statistical Analyses

Values are presented as mean ± S.E.M and all statistical analyses were performed using SAS 9.2 as described above (page 108). Briefly, the normality of the distribution of each variable distribution was verified. Data that were not normally distributed were 24-hour food and water intake, plasma insulin and TG concentrations at pre-diet to mid pregnancy, glucose and insulin concentrations during the OGTT, the weights of maternal organs and body weight, the level of TBARS in the liver, and the weight of the fetus and placenta on GD20. These variables were log-transformed and confirmed to be normally distributed prior to statistical analyses.

Group differences were assessed by two-way ANOVA and included repeated measures where appropriate as described above (i.e. body weight from pre-diet to late pregnancy, and plasma glucose, insulin and TG concentrations from pre-diet to late pregnancy). Pair-wise post hoc comparisons using a Tukey correction were used to identify individual group differences in analyses using repeated measures. A p-value of 0.05 was accepted as statistically significant.

Study C. The effects of high fructose intake on metabolic and physiologic profiles of female rats during two sequential pregnancies

Study C-1. Experimental Design and General Protocol

Five Dam-CONT and five Dam-FR from Study B littered out and remained on their assigned CONT or FR treatment during lactation. Seven extra Dam-CONT and nine extra Dam-FR that

were treated following the same protocol as in Study B were added to increase the sample size. Thus, Dam-CONT (n=12) and Dam-FR (n=14) were used in Study C. Once their pups were weaned, the Dam-FR and Dam-CONT received distilled water with food *ad libitum* for one week and were returned to their treatment group for three weeks. Thereafter, they were mated again to initiate second pregnancy. These Dams went through their second pregnancy beginning at ~23 weeks of age. Pregnancy was confirmed by vaginal lavage, and a positive sperm test was considered to represent GD0 (Figure 3-11). At the end of second pregnancy, all female and male rats were euthanized. Body weights were not recorded, plasma was not collected and body composition was not determined in male rats.

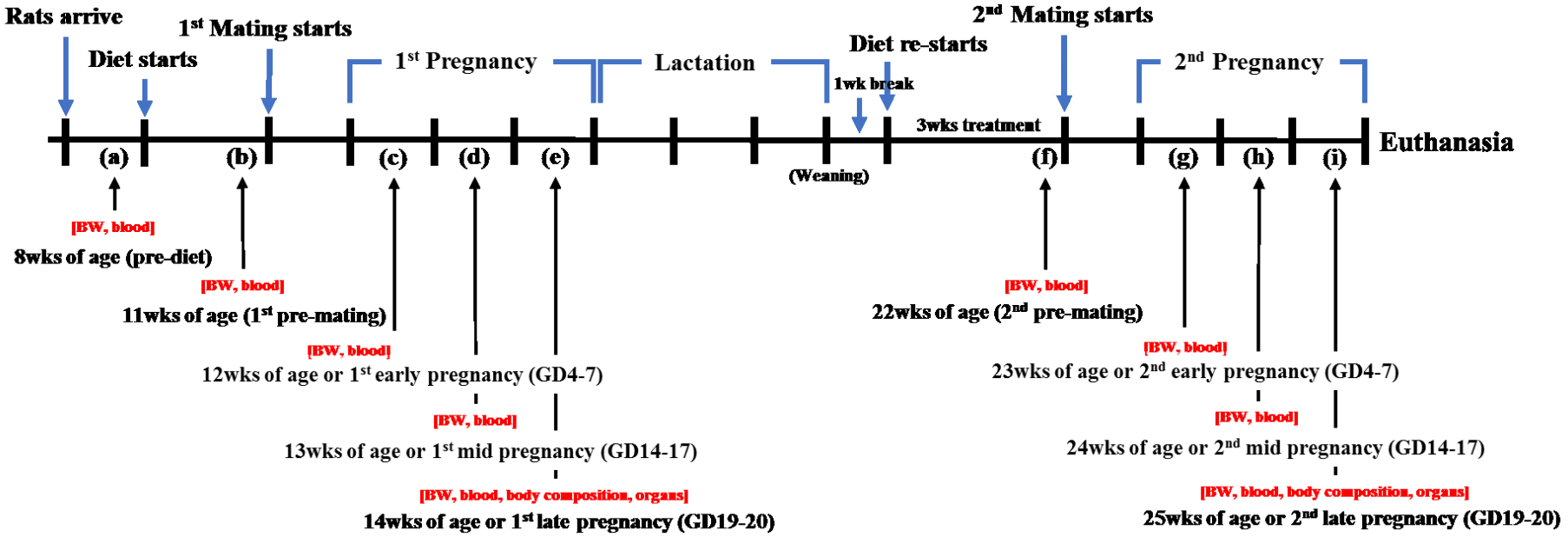


Figure 3-11: Animal model of study C. BW=body weight

Study C-2. Regular monitoring of body weight and metabolic profiles

Body weights were recorded and metabolic profiles were analyzed of Dam-CONT and Dam-FR during first pregnancy (Dam-CONT1, Dam-FR1) as described above. These same measurements were recorded for the Dam-CONT and Dam-FR during their second pregnancy (Dam-CONT2, Dam-FR2), beginning at (f) 22 weeks of age (Second pre-mating), (g) 23 weeks of age (GD4 – 7, second early pregnancy), (h) 24 weeks of age (GD14 – 17, second mid pregnancy) and (i) 25 weeks of age (GD19 – 20, second late pregnancy) (Figure 3-11, page 129).

Study C-3. Analyses in Plasma

Plasma glucose, insulin and TG concentrations were measured by the assays described above (page 103 – 108).

Study C-4. Determination of Body Composition and the Weights of Organs

Body composition (% fat and % lean of body weight) and body weight of rats were measured using quantitative magnetic resonance analysis (details described above, page 108) at GD21 during first and second pregnancy.

After measurements of body composition and body weight at GD21, rats were euthanized using CO_2. The pancreas and liver were excised and weighed.

Study C-5. Statistical Analyses

Values are presented as mean ± S.E.M and all statistical analyses were performed using SAS 9.2 as described above (page 108). The normality of the distribution of each variable distribution was verified prior to statistical analysis, and those that were not normally

distributed were log-transformed before analysis. Data that were not normally distributed were plasma glucose, insulin and TG concentrations at pre-diet to mid pregnancy.

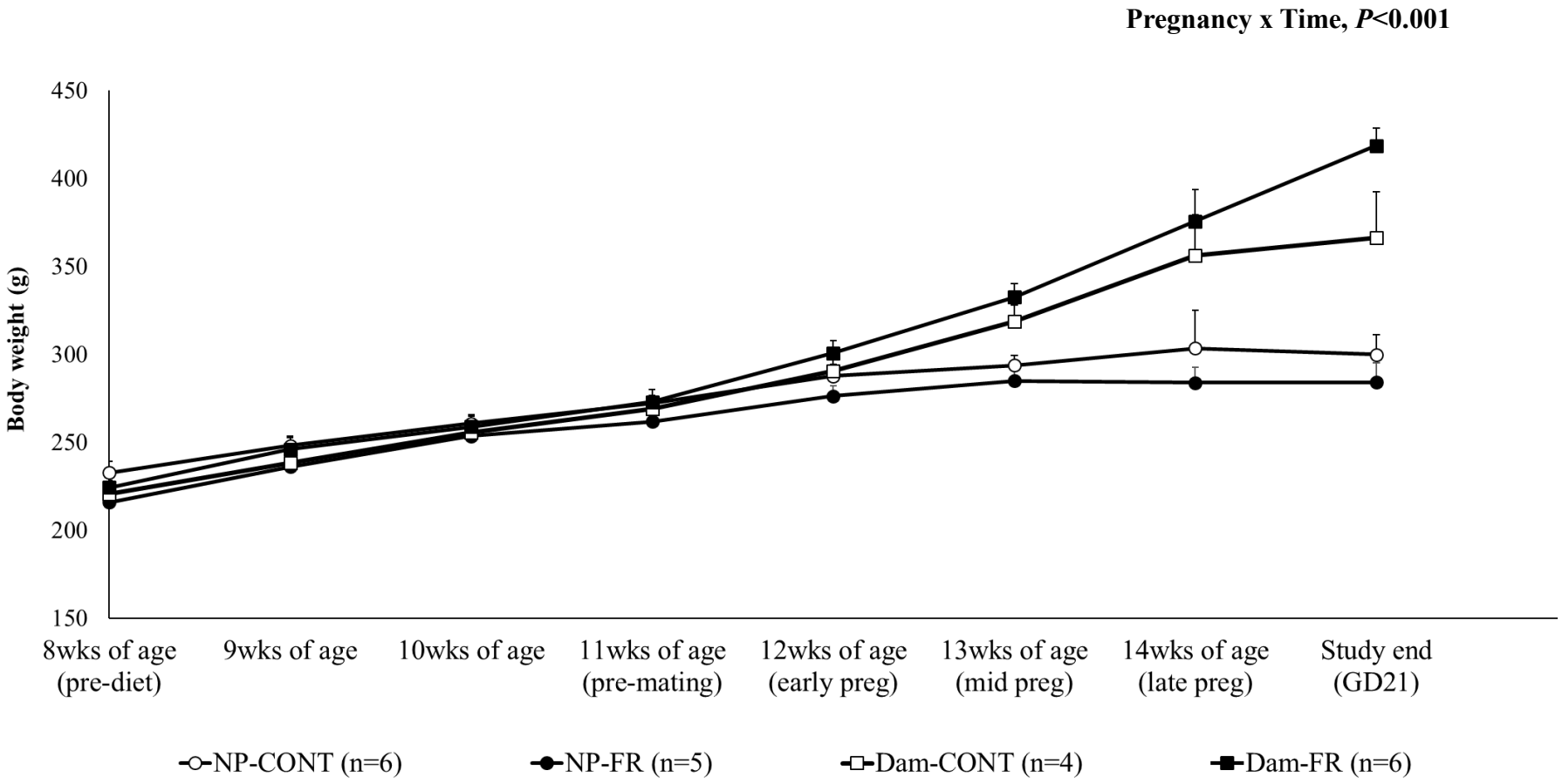
Group differences were assessed by two-way ANOVA and included repeated measures where appropriate, as described above (i.e. body weight from pre-diet to late pregnancy, and plasma glucose, insulin and TG concentrations from pre-diet to late pregnancy). Pair-wise post hoc comparisons using a Tukey correction were used to identify individual group differences in analyses using repeated measures. A p-value of 0.05 was accepted as statistically significant.

CHAPTER 4: RESULTS

Study A. The effects of high fructose intake on the body weight, metabolic profiles and fat mass of non-pregnant and pregnant rats

Study A. Body weights of non-pregnant and pregnant rats

Overall, body weights of non-pregnant rats (NPs) and Dams differed across the duration of the study from 8 weeks of age to the end of the study (main effect of time, $P<0.001$), and differed between the NP and pregnant groups of rat (main effect of pregnancy, $P=0.0169$) (Figure 4-1). There was no difference in body weights among groups after they had been acclimated for one week (at 8 weeks of age). Body weights of Dams were about 14 g, 36 g, 72 g and 110 g heavier than age-matched NPs at early ($P=0.0617$), mid ($P=0.0001$), late pregnancy ($P=0.0015$) and GD21 ($P<0.0001$) respectively. Body weight was not significantly affected by FR consumption in either the NPs and Dams groups at any time during this study.



Pregnancy x Time: interaction between pregnancy and time

	P-value
Time	**<0.0001**
Pregnancy (NP vs. Dam)	**0.0169**
Diet (CONT vs. FR)	0.8038
Pregnancy x Diet	0.1305
Diet x Time	0.9877
Pregnancy x Time	**<0.0001**
Pregnancy x Diet x Time	0.8747

Figure 4-1: Body weights of NPs and Dams fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age to 14 weeks of age. Each value is the mean ± S.E.M. 8wks of age (pre-diet): Body weight was measured on the day before they were eight weeks of age and all were still receiving solid food *ad libitum* and distilled water. At 8 wks of age: rats started to receive either distilled water or a 10 % (w/v) fructose solution. At 11wks of age (pre-mating): NPs were 11 weeks of age and Dams were co-housed with male rats. At 12wks of age (early pregnancy): NPs were 12 weeks of age and Dams had completed ~ 4 – 7days of pregnancy. At 13wks of age (mid pregnancy): NPs were 13 weeks of age and Dams had completed 10 – 12days of pregnancy. At 14wks of age (late pregnancy): NPs were 14 weeks of age and Dams had completed 17 – 19days of pregnancy. At Study end: NPs were the same age as Dams and Dams were at GD21.

Study A. Metabolic profiles of non-pregnant and pregnant rats

Non-fasting glucose, insulin and TG concentrations of rats were recorded from 8 (pre-diet) to 13 weeks of age (mid pregnancy for dams) (Table 4-1). Plasma concentrations of these same metabolites were measured during an OGTT at 14 weeks of age only (late pregnancy for dams) (Figure 4-2).

Non-fasting plasma glucose concentrations changed significantly over the time (main effect of time, P=0.0002) (Table 4-1). Glucose concentrations were similar among all groups at 8 weeks of age and at the time of mating, i.e. 11 weeks of age. At 12 weeks of age (early pregnancy), NP and Dams in the FR group had significantly higher plasma glucose concentrations than those in the CONT.

Non-fasting plasma insulin concentrations also varied significantly across the study (main effect of time, P<0.0001) and insulin concentrations generally rose in all groups as the study went on (Table 4-1). When considering each time point, rats in the FR group had insulin concentrations that were approximately 2-fold higher than those in the CONT group at 12 weeks of age (early pregnancy) (P=0.0303). As pregnancy progressed, Dam-CONT rats experienced increased insulin concentrations, as expected during pregnancy, and by mid-pregnancy there were no longer significant differences between diet groups. Insulin concentrations appeared to increase slightly in the Dams during pregnancy compared to the age-matched NP group, although the differences were not statistically significant.

Pregnant and NP rats had a significantly different time course in TG concentrations during this study (P<0.0001) (Table 4-1). NP and Dams had similar TG concentrations from the beginning of

the study until early pregnancy (12 weeks of age), but Dams had significantly increased TG concentrations during mid-pregnancy compared to the age-matched NP rats ($P<0.0030$). Overall, TG concentrations were higher in the FR vs. CONT rats ($P=0.0476$) with the FR group specifically having TG concentrations that were ~2-fold higher than the CONT group in early pregnancy (1.8-fold, $P=0.0012$). This diet effect was not evident at mid-pregnancy.

Table 4-1: Plasma glucose, insulin and triglyceride concentrations measured regularly in NPs and Dams fed distilled water (CONT) or the fructose solution (FR)

	NP-CONT (n=6)	NP-FR (n=5)	Dam-CONT (n=4)	Dam-FR (n=6)
Glucose (mmol/L)				
8wks of age (pre-diet)	8.2 ± 0.2	8.4 ± 0.4	8.6 ± 0.3	8.5 ± 0.3
11wks of age (pre-mating)	8.4 ± 0.3	8.3 ± 0.2	8.7 ± 0.5	8.4 ± 0.4
12wks of age (early pregnancy)	8.1 ± 0.2	8.4 ± 0.4	7.5 ± 0.3	8.4 ± 0.3
13wks of age (mid pregnancy)	7.7 ± 0.2	8.6 ± 0.3	6.7 ± 0.3	7.9 ± 1.0
14wks of age* (late pregnancy)	7.9 ± 0.7	7.3 ± 0.4	6.6 ± 0.7	7.0 ± 0.7
Insulin (ng/mL) [b]				
8wks of age (pre-diet)	0.9 ± 0.1	1.6 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
11wks of age (pre-mating)	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.2	1.0 ± 0.2
12wks of age (early pregnancy)	1.2 ± 0.2	2.1 ± 0.7	0.6 ± 0.2	1.9 ± 0.6
13wks of age (mid pregnancy)	2.2 ± 0.3	3.1 ± 0.7	3.0 ± 0.5	3.7 ± 0.3
14wks of age* (late pregnancy)	1.5 ± 0.3	1.2 ± 0.1	1.4 ± 0.1	1.7 ± 0.3
Triglyceride (mmo/L) [a, b]				
8wks of age (pre-diet)	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.2
11wks of age (pre-mating)	1.0 ± 0.1	1.6 ± 0.2	1.3 ± 0.2	1.6 ± 0.4
12wks of age (early pregnancy)	0.9 ± 0.3	1.5 ± 0.2	1.0 ± 0.1	2.0 ± 0.2
13wks of age (mid pregnancy)	1.1 ± 0.1	1.4 ± 0.2	3.7 ± 0.7	5.2 ± 1.2
14wks of age* (late pregnancy)	0.7 ± 0.1	1.4 ± 0.3	5.2 ± 1.4	5.6 ± 1.4

Each value is the mean ± S.E.M. Samples collected from 8wks to 13wks of age were measured in the non-fasting state. At 14wks of age samples were collected after a 4-hour fast.

[a]*p*-value from the main effect of pregnancy

[b]*p*-value from the main effect of diet

		***P*-value**		
		Glucose	**Insulin**	**Triglyceride**
	Time	**0.0002**	**<0.0001**	**<0.0001**
P^a	**Pregnancy (NP vs. Dam)**	0.4996	0.6290	**0.0047**
P^b	**Diet (CONT vs. FR)**	0.0944	**0.0031**	**0.0476**
	Pregnancy x Diet	0.7939	0.8248	0.6031
	Diet x Time	0.2445	0.6315	0.2648
	Pregnancy x Time	0.2500	0.0859	**<0.0001**
	Pregnancy x Diet x Time	0.7726	0.5091	0.8136

It is well known that glucose concentrations decrease in rats and other rodents during pregnancy due to the high glucose utilization by enlarged organs, placental growth and the high fetal mass relative to the maternal mass (291). At mid-pregnancy, glucose concentrations were ~1.0 mM lower in Dams than NP, although this decrease was not statistically significant. During the OGTT in late pregnancy, there was no difference in fasting glucose concentrations among groups, although glucose concentrations were ~1.3-fold lower in Dams than NPs at 15 minutes (*P*=0.0367), 60 minutes (*P*=0.0240) and 90 minutes (*P*=0.0159) (Fig 4-2 A). FR intake did not alter glucose responses during the OGTT in either NPs or Dams, and the total $AUC_{Glucose}$ was also not different between NP vs. Dam or between diet groups (Fig 4-2 B).

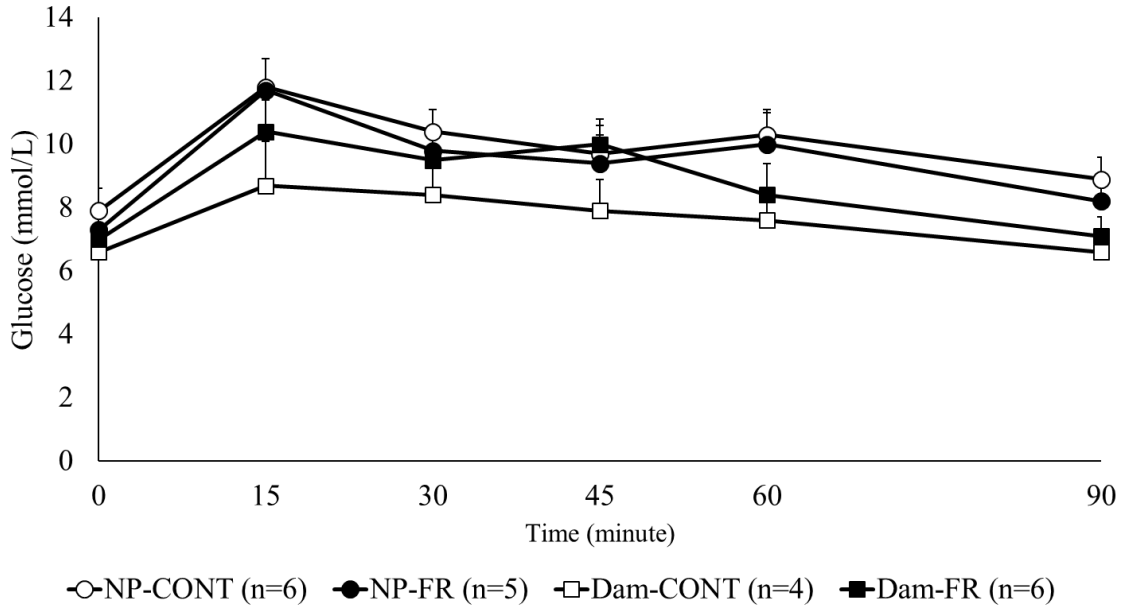
Insulin concentrations during the OGTT were generally not different between NP and Dams in either the FR or CONT groups (Figure 4-2 C + D). Fasting insulin concentrations were similar,

and differences between groups were not significant except at the 90 min timepoint. At this final time in the OGTT, insulin was 2-fold higher in Dams than NPs (P=0.0066).

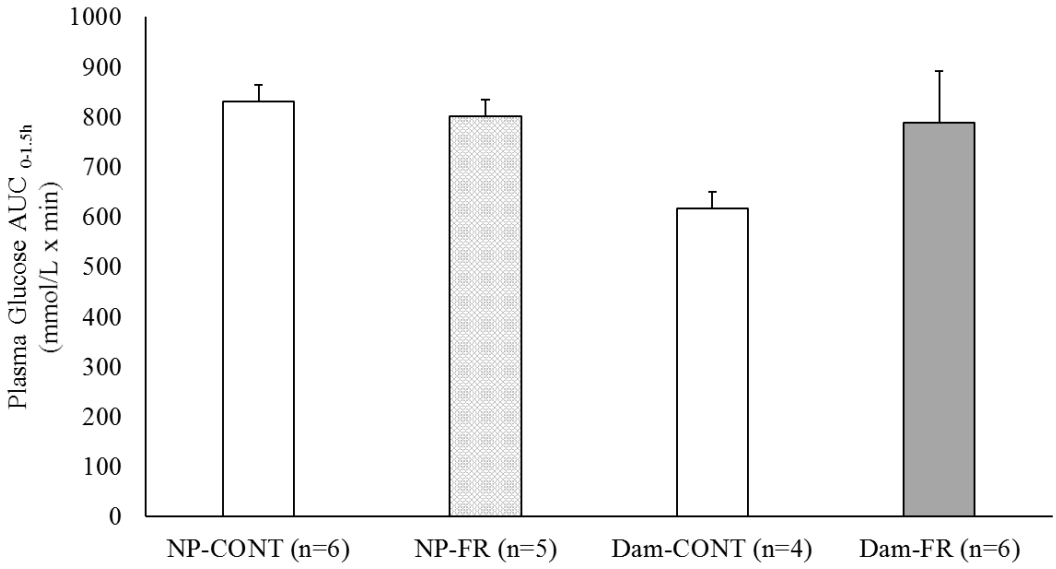
Insulin resistance was estimated in NPs and Dams using fasting glucose and insulin in the HOMA-IR model (Figure 4-2). There were no significant differences between pregnancy (NP vs. Dam) or diet (CONT vs. FR). There was also no interaction between pregnancy and diet using HOMA-IR.

The elevated plasma TG concentration observed between Dams and NP rats at the mid-pregnancy mark continued through late pregnancy (Figure 4-2). Fasting TG concentrations were increased by 5.1-fold (P=0.0005) in Dams vs NP, and these large differences were evident throughout the OGTT (15 minutes (P=0.0031); 30 minutes (P=0.0036); 45 minutes (P=0.0023); 60 minutes (P=0.0114); and 90 minutes (P=0.0027). The AUC_{TG} reflects these differences in Dams compared to NPs. While there were no statistically significant effects of FR at any individual time point within the NP and Dams groups, the AUG_{TG} reflects the fact that rats in the FR group had higher TG concentrations throughout the OGTT than the CONT group (P=0.0403) (Fig 4.2 E + F).

(A)

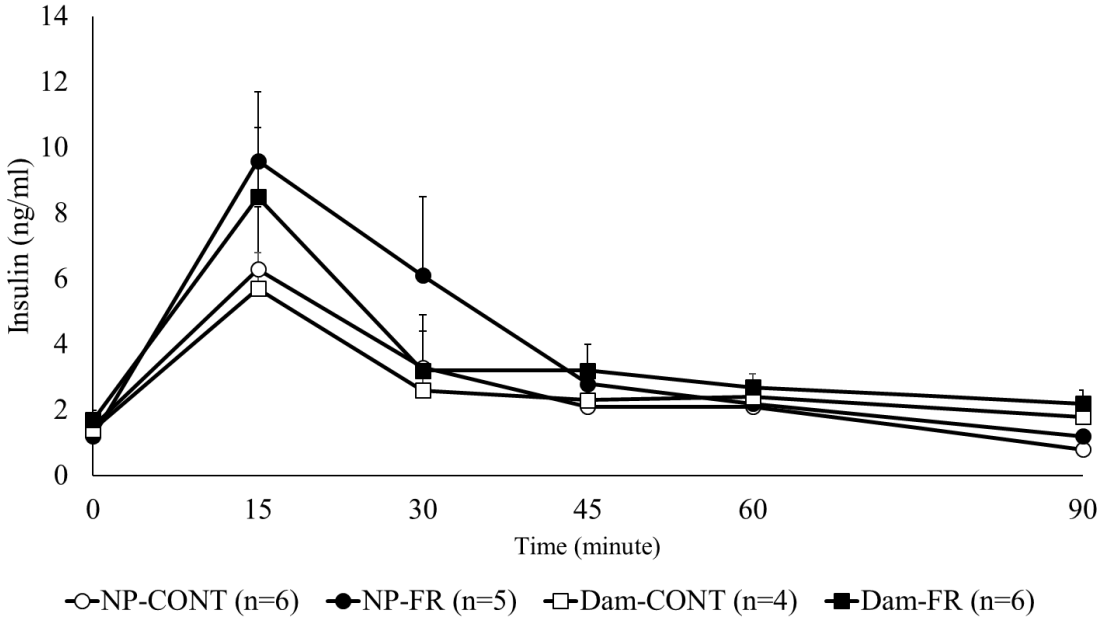


(B)

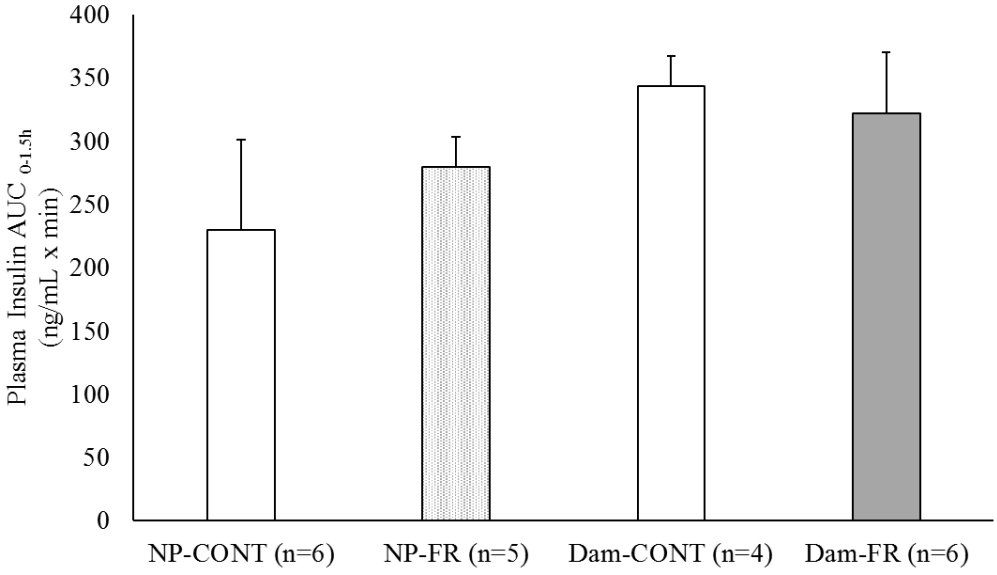


	$AUC_{Glucose}$
Pregnancy (NP vs. Dam)	0.1935
Diet (CONT vs. FR)	0.4373
Pregnancy x Diet	0.2810

(C)



(D)

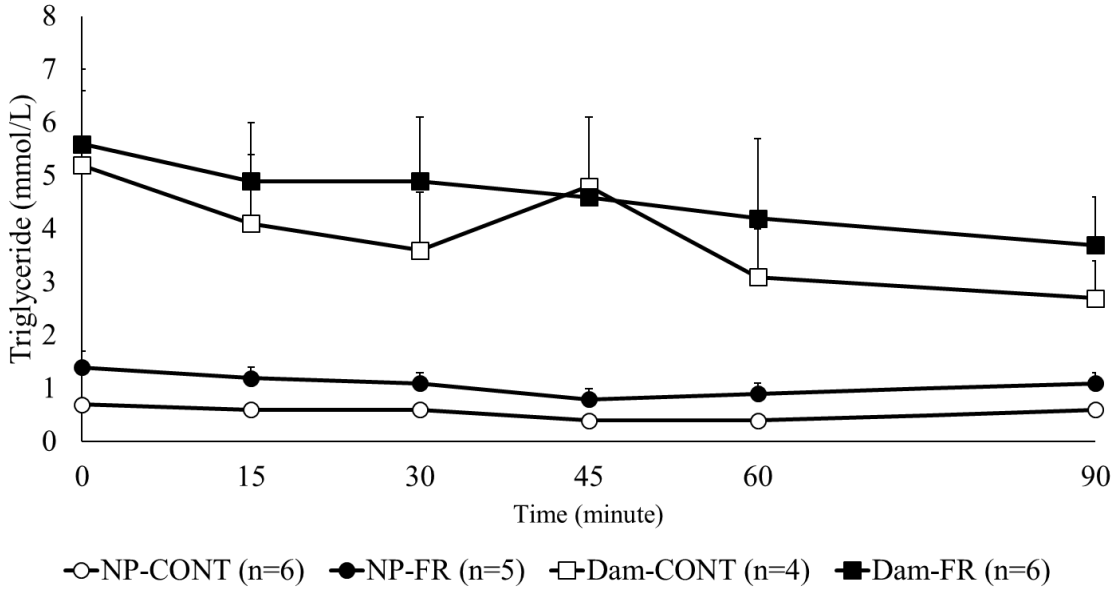


	$AUC_{Insulin}$
Pregnancy (NP vs. Dam)	0.2160
Diet (CONT vs. FR)	0.6965
Pregnancy x Diet	0.4535

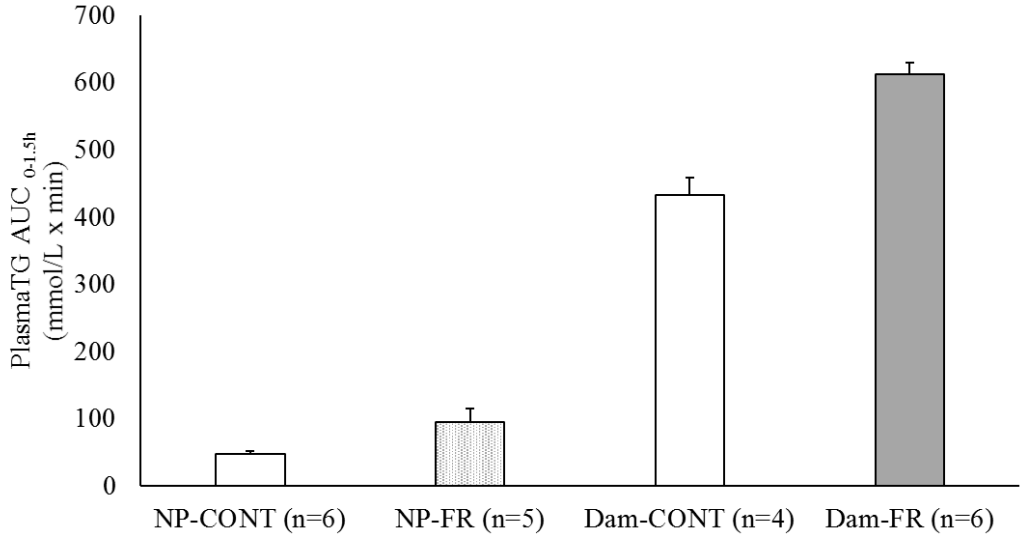
	NP-CONT (n=6)	NP-FR (n=5)	Dam-CONT (n=4)	Dam-FR (n=6)
HOMA-IR	10.1 ± 2.0	17.9 ± 4.9	13.8 ± 4.0	12.4 ± 2.3

	HOMA-IR
Pregnancy (NP vs. Dam)	0.6215
Diet (CONT vs. FR)	0.2896
Pregnancy x Diet	0.4191

(E)



(F)

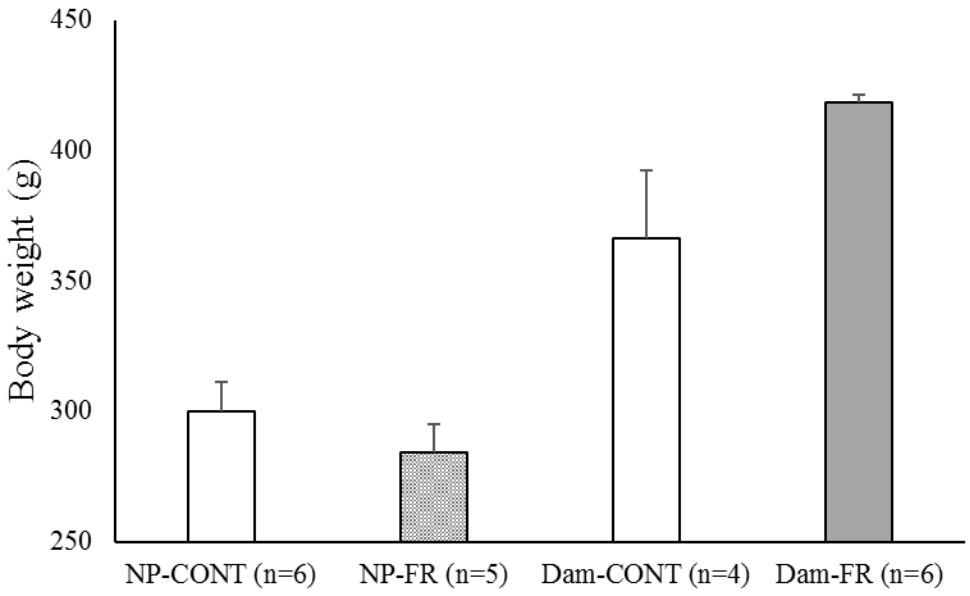


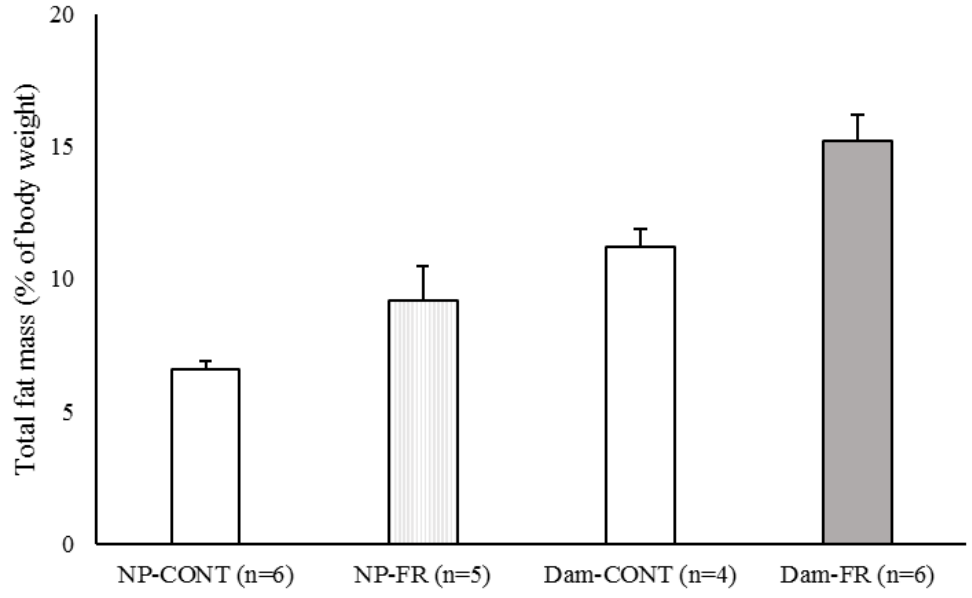
	AUC_{TG}
Pregnancy (NP vs. Dam)	**<0.0001**
Diet (CONT vs. FR)	**0.0403**
Pregnancy x Diet	0.4830

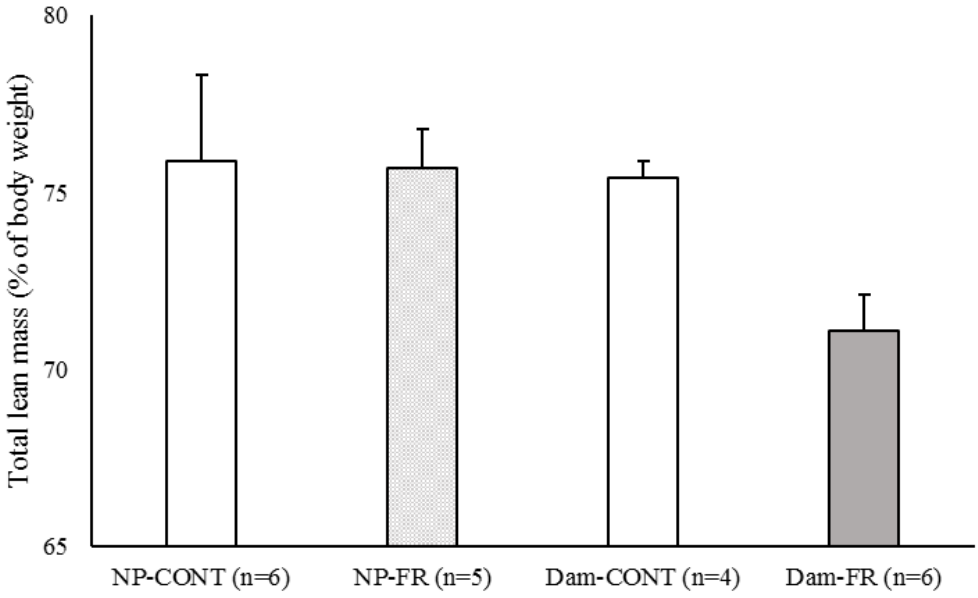
Figure 4-2: Plasma glucose, insulin, HOMA-IR and TG responses during an oral glucose tolerance test (OGTT) at 14 weeks of age (late pregnancy) in NPs and Dams fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age to 14 weeks of age. (A): Plasma glucose concentrations of NPs and Dams during the OGTT, (B): $AUC_{Glucose}$ during the OGTT of NPs and Dams, (C): Plasma insulin concentrations of NPs and Dams during the OGTT, (D): $AUC_{Insulin}$ during the OGTT of NPs and Dams, (E): Plasma TG concentrations of NPs and Dams during the OGTT, (B): AUC_{TG} during the OGTT of NPs and Dams.

Study A. Body composition and body weight of non-pregnant and pregnant rats at GD21

Body composition and body weight was measured at the end of pregnancy (Dams) or 14 weeks of age (NP). The proportion of fat and lean tissue relative to body weight and did not differ among groups (Figure 4-3). However, body weight (g) and lean mass (g) were higher in Dams than NP at GD21 (main effect of pregnancy, $P<0.0001$). Fructose intake was positively associated with the fat mass (g) in both NP and Dams (main effect of diet, $P<0.0001$).




20
15
10
5
0
Total fat mass (% of body weight)
NP-CONT (n=6)
NP-FR (n=5)
Dam-CONT (n=4)
Dam-FR (n=6)


80
75
70
65
Total lean mass (% of body weight)
NP-CONT (n=6)
NP-FR (n=5)
Dam-CONT (n=4)
Dam-FR (n=6)

	P-value		
	Proportion fat mass (% body weight)	**Proportion lean mass (% body weight)**	**Body weight (g)**
Pregnancy (NP vs. Dam)	0.0551	0.1403	**<0.0001**
Diet (CONT vs. FR)	0.3096	0.4354	0.2897
Pregnancy x Diet	0.5742	0.4604	0.0594

	NP-CONT (n=6)	**NP-FR (n=5)**	**Dam-CONT (n=4)**	**Dam-FR (n=6)**
Total fat mass (g)	25.8 ± 5.8	26.1 ± 4.1	44.3 ± 3.1	63.9 ± 4.2
Total lean mass (g)	225.5 ± 14.4	215.0 ± 7.8	298.4 ± 11.2	301.3 ± 7.9

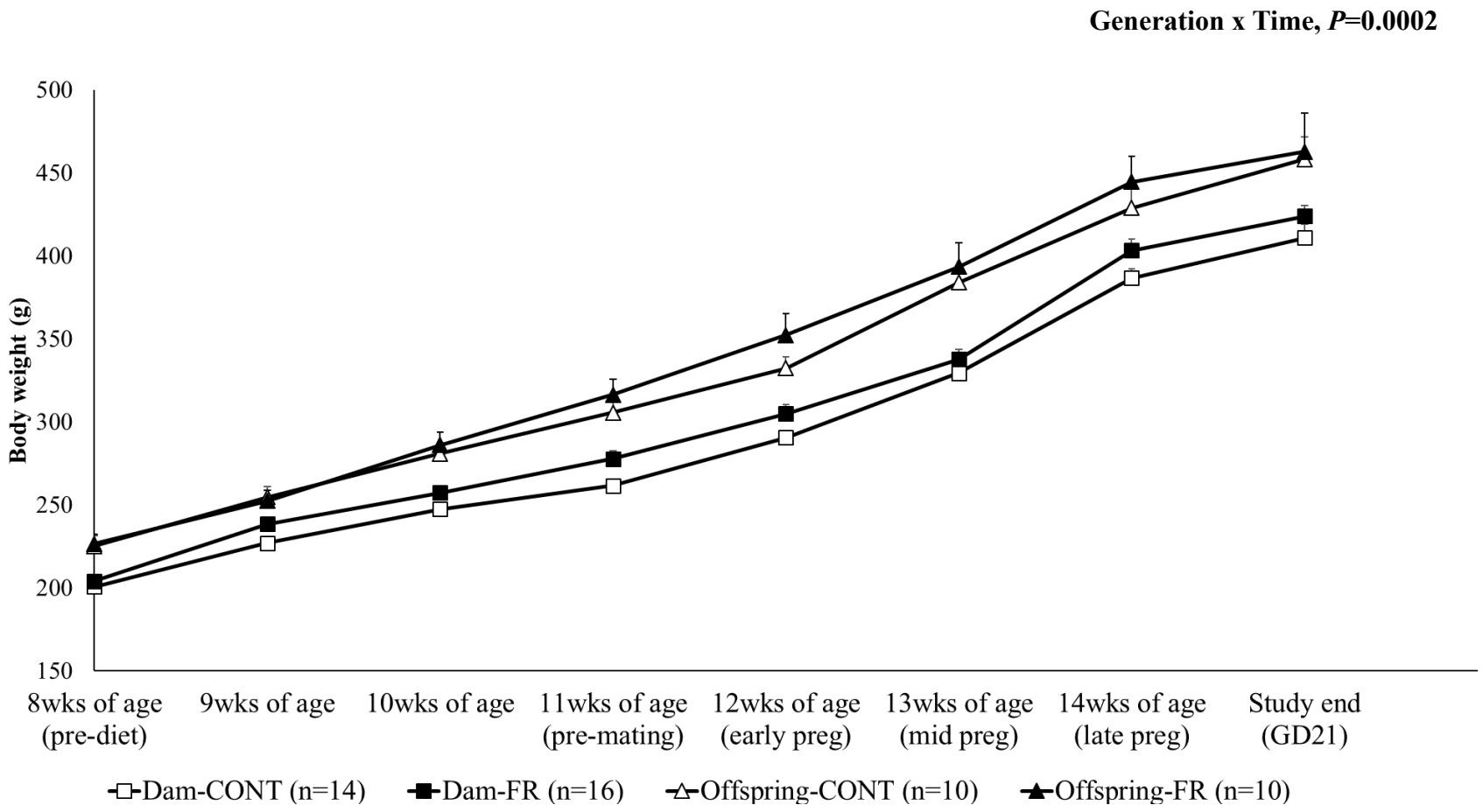
	P-value	
	Fat mass (g)	**Lean mass (g)**
Pregnancy (NP vs. Dam)	0.0904	**<0.0001**
Diet (CONT vs. FR)	**<0.0001**	0.7375
Pregnancy x Diet	0.1573	0.5445

Figure 4-3: Body weight and fat and lean mass (expressed relative to body weight and in absolute terms) at GD21 in NPs and Dams fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age 14 weeks of age. Each value is the mean ± S.E.M.

Study B. The effects of high fructose intake on metabolic and physiologic characteristics of rat dams and placentae across two generations

Study B. Body weights of Dams and Offspring

Body weights of Dams and Offspring differed across the study (main effect of time, $P<0.0001$; Figure 4-4). Starting at 8 weeks of age, Offspring weighed 23 g more than Dams ($P<0.0001$), and this continued throughout the study (main effect of generation, $P=0.0063$). Offspring were 21 g, 31 g, 41 g, 45 g, 55 g, 42 g and 43 g heavier than age-matched Dams at 9 weeks of age ($P<0.0001$), 10 weeks of age ($P<0.0001$), pre-mating ($P<0.0001$), early pregnancy ($P<0.0001$), mid pregnancy ($P<0.0001$), late pregnancy ($P<0.0001$) and GD21 ($P=0.0006$) respectively. Rats in the FR groups weighed significantly more than CONT groups during the study (main effect of diet, $P=0.0003$), particularly during pre-mating ($P=0.0202$) and early pregnancy ($P=0.0063$).



Generation x Time: The interaction between Generation and Time

	***P*-value**
Time	**<0.0001**
Generation (Dams vs. Offspring)	**0.0063**
Diet (CONT vs. FR)	**0.0003**
Generation x Diet	0.5714
Diet x Time	0.3509
Generation x Time	**0.0002**
Generation x Diet x Time	0.9677

Figure 4-4: Body weights of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to GD21 (end of late pregnancy). Each value is the mean ± S.E.M. 8wks of age (pre-diet): The day before eight weeks of age (rats received distilled water with food *ad libitum*). Rats started to receive either distilled water or a 10 % (w/v) fructose solution at 8 weeks of age. 11wks of age (pre-mating): Rats were co-housed with male rats. 12wks of age (early pregnancy): Rats had completed 4 – 7days of pregnancy. 13wks of age (mid pregnancy): Rats had completed 10 – 12days of pregnancy. 14wks of age (late pregnancy): Rats had completed 17 – 19days of pregnancy. Study end: Rats were at GD21 (end of late pregnancy).

Study B. Food and water intake of Dams and Offspring

The intake of food (g) significantly increased over time (main effect of time, $P<0.0001$; Table 4-2) and did not differ between Dams and Offspring, although was significantly higher in rats fed CONT vs. FR (main effect of diet, $P<0.0001$). Rats in CONT groups consumed approximately 2-fold more solid food than rats in the FR groups. The intake of water (g) did not change over the time of the study and it did not differ between Dams and Offspring. However, rats in FR groups consumed 2.8-fold times more fluid (i.e. fructose solution) than rats in CONT groups from pre-mating to mid pregnancy (main effect of diet, $P<0.0001$).

Table 4-2: Daily food intake (g/day) of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 11 weeks of age (pre-mating) to 13 weeks of age (mid-pregnancy)

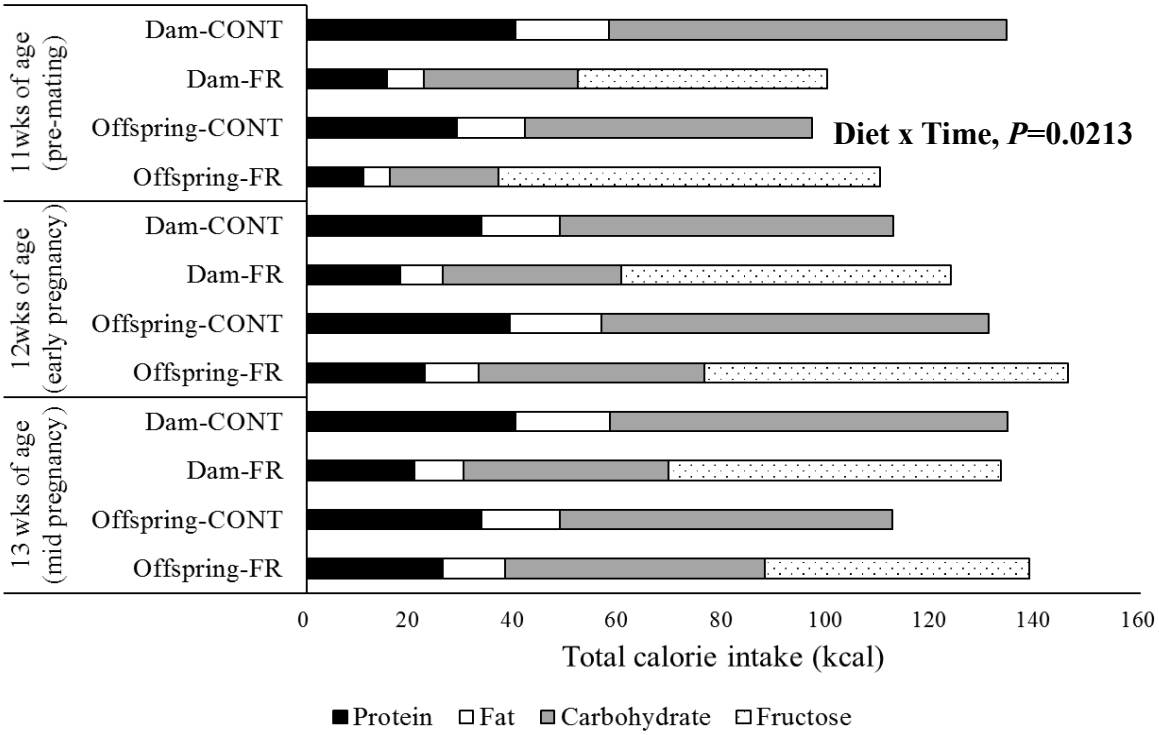
	Dam-CONT (n=14)	Dam-FR (n=16)	Offspring-CONT (n=10)	Offspring-FR (n=10)
Solid food intake (g) [a]				
11wks of age (pre-mating)	32.9 ± 3.3	12.8 ± 1.9	23.7 ± 1.5	9.0 ± 0.0
12wks of age (early pregnancy)	27.6 ± 0.6	14.8 ± 0.7	32.0 ± 1.9	18.7 ± 0.3
13wks of age (mid pregnancy)	32.4 ± 1.8	17.0 ± 1.2	32.2 ± 1.5	21.5 ± 1.7
Fluid intake (g) [a]				
11wks of age (pre-mating)	51.1 ± 4.9	119.4 ± 17.5	46.3 ± 4.5	183.0 ± 1.0
12wks of age (early pregnancy)	47.6 ± 3.1	157.9 ± 14.0	58.5 ± 8.5	174.3 ± 23.9
13wks of age (mid pregnancy)	58.0 ± 5.4	159.3 ± 5.8	68.3 ± 5.8	126.8 ± 20.2

Each value is the mean ± S.E.M. [a] *p*-value from the main effect of diet

		***P*-value**	
		Food intake (g)	**Water intake (g)**
	Time	**<0.0001**	0.1095
	Generation (Dams vs. Offspring)	0.8873	0.4448
P^a	**Diet (FR vs. CONT)**	**<0.0001**	**<0.0001**
	Generation x Diet	0.2239	0.7081
	Diet x Time	**<0.0001**	**<0.0001**
	Generation x Time	**0.0010**	0.4177
	Generation x Diet x Time	0.7565	**0.0323**

The intake of carbohydrate, protein and fat (as % energy intake) was determined according to the information on the nutrient composition of Laboratory Rodent Diet (Purina 5001) (Table 3-1, page 100), and the % calorie intake from fructose was calculated for rats fed the 10 % (w/v) fructose solution. Since rats in CONT groups did not consume energy from the liquid and consumed a significantly higher amount of solid food than rats in FR groups, intake of carbohydrate was lower and intake of protein and fat was significantly higher in rats fed CONT than rats fed FR (main effect of diet, $P<0.0001$). Total energy intake was not different between FR and CONT groups since rats in FR groups received additional energy from the fructose solution (Fig 4-5).

The intake of food and water is not available at the rest of the time points (pre-diet and late pregnancy) due to difficulties in obtaining accurate data for food and fluid intake (i.e. leaky bottles containing water or fructose solution).



Diet x Time: The interaction between Diet and Time

	P-value			
	Protein	**Fat**	**Carbohydrate**	**Total energy intake**
Time	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Generation (Dams vs. Offspring)	0.9756	0.9637	0.5166	0.6569
Diet (FR vs. CONT)	**<0.0001**	**<0.0001**	**<0.0001**	0.3809
Generation x Diet	0.1909	0.1897	0.1703	0.1342
Diet x Time	**<0.0001**	**<0.0001**	0.0818	**0.0338**
Generation x Time	**0.0013**	**0.0012**	0.2191	0.0774
Generation x Diet x Time	0.5068	0.5051	0.2454	0.2790

Figure 4-5: Calorie intake from protein, fat, carbohydrate and fructose of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 11weeks of age (pre-mating) to 13 weeks of age (mid pregnancy). Protein, fat, carbohydrate as % energy intake.

Study B. Metabolic profiles of Dams and Offspring

Overall, plasma glucose concentrations changed over the time of the study (main effect of time, $P<0.0001$; Table 4-3). Glucose concentrations did not differ between Dams and Offspring, but were elevated in the FR groups relative to the CONT groups throughout pregnancy (main effect of diet, $P<0.0001$). Plasma glucose concentrations were not different among groups before FR treatment began, but were significantly increased in the rats given FR at pre-mating ($P=0.0003$), early pregnancy ($P=0.0005$) and late pregnancy ($P=0.0133$) time points.

Plasma insulin concentrations also changed over time (main effect of time, $P<0.0001$) and were different between generations (main effect of generation, $P=0.0004$) and diets (main effect of diet, $P<0.0001$; Table 4-3). Post hoc analyses indicated that FR groups had significantly higher insulin concentrations during early ($P=0.0015$) and mid pregnancy compared to CONT ($P=0.0015$).

TG concentrations differed across time and by diet and generation (time x diet x generation interaction, $P=0.0178$; Table 4-3). The changes over time were expected since TG concentrations increase with pregnancy, and the patterns of change differed between the two generations and between the two diet groups. Plasma TG rose sharply in pregnancy in Dams-FR, but was even more elevated in Offspring-FR while TG increased with pregnancy in Dams-CONT and to a similar and lower degree in Offspring-FR.

Plasma protein concentrations were different between Dams and Offspring during the course of the study (generation x time interaction, $P<0.0001$; Table 4-3) since it decreased significantly during pregnancy in the Dams, but remained high in the Offspring, particularly during mid pregnancy ($P=0.0381$). Plasma protein concentrations were also significantly higher in the FR

compared to the CONT groups (P=0.0056).

Table 4-3: Plasma glucose, insulin, triglyceride and protein concentrations measured regularly in Dams and Offspring fed distilled water (CONT) or the fructose solution (FR)

	Dam-CONT (n=14)	Dam-FR (n=16)	Offspring-CONT (n=10)	Offspring-FR (n=10)
Glucose (mmol/L) [b]				
8wks of age (pre-diet)	7.8 ± 0.2	8.0 ± 0.3	7.4 ± 0.2	7.2 ± 0.2
11wks of age (pre-mating)	8.1 ± 0.2	8.4 ± 0.2	7.1 ± 0.1	8.5 ± 0.2
12wks of age (early pregnancy)	7.5 ± 0.3	8.4 ± 0.3	6.8 ± 0.2	7.7 ± 0.2
13wks of age (mid pregnancy)	6.5 ± 0.3	6.9 ± 0.3	5.5 ± 0.2	6.6 ± 0.3
Insulin (ng/mL) [a, b]				
8wks of age (pre-diet)	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	1.1 ± 0.1
11wks of age (pre-mating)	0.9 ± 0.2	1.0 ± 0.2	1.5 ± 0.2	2.6 ± 0.4
12wks of age (early pregnancy)	0.6 ± 0.2	1.7 ± 0.5	1.0 ± 0.2	2.8 ± 0.5
13wks of age (mid pregnancy)	0.6 ± 0.1	2.1 ± 0.5	1.0 ± 0.2	2.8 ± 0.6
Triglyceride (mmol/L) [b, c]				
8wks of age (pre-diet)	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
11wks of age (pre-mating)	1.1 ± 0.1	1.6 ± 0.2	1.0 ± 0.1	3.5 ± 0.4
12wks of age (early pregnancy)	1.0 ± 0.1	2.0 ± 0.2	0.9 ± 0.1	2.8 ± 0.3
13wks of age	2.2 ± 0.3	3.6 ± 0.9	2.4 ± 0.3	4.3 ± 0.5

	Dam-CONT (n=14)	Dam-FR (n=16)	Offspring-CONT (n=10)	Offspring-FR (n=10)
(mid pregnancy)				
Protein (g/dL) [b]				
8wks of age (pre-diet)	7.0 ± 0.1	7.3 ± 0.5	7.0 ± 0.2	6.9 ± 0.2
11wks of age (pre-mating)	7.7 ± 0.4	8.0 ± 0.7	7.5 ± 0.2	8.4 ± 0.3
12wks of age (early pregnancy)	6.3 ± 0.5	7.5 ± 0.5	7.5 ± 0.1	8.3 ± 0.3
13wks of age (mid pregnancy)	5.0 ± 0.2	6.1 ± 0.3	8.2 ± 0.2	8.3 ± 0.1

Each value is the mean ± S.E.M

[a] *p*-value from the main effect of generation

[b] *p*-value from the main effect of diet

[c] Significant interaction between generation x diet

		***P*-value**			
		Glucose	**Insulin**	**Triglyceride**	**Protein**
	Time	**<0.0001**	**<0.0001**	**<0.0001**	**0.0001**
P^a	**Generation (Dams vs. Offspring)**	0.4233	**0.0004**	0.5706	0.1342
P^b	**Diet (CONT vs. FR)**	**0.0001**	**<0.0001**	**<0.0001**	**0.0056**
P^c	**Generation x Diet**	0.3897	0.3118	**0.0099**	0.3777
	Diet x Time	**0.0107**	**0.0132**	**<0.0001**	0.3050
	Generation x Time	0.7719	0.1758	**0.0046**	**<0.0001**
	Generation x Diet x Time	0.0704	0.8166	**0.0178**	0.3045

During late pregnancy, glucose tolerance of Dams and Offspring was assessed by measuring plasma glucose and insulin concentrations during an OGTT.

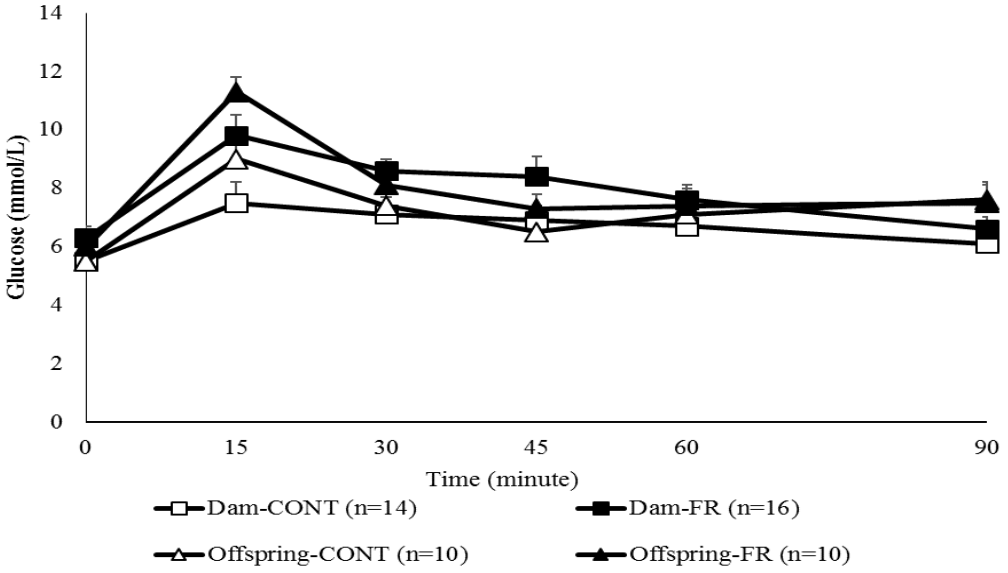
Fasting glucose concentrations did not differ between generations (Dams vs. Offspring) or diets (CONT vs. FR; Figure 4-6). At 15 minutes post glucose bolus, plasma glucose in Offspring was 1.2-fold higher than in Dams (effect of generation, P=0.0128) and 1.3-fold higher in FR rats than CONT rats (effect of diet, P=0.0006). At 30 minutes, rats fed FR had an increase in plasma glucose concentration compared to rats fed CONT (effect of diet, P=0.0432). At the end of the OGTT, Offspring had significantly higher glucose concentrations than Dams (effect of generation, P=0.0208), but no other significant differences were observed in glucose concentration between diet groups during the remainder of the OGTT. The $AUC_{glucose}$ was higher in FR groups than CONT groups (main effect of diet, P=0.0024), but was not different between Dams and Offspring.

Plasma insulin was significantly higher in Offspring than Dams at 15 minutes (P=0.0089), 30 minutes (P<0.0001) and 90 minutes (P=0.0008) post-glucose bolus. Compared to CONT rats, FR rats had ~3.2-fold increased insulin concentrations at fasting (P=0.0204), 15 minutes (P<0.0001), 60 minutes (P=0.0005) and 90 minutes (P=0.0062). There was no significant interaction between diet and generation for the The $AUC_{insulin}$ measurement. $AUC_{insulin}$ was significantly higher in FR groups than CONT groups (main effect of diet, P=0.0132) and in Offspring than Dams (main effect of generation, P=0.0032).

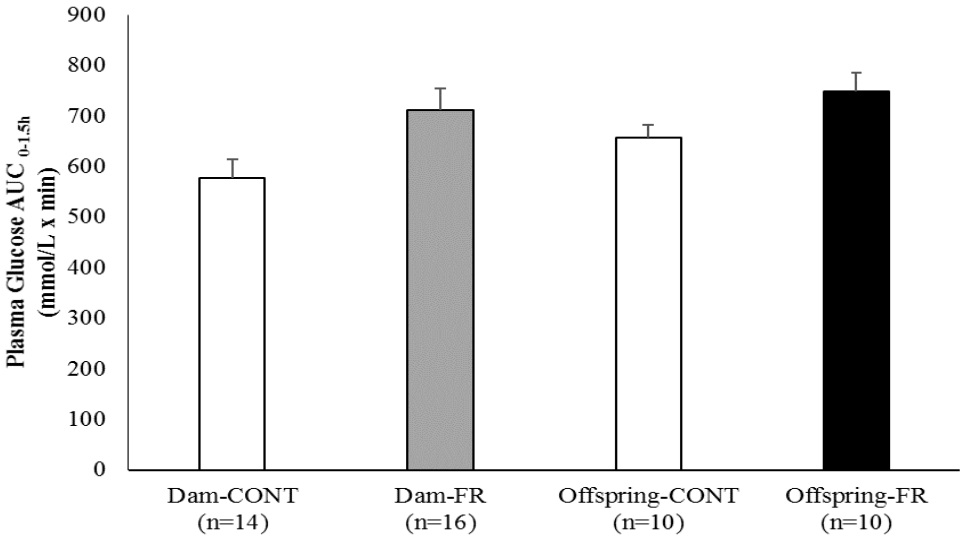
An additional marker of iInsulin resistance was estimated in both Dams and Offspring using fasting glucose and insulin in the HOMA-IR model (Figure 4-6). The HOMA-IR did not differ

between generations (Dam vs. Offspring) or diet groups (CONT vs. FR). There was also no interaction between generation and diet.

(A)

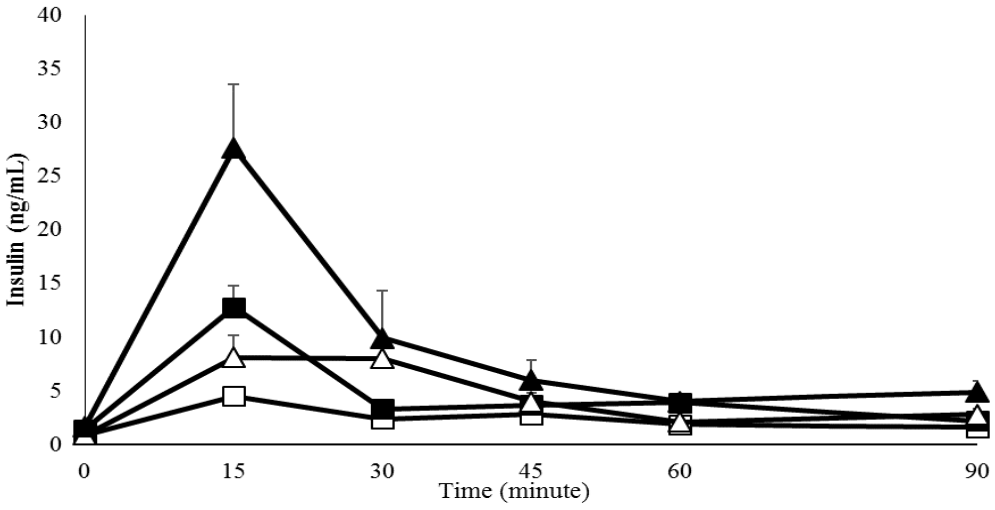


(B)

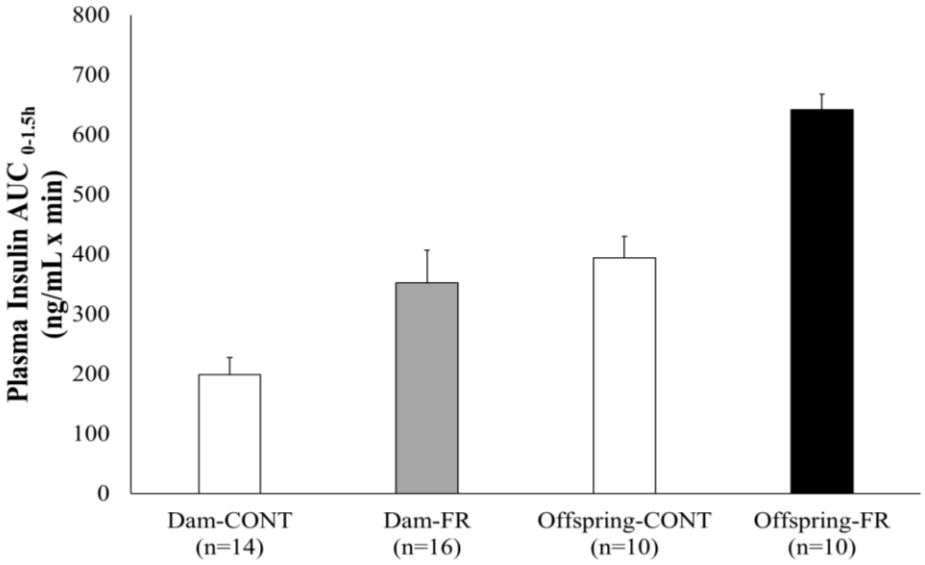


	$AUC_{glucose}$
Generation (Dams vs. Offspring)	0.0664
Diet (CONT vs. FR)	**0.0024**
Generation x Diet	0.4094

(C)



(D)



	$AUC_{Insulin}$
Generation (Dams vs. Offspring)	**0.0032**
Diet (CONT vs. FR)	**0.0132**
Generation x Diet	0.8488

	Dam-CONT (n=14)	Dam-FR (n=16)	Offspring-CONT (n=10)	Offspring-FR (n=10)
HOMA-IR	15.8 ± 5.2	10.9 ± 2.0	7.5 ± 2.6	14.7 ± 5.2

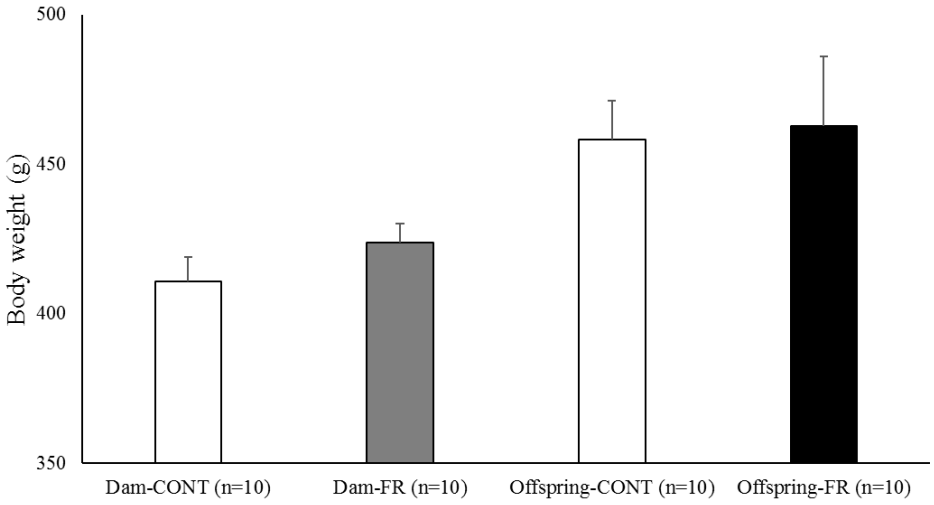
	HOMA-IR
Generation (Dams vs. Offspring)	0.8329
Diet (CONT vs. FR)	0.6609
Generation x Diet	0.3730

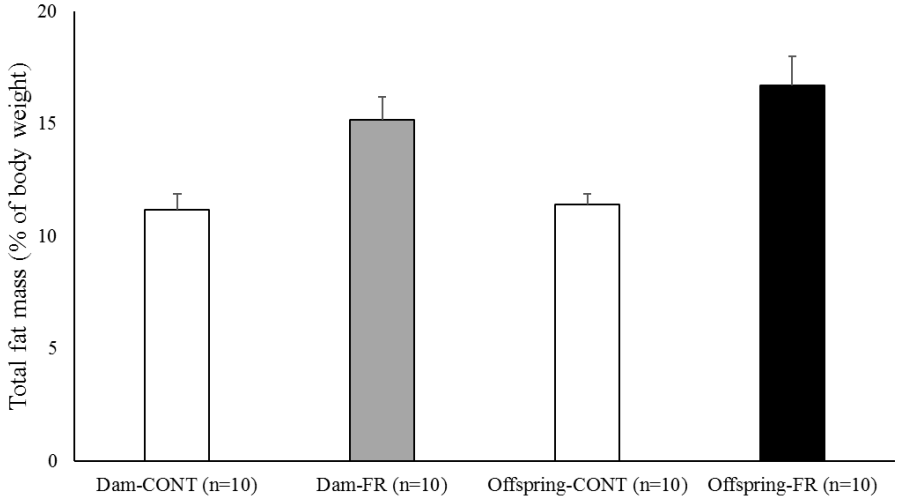
Figure 4-6: Plasma glucose and insulin responses during OGTT and HOMA-IR on GD19 in Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). (A): Plasma glucose concentrations of Dams and Offspring during the OGTT, (B): $AUC_{Glucose}$ during the OGTT of Dams and Offspring, (C): Plasma insulin concentrations of Dams and Offspring during the OGTT, (D): $AUC_{Insulin}$ during the OGTT of Dams and Offspring.

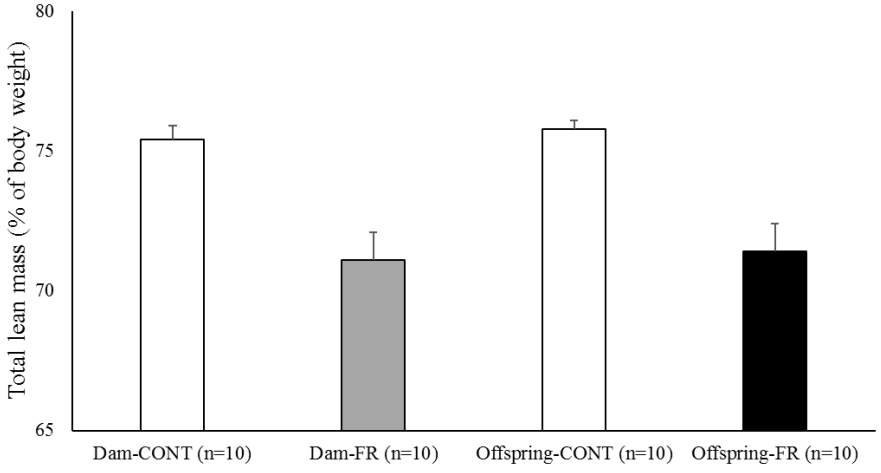
Study B. Body composition, body weight and the weights of organs in Dams and Offspring

Body composition (proportion of fat and lean mass) of Dams and Offspring was measured at GD21. Fat mass (%, relative to body weight) was 1.4-fold higher and lean mass (%, relative to body weight) was 0.9-fold lower in the FR groups than in the CONT groups (both fat and lean mass: main effect of diet, $P<0.0001$; Figure 4-7).

The absolute fat mass (g) was higher in Dam-FR than in rats in the CONT groups, but was even heavier in Offspring-FR (significant generation x diet interaction, $P<0.0001$; Figure 4-7).


500
450
400
350
Body weight (g)
Dam-CONT (n=10)
Dam-FR (n=10)
Offspring-CONT (n=10)
Offspring-FR (n=10)


20
15
10
5
0
Total fat mass (% of body weight)
Dam-CONT (n=10)
Dam-FR (n=10)
Offspring-CONT (n=10)
Offspring-FR (n=10)


80
75
70
65
Total lean mass (% of body weight)
Dam-CONT (n=10)
Dam-FR (n=10)
Offspring-CONT (n=10)
Offspring-FR (n=10)

	P-value		
	Proportion fat mass (% body weight)	**Proportion lean mass (% body weight)**	**Body weight (g)**
Generation (Dams vs. Offspring)	0.3310	0.8562	**0.0006**
Diet (CONT vs. FR)	**<0.0001**	**<0.0001**	0.4515
Generation x Diet	0.4805	0.9749	0.7129

	Dam-CONT (n=10)	**Dam-FR (n=10)**	**Offspring-CONT (n=10)**	**Offspring-FR (n=10)**
Total fat mass (g)	44.3 ± 3.1	63.9 ± 4.2	52.6 ± 3.4	75.3 ± 9.3
Total lean mass (g)	298.4 ± 11.2	301.3 ± 7.9	348.1 ± 11.1	332.3 ± 15.0

	P-value	
	Fat mass (g)	**Lean mass (g)**
Generation (Dams vs. Offspring)	0.9948	**0.0011**
Diet (CONT vs. FR)	**<0.0001**	0.5694
Generation x Diet	**<0.0001**	0.4107

Figure 4-7: Proportions of fat and lean tissue (expressed relative to body weight and in absolute terms) at GD21 in Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M.

On GD21, the weights of the pancreas and the liver relative to body weight were not different among groups (Table 4-4). FR consumption significantly increased the relative weights of intrascapular brown fat and uterine fat pads (uterine: main effect of diet, P=0.0001, and brown fat: main effect of diet, P=0.0015). The relative weight of retroperitoneal fat pads was affected by both generation (P<0.0001) and diet (P<0.0001). The weight of these fat pads was highest in offspring fed FR compared to the all other groups.

Table 4-4: Organ weights relative to final body weight at GD21 in Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age to 14 weeks of age

	Dam-CONT (n=10)	Dam-FR (n=10)	Offspring-CONT (n=10)	Offspring-FR (n=10)
Pancreas to body weight ratio (%)	0.27 ± 0.02	0.30 ± 0.02	0.28 ± 0.01	0.25 ± 0.03
Liver to body weight ratio (%)	4.47 ± 0.12	4.57 ± 0.14	4.61 ± 0.14	4.88 ± 0.17
Uterine fat to body weight ratio (%) [b]	0.66 ± 0.04	1.03 ± 0.16	1.44 ± 0.09	2.39 ± 0.17
Retroperitoneal fat to body weight ratio (%) [a, b, c]	0.48 ± 0.04	0.82 ± 0.14	1.09 ± 0.09	2.28 ± 0.20
Brown fat to body weight ratio (%) [b]	0.10 ± 0.00	0.18 ± 0.03	0.13 ± 0.02	0.23 ± 0.03

[a] Each value is the mean ± S.E.M; *p*-value from the main effect of generation

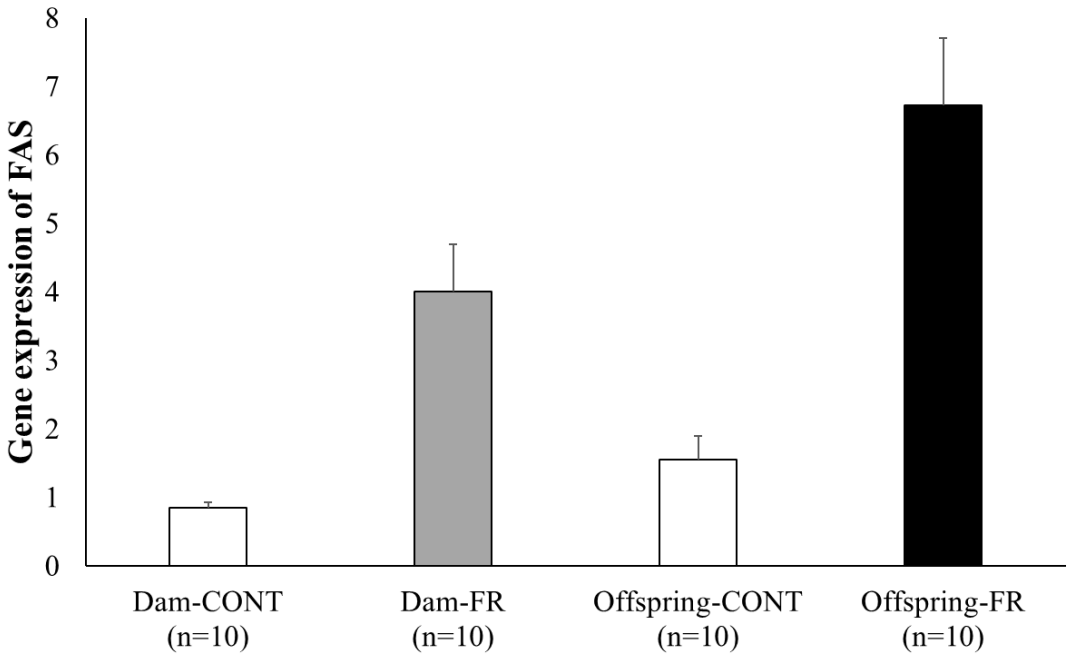
[b] *p*-value from the main effect of diet

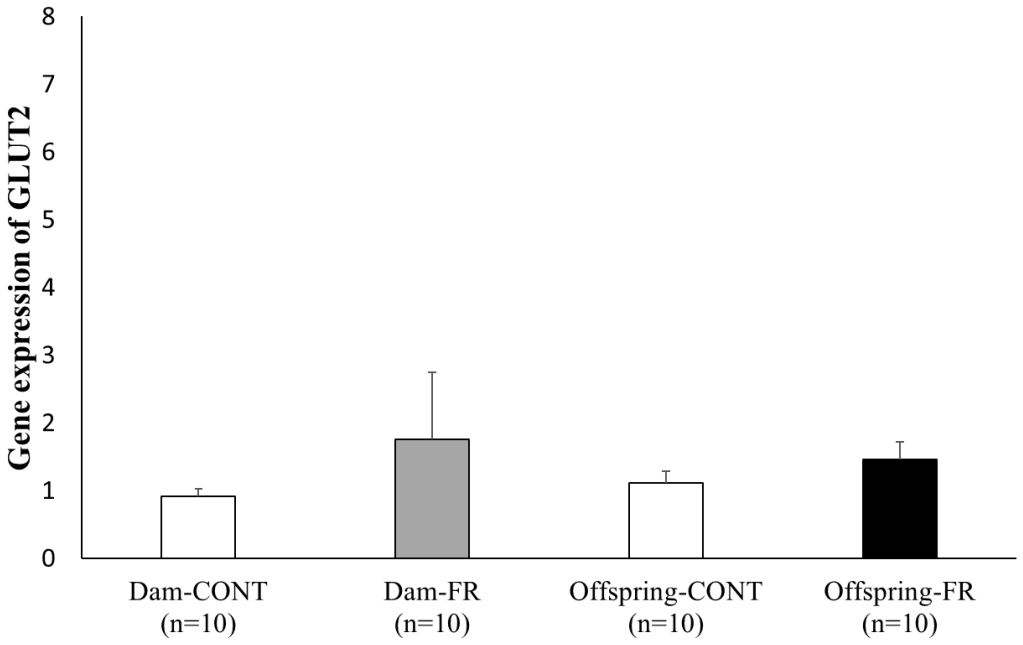
[c] Significant interaction between generation and diet

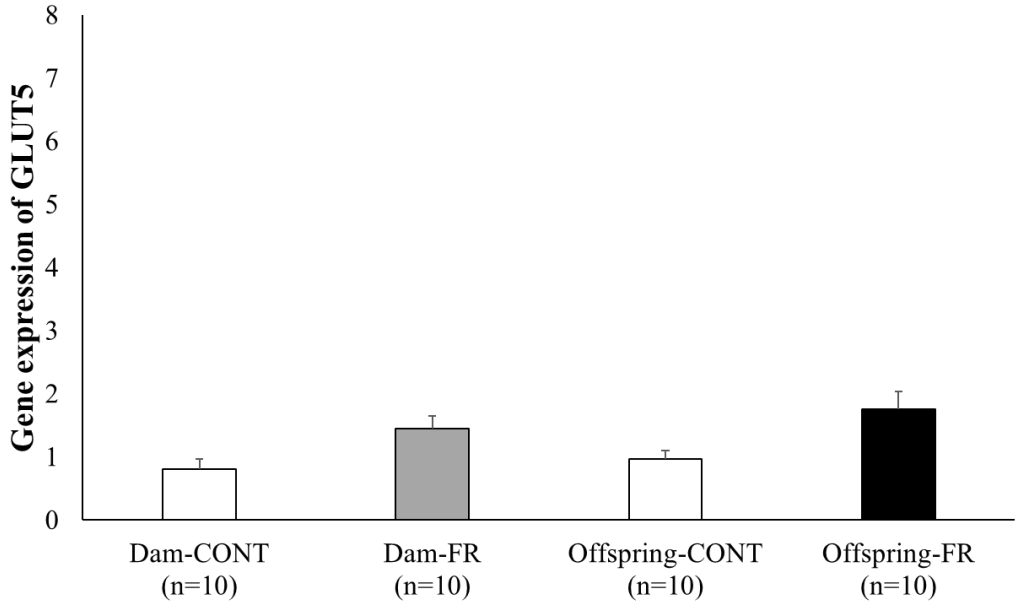
		***P*-value**				
		Pancreas	**Liver**	**Uterine fat**	**Retroperitoneal fat**	**Brown fat**
***P*[a]**	**Generation (Dams vs. Offspring)**	0.3762	0.1529	0.1217	**<0.0001**	0.1367
***P*[b]**	**Diet (CONT vs. FR)**	0.8986	0.2366	**0.0001**	**<0.0001**	**0.0015**
***P*[c]**	**Generation x Diet**	0.1690	0.6054	0.2218	**0.0079**	0.8654

Study B. Hepatic gene expression and oxidative stress in Dams and Offspring

Firstly, the mRNA expression of FAS, GLUT2 and GLUT5 was measured in the liver of Dams and Offspring (Figure 4-8). mRNA expression of FAS was 1.7-fold higher in Offspring vs. Dams (main effect of generation, P=0.0042) and 4.5-fold higher in FR groups vs. CONT groups (main effect of diet, P<0.0001). The expression of GLUT2 was not different between generations (Dams vs. Offspring) or diets (CONT vs. FR). The mRNA expression of GLUT5 did not differ between Dams and Offspring, but it was significantly increased in the liver of rats fed FR compared to that of rats fed CONT (main effect of diet, P=0.0061).







	***P*-value**		
	FAS	**GLUT2**	**GLUT5**
Generation (Dams vs. Offspring)	**0.0042**	0.9420	0.3049
Diet (CONT vs. FR)	**<0.0001**	0.0935	**0.0061**
Generation x Diet	0.4649	0.4513	0.7367

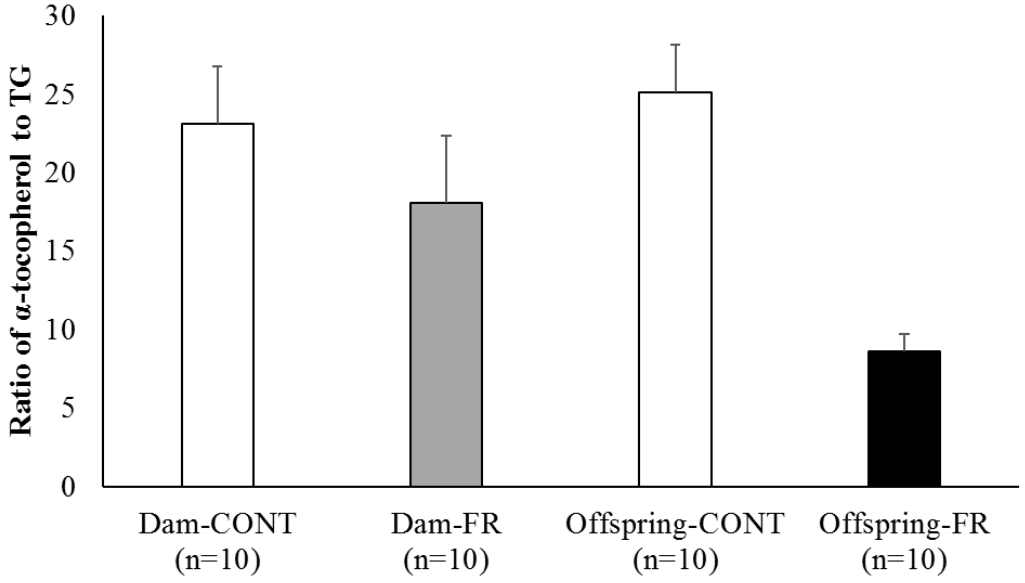
Figure 4-8: Relative expression of FAS, GLUT2 and GLUT5 at GD21 in the liver of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M. Relative expression vs. Dam-CONT. All values are adjusted to the reference genes GAPDH and RPLP0.

The degree of oxidative stress in the liver was determined by measuring vitamin E concentrations (α-tocopherol acetate, α, β, γ and δ-tocopherols) and lipid peroxidation (TBARS) in this tissue.

Concentrations of α-tocopherol were measured as an indicator of hepatic antioxidant status. Since α-tocopherol is a lipid-soluble vitamin and more may be required to control oxidative stress in situations when TG concentrations are high (69, 70), hepatic TG content was also measured and the ratio between α-tocopherol concentrations and hepatic TG content was calculated. Table 4-5 indicates the concentrations of TG and α-tocopherol from animals in this study. Hepatic concentrations of TG and α-tocopherol were not different between generations (Dams vs. Offspring), but TG contents were increased and α-tocopherol was decreased in the FR compared to the CONT group (main effect of diet, TG: P=0.0455; main effect of diet, α-tocopherol: P=0.0001). Thus, the ratio between α-tocopherol and TG in the liver was 0.5-fold lower in FR groups than CONT groups (main effect of diet, P=0.0062). α-tocopherol acetate, β, γ and δ-tocopherols were all below the level of detection in the liver using this assay.

Table 4-5: The concentration of TG (mmol/L) and α-tocopherol (nmol/g) and ratio of α-tocopherol to TG in the liver of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy)

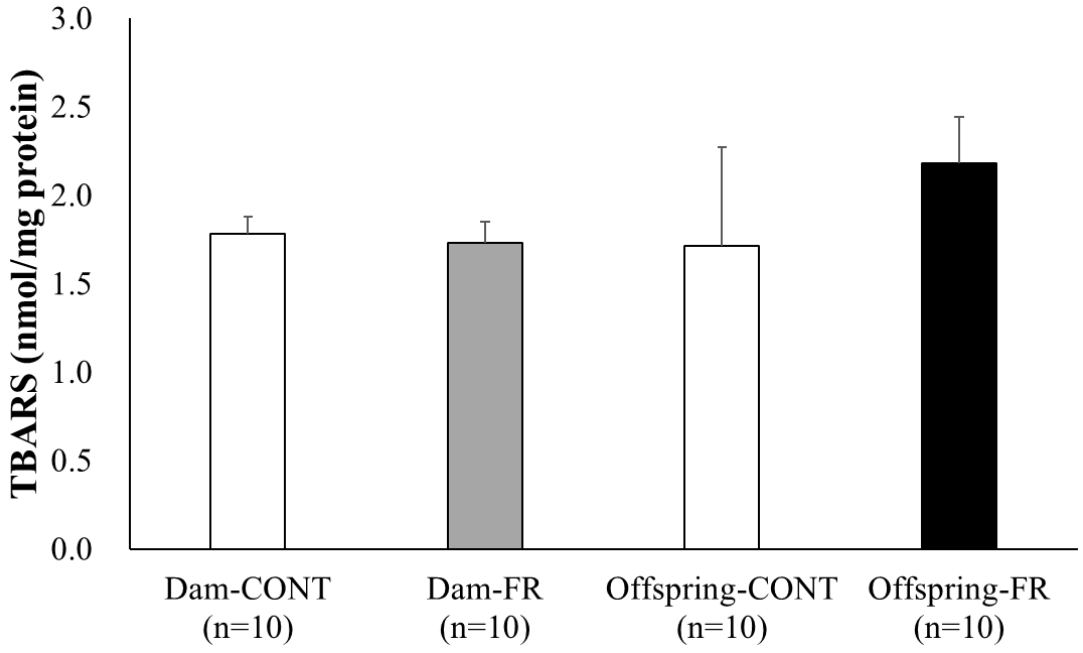
	Dam-CONT (n=10)	Dam-FR (n=10)	Offspring-CONT (n=10)	Offspring-FR (n=10)
TG (mmol/L)	1.48 ± 0.13	1.59 ± 0.20	1.30 ± 0.15	1.90 ± 0.20
α-tocopherol (nmol/g)	30.40 ± 2.51	24.07 ± 2.55	29.81 ± 2.58	15.41 ± 0.74



	P-value		
	TG	**α-tocopherol**	**Ratio of α-tocopherol and TG**
Generation (Dams vs. Offspring)	0.6947	0.4567	0.6948
Diet (FR vs. CONT)	**0.0455**	**0.0001**	**0.0062**
Generation x Diet	0.1547	0.0988	0.1192

Each value is the mean ± S.E.M.

The level of lipid peroxidation in the liver was assessed by the TBARS assay. The concentration of TBARS in the liver did not differ between generations (Dams vs. Offspring) or diets (CONT vs. FR) (Figure 4-9). There was also no interaction between generation and diet on the level of TBARS in the liver of Dams and Offspring.



	***P*-value**
Generation (Dams vs. Offspring)	0.1942
Diet (CONT vs. FR)	0.2178
Generation x Diet	0.0892

Figure 4-9: The concentration of TBARS (nmol/mg protein) at GD21 in the liver of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy)

Study B. The weights of the fetuses and placentae in Dams and Offspring at GD21

Fetal growth was determined by the measurement of their weights, whereas placental characteristics were examined by measuring their weights, and by determining expression of genes related for nutrient transport, angiogenesis, and the vascular network at GD21.

Table 4-6 indicates the number of pups/litter and the weights of the fetuses and the placentae. The pups were counted and the placentae and the fetuses from each pregnant rat were weighed. The number of pups/litter was not different among groups. The weights of the placentae and fetuses did not differ between Dams and Offspring, but these weights were lower in rats fed FR than rats fed CONT (main effect of diet, placentae, P=0.0002; fetuses, P=0.0004). The placental:fetal weight ratios were not different between generations (Dams vs. Offspring) or diet groups (CONT vs. FR).

Table 4-6: Number of pups, placental weights, fetal weights and the placental:fetal weight ratios at GD21 in Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy)

	Dam-CONT (n=14)	Dam-FR (n=16)	Offspring-CONT (n=10)	Offspring-FR (n=10)
Average number of pups/litter	15.5 ± 1.7	16.0 ± 3.2	16.3 ± 2.3	17.0 ± 3.3
Average placental weight/ litter (g)	0.53 ± 0.02	0.48 ± 0.02	0.57 ± 0.02	0.47 ± 0.01
Average fetal weight /litter (g)	3.88 ± 0.19	3.36 ± 0.14	4.07 ± 0.05	3.57 ± 0.09
Placental:fetal weight ratio	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.00	0.13 ± 0.01

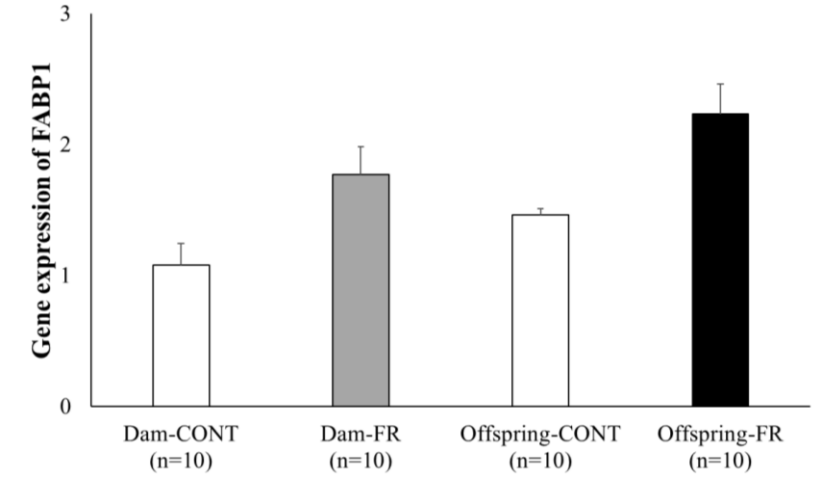
Each value is the mean ± S.E.M.

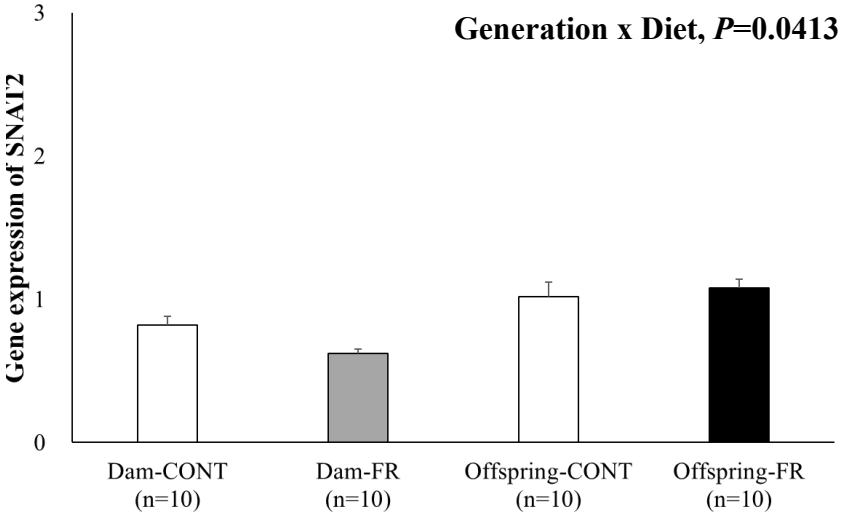
	P-value			
	Number of pups	**Placental weight**	**Fetal weight**	**Placental:fetal weight ratio**
Generation (Dams vs. Offspring)	0.6950	0.8640	0.7160	0.7368
Diet (FR vs. CONT)	0.5689	**0.0002**	**0.0004**	0.9076
Generation x Diet	0.2363	0.1857	0.8787	0.1355

Study B. The expression of nutrient transporters in the placentae of Dams and Offspring

The mRNA expression of FABP1, SNAT2 and the expression of GLUT1 at GD21 were measured to examine the impacts of fructose intake on placental nutrient transporters, which may influence nutrient transfer capacity from the mother to fetus during pregnancy.

The expression of FABP1 was not different between the placentae of Dams and Offspring, but it was 1.6-fold higher in the placentae of rats fed FR than rats fed CONT (main effect of diet, *P*=0.0004; Figure 4-10). The mRNA expression of placental SNAT2 varied by generation and diet group (generation*diet, *P*=0.0413). SNAT 2 was highest in the Offspring-FR group and lowest in the Dam-FR group (Offspring-FR=1.08 ± 0.06, Dam-FR=0.62 ± 0.03).



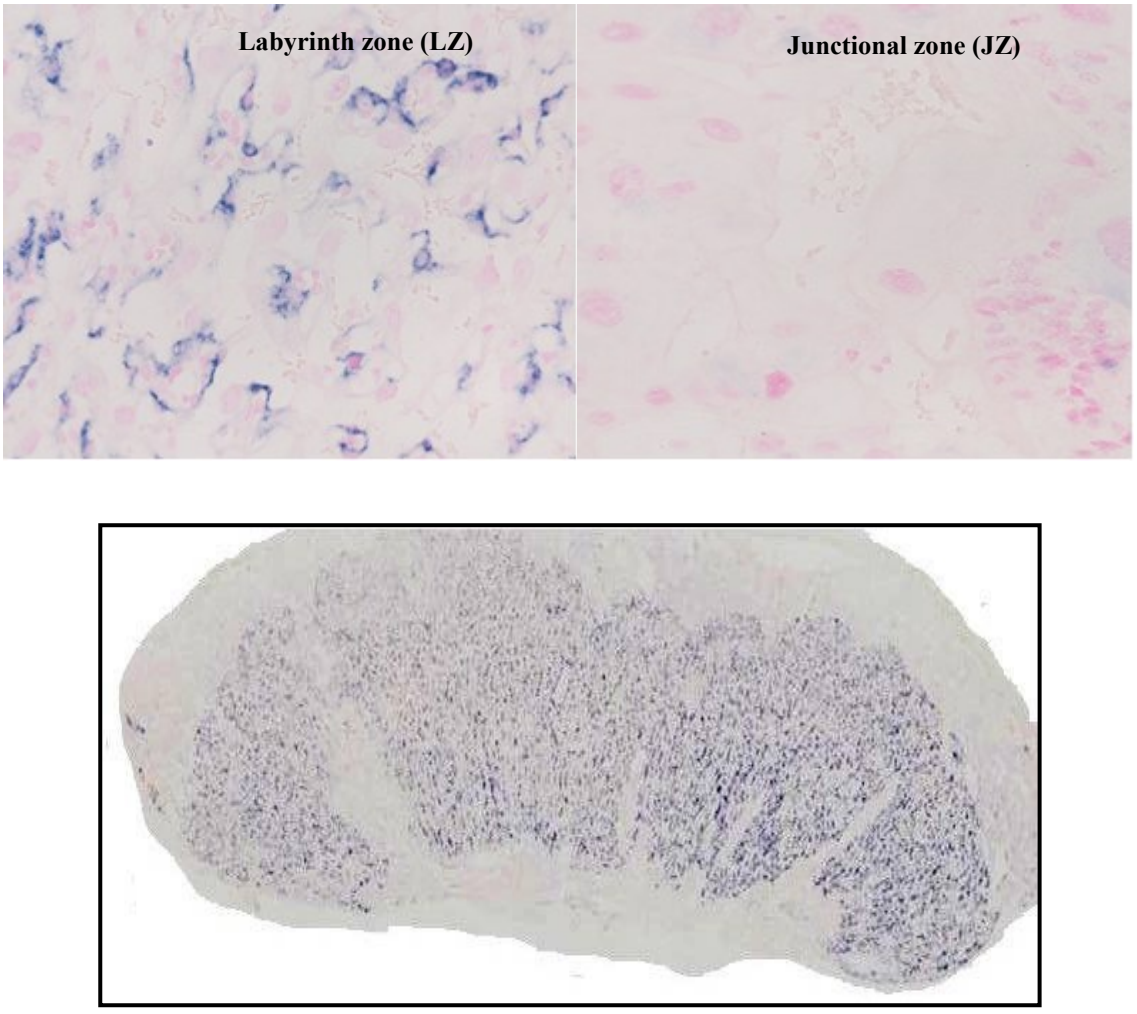


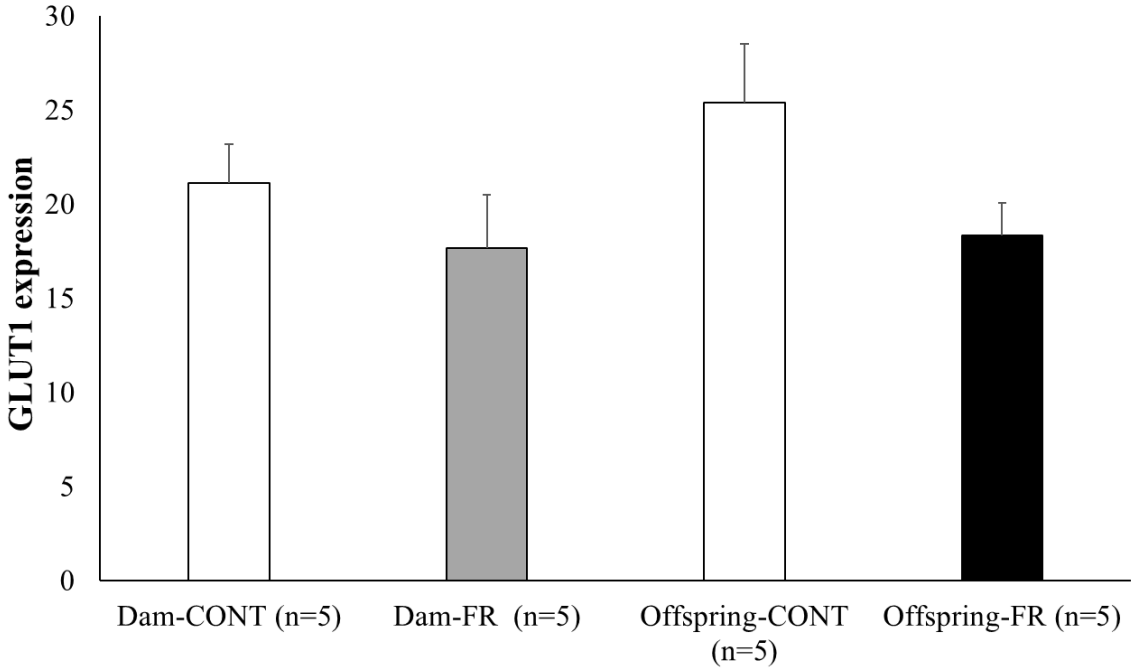
Generation x Diet: interaction between generation and diet

	P-value	
	FABP1	**SNAT2**
Generation (Dams vs. Offspring)	0.3624	**<0.0001**
Diet (CONT vs. FR)	**0.0004**	0.2528
Generation x Diet	0.8201	**0.0413**

Figure 4-10: Relative expression of FABP1 and SNAT2 at GD21 in the placentae of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M. Relative expression vs. Dam-CONT. All expression values are adjusted to the reference genes GAPDH and TBP.

The expression of GLUT1, the most abundant glucose transporter in the placenta during late pregnancy (231), was measured in the placentae at GD21. GLUT1 was primarily expressed in LZ (Figure 4-11). In previous work in our lab, we found that total placental expression of GLUT1 was reduced in rats fed FR vs. CONT (163) and in this study, there were also reductions in GLUT1 expression in the LZ, although the difference was not quite statistically significant (Figure 4-11).





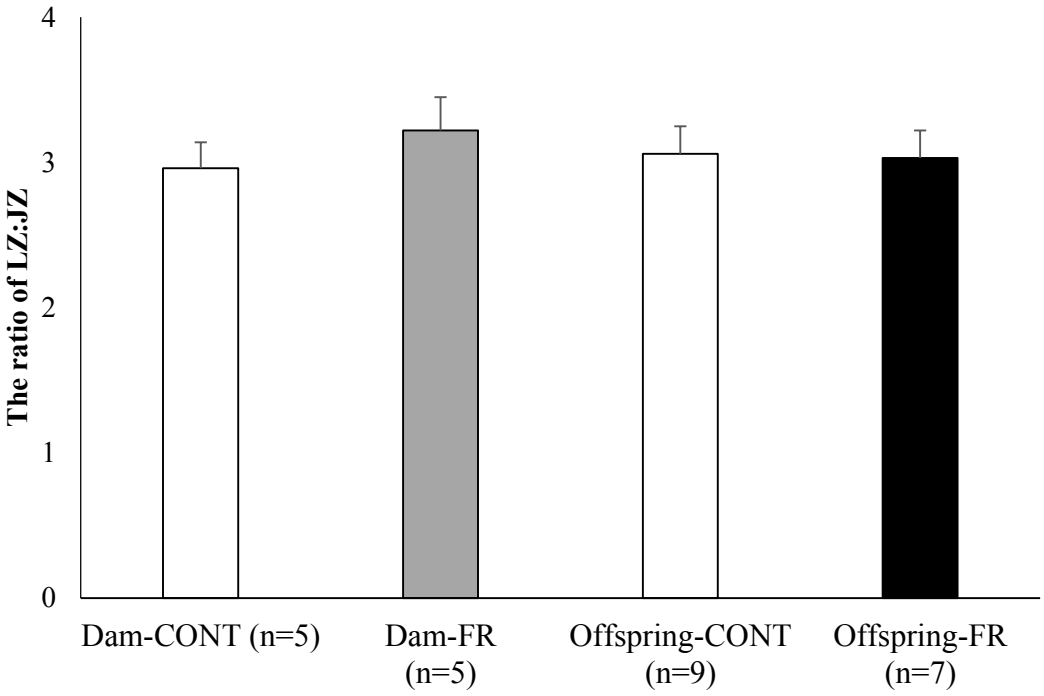
	P-value
Generation (Dams vs. Offspring)	0.4800
Diet (CONT vs. FR)	0.0685
Generation x Diet	0.6340

Figure 4-11: The expression of GLUT1 expression measured using in situ hybridization (ISH) in the placentae collected at GD21 of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M.

Study B. The ratio of the LZ area to the JZ area in the placentae of Dams and Offspring

In addition to measuring the expression of nutrient transporters in the placenta, the ratio of the LZ area to the JZ area in the placentae was used as an indicator of the capacity for nutrient transfer (main function of LZ) relative to endocrine function (main function of JZ) (292). In the placentae stained with H&E, the ratio of LZ area to JZ area was not different between

generations (Dams vs. Offspring) or diets (CONT vs. FR) (Figure 4-12).



	P-value
Generation (Dams vs. Offspring)	0.8186
Diet (CONT vs. FR)	0.6347
Generation x Diet	0.5676

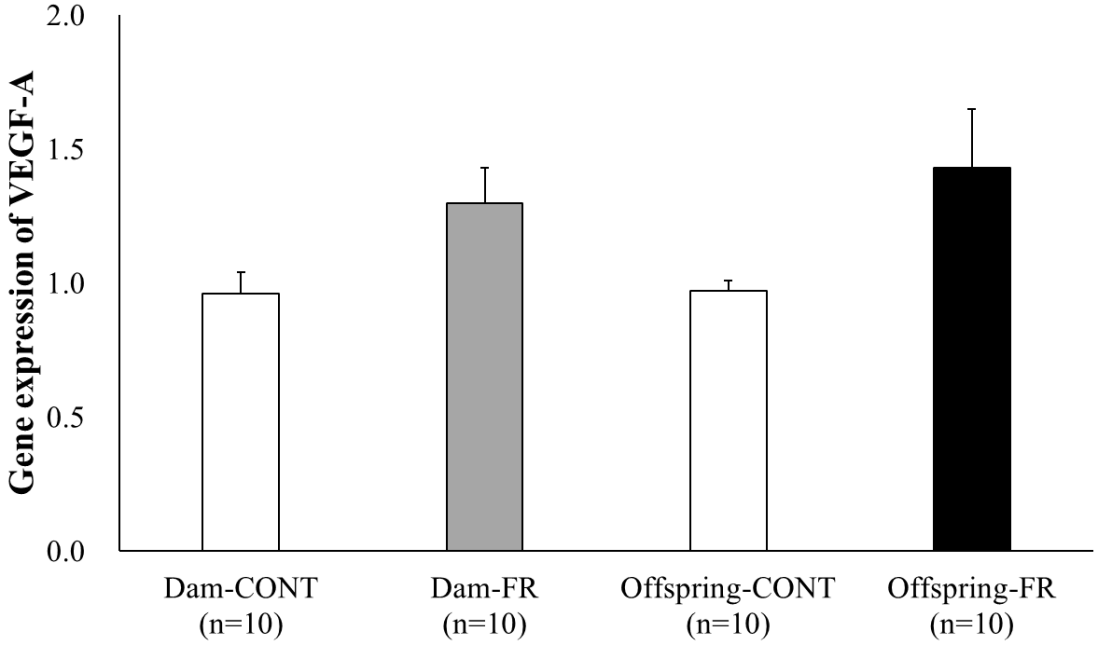
Figure 4-12: The ratio of the LZ area to the JZ area in the placentae collected at GD21 of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M.

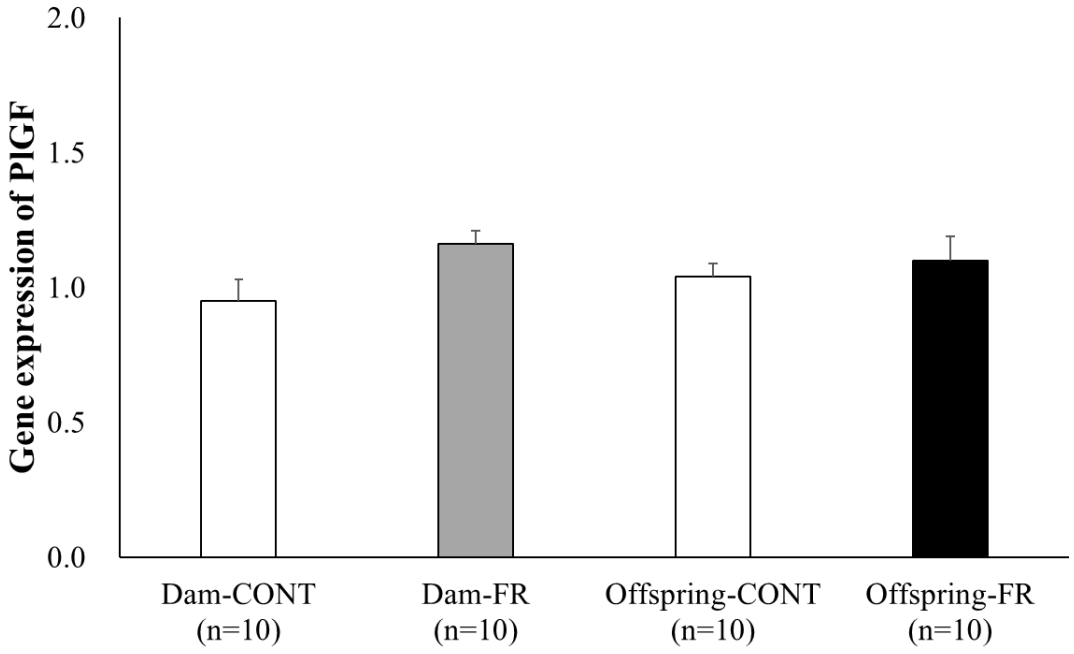
Study B. The expression of angiogenic factors and vascular development in the placentae of Dams and Offspring

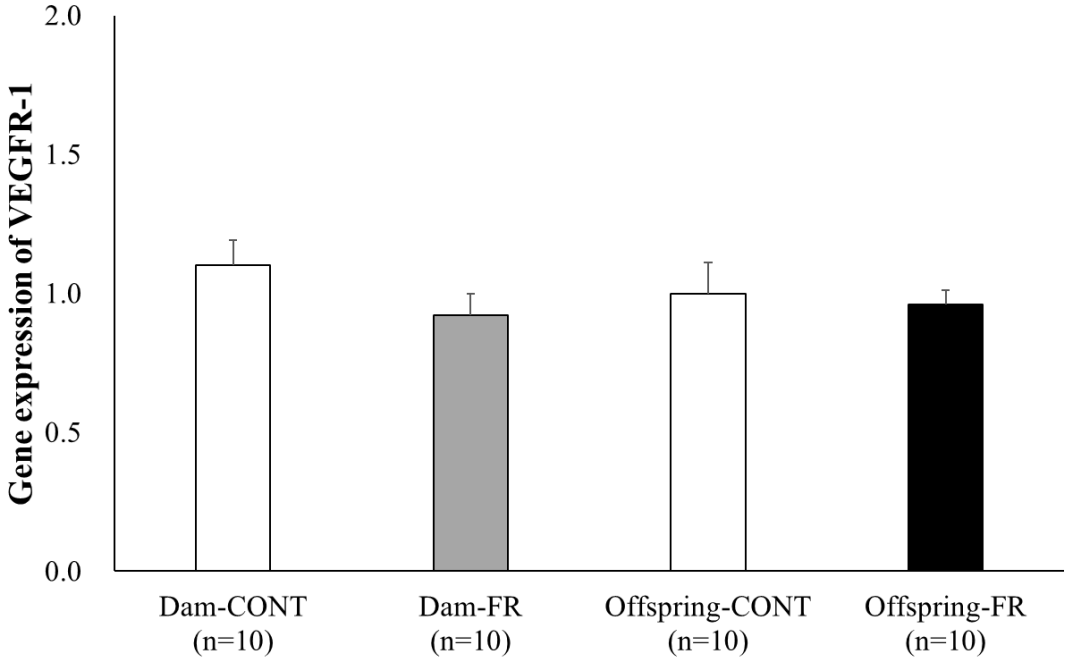
To examine whether placental vascular development could be affected by fructose intake, the expression of angiogenic growth factors (VEGF-A and PlGF), their receptors (VEGFR-1 and

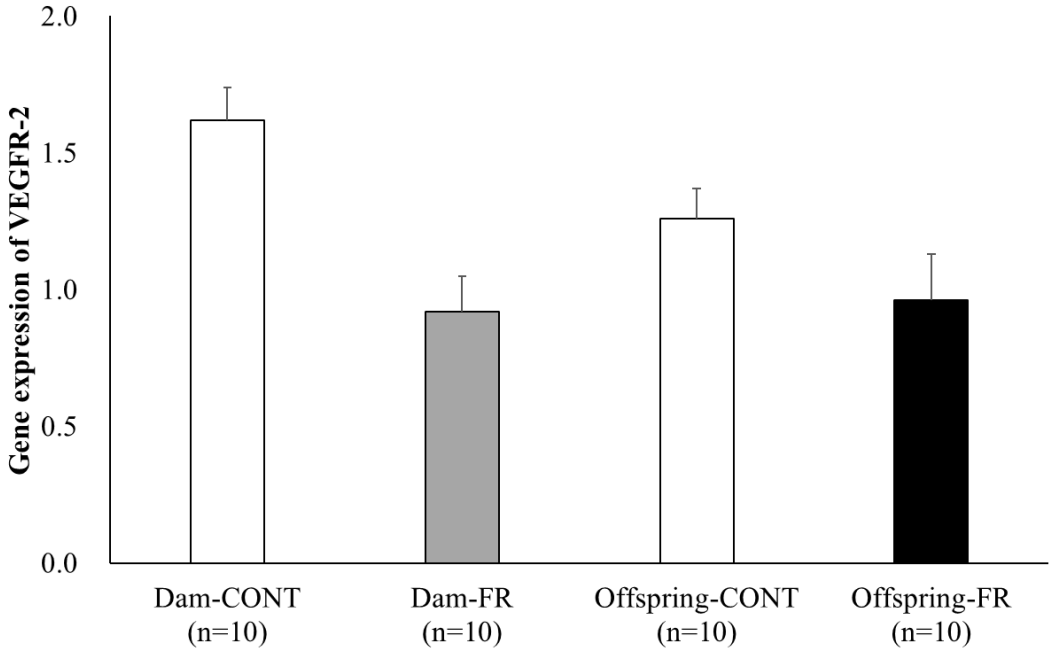
VEGFR-2) and the vascular network (using CD31 staining) were measured in the placentae of Dams and Offspring at GD21.

The mRNA expression of VEGF-A was not different between the placentae of Dams and Offspring, but it was increased by 1.4-fold in the placentae of rats fed FR compared to the placentae of rats fed CONT (main effect of diet, P=0.0015; Figure 4-13). The expression of PlGF and VEGFR-1 was not different between generations or diet groups. However, FR groups had lower expression of VEGFR-2 in the placentae than CONT groups (main effect of diet, P=0.0014).




Gene expression of PlGF
2.0
1.5
1.0
0.5
0.0
Dam-CONT (n=10)
Dam-FR (n=10)
Offspring-CONT (n=10)
Offspring-FR (n=10)

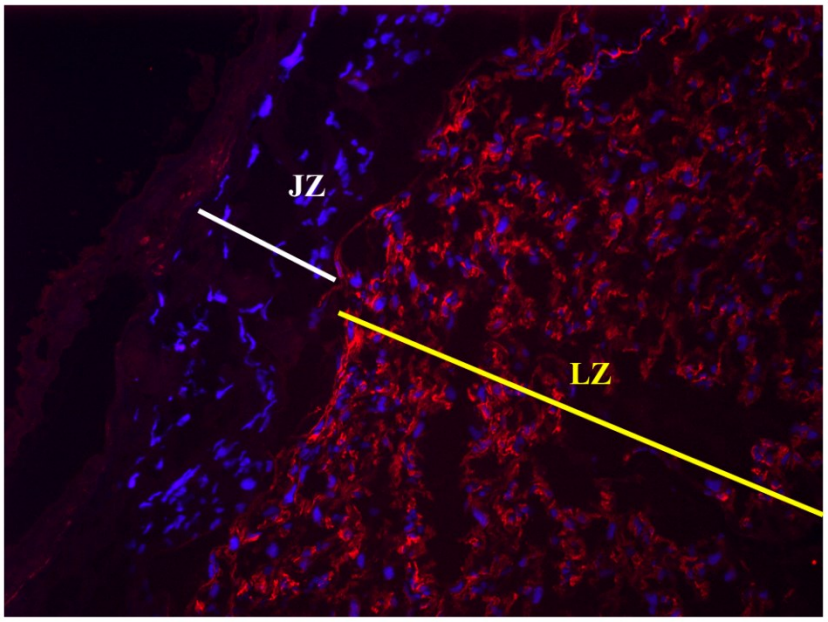

Gene expression of VEGFR-1
2.0
1.5
1.0
0.5
0.0
Dam-CONT (n=10)
Dam-FR (n=10)
Offspring-CONT (n=10)
Offspring-FR (n=10)

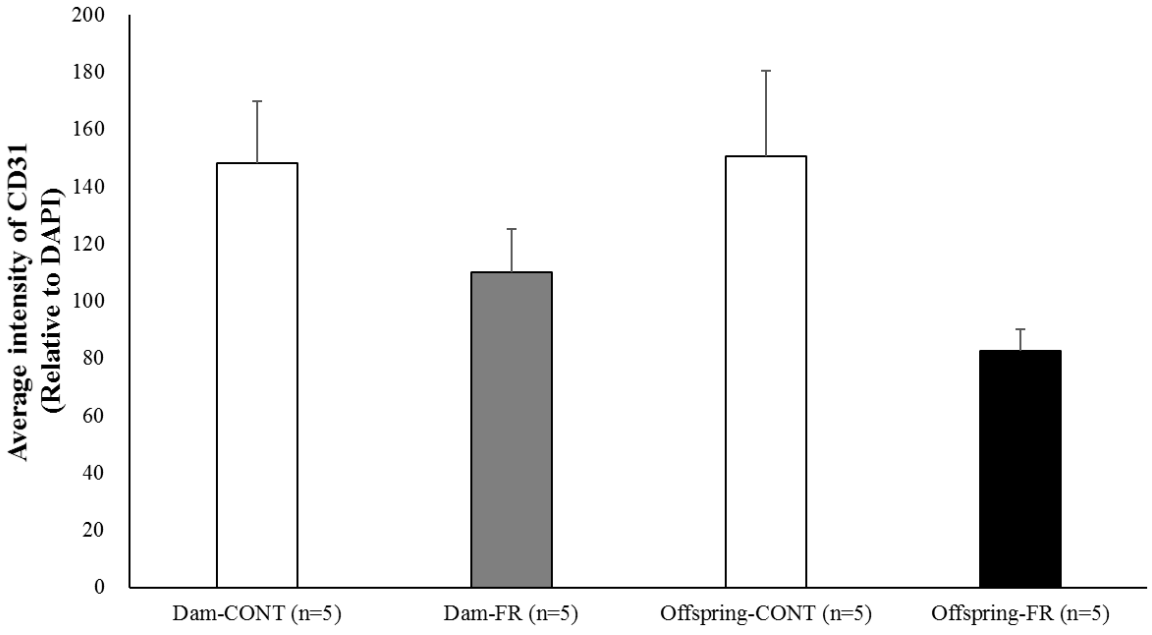


	P-value			
	VEGF-A	**PlGF**	**VEGFR-1**	**VEGFR-2**
Generation (Dams vs. Offspring)	0.7408	0.8445	0.7527	0.2319
Diet (CONT vs. FR)	**0.0015**	0.0607	0.3212	**0.0014**
Generation x Diet	0.6632	0.2834	0.4603	0.1393

Figure 4-13: Relative expression of VEGF-A, PlGF, VEGFR-1 and VEGFR-2 at GD21 in the placentae of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age to 14 weeks of age. Each value is the mean ± S.E.M. Relative expression vs. Dam-CONT. All values are adjusted to the reference genes GAPDH and TBP.

CD31 was used to evaluate vascular development in the placenta. Since there was no positive staining in the JZ, we focused exclusively on the LZ. The Average intensity of CD31 was reduced by 0.6-fold in the placentae of rats fed FR compared with rats fed CONT (main effect of diet, *P*=0.0129; Figure 4-14), but it was not affected by generation.


JZ
LZ


Average intensity of CD31 (Relative to DAPI)
200
180
160
140
120
100
80
60
40
20
0
Dam-CONT (n=5)
Dam-FR (n=5)
Offspring-CONT (n=5)
Offspring-FR (n=5)

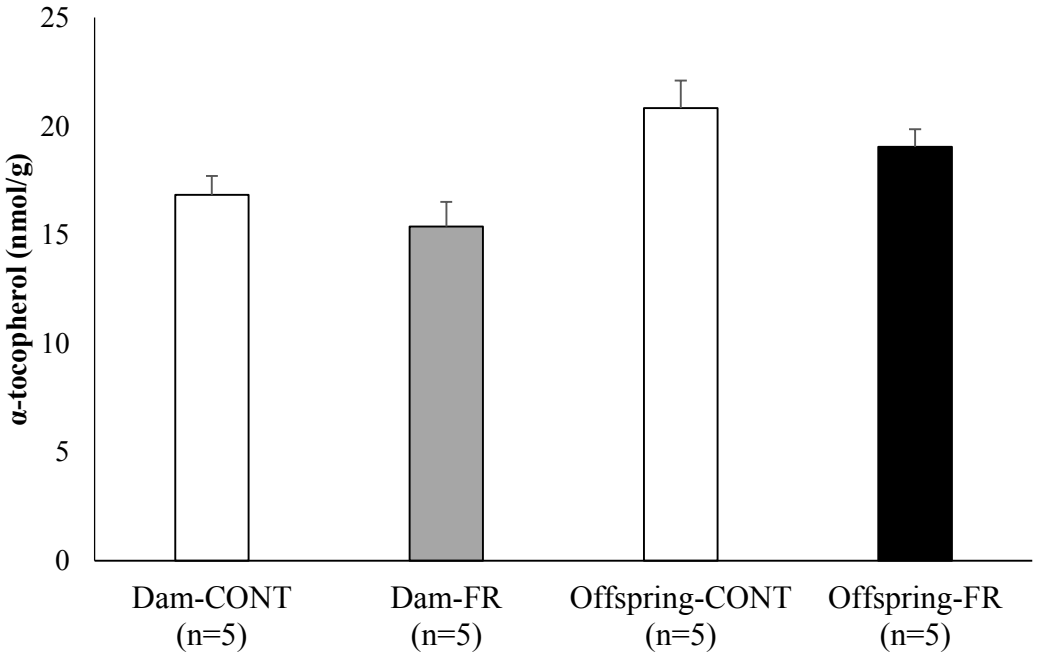
	P-value
Generation (Dams vs. Offspring)	0.5269
Diet (CONT vs. FR)	**0.0129**
Generation x Diet	0.4292

Figure 4-14: % CD31 (endothelial cells) area (relative to DAPI, cell nuclei) in the placentae collected at GD21 of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M. Blue staining: DAPI staining (nuclei staining), Red staining: CD31 staining (endothelial cells)

Study B. Oxidative stress in the placentae of Dams and Offspring

Oxidative stress is known to play a critical role in placental vascular development (described above; pages 66 – 69). Thus, the level of oxidative stress was examined in conjunction with alterations in the vascular network of the placenta.

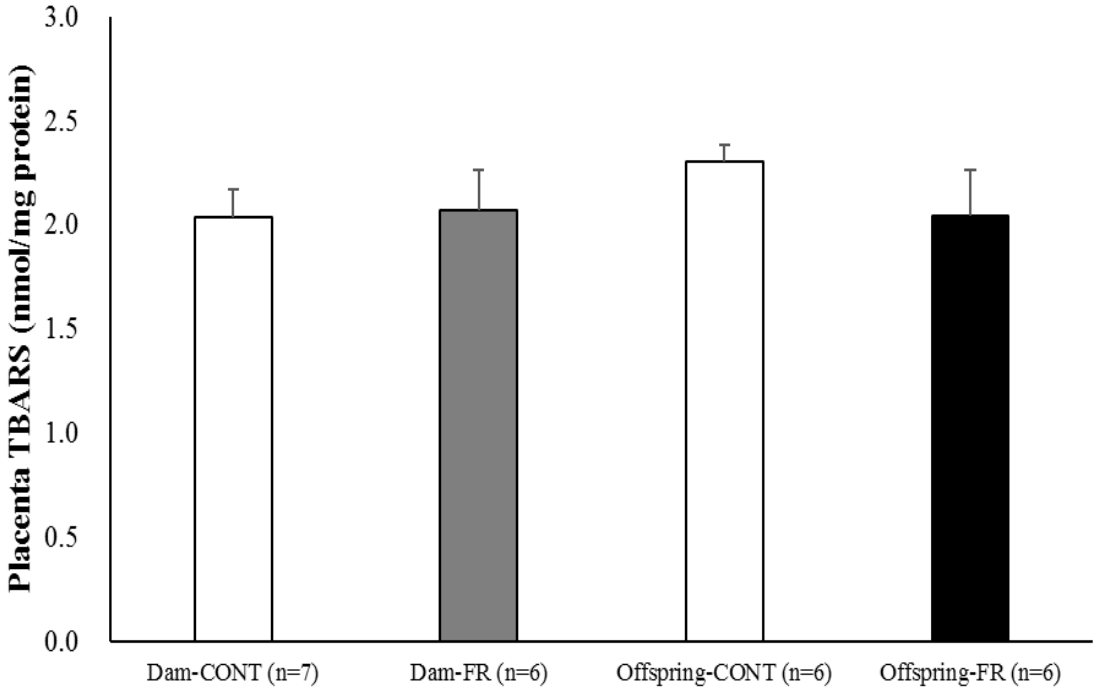
In the placentae, α-tocopherol concentrations were higher in Offspring than Dams (main effect of generation, P=0.0038; Figure 4-15) but not different between FR and CONT groups. As in the liver, α-tocopherol acetate, β, γ and δ-tocopherols were all below the level of detection using this assay.



	***P*-value**
Generation (Dams vs. Offspring)	**0.0038**
Diet (CONT vs. FR)	0.1807
Generation x Diet	0.8930

Figure 4-15: The concentration of α-tocopherol (nmol/g) at GD21 in the placentae of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age to 14 weeks of age. Each value is the mean ± S.E.M.

Secondly, the level of lipid peroxidation was assessed by measuring the concentration of TBARS in the placentae. The level of TBARS in the placentae did not differ between generations or between diet groups (Figure 4-16).

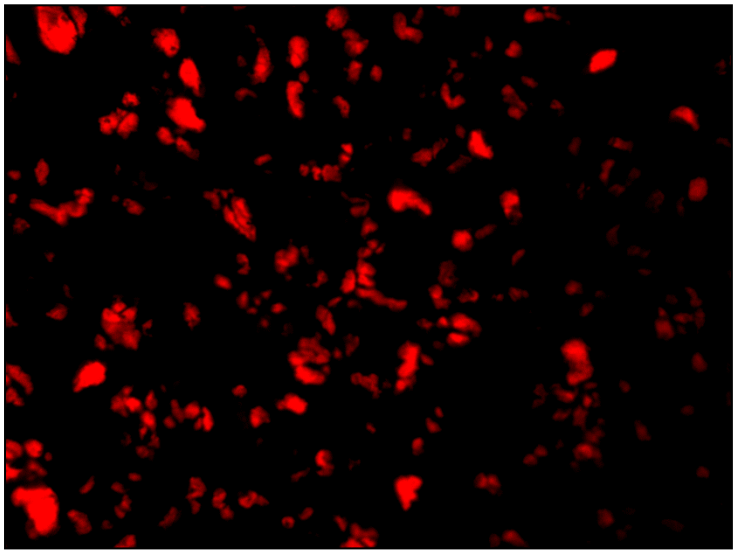


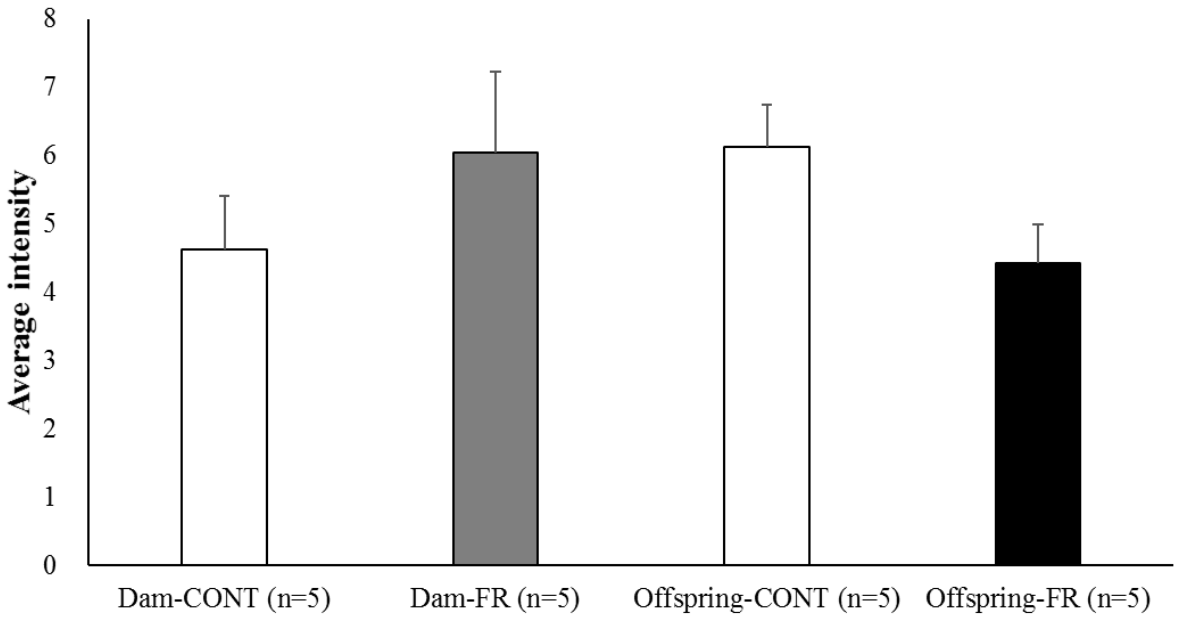
	P-value
Generation (Dams vs. Offspring)	0.4834
Diet (CONT vs. FR)	0.2660
Generation x Diet	0.1457

Figure 4-16: The concentration of TBARS (nmol/mg protein) at GD21 in the placentae of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M.

Lastly, oxidative stress was assessed in the frozen placentae by measuring the proportions of cells expressing superoxide (fluorescence of DHE, Figure 4-17) and peroxynitrite (fluorescence of DHR, Figure 4-18). The proportion of cells expressing superoxide in the placenta was not affected by either generation or diet and the fluorescence intensity of peroxynitrite was also not

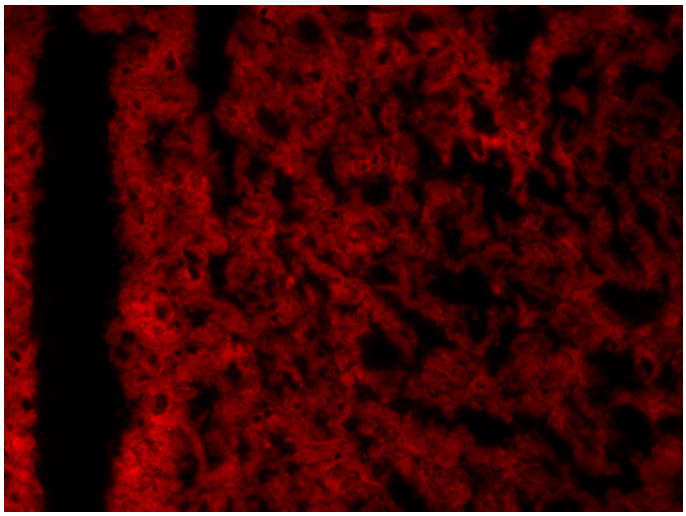
different between FR and CONT groups. However, the intensity of peroxynitrite was significantly lower in Offspring than Dams (P=0.0043).

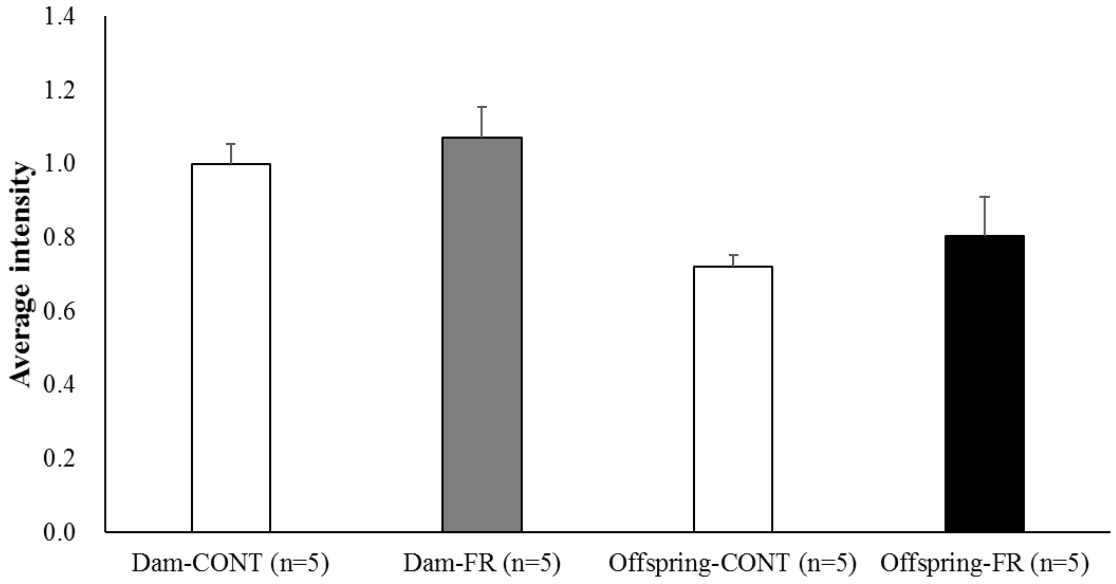




	P-value
Generation (Dams vs. Offspring)	0.9293
Diet (CONT vs. FR)	0.7601
Generation x Diet	0.3128

Figure 4-17: The fluorescence area (%) of DHE in the placentae collected at GD21 of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M.





	P-value
Generation (Dams vs. Offspring)	**0.0043**
Diet (CONT vs. FR)	0.4915
Generation x Diet	0.9363

Figure 4-18: The fluorescence intensity of DHR in the placentae collected at GD21 of Dams and Offspring fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (when diet started) to 14 weeks of age (late pregnancy). Each value is the mean ± S.E.M. All fluorescence data are expressed as fold changes relative to Dam-CONT.

Study C. The effects of high fructose intake on metabolic profiles of female rats during two sequential pregnancies

Study C. The success rate of second pregnancies

In Study C, Dam-CONT and Dam-FR were mated a second time at 22 weeks of age. The success rate of first pregnancy was 100 % for both Dam-CONT and Dam-FR. All Dam-CONT were successfully mated for a second pregnancy, whereas three out of nine Dam-FRs did not get

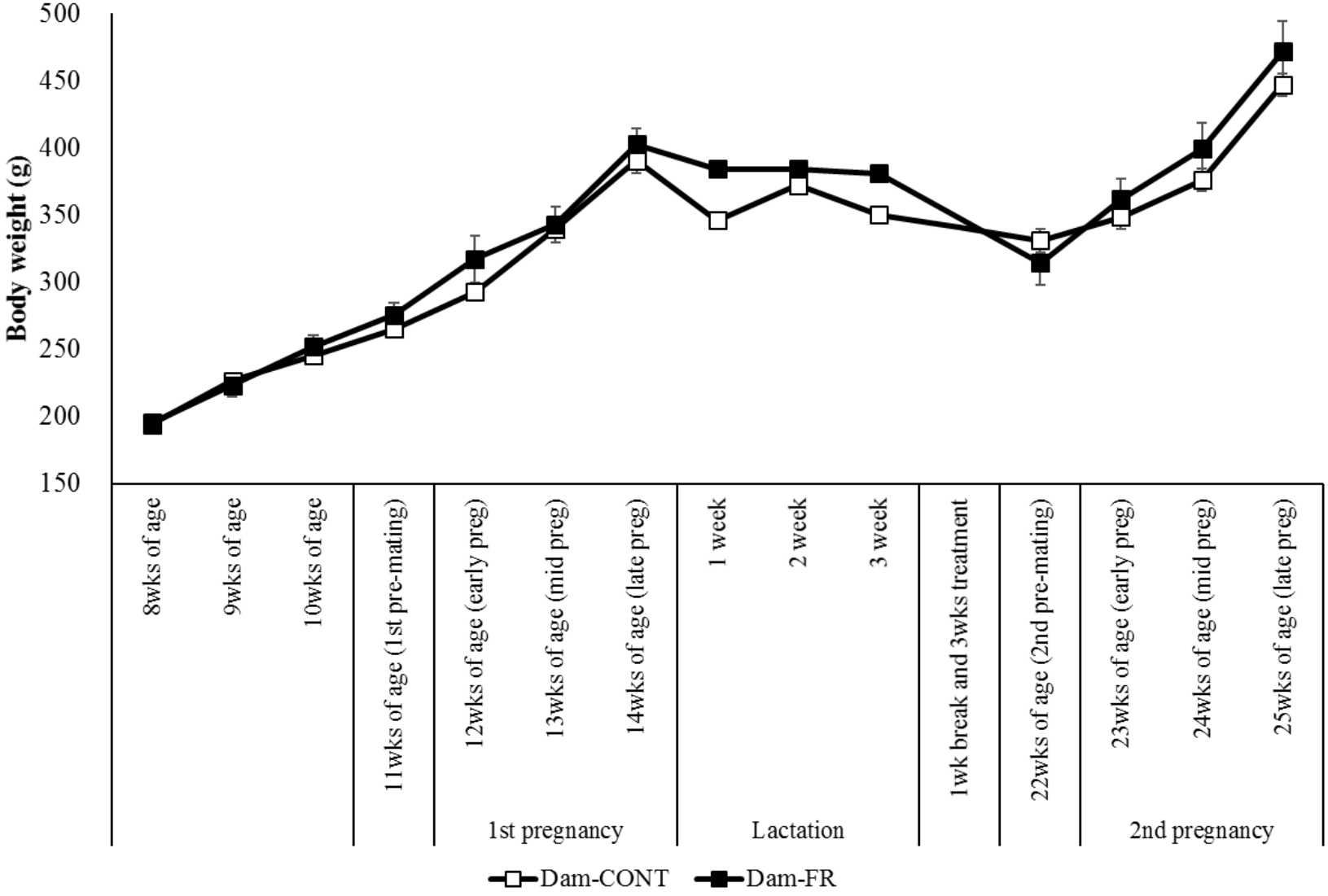
pregnant a second time (success rate of second pregnancy, 66.7 %; Table 4-7).

Table 4-7: The success rate of the 1st and 2nd pregnancy

	First pregnancy (n)	Second pregnancy (n)
Dam-CONT	100 % (7)	100 % (7)
Dam-FR	100 % (9)	66.7 % (6)

Study C. Body weights of Dams during two sequential pregnancies

Figure 4-19 shows the body weights of Dams-FR and Dams-CONT from 8 weeks to 25 weeks of age, including during their two sequential pregnancies. Body weights of all rats increased with time (main effect of time, $P<0.0001$; Figure 4-19). Body weights of Dams were about 50 g heavier entering the 2nd pregnancy than entering their 1st pregnancy (early pregnancy; 12 weeks of age vs. 23 weeks of age; $P<0.0001$) and this continued through the rest of pregnancy. Dams-FR had significantly higher body weights in mid- 2nd pregnancy than Dams-CONT (main effect of diet, $P<0.0001$).


Body weight (g)
500
450
400
350
300
250
200
150
8wks of age
9wks of age
10wks of age
11wks of age (1st pre-mating)
12wks of age (early preg)
13wks of age (mid preg)
14wks of age (late preg)
1 week
2 week
3 week
1wk break and 3wks treatment
22wks of age (2nd pre-mating)
23wks of age (early preg)
24wks of age (mid preg)
25wks of age (late preg)
1st pregnancy
Lactation
2nd pregnancy
Dam-CONT
Dam-FR

	***P*-value**
Time	**<0.0001**
Pregnancies (1preg vs. 2preg)	**<0.0001**
Diet (CONT vs. FR)	0.0609
Pregnancies x Diet	0.6750
Diet x Time	0.2727
Pregnancies x Time	0.7835
Pregnancies x Diet x Time	0.2740

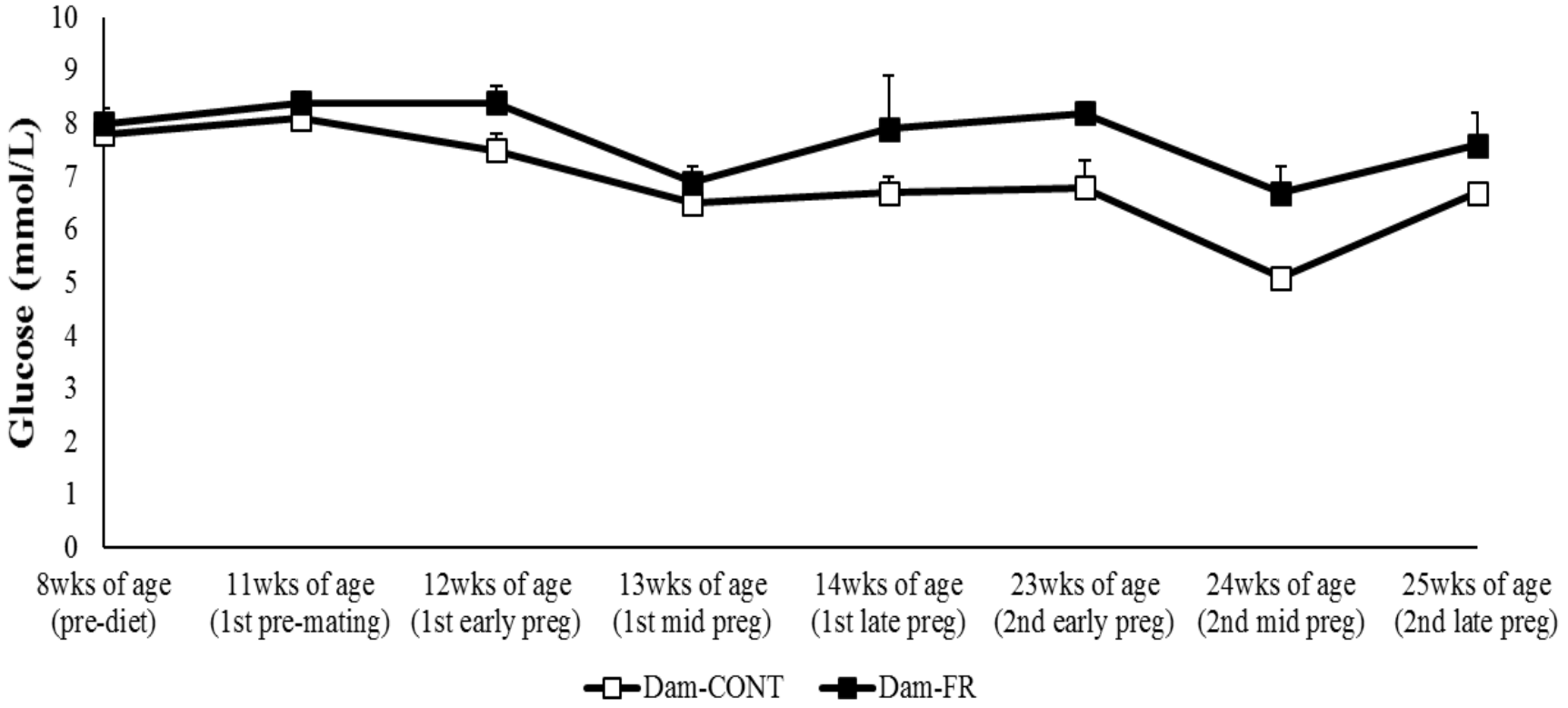
Figure 4-19: Body weights of Dams fed distilled water (CONT) or fructose solution (FR) from 8 weeks of age (pre-diet) to 25 weeks of age (2nd late pregnancy). Each value is the mean ± S.E.M. 8wks of age (pre-diet): The day before eight weeks of age (rats received distilled water with food *ad libitum*). Rats started to receive either distilled water or a 10 % (w/v) fructose solution at 8 weeks of age. 1st pregnancy was from 12 weeks of age to 14 weeks of age (Dam-CONT, n=12; Dam-FR, n=14; during 1st pregnancy). Rats remained on their assigned diet during 3 weeks of lactation. Dam-FR had one week break from the fructose solution, and then received FR for 3 weeks prior to their 2nd pregnancy. The 2nd pregnancy was from ~ 23 weeks of age to 25 weeks of age (Dam-CONT, n=7; Dam-FR, n=6; during 2nd pregnancy).

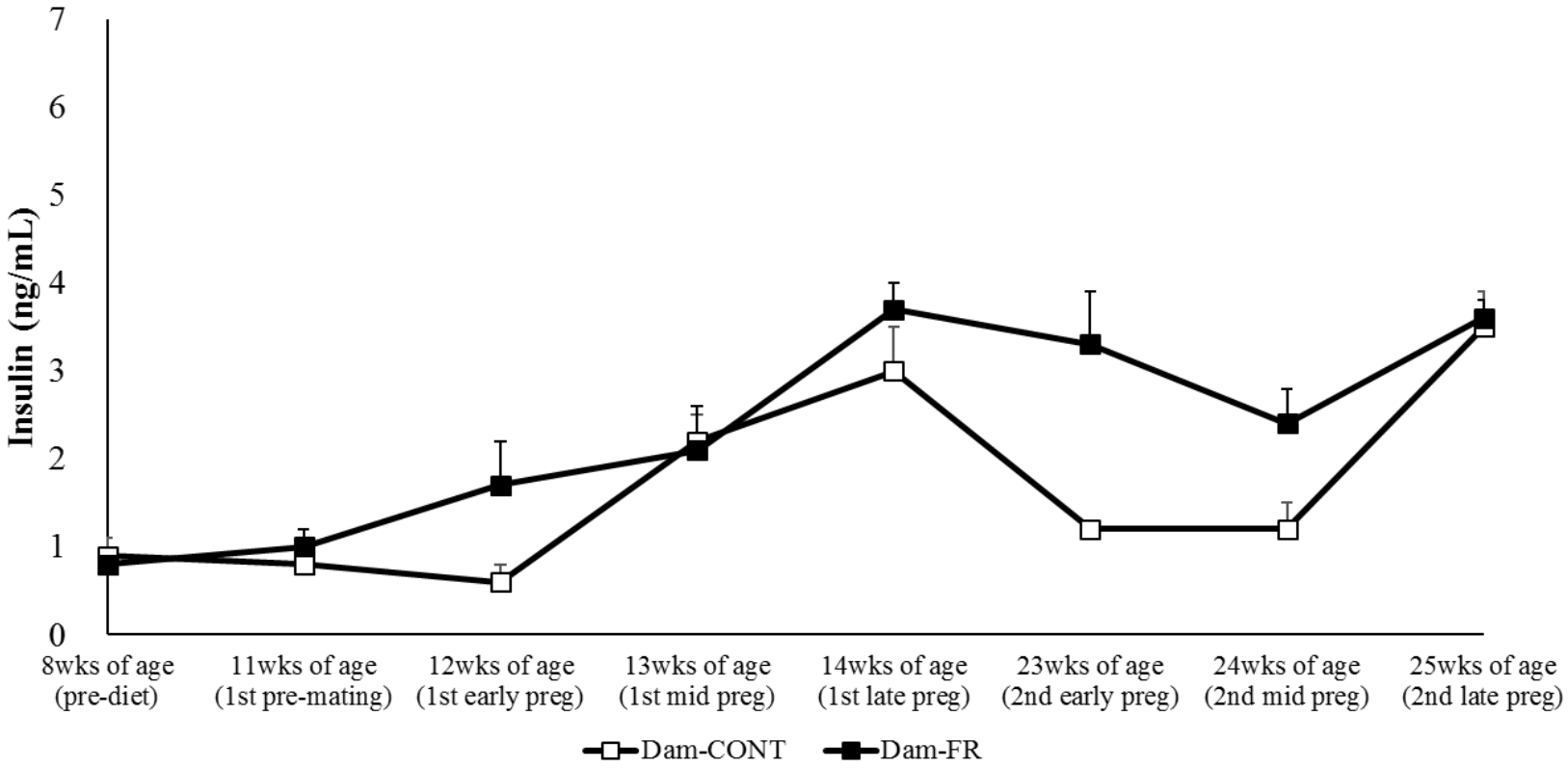
Study C. Metabolic profiles of Dams during two sequential pregnancies

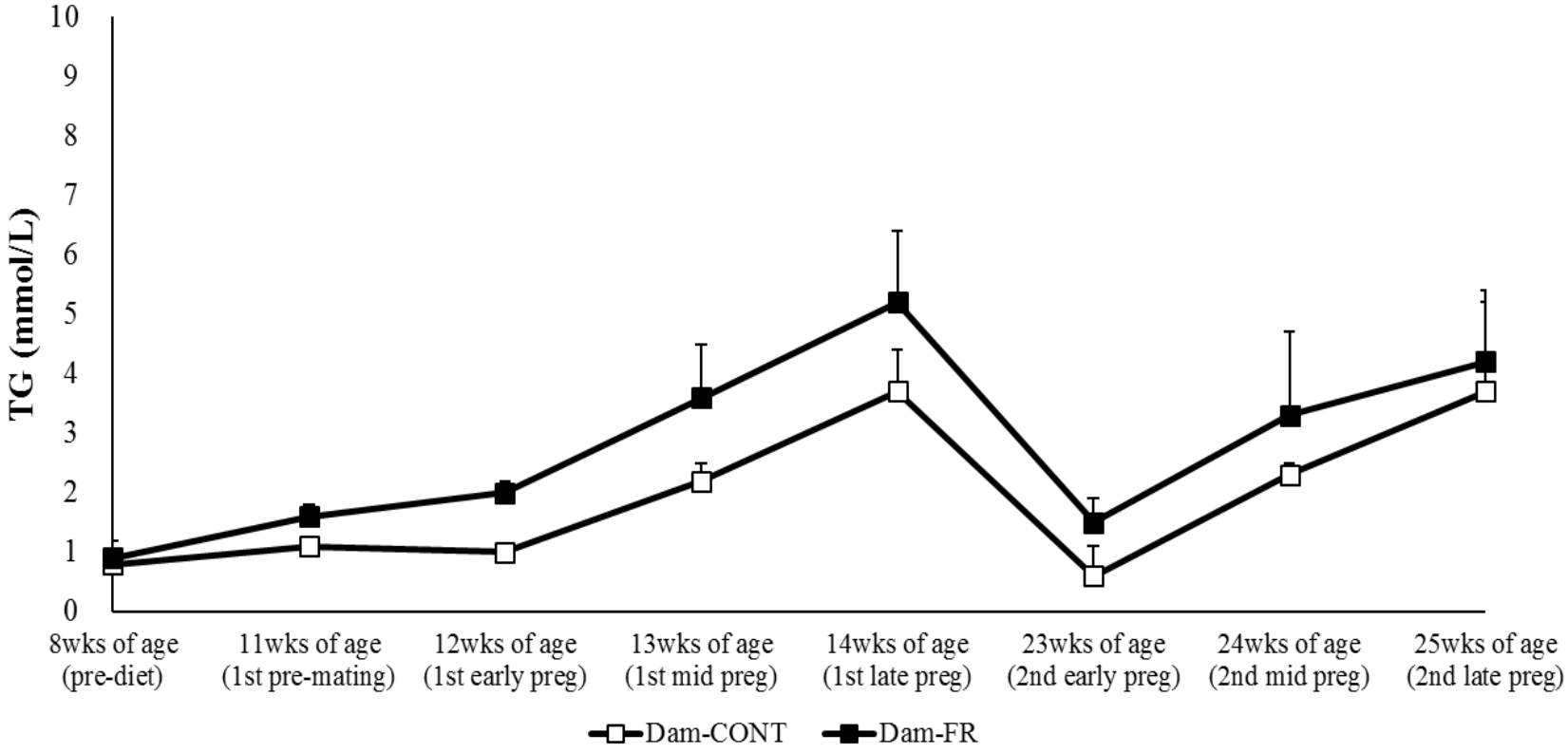
Non-fasting plasma glucose concentrations were ~1.2-fold higher in Dams fed FR than Dams fed CONT (main effect of diet, P=0.0062) throughout the study (Figure 4-20). In particular, fructose intake had a significant effect on plasma glucose concentrations during early (main effect of diet, P=0.0136) and mid (main effect of diet, P=0.0164) pregnancy. In mid- pregnancy, glucose concentrations were 0.8-fold lower in rats during their 2nd pregnancy than their 1st pregnancy (main effect of pregnancy, P=0.0341).

Non-fasting plasma insulin concentrations increased more dramatically across the study in the Dam-FR than they did in the Dam-CONT group (main effect of diet, P=0.0020; Figure 4-20). In the 1st and 2nd pregnancies, plasma insulin was 3-fold higher in the Dam-FR compared to the Dam-CONT in early and mid-pregnancy (early pregnancy, P=0.0183; mid-preganancy: P=0.0056), but not in late pregnancy. Insulin concentrations were significantly increased in the 2nd pregnancy compared to the 1st pregnancy (main effect of pregnancy, P=0.0272).

TG concentrations in Dams did not differ between 1st and 2nd pregnancies, but generally was ~2-fold higher in Dam-FR than Dam-CONT (main effect of diet, P=0.0070; Figure 4-20). Differences were most pronounced in early and mid-pregnancy for both pregnancies although by the end of the 2nd pregnancy, TG concentrations did not differ between diet groups. TG did not differ significantly between diet groups at any other time in this study.


Glucose (mmol/L)
10
9
8
7
6
5
4
3
2
1
0
8wks of age (pre-diet)
11wks of age (1st pre-mating)
12wks of age (1st early preg)
13wks of age (1st mid preg)
14wks of age (1st late preg)
23wks of age (2nd early preg)
24wks of age (2nd mid preg)
25wks of age (2nd late preg)
Dam-CONT
Dam-FR


Insulin (ng/mL)
7
6
5
4
3
2
1
0
8wks of age (pre-diet)
11wks of age (1st pre-mating)
12wks of age (1st early preg)
13wks of age (1st mid preg)
14wks of age (1st late preg)
23wks of age (2nd early preg)
24wks of age (2nd mid preg)
25wks of age (2nd late preg)
Dam-CONT
Dam-FR

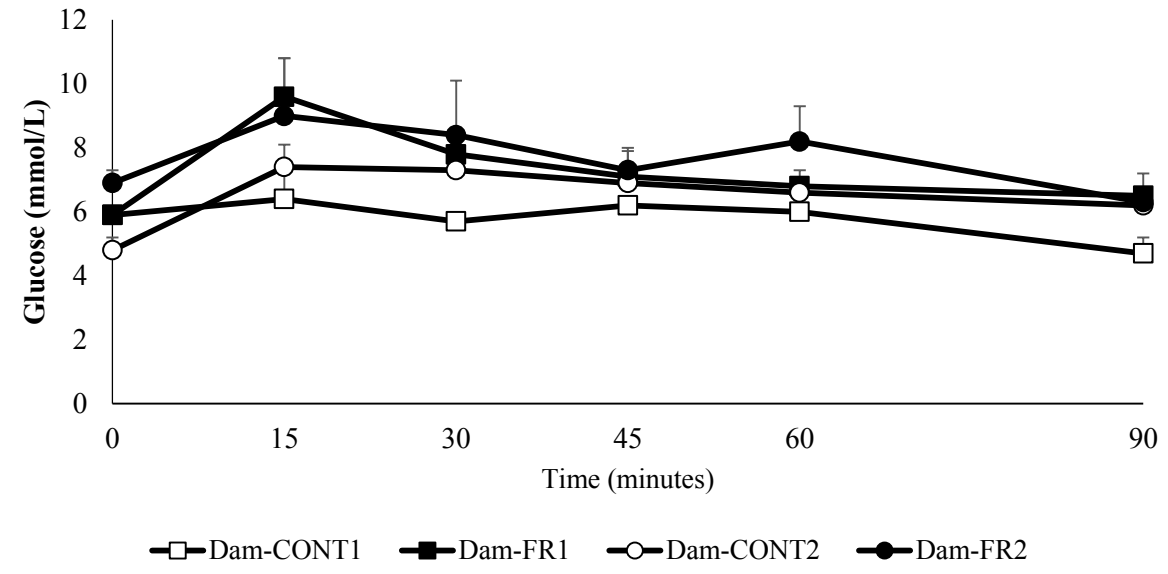

TG (mmol/L)
10
9
8
7
6
5
4
3
2
1
0
8wks of age (pre-diet)
11wks of age (1st pre-mating)
12wks of age (1st early preg)
13wks of age (1st mid preg)
14wks of age (1st late preg)
23wks of age (2nd early preg)
24wks of age (2nd mid preg)
25wks of age (2nd late preg)
Dam-CONT
Dam-FR

	P-value		
	Glucose	**Insulin**	**TG**
Time	**<0.0001**	**<0.0001**	**<0.0001**
Pregnancies (1preg vs. 2preg)	0.1011	**0.0182**	0.2481
Diet (CONT vs. FR)	**0.0062**	**0.0020**	**0.0070**
Pregnancies x Diet	0.5264	0.9325	0.7572
Diet x Time	0.6041	**0.0206**	0.0992
Pregnancies x Time	0.6099	0.2684	0.4702
Pregnancies x Diet x Time	0.5323	0.5682	0.7632

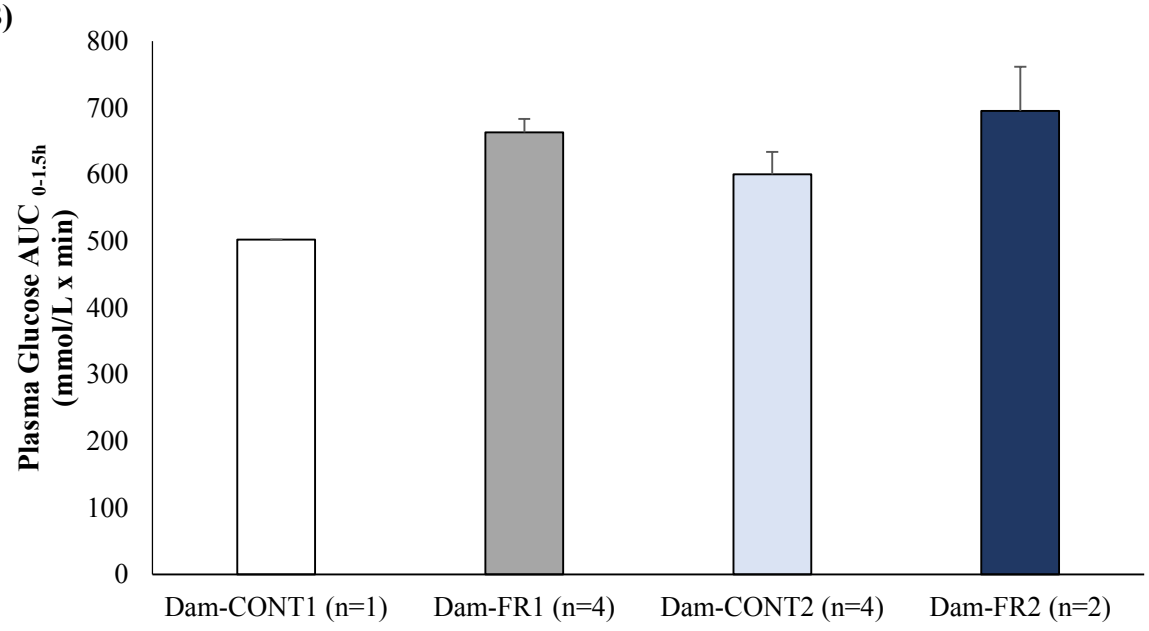
Figure 4-20: Plasma glucose, insulin and TG concentrations measured regularly in Dams fed distilled water (CONT) or the fructose solution (FR) from 8 weeks of age (when diet started) to 25 weeks of age (2nd late pregnancy). Each value is the mean ± S.E.M. 1st pregnancy was from 12weeks of age to 14 weeks of age (Dam-CONT, n=12; Dam-FR, n=14; during 1st pregnancy). 2nd pregnancy was from 23 weeks of age to 25 weeks of age (Dam-CONT, n=7; Dam-FR, n=6; during 2nd pregnancy).

During late pregnancy, glucose tolerance of Dams during the 1st and 2nd pregnancies was assessed by measuring plasma glucose and insulin concentrations during an OGTT. However, due to a number of technical difficulties, very few samples were available for these analyses (Dam-CONT1, n=1; Dam-FR1, n=4; Dam-CONT2, n=4; Dam-FR2, n=2) and therefore results from the OGTT should be considered as preliminary. Statistical analyses were not conducted on these data (Figure 4-21).

(A)


12
10
8
6
4
2
0
Glucose (mmol/L)
0
15
30
45
60
90
Time (minutes)
Dam-CONT1
Dam-FR1
Dam-CONT2
Dam-FR2

(B)


800
700
600
500
400
300
200
100
0
Plasma Glucose AUC 0-1.5h (mmol/L x min)
Dam-CONT1 (n=1)
Dam-FR1 (n=4)
Dam-CONT2 (n=4)
Dam-FR2 (n=2)

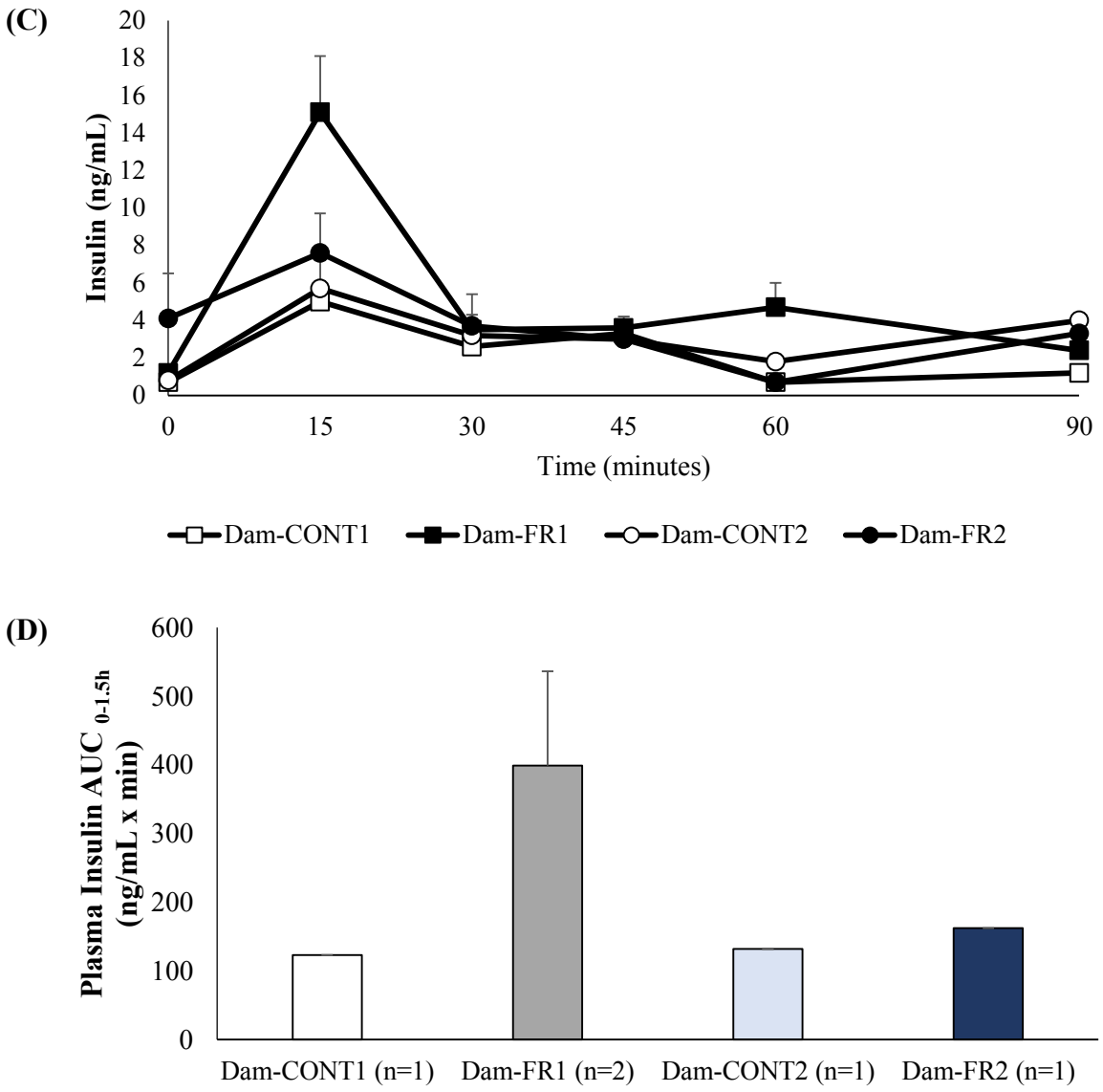


Figure 4-21: Plasma glucose and insulin concentrations measured during OGTT in Dams fed distilled water (CONT) or the fructose solution (FR) from 8 weeks of age (when diet started) to 25 weeks of age (2nd late pregnancy). Each value is the mean ± S.E.M. Dam-CONT1=Dam fed CONT during 1st pregnancy, Dam-FR1=Dam fed FR during 1st pregnancy, Dam-CONT2=Dam fed CONT during 2nd pregnancy, Dam-FR2=Dam fed FR during 2nd pregnancy, (A): Plasma glucose concentrations during the OGTT in Dams during 1st and 2nd pregnancies, (B): $AUC_{Glucose}$ during the OGTT of Dams during 1st and 2nd pregnancies, (C): Plasma insulin concentrations during the OGTT in Dams during 1st and 2nd pregnancies, (D): $AUC_{Insulin}$ during the OGTT of Dams during 1st and 2nd pregnancies.

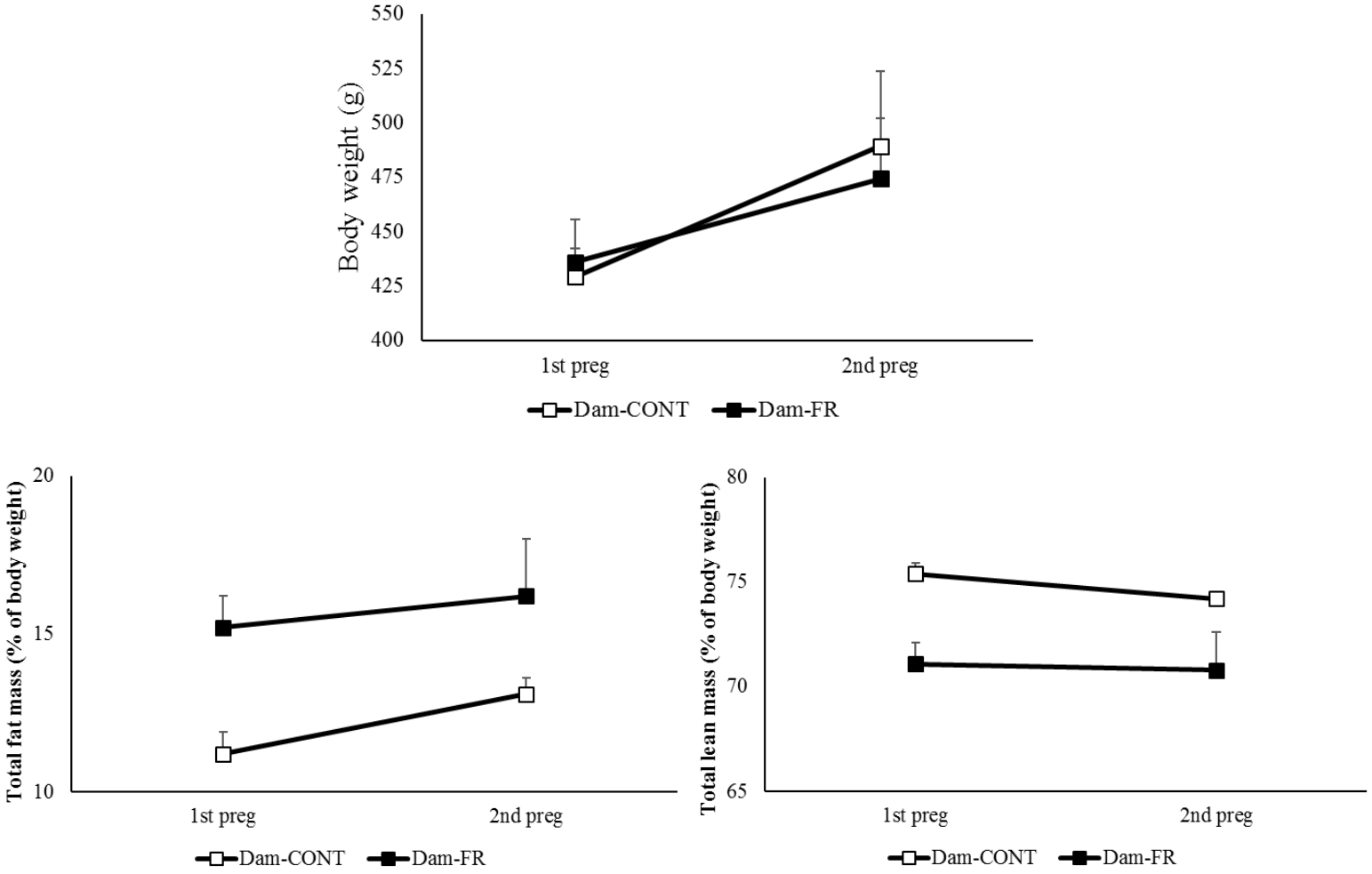
Study C. Body composition and the weight of organs of Dams at GD21 in 2 sequential pregnancies

Body composition was measured on GD21 in rats' 1st and 2nd pregnancy. Total fat mass was higher and lean mass (%, relative to body weights) was lower in Dam-FR than in Dam-CONT in both pregnancies (total fat mass: main effect of diet, P=0.0046; lean mass: main effect of diet, P=0.0016, Table 4-8). Both fat mass and lean mass during the 2nd pregnancy were not different from those during the 1st pregnancy.

The absolute fat mass (g) was heavier in Dam-FR than in Dam-CONT (main effect of diet, P=0.0137) and during 2nd pregnancy than 1st pregnancy (main effect of pregnancies, P=0.0123). Body weight (g) and the absolute lean mass (g) was increased in the rats during 2nd pregnancy compared to 1st pregnancy (main effect of pregnancies, P=0.0023), but those were not affected by fructose intake.

The weights of the pancreas and the liver (relative to body weight) were not affected by diet. Only the weight of the pancreas was lower in the 2nd pregnancy than the 1st pregnancy (main effect of pregnancy, P=0.0504).

Table 4-8: Total fat mass and lean mass (expressed relative to body weight and in absolute terms) at GD21 in Dams fed distilled water (CONT, n=7) or the fructose solution (FR, n=6) from 8 weeks of age (when diet started) to 25 weeks of age (2nd late pregnancy)



	P-value		
	Proportion fat mass (% body weight)	**Proportion lean mass (% body weight)**	**Body weight (g)**
Pregnancies (1preg vs. 2preg)	0.2085	0.4847	**0.0003**
Diet (CONT vs. FR)	**0.0046**	**0.0016**	0.8410
Pregnancies x Diet	0.7278	0.6398	0.2547

	1st pregnancy		**2nd pregnancy**	
	Dam-CONT	**Dam-FR**	**Dam-CONT**	**Dam-FR**
Total fat mass (g)	44.3 ± 3.1	63.9 ± 4.2	64.2 ± 4.5	79.2 ± 16.0
Total lean mass (g)	298.4 ± 11.2	301.3 ± 7.9	363.8 ± 13.0	333.7 ± 27.7
Pancreas to body weight ratio (%)	0.27 ± 0.02	0.3 ± 0.02	0.23 ± 0.03	0.23 ± 0.03
Liver to body weight ratio (%)	4.5 ± 0.1	4.6 ± 0.1	4.4 ± 0.1	4.6 ± 0.2

	P-value			
	Total fat mass (g)	**Total lean mass (g)**	**(% of body weight)**	
			Pancreas	**Liver**
Pregnancies (1preg vs. 2preg)	**0.0123**	**0.0023**	**0.0504**	0.9882
Diet (CONT vs. FR)	**0.0137**	0.3530	0.4349	0.3753
Pregnancies x Diet	0.7169	0.2618	0.6432	0.8099

Each value is the mean ± S.E.M. Dam-CONT1: Dam fed CONT during 1st pregnancy, Dam-FR1: Dam fed FR during 1st pregnancy, Dam-CONT2: Dam fed CONT during 2nd pregnancy, Dam-FR2: Dam fed FR during 2nd pregnancy.

CHAPTER 5: DISCUSSION

High fructose intake is recognized as a potential health concern since daily sugar consumption from sugar-sweetened beverages and soft drinks has increased significantly over several decades (7). Epidemiological evidence suggests that high consumption of fructose, in part due to intake of sugar-sweetened beverages, is associated with increased incidence of disrupted glucose homeostasis, including development of type 2 diabetes, dyslipidemia, weight gain and obesity among men and non-pregnant women (20, 293, 294, 295). The extent to which fructose has an impact on pregnancy is an evolving investigation and the subject of a growing number of studies, including those described in the literature of this thesis.

Pregnancy is a sensitive period of growth and development for both the mother and developing fetus, with the potential to influence long-term health. The concept of early life programming, also known as the DOHaD hypothesis, suggests that a less than optimal maternal dietary intake in pregnancy, and even prior to pregnancy, can lead to deleterious effects on fetal development, and can have lasting adverse effects on the risk of disease in the offspring in later life (296). Results from this thesis indicate that the high intakes of refined fructose, in particular a 10 % purified fructose solution, is detrimental to pregnant dams and their female offspring. The adverse effects on glucose homeostasis and lipid profiles that have been previously described in male rodents are magnified in female during pregnancy and in their offspring when the offspring are pregnant. These findings highlight the need for additional studies in pregnant women and their offspring to explore the applicability of these findings and examine potential health impacts in humans in more detail.

Study A. The effects of high fructose intake on the body weight, metabolic profiles and fat mass of non-pregnant and pregnant rats

This initial study was used to examine the effects of intake of a 10 % fructose solution before and during pregnancy on key physiological indicators of health, namely body weight, body composition, non-fasting metabolic profiles of glucose, insulin and TG during the study and during an oral glucose tolerance test. A small number of animals were used in this study, thus the results should be interpreted with caution. Overall, these results indicated that fructose intake leads to hyperinsulinemia and hypertriglyceridemia and that these effects were observed in pregnant rats. A summary of statistical analysis is shown in Table 5-1, and all of these observations are discussed in more detail below.

Table 5-1: A summary table of statistical analyses in Study A

	Interaction (Diet x Pregnancy)	Main effect of diet (CONT vs. FR)	Main effect of pregnancy (NP vs. Preg)
Body weight	0.1305	0.8038	**0.0169**
Metabolic profiles			
Plasma glucose	0.7939	0.0944	0.4996
Plasma insulin	0.8248	**0.0031**	0.6290
Plasma TG	0.6031	**0.0476**	**0.0047**
$AUC_{glucose}$	0.2810	0.4373	0.1935
$AUC_{insulin}$	0.4535	0.6965	0.2160
HOMA-IR	0.4191	0.2896	0.6215
AUC_{TG}	0.4830	**0.0403**	**<0.0001**
Body composition			
Total fat mass (%, relative to body weight)	0.5742	0.3096	0.0551
Total lean mass (%, relative to body weight)	0.4604	0.4354	0.1403
Fat mass (g)	0.1573	**<0.0001**	0.0904
Lean mass (g)	0.5445	0.7375	**<0.0001**

Fructose intake, body weight and body composition in non-pregnant and pregnant rats

Study A showed that fructose intake did not increase body weights of either non-pregnant rats or pregnant rats from 8 weeks of age (when rats started to receive the fructose solution) to the end of the study (GD21 for pregnant rats). These results add to the literature examining whether fructose intake specifically affects body weight, and suggest that it does not affect female rats' body weights in the non-pregnant state, although it may lead to small increases in body weights at the end of pregnancy. Results from other studies in the literature that have examined the

effects of high fructose intake on body weight in male and non-pregnant female rodents have been inconsistent. Some studies have shown that short (≤10 weeks) and long-term (six to seven months) HFCS intake increases the body weights of male rats (25) while others did not observe significant differences between body weights of female pregnant rats (28, 161, 163, 178, 180, 183, 184, 297). The reasons for these inconsistencies are not known, but variation in the amount, form of fructose (solid vs. liquid), or duration of fructose consumption may contribute to the inconsistencies in the effects on body weight. Considering the males and non-pregnant females, there may be differences between genders, as male rats housed in animal facilities continue to put on weight throughout their adult life, while female rats tend to maintain a lower body weight than males post-weaning, female rats put on weight at a slower rate than males, and they do not achieve the high weights observed in males at any time. The extent to which variation in the amount, form (solid vs. liquid), or duration of fructose consumption may contribute to the inconsistencies in the effects on body weight is also unclear. It may be possible to unravel some of these questions as the literature in this area grows. Considering pregnant female rodents, there are a few studies in this area to date, and they all use different experimental protocols.

Extrapolation to humans must be made with caution. Obviously dietary intake and energy expenditure of humans are both complex behaviors that significantly impact body weight and are difficult to account for, even in controlled clinical trials. A recent meta-analysis examined the effects of regular fructose intake on body weight in humans and concluded that fructose has no effect on body weight in non-pregnant adult humans when compared with isocaloric diets substituted with other carbohydrates (298). Further their results indicated that studies that observed increased body weight with high fructose intake was most likely the result of increased

calorie intake. For example, the authors of another meta-analysis noted that the positive association between fructose consumption and body weight disappeared when fructose consumption was less than 19% of energy intake in adults (299).

In Study A, there were no clear differences in the proportion of fat or lean mass of fructose-fed rats. In fact, a number of studies indicated a higher proportion of fat among rats consuming fructose (161, 164, 165, 178, 179, 180). We did not observe such changes in either pregnant or non-pregnant rats. This lack of effect in our study could reflect the small numbers in our sample size and an inability to detect statistically significant differences. It is also possible that the increased fat mass with pregnancy masked further effects of fructose.

Fructose intake and metabolic profiles in non-pregnant and pregnant rats

Results from Study A confirm that fructose consumption before and during pregnancy leads to hyperinsulinemia and hypertriglyceridemia in non-pregnant and pregnant rats. Many studies have shown similar results of fructose consumption on insulin and TG concentration in male and non-pregnant rats (27, 40, 41, 300). Fructose intake is known to increase insulin concentrations in blood through a combination of mechanisms including down-regulation of insulin receptors in tissues (i.e. skeletal muscle, liver) and an elevation of FFA concentrations that inhibit insulin access to the liver via changing the level of insulin receptor and glucose metabolism in tissues (i.e. muscle, adipose cells, liver) (44). In addition, increased TG concentrations are the result of up-regulation of *de novo* lipogenesis (36, 37, 38). Thus, the present data are consistent with these previous findings.

In Study A, both non-pregnant and pregnant rats had significantly increased plasma insulin

concentrations as a result of fructose intake but neither plasma glucose nor insulin concentrations were different between NP and Dams, nor was there any interaction between pregnancy and diet. Pregnancy is known to be an insulin resistant state. Insulin resistance in pregnancy comes about through the actions of several pregnancy hormones including placental lactogen which also increases oxidative stress in adipocytes (87). In rats, despite the insulin resistance in pregnancy, glucose concentrations decrease toward the end of pregnancy due to increased glucose utilization by the large fetal mass relative to maternal size and enlarged maternal organs (291). However, Study A did not find significant differences in glucose and insulin concentrations between non-pregnant rats and pregnant rats. It is possible that the differences were not observed due to the small number of rats/group or due to the similar effects of fructose intake on plasma glucose and insulin concentrations in both pregnant and non-pregnant rats.

Plasma TG concentrations were significantly high in pregnant rats compared to those in non-pregnant rats given fructose. Increased TG concentrations during pregnancy are due to an elevation of plasma lipid concentrations (FFA, TG, cholesterol and phospholipids) for the utilization of substrates by the mother (107). Fructose intake increased TG concentrations as well, but the effects of fructose on plasma TG concentrations were not different between pregnant vs non-pregnant rats. Similar to glucose and insulin, fructose also increases circulating TG concentrations, but it does not appear to have synergistic effects with pregnancy. In previous studies, fructose intake increased TG concentrations in either non-pregnant rats or pregnant rats (40, 41). However, none of these studies have compared TG concentrations between non-pregnant status and pregnancy. Thus, Study A is the first study that showed that the effects of fructose intake on plasma lipids are not pronounced during pregnancy.

In summary, this study compared the effects of fructose intake in pregnancy vs. non-pregnancy. This study indicated that fructose intake increased insulin and TG concentrations in both non-pregnant rats and pregnant rats, but that those effects were not more pronounced in pregnant rats.

Study A, however, as a part of a larger study (Islet study, mentioned in page 101), was limited to providing basic information about physiological changes in pregnant rats carried by fructose intake. Study A showed the effects of fructose intake on metabolic profiles but this study did not measure further pregnancy outcomes. Thus, to understand how fructose consumption can affect pregnancy, more detailed investigations are required. The next study conducted more extensive measures related to hepatic expression of genes involved in lipid metabolism, pregnancy outcomes including fetal and placental weights and novel aspects of placental structure and nutrient transport. It also included an investigation of developmental programming effects of fructose by examining lasting effects of maternal fructose intake on offspring health in pregnancy.

Study B. The effects of high fructose intake on metabolic and physiologic characteristics of rat dams and placentae across two generations

To our knowledge, this is the first study to examine the effects of fructose intake on pregnant dams and adult female offspring during pregnancy. Rat dams exposed to the fructose solution prior to and during pregnancy experienced hyperglycemia, insulin resistance, hypertriglyceridemia, increased fat mass and increased hepatic expression of FAS and GLUT 5. Pregnant female offspring from these dams given fructose solution had dramatically higher plasma TG concentrations than all other groups. This strongly suggests that the effect of dietary

programming on lipid metabolism occurs in conjunction with a physiological challenge and points to the potential of dietary intake of fructose to adversely influence offspring metabolism over multiple generations.

Fructose intake also induced changes in the placentae of both generations that are consistent with reduced nutrient transport to the developing fetus and reduced vascularity. These alterations could help to account for the lower weights of the placentae and the fetuses in the groups given fructose. A summary of statistical analysis is shown in Table 5-2, and all of these observations are discussed in more detail below.

Table 5-2: A summary table of statistical analyses in Study B

	Interaction (Diet x Generation)	**Main effect of diet (CONT vs. FR)**	**Main effect of pregnancy (1gen vs. 2gen)**
Body weight	0.5714	**0.0003**	**0.0063**
24hr food and water intake			
Food intake (g/day)	0.2239	**<0.0001**	0.8873
Water intake (g/day)	0.7081	**<0.0001**	0.4448
Energy intake (kcal/day)	0.1256	0.8276	0.7837
Metabolic profiles			
Plasma glucose	0.3897	**0.0001**	0.4233
Plasma insulin	0.3118	**<0.0001**	**0.0004**
Plasma TG	**0.0099**	**<0.0001**	0.5706
Plasma protein	0.3777	**0.0056**	0.1342
$AUC_{glucose}$	0.4094	**0.0024**	0.0664
$AUC_{insulin}$	0.8668	0.0603	**0.0156**
HOMA-IR	0.3730	0.6609	**0.8329**
Body composition			
Total fat mass (%, relative to body	0.4805	**<0.0001**	0.3310

	Interaction (Diet x Generation)	Main effect of diet (CONT vs. FR)	Main effect of pregnancy (1gen vs. 2gen)
weight)			
Total lean mass (%, relative to body weight)	0.9749	**<0.0001**	0.8562
Fat mass (g)	**<0.0001**	**<0.0001**	0.9948
Lean mass (g)	0.4107	0.5694	**0.0011**
Organ weights (relative to final body weight) at GD21			
Pancreas	0.1690	0.8986	0.3762
Liver	0.6054	0.2366	0.1529
Uterine fat	0.2218	**0.0001**	0.1217
Retroperitoneal fat	**0.0079**	**<0.0001**	**<0.0001**
Brown fat	0.8654	**0.0015**	0.1367
Hepatic gene expression at GD21			
FAS	0.4649	**<0.0001**	**0.0042**
GLUT2	0.4513	0.0935	0.9420
GLUT5	0.7367	**0.0061**	0.3049
Oxidative stress in the liver			
Ratio of α-tocopherol to TG	0.1192	**0.0062**	0.6948
TBARS	0.0892	0.2178	0.1942
Feto-placental unit			
Number of pups	0.2363	0.5689	0.6950
Placental weight	0.1857	**0.0002**	0.8640
Fetal weight	0.8787	**0.0004**	0.7160
Placental:fetal weight ratio	0.1355	0.9076	0.7368
Nutrient transporters			
FABP1	0.8201	**0.0004**	0.3624
SNAT2	**0.0413**	0.2528	**<0.0001**
GLUT1	0.6340	0.0685	0.4800
The ratio of LZ:JZ	0.5676	0.6347	0.8186
Angiogenic factors			

	Interaction (Diet x Generation)	Main effect of diet (CONT vs. FR)	Main effect of pregnancy (1gen vs. 2gen)
VEGF-A	0.6632	**0.0015**	0.7408
PlGF	0.2834	0.0607	0.8445
VEGFR-1	0.4603	0.3212	0.7527
VEGFR-2	0.1393	**0.0014**	0.2319
Vascularity (Intensity of CD31)	0.4292	**0.0129**	0.5269
Oxidative stress in the placenta			
α-tocopherol	0.8930	0.1807	**0.0038**
TBARS	0.1457	0.2660	0.4834
Superoxide (Intensity of DHE)	0.3128	0.7601	0.9293
Peroxynitrite (Intensity of DHR)	0.9363	0.4915	**0.0043**

Fructose intake and body weights of dams and adult offspring during pregnancy

There is debate over whether a high fructose diet increases body weight as discussed above. Pre-pregnancy body weight is an important predictor of maternal and fetal health in humans (301) and there is also evidence that excessive weight gain in early pregnancy is detrimental to pregnancy outcomes (302). Either or both of these effects could be a means through which fructose intake negatively affects maternal and fetal health since pregnant dams and offspring given fructose weighed more than the control dams and offspring in pre-mating and early pregnancy. It is interesting that the effects of fructose on body weight were restricted to these two-time points. Body weights of fructose-fed and control rats may have been similar in middle and late pregnancy because the fructose-fed rats had reduced fetal and placental weights and a lower proportion of lean mass, but an increased proportion of fat mass. It should be also noted that the body weights of dams and offspring were significantly different during the study.

Although causes for this are not clear, the body weights of dams and offspring could have been affected by different environmental exposures. For example, Dams were purchased from a commercial breeding facility and transported over several days to the Animal Facility at the University of Alberta where they were bred. In contrast, the offspring were bred, raised and then bred again at the University of Alberta. They therefore were not exposed to the stress of transport, changes in facilities, etc.

Total energy intake of rats fed the fructose solution was not different from rats fed distilled water, although fluid consumption was significantly higher in fructose-fed rats than control rats. Higher fluid consumption (containing fructose, and therefore energy) resulted in a significant reduction in solid food intake of rats. The fact that energy intake was similar in the fructose and control groups likely explains the lack of difference in body weights between diet groups. The lower food intake was cause for concern about whether fructose-fed rats may have been at risk for lower protein status, particularly during pregnancy. Although the proportion of calories intake from carbohydrates was higher due to intake of the fructose solution while fat and protein intake was lower in the fructose group than the control group, intake of macronutrients was within ranges used by other studies and consistent with healthy pregnancy in rats (303). We examined plasma protein concentrations as a means of better understanding whether rats given fructose were at increased risk for low protein status. Since plasma protein concentrations were slightly higher in dams given fructose vs. dams given distilled water and not different between Offspring-CONT and Offspring-FR, it does not appear that rats in the FR groups had reduced protein status. Plasma protein concentrations in both groups remained within normal ranges seen in health animals, thus protein status was not compromised in the group consuming the fructose

solution. We are not the first investigative group to observe reductions in food intake in rats given fructose solution in their drinking water (162, 163, 178, 179, 183), however we are the first to confirm that rats' protein status does not appear to have been compromised; other research groups have not examined or reported this important marker of health.

Fructose intake and hepatic lipid peroxidation in dams and adult offspring during pregnancy

Lowered micronutrient status may have been another consequence of reduced solid food intake in the FR-rats. Optimal antioxidant status is particularly important in situations with high FR intake since many studies using a rat model with high fructose consumption report increases in the production of oxidative stress and reduced activities of antioxidant enzymes (66). The level of TBARS, an indicator of lipid peroxidation, was significantly increased by consumption of a 60 % (w/v) fructose solution for 42 days (66), consumption of 65 % (w/w) fructose diet for eight weeks (67) or intake of a 25 % (w/v) fructose solution for two months (59). In addition, increased oxidative stress has been shown to negatively impact pregnancy outcomes in several animal models and human conditions (123, 294).

Vitamin E is a well-known antioxidant that protects lipid and lipoproteins from oxidative damage (116, 118, 119). α-tocopherol, in particular, reacts with lipid radicals in the lipid peroxidation chain reaction (70). In our study, hepatic α-tocopherol concentrations were lower in fructose-fed rats than control rats. This may be a consequence of consuming less vitamin E from solid food throughout the study, or could indicate increased loss of vitamin E due to using up the available vitamin E as a result of the increased oxidative stress in animals given fructose. Generally, vitamin E deficiency is rare and not readily induced in animal models with short interventions

(223). In humans, severe vitamin E deficiency is also rare, but suboptimal vitamin E status is widespread, and the risk of miscarriage is doubled in pregnant women who have low vitamin E intake (304, 305). Therefore, a reduction in hepatic α-tocopherol concentrations in fructose-fed rats might have a role in adverse pregnancy outcomes, such as miscarriage, or could contribute to the reduced weights of the placentae and the fetuses. There was no relationship between α-tocopherol concentrations in the liver and litter size in CONT and FR groups in the current study. This suggests that although lower concentrations of hepatic α-tocopherol concentrations were observed, the effects did not carry over to affect general pregnancy outcomes.

We did not observe any increase in lipid peroxidation in the liver of FR rats compared with control rats. Since α-tocopherol has strong antioxidant activities, we expected to observe increased indicators of lipid peroxidation in rats given fructose, however the level of TBARS in the liver was not different between the fructose group and the control group. Other studies in the literature reported increased TBARS in the liver of rats given dietary fructose concentrations of 25 % (w/v) in the drinking water and a 65 % (w/w) fructose diet (59, 66, 67). It is possible that the present study showed that a lower concentration of fructose (10 % (w/v) did not produce a dramatic oxidative load and hepatic anti-oxidant mechanisms were able to overcome adverse effects. In addition, the breeding age of our rats (12 to 14 weeks of age for rats in Study B) may be within an age range in which animals are able to can control their level of oxidative stress. This is because aging is considered as one of the major factors that increases oxidative stress. However, further investigations are required to confirm this.

The lack of differences in lipid peroxidation could be due to the fact that there are a number of factors that regulate lipid peroxidation and that many micronutrients work in synergy and with

redundancy in cells and tissues. In addition, vitamin E may not directly influence the level of lipid peroxidation, but it may regulate the level of lipid peroxidation indirectly through interaction with other antioxidants. Similarly, Zagrodzki et al. (2012) found that rats fed 31 % (w/w) fructose diet did not increase MDA in plasma (68). Moreover, in Lozano's study, the rats that were fed a 25 % (w/v) fructose solution for eight months had increased levels of TBARS in plasma at two months of age, but there was no elevation of plasma TBARS levels at eight months of age (59). In Faure's study (2004), supplementation of vitamin E, zinc and selenium increased GSH/GSSG ratio, but did not reduce the level of TBARS in plasma in rats fed a 58 % (w/w) fructose diet (69). Therefore, we can consider that the effects of fructose intake on the level of oxidative stress may vary and many factors contribute to the regulation of oxidative stress.

Fructose intake and plasma glucose and insulin concentrations in dams and offspring during pregnancy

Long-term fructose intake induced insulin resistance in rats in both generations. As described above (page 54 – 59), previous studies have reported that long-term fructose consumption is associated with insulin resistance, possibly through reductions in leptin production, down-regulation of insulin receptors in tissues (i.e. skeletal muscle, liver) and alterations in lipid metabolism (22, 44, 306). In Study B, plasma glucose concentrations were also significantly elevated in fructose-fed rats compared to control rats. A similar result was found in Rawana's study in that female Sprague-Dawley rats fed a 10% (w/v) fructose solution had increased plasma glucose concentrations at GD19 (178). These authors found that gluconeogenesis was promoted by the elevation of phosphoenolpyruvate carboxykinase in the liver, which could

increase the amount of gluconeogenic substrates (178). Fructose may be metabolized to glucose, resulting in increased glucose concentrations in plasma (178). In Study B, therefore, fructose intake may be associated with increased plasma glucose concentrations, despite insulin concentrations being increased.

Fructose intake and lipid metabolism in dams and offspring during pregnancy

Once fructose is transported to the liver, excess amounts of fructose are rapidly converted to triglycerides since fructose substrates bypass the rate-limiting step of phosphofructokinase-1 in glycolysis. As described above, a number of studies have shown that fructose consumption increased plasma TG concentrations in rats (27, 40, 41, 161, 163, 300). Our results are consistent with these previous observations since we observed a significant increase in plasma TG concentrations in dams and offspring in the fructose groups. Furthermore, offspring fed fructose had even higher plasma TG concentrations than their dams fed fructose during the study. A few recent studies found that concentrations of TG were increased in offspring born to fructose-fed dams (165, 179). In Rodriguez's study (2016), offspring born to rat dams fed either a standard chow diet or a standard chow diet with a 10 % (w/v) fructose solution were kept on a standard chow diet with a 10 % (w/v) fructose solution (179). At 240 days of age, the fructose-fed offspring that were born to fructose-fed dams had significant increases in plasma TG and FFA concentrations and hepatic TG concentrations compared to fructose-fed offspring born to dams that did not receive the fructose solution (179). In addition to increased plasma TG concentrations in offspring fed fructose, the weight (g) of fat mass was significantly higher in offspring fed fructose and it was even higher than dams fed fructose. The mechanisms contributing to these effects are not well understood but appear to be an example of early

programming and could involve changes in maternal lipid metabolism, fetal lipid metabolism, effects on the placenta, or a combination of these factors (187).

Hepatic FAS may be a potential enzyme to consider as a target of early programming effects. We found that mRNA expression of FAS was increased in the liver of fructose-fed rats and was higher in Offspring than in Dams. FAS is an enzyme that stimulates *de novo* lipogenesis by catalyzing the steps in the conversion of malonyl-CoA to palmitate (6) and its expression is positively associated with lipid synthesis. Increased expression of FAS following fructose intake was also found in previous studies (161). Rodriguez found that intake of a 10 % (w/v) fructose solution increased hepatic expression of FAS, along with ACC and carbohydrate-responsive element-binding protein (ChREBP) in rats at GD21 (161). In addition, we also observed that FR intake resulted in increased circulating TG concentrations, hepatic TG content, a higher proportion of fat (both fat mass (%) relative to body weight and the fat mass (g)) in the body and increased weight of adipose tissues in different body depots (i.e. uterine, retroperitoneal and brown/interscapular) in pregnant rats at GD21.

Additional effects of fructose intake on body composition

Relatively little is known about lean mass of rats given fructose. Only one previous study measured the proportion of lean mass in fructose-fed rats and they did not find any differences between rats fed fructose (20 % of calorie intake) and rats fed a chow diet at GD21 and postnatal day 10 (184). Data from our study suggests that regular fructose intake negatively impacts the proportion of lean mass, while it increased the proportion of fat mass at GD21. The weight (g) of lean mass was positively associated with body weight (g). For example, both lean mass (g) and body weight (g) were higher in offspring than in dams at GD21.

Fructose intake and products of conception

In Study B, weights of the fetuses and the placentae were lower in rats fed fructose than CONT. The effects of feeding high-fructose on fetal and placental weights vary among previous studies. Lineker et al. (2015) and Rodriguez et al. (2016) found that maternal consumption of a 10 % (w/v) fructose solution during pregnancy did not change litter size, weights of the fetuses and or placentae on GD20 (163, 179). Alzamendi et al. (2012) showed an increase in fetal weight, but no changes in placental weights resulting in an increase in the fetal:placental weight ratio in rats fed a 10 % (w/v) fructose solution during pregnancy (162). Zhang et al. (2011) reported that rats exposed to a 10 % (w/v) fructose beverage during gestation had a low birth weight (307). It is possible that some of the variation among studies examining fetal and placental weights are due to a lack of reporting these outcomes according to sex, since a few studies showed changes in female but not male offspring (182, 183). Vickers et al. (2011) found that intake of fructose (20 % of caloric intake) did not change fetal weight, but reduced placental weight of female fetuses, which resulted in an increase in the fetal:placental weight ratios at GD21 (303). Vickers et al. suggested that the placentae of a female fetuses may be more sensitive to fructose transfer, growth factors (i.e. IGF) or other placental transporters than the placentae of a male fetuses (303). In the present study, we observed reductions in both fetal and placental weight and no difference in fetal:placental weight ratios between fructose and control groups. Since the sex of the fetuses was not determined in our study, we cannot speculate whether sex-specific differences exist here. This could be further explored in future studies.

The reduction in fetal and placental weights may be related to increased insulin and TG in the mother. Buresova et al. (2006) suggested either insufficient or excess maternal nutritional intake

could result in low birth weights (308). This author found that reduced fetal weights were associated with increased insulin responses following a glucose challenge, elevated serum TG concentrations and hepatic TG content in BXH/HXB RI mice (note: this strain of mouse is used for correlation-based genetic analysis of the "thrifty phenotype" hypothesis) suggesting that this type of metabolic profile could predispose mice to disease (308). Thus, in Study B, reductions in fetal weights in fructose-fed rats may be associated with increased plasma insulin and TG (and maybe glucose) concentrations during pregnancy.

Fructose intake and the placental development

Very little is known about the effects of maternal fructose intake on placental development or structure, thus results from our experiment contribute novel findings to the literature in this area and may help to identify potential targets for future study. We examined aspects of placental morphology (the areas of LZ and JZ and the ratio of these two areas), mRNA expression of key macronutrient transporters (including the physical distribution of GLUT1) and angiogenic factors (as indicators of vascular growth) and oxidative stress in the placenta. These indicators were measured in dams and their pregnant female offspring that were fructose or control-fed in order to examine interactions between fructose feeding and generation on placental development; the independent impact of each of these factors was considered when interactions were not significant.

Fructose intake and the ratio of LZ:JZ areas in the placenta

Two fetal compartments of rodent placenta, labyrinth zone (LZ) and junctional zone (JZ), are known to be responsible for nutrient transfer and hormone secretion during pregnancy (244).

Other investigators have proposed that the area, or volume, of the LZ or JZ could be indicative of the size and distribution of different cell populations (i.e. glycogen cells) or could underlie the nutrient transfer capacity of the placenta (292). We did not observe any differences in the area of the LZ or JZ between experimental groups, nor were there any differences in the LZ:JZ ratio, suggesting that the hormone-secreting and nutrient transfer areas were not adversely affected by the fructose treatment and do not differ between generations. Coan et al. (2011) note that the LZ volume (as measured by histology) was highest in placentas from pregnant female mice that received a 23 % (w/w) protein diet compared to mice fed 9 % (w/w) and 18 % (w/w) protein diets while the JZ volume was lowest in mice fed 9 % protein compared to mice fed 18 % (w/w) and 23 % (w/w) protein diets (292). Interestingly, mice fed 18 % protein had the lowest LZ volume and a lower LZ:JZ ratio compared with the other two groups (292). Other groups (186, 303) found that the LZ area was reduced in rats fed an obesogenic diet (30 % of kcal from fat, 36 % of kcal from simple sugars) compared to a control diet (11 % fat, 7 % simple sugars). Together these observations suggest that exposure to either high or low protein intakes or high fat intake during pregnancy may induce the placenta to adapt, presumably to maintain critical processes including endocrine secretion, nutrient exchange to the fetus, and maternal metabolic adaptations to pregnancy (292). Thus, while changes in protein intake may change the structure of the placenta, high fructose feeding alone does not appear to do so.

Fructose intake, the expression of placental nutrient transporters (GLUT1, SNAT2, FABP1) and fetal growth

GLUT1 is the primary glucose transporter in the placenta of humans and rodents in late pregnancy (190, 231, 309, 310), although it remains controversial whether placental GLUT1

expression is directly associated with fetal growth (258, 311 312, 313, 314). *In vitro* studies suggest that high glucose concentrations reduce glucose uptake in human term trophoblast cells by reducing GLUT1 expression and by reducing GLUT1 translocation to the cell surface of the cells (315, 316). Thus, a reduction in expression of GLUT1 under conditions of high glucose concentrations may limit glucose transfer to the fetus and could contribute to fetal growth restriction (163). In the current study, GLUT1 expression and distribution in the placentae were examined by ISH. The results showed no changes in GLUT1 expression in the placentae of rats fed fructose compared to rats fed CONT. The results from Study B were different from previous findings in our lab in which there were no differences in fetal weight between rats given a 10 % fructose solution only during prenancy (ie. Not prior to, and during, pregnancy), but GLUT1 expression in whole placenta homogenates was reduced. Reasons for the differences between these studies might be that GLUT1 expression is affected by the pre-pregnancy or very early pregnancy metabolic state of the mother and this question deserves further study. If this was the case, it could have implications for the types of placental changes to examine in women with pre-existing insulin resistance, Type 2 diabetes, or hyperlipidemia compared to women who develop gestational diabetes. It is also important to consider that the methods used to examine GLUT1 expression could have contributed to the differences between these studies. Further study of placental glucose transporters would help to better elucidate the association between these transporters and fetal growth.

SNAT2 is an amino acid transporter, and its expression was lower in Dams-FR compared with Dams-CONT but not different between Offspring in the FR and CONT groups. Several investigators have suggested that reductions in SNAT2 expression might be a cause of lower

birth weights (239) but given the lower fetal weights without reductions in SNAT2 in the Offspring-FR, other factors must also contribute to fetal weights at term. Sferruzzi-Perri et al. (2015) reported that fetal weights from dams fed a high fat/high sugar diet were not different from controls and placental expression of SNAT2 was also not different between the diet intervention and control rats (186). The expression of SNAT2 might be sensitive to the timing in changes of nutrient availability thus could have contributed to the differences between offspring and dams. Further work is needed to better understand the nutritional regulation of this key placental transporter.

FABP1 expression in the placenta was significantly increased in rats fed the fructose solution compared to rats fed CONT. While FABP1 is clearly important in transporting fatty acids from mother to the placenta and fetus (231), it may also have a role to play in other processes related to development. In the liver, functions of FABP1 include fatty acid uptake, regulating lipid metabolism and cellular signaling pathways (317). The impact of increased placental FABP1 that occurs with FR feeding remains to be clarified.

Fructose intake and oxidative stress

In Study B, oxidative stress was measured in the placentae by analyzing the following markers: DHE as an indicator of superoxide production, DHR as an indicator of peroxynitrite production, TBARS as an indicator of lipid peroxidation and α-tocopherol as an indicator of antioxidant status. We contrasted these measures in the placenta with assessment of α-tocopherol and TBARS in the liver. Based on previous studies, we hypothesized that fructose intake might reduce antioxidant status in the placenta and liver since a number of previous studies showed that fructose feeding increased production of ROS and reduced the activity of antioxidant enzymes in

plasma and the liver of fructose-fed rats (59, 66, 67, 68, 69). Interestingly, levels of DHE, DHR, TBARS and α-tocopherol in the placentae were not different between diet groups or between generations, while in the liver, α-tocopherol concentrations were reduced in FR groups, and there was a suggestion that oxidative damage (TBARS) was increased in Offspring FR compared with other groups (Generation x Diet, *P*0.089). These results suggest that the placenta may be somewhat protected from low α-tocopherol status, even when liver α-tocopherol is low. No other studies to date have examined oxidative stress and fructose feeding in pregnancy, although a few have shown a positive relationship between the level of oxidative stress resulting from maternal high fat intake in the placenta (256). Saad et al. (2016) showed that intake of a high-fat diet (known as an obesogenic diet) like a high fructose diet, increased the level of oxidative stress by increasing GPX and decreasing glutathione S transferase in the placentae of obese rats (256). In humans, the placentae of obese women had significantly increased concentrations of nitrotyrosine compared to those from lean and overweight women at delivery (255). However, no differences were found in the levels of antioxidant enzyme activities of SOD and GPX (255).

There could be additional reasons for why the placentae of fructose-fed rats did not have extensive changes in oxidative stress. For example, the levels of both pro-oxidants and antioxidants might not be changed because the placenta may control the level of ROS production better than other tissues. However, this should be supported by further studies examining placental mechanisms to limit oxidative stress relative to other tissue such as the liver.

In the liver of fructose-fed rats, lipid peroxidation was also not changed, results which differed from previous studies that showed alterations in the level of oxidative stress and antioxidants in blood and the liver in rats (Appendix 1). In these studies, rats that received a diet or solution

containing 25 – 60 % (w/v) fructose had an increased level of TBARS in the liver (a 60 % (w/v) fructose solution for 42 days, 59; a 65 % (w/w) fructose diet for eight weeks, 66). Although treatment duration in those studies was similar to the duration used in Study B (six weeks), fructose concentrations in these previous studies were higher than the diet (fructose solution) in Study B. Thus, intake of a 10 % (w/v) fructose solution in this study may have less effects on oxidative stress in the liver and the placentae than previous studies.

We must also consider whether the absence of increased oxidative stress in placenta from FR groups is due to inappropriate sample preparation. Biomarkers of oxidative stress are sensitive to sample storage and preparation methods. Since decisions to measure these outcomes were initiated after the samples had been collected, storage and preparations methods may not have been optimal for detection of oxidative stress (318). We cannot rule out the possibility that lack of differences observed in the placenta could reflect an artifact or degradation of the indicator molecules (318, 319). In many labs, the TBARS assay is run immediately after sample collection (plasma, serum and urine) and for frozen tissues, samples should be stored at -70 ℃ until analysis to prevent loss of sample oxidation (instruction; Oxford Biomedical Research). It is recommended that frozen samples are analyzed within one to two months after the collection (instruction for TBARS; Cell Biolabs). Although the placentae in this study were stored at -80 ℃ immediately after collection and not exposed to thawing or light before these analyses, they were stored for longer than is recommended. Results of the oxidative stress measurements should be confirmed in studies that have included these measures from the start so as to avoid these potential pitfalls.

Interestingly, in the Offsprings placentae, the levels of peroxynitrite were lower and the

concentration of α-tocopherol was higher than in the Dam placentae. In cell membranes, peroxynitrite rapidly oxidizes α-tocopherols to α-tocopheryl quinone which is a form of α-tocopherol that is not extensively regulated by cellular reducing agents (320). Thus, the level of peroxynitrite is inversely associated with the concentration of α-tocopherol, and this is consistent with our observations in the placentae. It remains unclear as to why placentae from Offspring had a decreased level of peroxynitrite and increased concentrations of α-tocopherol compared with their dams. The extent to which these observations are the result of early programming of antioxidant processes could be of interest to follow up.

Fructose intake, placental angiogenesis and placental vascular development

The final set of analyses conducted in this study relate to angiogenesis within the placenta. Angiogenesis requires initiation by factors such as VEGF-A, PlGF and their receptors (197, 198). Appropriate activation and timing of angiogenesis is essential for embryogenesis (321) and inappropriate angiogenesis resulting from either excessive or insufficient levels of angiogenic factors contributes to immature vessel development (211). Perturbations in angiogenesis reduce the formation of vascular networks, and CD31 is a common biomarker used to measure endothelial cells which represent vascular growth (250). Decreased expression of CD31 is indicative of endothelial dysfunction and vasculopathy (250).

In the current study, mRNA expression of PlGF and VEGFR-1 was not changed, but the expression of VEGF-A was higher and VEGFR-2 was lower in the placentae from fructose-fed rats than rats fed CONT. Placentae from FR rats also had lower CD31 staining than the placentae from CONT rats.

Inappropriate regulation of the VEGF pathway (HIF-VEGF/VEGFR2 signaling pathway) is

known to induce immature vascular structure (199, 200, 205, 322). In other studies, it has been reported that placentae from mice fed a high-fat diet (40 % of total energy intake as fat) in pregnancy have elevated expression of HIF-1α and VEGF-A, but only a 1.3-fold increase in CD31 expression (254). This suggests that vascular development may be delayed in rats with this diet treatment. Thus, the increased expression of VEGF-A and reduced expression of VEGFR-2 observed in placenta from our study may reflect imbalanced regulation of angiogenesis which may contribute to inappropriate vessel growth in the placentae of fructose-fed rats. Lower intensity of CD31 staining has also been observed in term placentae from a mouse model of GDM (GDM was developed by intake of 60 % of the calorie as fat) (250). The authors suggested that the reduction in CD31 staining in GDM mice may have been the result of increased oxidative stress-induced trophoblast loss and endothelial necrosis (250). Similar to our study, GDM in this mouse model also lead to reduced placental weights and reduced fetal weight (250). Thus, in Study B, inappropriate vessel growth in the placenta by maternal fructose intake was associated with a reduction in the weight of the fetuses and the placentae. Although the level of placental oxidative stress was not changed, this may be due to the trophoblast loss and endothelial necrosis. Further investigations are required to determine how maternal fructose intake reduces the weight of the fetuses and the placentae.

A possible mechanism between the level of oxidative stress and angiogenesis in the placenta

It is known that both exogenous and intracellular ROS stimulate VEGF. Increased VEGF further stimulates ROS production via activation of NADPH oxidase (321, 323). VEGFR-2 is known to be increased by components of NADPH oxidase, Nox1, via ROS production in endothelial cells of tumor vessels (324) and positively associated with the production of oxidation by oxidized

phospholipids through HIF/VEGF pathway (325). In addition, α-tocopheryl phosphate also stimulates Pl3K/Akt signaling pathway that increases HIF-1α/PGC-1α, leading to an increased expression of VEGF (224). Thus, in Study B, we expected to see alterations in both oxidative stress (superoxide, peroxynitrite, lipid peroxidation, α-tocopherols) and angiogenic factors (VEGF-A, PlGF, VEGFR-1, VEGFR-2).

In Study B, however, fructose intake altered the expression of VEGF-A and VEGFR-2 only, and there was no change in the level of oxidants and a specific antioxidant in the placenta. This may suggest that measuring accurate levels of oxidants was limited in our samples as discussed earlier or that placental angiogenesis is affected by other factors such as other antioxidant enzymes and the VEGF-independent signaling pathway rather than the level of superoxide, peroxynitrite, lipid peroxidation and α-tocopherols. Indeed, there are a number of factors involved in stress-activated signaling pathways (i.e. VEGF/VEGFR-2 signaling pathway). For example, the expression of VEGFR-2 is associated with the production of oxidation by oxidized phospholipids through HIF/VEGF pathway (325). VEGFR-2 is also increased by components of NADPH oxidase, Nox1, via ROS production in endothelial cells (324). α-tocopheryl phosphate stimulates Pl3K/Akt signaling pathway that increases HIF-1α/PGC-1α, leading to an increased expression of VEGF (224). Increased VEGF further stimulates ROS production via activation of NADPH oxidase (321, 323). Thus, it is hard to fully analyze the level of oxidative stress in the placenta by measuring only a few factors. Future studies are needed to analyze more oxidants and antioxidant enzymes that are related to both oxidative stress and angiogenesis in the placenta. This may help our understanding of alterations in stress-induced placental vascular development in rats fed fructose.

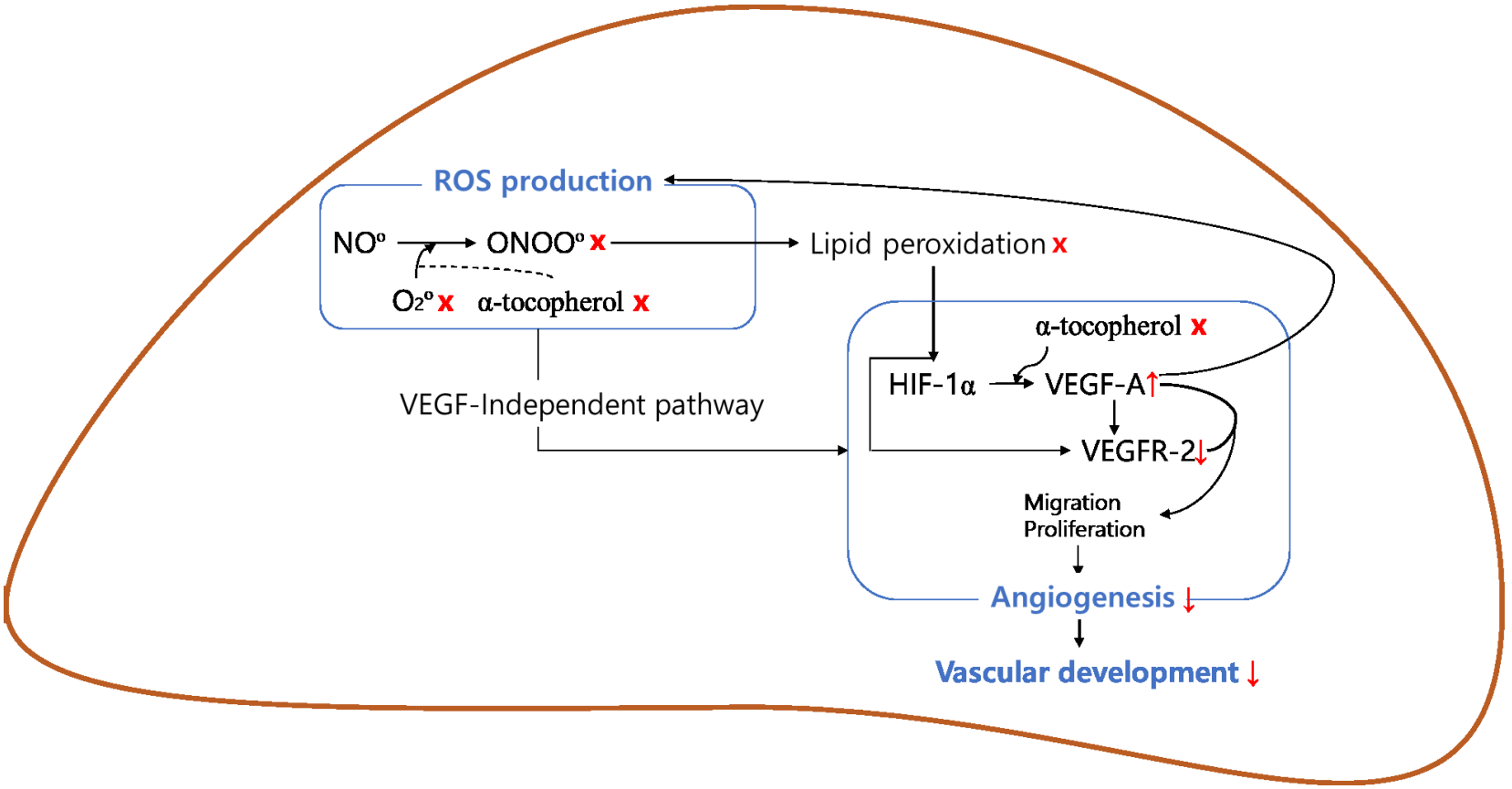


Figure 5-1: Possible mechanisms that are involved in oxidative stress, angiogenesis and vascular development in the placenta.

NO°, Nitric oxide; O_2^{-}, Superoxide anion; $ONOO^{-}$, Peroxynitrite; Pathway was drawn based on the information from previous studies (224, 321, 323, 324, 325); Red arrows and x are the results found from Study B. This figure explains a possible relationship among the factors mentioned above. Study B does not provide information about a cause and effect relationship among the factors.

Study C. The effects of high fructose intake on metabolic profiles of female rats during two sequential pregnancies

There has been no study to assess alterations in maternal physiology by diets during two sequential pregnancies and Study C is the first to examine the effects of fructose intake on two sequential pregnancies. The goal of this study was to test whether alterations in metabolic profiles and body composition by fructose intake were aggravated in rats during second pregnancy compared with their first pregnancy. As shown in Study A and Study B, fructose intake increased plasma glucose, insulin and TG concentrations and increased the proportion of fat mass in rats during late pregnancy. During the second pregnancy, body weights were heavier than first pregnancy. Plasma glucose concentration during mid pregnancy only, insulin concentrations during pregnancy and TG concentrations during mid pregnancy only were higher during the second pregnancy than the first pregnancy. However, fructose did not impact these effects to any greater degree in the second pregnancy compared to first pregnancy. A summary of statistical analysis is shown in Table 5-3, and all of these observations are discussed in more detail below.

Table5-3: A summary table of statistical analyses in Study C

	Interaction (Diet x Pregnancy)	Main effect of diet (CONT vs. FR)	Main effect of pregnancy (1^{st} preg vs. 2^{nd} preg)
Body weight	0.6750	0.0609	**<0.0001**
Metabolic profiles			
Plasma glucose	0.5264	**0.0062**	0.1011
Plasma insulin	0.9325	**0.0020**	**0.0182**
Plasma TG	0.7572	**0.0070**	0.2481
Body composition at GD21			
Total fat mass (%, relative to body weight)	0.7278	**0.0046**	0.2085
Total lean mass (%, relative to body weight)	0.6398	**0.0016**	0.4847
Fat mass (g)	0.7169	**0.0137**	**0.0123**
Lean mass (g)	0.2618	0.3530	**0.0023**
Organ weights at GD21			
Pancreas	0.6432	0.4349	**0.0504**
Liver	0.8099	0.3753	0.9882

It was not surprising that intake of a 10 % (w/v) fructose solution increased glucose, insulin and TG concentrations in plasma and increased the proportion of fat mass during pregnancy in both first and second pregnancies as has been discussed earlier in this thesis.

The proportion of lean mass was lower and total fat mass was higher in rats fed the fructose solution compared to rats fed control in both first and second pregnancy. However, those did not differ between the first and second pregnancy.

An increase in plasma insulin concentrations and body weights during second pregnancy may increase the chances of adverse pregnancy outcomes. However, there has been no previous study

that investigated physical and metabolic changes during two sequential pregnancies in response to increased fructose feeding. Only a few studies have examined the the risk of pregnancy complications in sequential pregnancies. Daltveit et al. (2008) conducted a cohort study and found that a cesarean delivery at the first birth increased the risk of complications including preeclampsia (OR 2.9; CI 2.8 – 3.1), SGA (OR 1.5; CI 1.4 – 1.5), placenta previa (OR 1.5; CI 1.3 – 1.8), placental abruption (OR 2.0; CI 1.8 – 2.2) and uterine rupture (OR 37.4; CI 24.9 – 56.2) (342) in the next pregnancy. Sibai et al (1995), found that women who had chronic hypertension during their previous pregnancies had an increased rate of preeclampsia (75%), preterm delivery (80%), IUGR (45%) and perinatal death (40%) in future pregnancies (343). These studies indicated that adverse maternal conditions during a previous pregnancy are associated with increased risk for complicatons in subsequent pregnancies. These interesting finding suggest that it is important to evaluate the role that nutrition may play in the expression of these complications in animal models. No other study to date has investigated whether alterations in maternal physiology by diets during previous pregnancy are aggravated in their subsequent pregnancies. The preliminary nature of our findings and the relatively small sample size in Study C makes it difficult to interpret the current findings. Larger numbers of animals in each of these groups would help to identify relationships between important metabolic variables.

Limitations of Studies and Future Directions

Several limitations of this study should be noted. Previous studies have supported the idea that fetal programming is affected by exposure to environmental factors, including maternal nutrition, in utero. However, a number of other studies have also proposed that environmental factors in postnatal life can affect the developmental programming of offspring (326). In Study B, dams

began their assigned treatment prior to pregnancy and continued it during pregnancy and lactation. Thus, it is not clear whether the hypertriglyceridemia observed in pregnant offspring was the result of maternal fructose intake before or during pregnancy or during lactation or a combination of these. From a practical point of view, strict delineation of these specific time points may not be important since women do not generally make abrupt changes to their nutritional intake or status during pregnancy and lactation. Such studies are important from a mechanistic perspective since they can help identify pathways that are affected and potential areas for effective interventions. Future studies could use a cross-fostering model (i.e. in which half of offspring from fructose-fed dams are nursed by a dam provided with distilled water, and half of offspring from dams fed CONT are nursed by a dam provided with fructose) to provide a clearer understanding of whether the timing of the fructose exposure during pregnancy or lactation alone has the potential to disrupt lipid homeostasis in the offspring.

A second consideration is that rats were delivered from a commercial breeding facility (Charles River Canada, Montreal, Quebec) at seven weeks of age. Transportation is undoubtedly stressful to these animals and can result in lower food and fluid intake during the days spent in transport. This may contribute to differences in body weights betwen dams and their offspring at the start of the study and the intervention. Castelhano-Carlos and Baumans (2009) summarized the impact of environmental factors on the behavior and the health of laboratory rats (327). In this review, the authors noted that environmental factors such as cage size, CO_2 levels, light, sounds, temperature and transportation can interfere with the homeostasis of the animals. In particular, transportation can cause acute stress responses such as hormonal changes, and increases in hemoglobin and plasma metabolites (327). We did not measure any indicators of physiological

stress in the animals that were delivered from another facility. These rats were given one week to acclimate to the local animal housing facilities, which is standard practice, but may not be enough time to adjust and rat dams may have been more stressed at eight weeks of age compared with offspring of the same age that were born in the local animal facility. Subtle differences in the effects of the external environment on animals and their responses to dietary interventions could contribute to variation in results in different labs or within a lab setting if the protocol changes slightly or if even the same strain of rats have to be sourced from different locations (i.e. within an institution or from a commercial supplier). Thus, future studies may consider using rats all bred and raised within the same facility to examine this phenomenon more broadly. For this reason, Dunn and Bale (2011) used second and third generations of mice to examine the effects of maternal high-fat diet in body size and insulin sensitivity across two generations. The authors indicate that this procedure avoids exposure of the first generation's germ cells to unnecessary environmental stressors (296).

Third, rats given the fructose solution reduced the calories they consumed from solid food. Purina 5001 is a complete chow, containing required amounts of micronutrients to support rodent growth and health. Reduced intake of solid food also resulted in reduced intake of all micronutrients (vitamins and minerals) among the rats in the fructose group. A detailed analysis of nutritional status was beyond the scope of these studies, although detailed analyses of plasma protein and hepatic vitamin E status were undertaken. Overall, all rats in this study appeared to be healthy, grew normally, and supported pregnancies that resulted in a normal number of pups. We must consider the possibility, however, that the effects that we are attributing to fructose intake during pregnancy could be due to increased fructose intake and lowered intake or status of

important micronutrients. Future studies should consider providing additional micronutrients to fructose-fed rats to avoid the potential for confounding.

The expression of nutrient transporters and angiogenic factors in the placenta were measured by qPCR. However, measurement of mRNA expression by qPCR does not represent the level of protein expression. The correlation between gene and protein expressions varies, depending on the system (328). There are many processes between mRNA transcription and protein translation, and directly inferring the level of protein expression from mRNA expression directly is not possible. In Study B, we only measured mRNA expressions of a few genes in the placenta, but future studies should consider measuring protein levels as well as their distribution to gain further insight into the process of placental development and function.

CHAPTER 6: GENERAL CONCLUSION

High fructose intake is recognized as a potential health concern as daily sugar consumption from SSB and soft drinks has increased significantly over several decades (7). Epidemiological evidence suggests that high consumption of fructose, in part due to intake of SSB has been associated with increased incidence of disrupted glucose homeostasis, including development of type 2 diabetes, dyslipidemia, weight gain and obesity among men and non-pregnant women (20, 293, 294, 295). The extent to which the effects of fructose seen in non-pregnant animal models and humans now extend to pregnancy has been the subject of a number of recent studies. Pregnancy is a sensitive period of growth and development for both the mother and developing fetus, with the potential to influence long-term health. The concept of early life programming, also known as the DOHaD hypothesis, suggests that a less than optimal maternal dietary intake in pregnancy, and even prior to pregnancy, can lead to deleterious effects on fetal development, with lasting adverse effects on the risk of disease in the offspring in later life (296). The effects of high intakes of refined carbohydrates, in particular fructose, is now coming to the forefront.

In this research, we found that fructose intake altered metabolic profiles (insulin and TG concentrations) in both non-pregnant and pregnant rats (Study A). In adult offspring of fructose-fed dams who were themselves fed fructose, hypertriglyceridemia was seem to be more aggravated than in their dams, which may suggest that maternal fructose intake during pregnancy adversely influences fetal programming of lipid synthesis (Study B). Fructose intake also adversely affected placental development. For example, fatty acid transporter gene expression was increased, and markers of angiogenesis and the vascular network were reduced in the placentae from fructose-fed rats during late pregnancy (Study B). These alterations may

contribute to lower fetal and placental weights and adversely influence developmental programming of the fetus. Lastly, Study C found significant changes in body weights, plasma insulin and TG concentrations, and body composition during second pregnancies compared to first pregnancies, but those alterations were not worsened by consuming the fructose solution during second pregnancies.

These three studies add a number of novel observations to the literature and strengthen our understanding of potential adverse effects of fructose during pregnancy on dams and offspring. Some of the strengths of these studies include the following: Firstly, body weights and metabolic profiles of rats were recorded weekly during the experiment and changes due to diet and generation were explored; changes over time were considered in instances where the data were available. Important outcomes were measured during pregnancy, including oral glucose tolerance, body composition and pregnancy outcomes. This study, therefore, provides one of the most extensive characterizations of how fructose intake affects maternal metabolic health prior to and during pregnancy. This level of detail is critical to advance our understanding of what metabolic changes may or may not be important in developmental programming. For example, there is a large body of literature exploring the lasting effects of maternal gestational diabetes (and changes in glycemic control) on offspring, but relatively little is known about the effects of changes in maternal lipid metabolism. Results from this study indicate that glucose and insulin responses to fructose feeding were a result of the dietary treatment but were similar between dams and offspring. Lipid metabolism, however, appears to worsen in offspring whose mothers had hypertriglyceridemia, and suggests that future studies should examine this area more closely.

Secondly, rats were provided fructose in a liquid form (a 10 % (w/v) fructose solution) with *ad*

libitum chow. Much of the existing data suggests that overconsumption of fructose from SSB and HFCS may play a critical contributing role in the epidemic of obesity and related chronic diseases (7). Elliott et al. (2002) also suggested that a liquid form of fructose may have more adverse effects on body weight than a solid source of fructose since humans do not compensate for their energy intake by reducing food intake when they consume fructose as liquid (6).

In conclusion, this research supports the suggestion that high fructose intake induces adverse effects on maternal and offspring health. In addition, this thesis indicates that maternal diet manipulation (high fructose solution in this study) has adverse impacts on their metabolic profile and placental development, and that those have long-term adverse effects on offspring in later life. These findings may have implications for humans consuming high fructose, and may help to understand the effects of maternal diets on fetal programming that can increase the risk of developing diseases in offspring in later life. Further studies are needed to establish underlying mechanisms of how maternal high fructose intake is associated with alterations in feto-placental unit and to further explore additional long-term influences on offspring in adult life.

REFERENCES

1. Hanover, L. M., & White, J. S. (1993). Manufacturing, composition, and applications of fructose. *The American Journal of Clinical Nutrition, 58*(5 Suppl), 724S-732S.

2. Fan, A. M. (1987), L. O. Nabors and R. C. Gelardi (eds). Alternative Sweeteners. Marcel Dekker, New York, 1986. 355 pp. £64.75 Hard cover. J. Appl. Toxicol., 7: 150. doi: 10.1002/jat.2550070216

3. Vos, M. B., Kimmons, J. E., Gillespie, C., Welsh, J., & Blanck, H. M. (2008). Dietary fructose consumption among US children and adults: The third national health and nutrition examination survey. *Medscape Journal of Medicine, 10*(7), 160.

4. US Department of Agriculture, Economic Research Service. Sugar and Sweeteners Yearbook 2008. Table 30 - US high fructose corn syrup (HFCS) supply and use. Table 51 - Refined cane and beet sugar: estimated number of per capita calories consumed daily, by calendar year. Table 52 - High fructose corn syrup: estimated number of per capita calories consumed daily, by calendar year. Table 53 - Other sweeteners: estimated number of per capita calories consumed daily, by calendar year. Available at: http://www.ers.usda.gov/briefing/sugar/data/data.htm Accessed June 20, 2008.

5. Park, Y. K., & Yetley, E. A. (1993). Intakes and food sources of fructose in the United States. *The American Journal of Clinical Nutrition, 58*(5 Suppl), 737S-747S.

6. Elliott, S. S., Keim, N. L., Stern, J. S., Teff, K., & Havel, P. J. (2002). Fructose, weight gain, and the insulin resistance syndrome. *The American Journal of Clinical Nutrition, 76*(5), 911-922.

7. Bray, G. A., Nielsen, S. J., & Popkin, B. M. (2004). Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. *The American Journal of Clinical Nutrition, 79*(4), 537-543.

8. Brisbois, T. D., Marsden, S. L., Anderson, G. H., & Sievenpiper, J. L. (2014). Estimated intakes and sources of total and added sugars in the canadian diet. *Nutrients,6*(5), 1899-1912. doi:10.3390/nu6051899 [doi]

9. Caudill MA and Stipanuk MH (2012). Biochemical, physiological, and molecular aspects of human nutrition, 3rd edition

10. Kellett, G. L., & Brot-Laroche, E. (2005). Apical GLUT2: A major pathway of intestinal sugar absorption. *Diabetes, 54*(10), 3056-3062. doi:54/10/3056 [pii]

11. Rumessen, J. J., & Gudmand-Hoyer, E. (1986). Absorption capacity of fructose in healthy adults. comparison with sucrose and its constituent monosaccharides. *Gut,27*(10), 1161-1168.

12. Latulippe, M. E., & Skoog, S. M. (2011). Fructose malabsorption and intolerance: Effects of fructose with and without simultaneous glucose ingestion. *Critical Reviews in Food Science and Nutrition, 51*(7), 583-592. doi:10.1080/10408398.2011.566646 [doi]

13. Skoog, S. M., & Bharucha, A. E. (2004). Dietary fructose and gastrointestinal symptoms: A review. *The American Journal of Gastroenterology, 99*(10), 2046-2050. doi:10.1111/j.1572-0241.2004.40266.x [doi]

14. Born, P., Zech, J., Lehn, H., Classen, M., & Lorenz, R. (1995). Colonic bacterial activity determines the symptoms in people with fructose-malabsorption. *Hepato-Gastroenterology, 42*(6), 778-785.

15. Kneepkens, C. M., Vonk, R. J., & Fernandes, J. (1984). Incomplete intestinal absorption of fructose. *Archives of Disease in Childhood, 59*(8), 735-738.

16. Wakil, S. J., & Abu-Elheiga, L. A. (2009). Fatty acid metabolism: Target for metabolic syndrome. *Journal of Lipid Research, 50 Suppl*, S138-43. doi:10.1194/jlr.R800079-JLR200 [doi]

17. Tordoff, M. G., & Alleva, A. M. (1990). Effect of drinking soda sweetened with aspartame or high-Fructose corn syrup on food intake and body weight. *The American Journal of Clinical Nutrition, 51*(6), 963-969.

18. Ludwig, D. S., Peterson, K. E., & Gortmaker, S. L. (2001). Relation between consumption of sugar-sweetened drinks and childhood obesity: A prospective, observational analysis. *Lancet (London, England), 357*(9255), 505-508. doi:S0140-6736(00)04041-1 [pii]

19. Vuilleumier, S. (1993). Worldwide production of high-fructose syrup and crystalline fructose. *The American Journal of Clinical Nutrition, 58*(5 Suppl), 733S-736S.

20. Malik, V. S., Pan, A., Willett, W. C., & Hu, F. B. (2013). Sugar-sweetened beverages and weight gain in children and adults: A systematic review and meta-analysis. *The American Journal of Clinical Nutrition, 98*(4), 1084-1102. doi:10.3945/ajcn.113.058362 [doi]

21. Flegal, K. M., Carroll, M. D., Ogden, C. L., & Johnson, C. L. (2002). Prevalence and trends in obesity among US adults, 1999-2000. *Jama, 288*(14), 1723-1727. doi:joc21463 [pii]

22. Tappy, L., & Le, K. A. (2010). Metabolic effects of fructose and the worldwide increase in obesity. *Physiological Reviews, 90*(1), 23-46. doi:10.1152/physrev.00019.2009 [doi]

23. Klurfeld, D. M., Foreyt, J., Angelopoulos, T. J., & Rippe, J. M. (2013). Lack of evidence for high fructose corn syrup as the cause of the obesity epidemic. *International Journal of Obesity (2005), 37*(6), 771-773. doi:ijo2012157 [pii]

24. Forshee, R. A., Storey, M. L., Allison, D. B., Glinsmann, W. H., Hein, G. L., Lineback, D. R., . . . White, J. S. (2007). A critical examination of the evidence relating high fructose corn syrup and weight gain. *Critical Reviews in Food Science and Nutrition, 47*(6), 561-582. doi:780775427 [pii]

25. Bocarsly, M. E., Powell, E. S., Avena, N. M., & Hoebel, B. G. (2010). High-fructose corn syrup causes characteristics of obesity in rats: Increased body weight, body fat and triglyceride levels. *Pharmacology, Biochemistry, and Behavior, 97*(1), 101-106. doi:10.1016/j.pbb.2010.02.012 [doi]

26. Light, H. R., Tsanzi, E., Gigliotti, J., Morgan, K., & Tou, J. C. (2009). The type of caloric sweetener added to water influences weight gain, fat mass, and reproduction in growing sprague-dawley female rats. *Experimental Biology and Medicine (Maywood, N.J.), 234*(6), 651-661. doi:10.3181/0812-RM-368 [doi]

27. Hwang, I. S., Ho, H., Hoffman, B. B., & Reaven, G. M. (1987). Fructose-induced insulin resistance and hypertension in rats. *Hypertension, 10*(5), 512-516.

28. Bell, R. C., Ryan, E. A., & Finegood, D. T. (1996). Consequences of high dietary fructose in the islet-transplanted rat with suboptimal beta-cell mass. *The American Journal of Physiology, 270*(2 Pt 1), E292-8.

29. DiMeglio, D. P., & Mattes, R. D. (2000). Liquid versus solid carbohydrate: Effects on food

intake and body weight. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 24*(6), 794-800.

30. Raben, A., Vasilaras, T. H., Moller, A. C., & Astrup, A. (2002). Sucrose compared with artificial sweeteners: Different effects on ad libitum food intake and body weight after 10 wk of supplementation in overweight subjects. *The American Journal of Clinical Nutrition, 76*(4), 721-729.

31. Havel, P. J. (2002). Control of energy homeostasis and insulin action by adipocyte hormones: Leptin, acylation stimulating protein, and adiponectin. *Current Opinion in Lipidology, 13*(1), 51-59.

32. Saad, M. F., Khan, A., Sharma, A., Michael, R., Riad-Gabriel, M. G., Boyadjian, R., . . . Kamdar, V. (1998). Physiological insulinemia acutely modulates plasma leptin.*Diabetes, 47*(4), 544-549.

33. Grant, A. M., Christie, M. R., & Ashcroft, S. J. (1980). Insulin release from human pancreatic islets in vitro. *Diabetologia, 19*(2), 114-117.

34. Sato, Y., Ito, T., Udaka, N., Kanisawa, M., Noguchi, Y., Cushman, S. W., & Satoh, S. (1996). Immunohistochemical localization of facilitated-diffusion glucose transporters in rat pancreatic islets. *Tissue & Cell, 28*(6), 637-643.

35. Havel, P. J. (2000). Role of adipose tissue in body-weight regulation: Mechanisms regulating leptin production and energy balance. *The Proceedings of the Nutrition Society, 59*(3), 359-371. doi:S0029665100000410 [pii]

36. Teff, K. L., Elliott, S. S., Tschop, M., Kieffer, T. J., Rader, D., Heiman, M., . . . Havel, P. J.

(2004). Dietary fructose reduces circulating insulin and leptin, attenuates postprandial suppression of ghrelin, and increases triglycerides in women. *The Journal of Clinical Endocrinology and Metabolism, 89*(6), 2963-2972. doi:10.1210/jc.2003-031855 [doi]

37. Faeh, D., Minehira, K., Schwarz, J. M., Periasamy, R., Park, S., & Tappy, L. (2005). Effect of fructose overfeeding and fish oil administration on hepatic de novo lipogenesis and insulin sensitivity in healthy men. *Diabetes, 54*(7), 1907-1913. doi:54/7/1907 [pii]

38. Le, K. A., Faeh, D., Stettler, R., Ith, M., Kreis, R., Vermathen, P., . . . Tappy, L. (2006). A 4-wk high-fructose diet alters lipid metabolism without affecting insulin sensitivity or ectopic lipids in healthy humans. *The American Journal of Clinical Nutrition, 84*(6), 1374-1379. doi:84/6/1374 [pii]

39. Hallfrisch, J., Reiser, S., & Prather, E. S. (1983). Blood lipid distribution of hyperinsulinemic men consuming three levels of fructose. *The American Journal of Clinical Nutrition, 37*(5), 740-748.

40. Koo, H. Y., Wallig, M. A., Chung, B. H., Nara, T. Y., Cho, B. H., & Nakamura, M. T. (2008). Dietary fructose induces a wide range of genes with distinct shift in carbohydrate and lipid metabolism in fed and fasted rat liver. *Biochimica Et Biophysica Acta, 1782*(5), 341-348. doi:10.1016/j.bbadis.2008.02.007 [doi]

41. Girard, A., Madani, S., El Boustani, E. S., Belleville, J., & Prost, J. (2005). Changes in lipid metabolism and antioxidant defense status in spontaneously hypertensive rats and wistar rats fed a diet enriched with fructose and saturated fatty acids. *Nutrition (Burbank, Los Angeles County, Calif.), 21*(2), 240-248. doi:S0899-9007(04)00245-X [pii]

42. Le, K. A., Ith, M., Kreis, R., Faeh, D., Bortolotti, M., Tran, C., . . . Tappy, L. (2009). Fructose overconsumption causes dyslipidemia and ectopic lipid deposition in healthy subjects with and without a family history of type 2 diabetes. *The American Journal of Clinical Nutrition, 89*(6), 1760-1765. doi:10.3945/ajcn.2008.27336 [doi]

43. Stanhope, K. L., Schwarz, J. M., Keim, N. L., Griffen, S. C., Bremer, A. A., Graham, J. L., . . . Havel, P. J. (2009). Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *The Journal of Clinical Investigation, 119*(5), 1322-1334. doi:10.1172/JCI37385 [doi]

44. Catena, C., Giacchetti, G., Novello, M., Colussi, G., Cavarape, A., & Sechi, L. A. (2003). Cellular mechanisms of insulin resistance in rats with fructose-induced hypertension. *American Journal of Hypertension, 16*(11 Pt 1), 973-978. doi:S0895706103010021 [pii]

45. Bezerra, R. M., Ueno, M., Silva, M. S., Tavares, D. Q., Carvalho, C. R., & Saad, M. J. (2000). A high fructose diet affects the early steps of insulin action in muscle and liver of rats. *The Journal of Nutrition, 130*(6), 1531-1535.

46. Carvalho, C. R., Bueno, A. A., Mattos, A. M., Biz, C., de Oliveira, C., Pisani, L. P., . . . Oyama, L. M. (2010). Fructose alters adiponectin, haptoglobin and angiotensinogen gene expression in 3T3-L1 adipocytes. *Nutrition Research (New York, N.Y.), 30*(9), 644-649. doi:10.1016/j.nutres.2010.06.012 [doi]

47. Hwang IS, Huang WC, Wu JN, Shian LR, Reaven GM. (1989). Effect of fructose-induced

hypertension on the renin-angiotensin-aldosterone system and atrial natriuretic factor. Am J Hypertens. Jun;2(6 Pt 1):424-7.

48. Reaven GM, Hoffman BB. (1989). Hypertension as a disease of carbohydrate and lipoprotein metabolism. Am J Med. Dec 8;87(6A):2S-6S. Review

49. Reaven GM. (1991). Insulin resistance, hyperinsulinemia, and hypertriglyceridemia in the etiology and clinical course ofhypertension. Am J Med. Feb 21;90(2A):7S-12S.

50. DiNicolantonio, J. J., & O'Keefe, J. H. (2016). Hypertension due to toxic white crystals in the diet: Should we blame salt or sugar? *Progress in Cardiovascular Diseases,* doi:S0033-0620(16)30056-1 [pii]

51. Pilic, L., Pedlar, C. R., & Mavrommatis, Y. (2016). Salt-sensitive hypertension: Mechanisms and effects of dietary and other lifestyle factors. *Nutrition Reviews, 74*(10), 645-658. doi:10.1093/nutrit/nuw028 [doi]

52. Dai S and McNeill JH (1995). Dai S and McNeill JH (1995). Fructose-induced hypertension in rats is concentration- and duration-dependent. *Journal of Pharmacological Toxicological Methods* 33(2), 101-107.in rats is concentration- and duration-dependent. *Journal of Pharmacological Toxicological Methods* 33(2), 101-107.

53. Kamide, K., Rakugi, H., Higaki, J., Okamura, A., Nagai, M., Moriguchi, K., . . . Ogihara, T. (2002). The renin-angiotensin and adrenergic nervous system in cardiac hypertrophy in fructose-fed rats. *American Journal of Hypertension, 15*(1 Pt 1), 66-71. doi:S0895706101022324 [pii]

54. Sanchez-Lozada, L. G., Tapia, E., Bautista-Garcia, P., Soto, V., Avila-Casado, C.,

Vega-Campos, I. P., . . . Johnson, R. J. (2008). Effects of febuxostat on metabolic and renal alterations in rats with fructose-induced metabolic syndrome. *American Journal of Physiology.Renal Physiology, 294*(4), F710-8. doi:10.1152/ajprenal.00454.2007 [doi]

55. Perez-Pozo, S. E., Schold, J., Nakagawa, T., Sanchez-Lozada, L. G., Johnson, R. J., & Lillo, J. L. (2010). Excessive fructose intake induces the features of metabolic syndrome in healthy adult men: Role of uric acid in the hypertensive response. *International Journal of Obesity (2005), 34*(3), 454-461. doi:10.1038/ijo.2009.259 [doi]

56. Brown, C. M., Dulloo, A. G., Yepuri, G., & Montani, J. P. (2008). Fructose ingestion acutely elevates blood pressure in healthy young humans. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 294*(3), R730-7. doi:10.1152/ajpregu.00680.2007 [doi]

57. Jalal, D. I., Smits, G., Johnson, R. J., & Chonchol, M. (2010). Increased fructose associates with elevated blood pressure. *Journal of the American Society of Nephrology : JASN, 21*(9), 1543-1549. doi:10.1681/ASN.2009111111 [doi]

58. Jayalath, V. H., Sievenpiper, J. L., de Souza, R. J., Ha, V., Mirrahimi, A., Santaren, I. D., . . . Jenkins, D. J. (2014). Total fructose intake and risk of hypertension: A systematic review and meta-analysis of prospective cohorts. *Journal of the American College of Nutrition, 33*(4), 328-339. doi:10.1080/07315724.2014.916237 [doi]

59. Lozano, I., Van der Werf, R., Bietiger, W., Seyfritz, E., Peronet, C., Pinget, M., . . . Dal, S. (2016). High-fructose and high-fat diet-induced disorders in rats: Impact on diabetes risk,

hepatic and vascular complications. *Nutrition & Metabolism, 13*, 15-016-0074-1. eCollection 2016. doi:10.1186/s12986-016-0074-1 [doi]

60. Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews, 82*(1), 47-95. doi:10.1152/physrev.00018.2001 [doi]

61. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*(1), 44-84. doi:S1357-2725(06)00219-6 [pii]

62. Bidlack WR. (1996). Interrelationships of food, nutrition, diet and health: the National Association of State Universities and Land Grant Colleges White Paper. J Am Coll Nutr 1996;15:422–33.

63. Djamali, A. (2007). Oxidative stress as a common pathway to chronic tubulointerstitial injury in kidney allografts. *American Journal of Physiology.Renal Physiology, 293*(2), F445-55. doi:00037.2007 [pii]

64. Irshad, M., & Chaudhuri, P. S. (2002). Oxidant-antioxidant system: Role and significance in human body. *Indian Journal of Experimental Biology, 40*(11), 1233-1239.

65. Fisher-Wellman, K. H., & Bloomer, R. J. (2010). Exacerbated postprandial oxidative stress induced by the acute intake of a lipid meal compared to isoenergetically administered carbohydrate, protein, and mixed meals in young, healthy men. *Journal of the American College of Nutrition, 29*(4), 373-381. doi:29/4/373 [pii]

66. Delbosc, S., Paizanis, E., Magous, R., Araiz, C., Dimo, T., Cristol, J. P., . . . Azay, J. (2005). Involvement of oxidative stress and NADPH oxidase activation in the development of

cardiovascular complications in a model of insulin resistance, the fructose-fed rat. *Atherosclerosis, 179*(1), 43-49. doi:S0021-9150(04)00556-8 [pii]

67. Zhou, K., Kumar, U., Yuen, V. G., & McNeill, J. H. (2012). The effects of phentolamine on fructose-fed rats. *Canadian Journal of Physiology and Pharmacology, 90*(8), 1075-1085. doi:10.1139/y2012-063 [doi]

68. P ZAGRODZKI (2007). High fructose model of oxidative stress and metabolic disturbances in rats. Part1. Antioxidant status of rats tissues, 2007

69. Faure, P., Barclay, D., Joyeux-Faure, M., & Halimi, S. (2007). Comparison of the effects of zinc alone and zinc associated with selenium and vitamin E on insulin sensitivity and oxidative stress in high-fructose-fed rats. *Journal of Trace Elements in Medicine and Biology : Organ of the Society for Minerals and Trace Elements (GMS), 21*(2), 113-119. doi:S0946-672X(07)00005-3 [pii]

70. Herrera, E., & Barbas, C. (2001). Vitamin E: Action, metabolism and perspectives. *Journal of Physiology and Biochemistry, 57*(2), 43-56.

71. Tan, E. K., & Tan, E. L. (2013). Alterations in physiology and anatomy during pregnancy. *Best Practice & Research. Clinical Obstetrics & Gynaecology, 27*(6), 791-802. doi:10.1016/j.bpobgyn.2013.08.001 [doi]

72. Susan Blackburn. Maternal, Fetal & Neonatal Physiology, 4th Edition.

73. Irani, R. A., & Xia, Y. (2008). The functional role of the renin-angiotensin system in pregnancy and preeclampsia. *Placenta, 29*(9), 763-771. doi:10.1016/j.placenta.2008.06.011 [doi]

74. Bernstein, I. M., Ziegler, W., & Badger, G. J. (2001). Plasma volume expansion in early pregnancy. *Obstetrics and Gynecology, 97*(5 Pt 1), 669-672. doi:S0029784400012229 [pii]

75. Hytten, F. (1985). Blood volume changes in normal pregnancy. *Clinics in Haematology, 14*(3), 601-612.

76. San-FRutos, L., Engels, V., Zapardiel, I., Perez-Medina, T., Almagro-Martinez, J., Fernandez, R., & Bajo-Arenas, J. M. (2011). Hemodynamic changes during pregnancy and postpartum: A prospective study using thoracic electrical bioimpedance. *The Journal of Maternal-Fetal & Neonatal Medicine : The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, 24*(11), 1333-1340. doi:10.3109/14767058.2011.556203 [doi]

77. Thornburg, K. L., Jacobson, S. L., Giraud, G. D., & Morton, M. J. (2000). Hemodynamic changes in pregnancy. *Seminars in Perinatology, 24*(1), 11-14.

78. Kallen, C. B. (2004). Steroid hormone synthesis in pregnancy. *Obstetrics and Gynecology Clinics of North America, 31*(4), 795-816, x. doi:S0889-8545(04)00095-6 [pii]

79. Speroff L., Glass R.H., Kase N.G.. Clinical Gynecologic Endocrinology and Infertility, 6th edition.

80. Norwitz, E. R., Schust, D. J., & Fisher, S. J. (2001). Implantation and the survival of early pregnancy. *The New England Journal of Medicine, 345*(19), 1400-1408. doi:10.1056/NEJMra000763 [doi]

81. Hart, M. V., Hosenpud, J. D., Hohimer, A. R., & Morton, M. J. (1985). Hemodynamics during pregnancy and sex steroid administration in guinea pigs. *The American Journal of*

Physiology, 249(2 Pt 2), R179-85.

82. Lopez-Jaramillo, P., Diaz, L. A., Pardo, A., Parra, G., Jaimes, H., & Chaudhuri, G. (2004). Estrogen therapy increases plasma concentrations of nitric oxide metabolites in postmenopausal women but increases flow-mediated vasodilation only in younger women. *Fertility and Sterility, 82*(6), 1550-1555. doi:S0015-0282(04)02350-7 [pii]

83. Joseph A Majzoub (2006). Corticotropin-releasing hormone physiology. *Eur J Endocrinol* 155, 71–S76.

84. Duthie, L., & Reynolds, R. M. (2013). Changes in the maternal hypothalamic-pituitary-adrenal axis in pregnancy and postpartum: Influences on maternal and fetal outcomes. *Neuroendocrinology, 98*(2), 106-115. doi:10.1159/000354702 [doi]

85. Zavalza-Gomez, A. B., Anaya-Prado, R., Rincon-Sanchez, A. R., & Mora-Martinez, J. M. (2008). Adipokines and insulin resistance during pregnancy. *Diabetes Research and Clinical Practice, 80*(1), 8-15. doi:10.1016/j.diabres.2007.12.012 [doi]

86. Ramos, M. P., Crespo-Solans, M. D., del Campo, S., Cacho, J., & Herrera, E. (2003). Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness. *American Journal of Physiology.Endocrinology and Metabolism, 285*(2), E318-28. doi:10.1152/ajpendo.00456.2002 [doi]

87. Seely EW, Solomon CG. (2003). Insulin resistance and its potential role in pregnancy-induced hypertension. J Clin Endocrinol Metab, 88(6):2393-8.

88. Cietak, K. A., & Newton, J. R. (1985). Serial quantitative maternal nephrosonography in pregnancy. *The British Journal of Radiology, 58*(689), 405-413.

doi:10.1259/0007-1285-58-689-405 [doi]

89. Gabbe SG, Niebyl JR, Simpson JL (2007). Obstetrics: normal and problem pregnancies, 5th edition.

90. Fried, A. M., Woodring, J. H., & Thompson, D. J. (1983). Hydronephrosis of pregnancy: A prospective sequential study of the course of dilatation. *Journal of Ultrasound in Medicine : Official Journal of the American Institute of Ultrasound in Medicine, 2*(6), 255-259.

91. Yeomans, E. R., & Gilstrap, L. C.,3rd. (2005). Physiologic changes in pregnancy and their impact on critical care. *Critical Care Medicine, 33*(10 Suppl), S256-8. doi:00003246-200510001-00003 [pii]

92. Izci, B., Vennelle, M., Liston, W. A., Dundas, K. C., Calder, A. A., & Douglas, N. J. (2006). Sleep-disordered breathing and upper airway size in pregnancy and post-partum. *The European Respiratory Journal, 27*(2), 321-327. doi:27/2/321 [pii]

93. Dzieciolowska-Baran, E., Teul-Swiniarska, I., Gawlikowska-Sroka, A., Poziomkowska-Gesicka, I., & Zietek, Z. (2013). Rhinitis as a cause of respiratory disorders during pregnancy. *Advances in Experimental Medicine and Biology, 755*, 213-220. doi:10.1007/978-94-007-4546-9_27 [doi]

94. Astbury, S., Mostyn, A., Symonds, M. E., & Bell, R. C. (2015). Nutrient availability, the microbiome, and intestinal transport during pregnancy. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition Et Metabolisme, 40*(11), 1100-1106. doi:10.1139/apnm-2015-0117 [doi]

95. Cripps, A. W., & Williams, V. J. (1975). The effect of pregnancy and lactation on food intake,

gastrointestinal anatomy and the absorptive capacity of the small intestine in the albino rat. *The British Journal of Nutrition, 33*(1), 17-32. doi:S0007114575000062 [pii]

96. Sabet Sarvestani, F., Rahmanifar, F., & Tamadon, A. (2015). Histomorphometric changes of small intestine in pregnant rat. *Veterinary Research Forum : An International Quarterly Journal, 6*(1), 69-73.

97. Parry, E., Shields, R., & Turnbull, A. C. (1970). Transit time in the small intestine in pregnancy. *The Journal of Obstetrics and Gynaecology of the British Commonwealth, 77*(10), 900-901.

98. Broussard, C. N., & Richter, J. E. (1998). Nausea and vomiting of pregnancy. *Gastroenterology Clinics of North America, 27*(1), 123-151.

99. Hytten F, Chamberlain G. Clinical physiology in obstetrics. Oxford, United Kingdom: Blackwell Scientific Publications, 1980.

100. King, J. C. (2000). Physiology of pregnancy and nutrient metabolism. *The American Journal of Clinical Nutrition, 71*(5 Suppl), 1218S-25S.

101. Institute of Medicine (US) and National Research Council (US) Committee to Reexamine IOM Pregnancy Weight Guidelines. (2009). doi:NBK32813 [bookaccession]

102. Cnattingius, S., Bergstrom, R., Lipworth, L., & Kramer, M. S. (1998). Prepregnancy weight and the risk of adverse pregnancy outcomes. *The New England Journal of Medicine, 338*(3), 147-152. doi:10.1056/NEJM199801153380302 [doi]

103. Stotland, N. E., Cheng, Y. W., Hopkins, L. M., & Caughey, A. B. (2006). Gestational weight

gain and adverse neonatal outcome among term infants. *Obstetrics and Gynecology, 108*(3 Pt 1), 635-643. doi:108/3/635 [pii]

104. Begum, F., Colman, I., McCargar, L. J., & Bell, R. C. (2012). Gestational weight gain and early postpartum weight retention in a prospective cohort of alberta women. *Journal of Obstetrics and Gynaecology Canada : JOGC = Journal d'Obstetrique Et Gynecologie Du Canada : JOGC, 34*(7), 637-647.

105. Mills, J. L., Jovanovic, L., Knopp, R., Aarons, J., Conley, M., Park, E., . . . Metzger, B. (1998). Physiological reduction in fasting plasma glucose concentration in the first trimester of normal pregnancy: The diabetes in early pregnancy study. *Metabolism: Clinical and Experimental, 47*(9), 1140-1144. doi:S0026-0495(98)90290-6 [pii]

106. Pedro Rosso (1995) Nutrition and Metabolism in Pregnancy: Mother and Fetus.

107. Herrera, E. (2002). Lipid metabolism in pregnancy and its consequences in the fetus and newborn. *Endocrine, 19*(1), 43-55. doi:ENDO:19:1:43 [pii]

108. Gernand, A. D., Schulze, K. J., Stewart, C. P., West, K. P.,Jr, & Christian, P. (2016). Micronutrient deficiencies in pregnancy worldwide: Health effects and prevention. *Nature Reviews.Endocrinology, 12*(5), 274-289. doi:10.1038/nrendo.2016.37 [doi]

109. Steegers-Theunissen, R. P., Twigt, J., Pestinger, V. & Sinclair, K. D. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. Hum. Reprod. Update 19, 640–655 (2013).

110. Sinclair, K. D., Allegrucci, C., Singh, R., Gardner, D. S., Sebastian, S., Bispham, J., . . . Young, L. E. (2007). DNA methylation, insulin resistance, and blood pressure in offspring

determined by maternal periconceptional B vitamin and methionine status. *Proceedings of the National Academy of Sciences of the United States of America, 104*(49), 19351-19356. doi:0707258104 [pii]

111. Pharoah, P., Buttfield, I. H., & Hetzel, B. S. (2012). Neurological damage to the fetus resulting from severe iodine deficiency during pregnancy. *International Journal of Epidemiology, 41*(3), 589-592. doi:10.1093/ije/dys070 [doi]

112. Potter BJ, Mano MT, Belling GB, Mclntosh GH, Cragg BG, Marshall J, Wellby ML, Hetzel BS. (1982). Retarded fetal brain development resulting from severe dietary iodine deficiency in sheep. Neuropathol Appl Neurobiol, 8(4):303-13.

113. McCann, J. C., & Ames, B. N. (2007). An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. *The American Journal of Clinical Nutrition, 85*(4), 931-945. doi:85/4/931 [pii]

114. Wong, E. M., Sullivan, K. M., Perrine, C. G., Rogers, L. M. & Pena-Rosas, J. P. Comparison of median urinary iodine concentration as an indicator of iodine status among pregnant women, school-age children, and nonpregnant women. *Food Nutr. Bull.* **32**, 206–212 (2011).

115. Prado, E. L., & Dewey, K. G. (2014). Nutrition and brain development in early life. *Nutrition Reviews, 72*(4), 267-284. doi:10.1111/nure.12102 [doi]

116. Gagne, A., Wei, S. Q., Fraser, W. D., & Julien, P. (2009). Absorption, transport, and bioavailability of vitamin e and its role in pregnant women. *Journal of Obstetrics and Gynaecology Canada : JOGC = Journal d'Obstetrique Et Gynecologie Du Canada :*

JOGC, 31(3), 210-217.

117. Leger CL, Dumontier C, Fouret G, Boulot P, Descomps B. A short-term supplementation of pregnant women before delivery does not improve significantly the vitamin E status of neonates-low efficiency of the vitamin E placental transfer. Int J Vitam Nutr Res 1998;68:293–9.

118. Borel, P., Preveraud, D., & Desmarchelier, C. (2013). Bioavailability of vitamin E in humans: An update. *Nutrition Reviews, 71*(6), 319-331. doi:10.1111/nure.12026 [doi]

119. Hacquebard, M., & Carpentier, Y. A. (2005). Vitamin E: Absorption, plasma transport and cell uptake. *Current Opinion in Clinical Nutrition and Metabolic Care, 8*(2), 133-138. doi:00075197-200503000-00005 [pii]

120. Saldeen, T., Li, D., & Mehta, J. L. (1999). Differential effects of alpha- and gamma-tocopherol on low-density lipoprotein oxidation, superoxide activity, platelet aggregation and arterial thrombogenesis. *Journal of the American College of Cardiology, 34*(4), 1208-1215. doi:S0735-1097(99)00333-2 [pii]

121. Jishage K-i, Tachibe T, Ito T, Shibata N, Suzuki S, Mori T, et al. (2005) Vitamin E is essential for mouse placentation but not for embryonic development itself. Biol Reprod, 73:983–7.

122. Brigelius-Flohe R, Kelly FJ, Salonen JT, Neuzil J, Zingg J-M, Azzi A. The European perspective on vitamin E: current knowledge and future research. Am J Clin Nutr 2002;76:703–16.

123. Shamim, A. A., Schulze, K., Merrill, R. D., Kabir, A., Christian, P., Shaikh, S., . . . West, K.

P.,Jr. (2015). First-trimester plasma tocopherols are associated with risk of miscarriage in rural bangladesh. *The American Journal of Clinical Nutrition, 101*(2), 294-301. doi:10.3945/ajcn.114.094920 [doi]

124. Barker, D. J., Winter, P. D., Osmond, C., Margetts, B., & Simmonds, S. J. (1989). Weight in infancy and death from ischaemic heart disease. *Lancet (London, England), 2*(8663), 577-580. doi:S0140-6736(89)90710-1 [pii]

125. Ravelli, A. C., van der Meulen, J. H., Michels, R. P., Osmond, C., Barker, D. J., Hales, C. N., & Bleker, O. P. (1998). Glucose tolerance in adults after prenatal exposure to famine. *Lancet (London, England), 351*(9097), 173-177. doi:S0140673697072449 [pii]

126. Roseboom, T. J., van der Meulen, J. H., Ravelli, A. C., Osmond, C., Barker, D. J., & Bleker, O. P. (2001). Effects of prenatal exposure to the dutch famine on adult disease in later life: An overview. *Molecular and Cellular Endocrinology, 185*(1-2), 93-98. doi:S0303720701007213 [pii]

127. Osmond, C., Barker, D. J., Winter, P. D., Fall, C. H., & Simmonds, S. J. (1993). Early growth and death from cardiovascular disease in women. *BMJ (Clinical Research Ed.), 307*(6918), 1519-1524.

128. Rich-Edwards, J. W., Stampfer, M. J., Manson, J. E., Rosner, B., Hankinson, S. E., Colditz, G. A., . . . Hennekens, C. H. (1997). Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *BMJ (Clinical Research Ed.), 315*(7105), 396-400.

129. Frankel, S., Elwood, P., Sweetnam, P., Yarnell, J., & Smith, G. D. (1996). Birthweight, adult risk factors and incident coronary heart disease: The caerphilly study. *Public Health, 110*(3),

139-143.

130. Eriksson, J., Forsen, T., Tuomilehto, J., Osmond, C., & Barker, D. (2001). Size at birth, childhood growth and obesity in adult life. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 25*(5), 735-740. doi:10.1038/sj.ijo.0801602 [doi]

131. Stein, C. E., Fall, C. H., Kumaran, K., Osmond, C., Cox, V., & Barker, D. J. (1996). Fetal growth and coronary heart disease in south india. *Lancet (London, England),348*(9037), 1269-1273. doi:S0140673696045473 [pii]

132. McCance, D. R., Pettitt, D. J., Hanson, R. L., Jacobsson, L. T., Knowler, W. C., & Bennett, P. H. (1994). Birth weight and non-insulin dependent diabetes: Thrifty genotype, thrifty phenotype, or surviving small baby genotype? *BMJ (Clinical Research Ed.), 308*(6934), 942-945.

133. Hales, C. N., Barker, D. J., Clark, P. M., Cox, L. J., Fall, C., Osmond, C., & Winter, P. D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ (Clinical Research Ed.), 303*(6809), 1019-1022.

134. Robinson, S., Walton, R. J., Clark, P. M., Barker, D. J., Hales, C. N., & Osmond, C. (1992). The relation of fetal growth to plasma glucose in young men. *Diabetologia,35*(5), 444-446.

135. Curhan, G. C., Willett, W. C., Rimm, E. B., Spiegelman, D., Ascherio, A. L., & Stampfer, M. J. (1996). Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. *Circulation, 94*(12), 3246-3250.

136. Barker, D. J., Godfrey, K. M., Osmond, C., & Bull, A. (1992). The relation of fetal length,

ponderal index and head circumference to blood pressure and the risk of hypertension in adult life. *Paediatric and Perinatal Epidemiology, 6*(1), 35-44.

137. Martyn, C. N., Barker, D. J., & Osmond, C. (1996). Mothers' pelvic size, fetal growth, and death from stroke and coronary heart disease in men in the UK. *Lancet (London, England), 348*(9037), 1264-1268. doi:S0140673696042572 [pii]

138. Barker, D. J., & Clark, P. M. (1997). Fetal undernutrition and disease in later life. *Reviews of Reproduction, 2*(2), 105-112.

139. King, J. C. (2003). The risk of maternal nutritional depletion and poor outcomes increases in early or closely spaced pregnancies. *The Journal of Nutrition, 133*(5 Suppl 2), 1732S-1736S.

140. Snell, L. H., Haughey, B. P., Buck, G., & Marecki, M. A. (1998). Metabolic crisis: Hyperemesis gravidarum. *The Journal of Perinatal & Neonatal Nursing, 12*(2), 26-37.

141. Holmes, M. C., Abrahamsen, C. T., French, K. L., Paterson, J. M., Mullins, J. J., & Seckl, J. R. (2006). The mother or the fetus? 11beta-hydroxysteroid dehydrogenase type 2 null mice provide evidence for direct fetal programming of behavior by endogenous glucocorticoids. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 26*(14), 3840-3844. doi:26/14/3840 [pii]

142. Gardner, D. S., Tingey, K., Van Bon, B. W., Ozanne, S. E., Wilson, V., Dandrea, J., . . . Symonds, M. E. (2005). Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 289*(4), R947-54. doi:00120.2005 [pii]

143. Desai, M., Crowther, N. J., Lucas, A., & Hales, C. N. (1996). Organ-selective growth in the offspring of protein-restricted mothers. *The British Journal of Nutrition, 76*(4), 591-603. doi:S0007114596001687 [pii]

144. Pond, W. G., Maurer, R. R., & Klindt, J. (1991). Fetal organ response to maternal protein deprivation during pregnancy in swine. *The Journal of Nutrition, 121*(4), 504-509.

145. Osgerby, J. C., Wathes, D. C., Howard, D., & Gadd, T. S. (2002). The effect of maternal undernutrition on ovine fetal growth. *The Journal of Endocrinology, 173*(1), 131-141. doi:JOE04547 [pii]

146. Bauer, M. K., Breier, B. H., Harding, J. E., Veldhuis, J. D., & Gluckman, P. D. (1995). The fetal somatotropic axis during long term maternal undernutrition in sheep: Evidence for nutritional regulation in utero. *Endocrinology, 136*(3), 1250-1257. doi:10.1210/endo.136.3.7867579 [doi]

147. Gallaher, B. W., Breier, B. H., Keven, C. L., Harding, J. E., & Gluckman, P. D. (1998). Fetal programming of insulin-like growth factor (IGF)-I and IGF-binding protein-3: Evidence for an altered response to undernutrition in late gestation following exposure to periconceptual undernutrition in the sheep. *The Journal of Endocrinology, 159*(3), 501-508.

148. Desai, M., Byrne, C. D., Meeran, K., Martenz, N. D., Bloom, S. R., & Hales, C. N. (1997). Regulation of hepatic enzymes and insulin levels in offspring of rat dams fed a reduced-protein diet. *The American Journal of Physiology, 273*(4 Pt 1), G899-904.

149. Snoeck, A., Remacle, C., Reusens, B., & Hoet, J. J. (1990). Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biology of the Neonate, 57*(2), 107-118.

150. Rasschaert, J., Reusens, B., Dahri, S., Sener, A., Remacle, C., Hoet, J. J., & Malaisse, W. J. (1995). Impaired activity of rat pancreatic islet mitochondrial glycerophosphate dehydrogenase in protein malnutrition. *Endocrinology, 136*(6), 2631-2634. doi:10.1210/endo.136.6.7750486 [doi]

151. Wu, G., Pond, W. G., Ott, T., & Bazer, F. W. (1998). Maternal dietary protein deficiency decreases amino acid concentrations in fetal plasma and allantoic fluid of pigs. *The Journal of Nutrition, 128*(5), 894-902.

152. Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nature Genetics, 33 Suppl*, 245-254. doi:10.1038/ng1089 [doi]

153. Wang, J., Wu, Z., Li, D., Li, N., Dindot, S. V., Satterfield, M. C., . . . Wu, G. (2012). Nutrition, epigenetics, and metabolic syndrome. *Antioxidants & Redox Signaling, 17*(2), 282-301. doi:10.1089/ars.2011.4381 [doi]

154. Bayol, S. A., Farrington, S. J., & Stickland, N. C. (2007). A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. *The British Journal of Nutrition, 98*(4), 843-851. doi:S0007114507812037 [pii]

155. Samuelsson, A. M., Matthews, P. A., Argenton, M., Christie, M. R., McConnell, J. M., Jansen, E. H., . . . Taylor, P. D. (2008). Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: A novel murine model of developmental programming. *Hypertension, 51*(2), 383-392.

doi:HYPERTENSIONAHA.107.101477 [pii]

156. Nivoit, P., Morens, C., Van Assche, F. A., Jansen, E., Poston, L., Remacle, C., & Reusens, B. (2009). Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia, 52*(6), 1133-1142. doi:10.1007/s00125-009-1316-9 [doi]

157. Knudsen, V. K., Orozova-Bekkevold, I. M., Mikkelsen, T. B., Wolff, S., & Olsen, S. F. (2008). Major dietary patterns in pregnancy and fetal growth. *European Journal of Clinical Nutrition, 62*(4), 463-470. doi:1602745 [pii]

158. Rattanatray, L., MacLaughlin, S. M., Kleemann, D. O., Walker, S. K., Muhlhausler, B. S., & McMillen, I. C. (2010). Impact of maternal periconceptional overnutrition on fat mass and expression of adipogenic and lipogenic genes in visceral and subcutaneous fat depots in the postnatal lamb. *Endocrinology, 151*(11), 5195-5205. doi:10.1210/en.2010-0501 [doi]

159. Srinivasan, M., Katewa, S. D., Palaniyappan, A., Pandya, J. D., & Patel, M. S. (2006). Maternal high-fat diet consumption results in fetal malprogramming predisposing to the onset of metabolic syndrome-like phenotype in adulthood. *American Journal of Physiology.Endocrinology and Metabolism, 291*(4), E792-9. doi:00078.2006 [pii]

160. Cerf, M. E., Williams, K., Nkomo, X. I., Muller, C. J., Du Toit, D. F., Louw, J., & Wolfe-Coote, S. A. (2005). Islet cell response in the neonatal rat after exposure to a high-fat diet during pregnancy. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 288*(5), R1122-8. doi:00335.2004 [pii]

161. Rodriguez, L., Panadero, M. I., Roglans, N., Otero, P., Alvarez-Millan, J. J., Laguna, J. C.,

& Bocos, C. (2013). Fructose during pregnancy affects maternal and fetal leptin signaling. *The Journal of Nutritional Biochemistry, 24*(10), 1709-1716. doi:10.1016/j.jnutbio.2013.02.011 [doi]

162. Alzamendi, A., Del Zotto, H., Castrogiovanni, D., Romero, J., Giovambattista, A., & Spinedi, E. (2012). Oral metformin treatment prevents enhanced insulin demand and placental dysfunction in the pregnant rat fed a fructose-rich diet. *ISRN Endocrinology, 2012*, 757913. doi:10.5402/2012/757913 [doi]

163. Lineker, C., Kerr, P. M., Nguyen, P., Bloor, I., Astbury, S., Patel, N., . . . Bell, R. C. (2015). High fructose consumption in pregnancy alters the perinatal environment without increasing metabolic disease in the offspring. *Reproduction, Fertility, and Development,*doi:10.1071/RD15119 [doi]

164. Fuente-Martin, E., Garcia-Caceres, C., Granado, M., Sanchez-Garrido, M. A., Tena-Sempere, M., Frago, L. M., . . . Chowen, J. A. (2012). Early postnatal overnutrition increases adipose tissue accrual in response to a sucrose-enriched diet. *American Journal of Physiology.Endocrinology and Metabolism, 302*(12), E1586-98. doi:10.1152/ajpendo.00618.2011 [doi]

165. Malo, E., Saukko, M., Santaniemi, M., Hietaniemi, M., Lammentausta, E., Blanco Sequeiros, R., . . . Kesaniemi, Y. A. (2013). Plasma lipid levels and body weight altered by intrauterine growth restriction and postnatal fructose diet in adult rats. *Pediatric Research, 73*(2), 155-162. doi:10.1038/pr.2012.173 [doi]

166. Igosheva, N., Abramov, A. Y., Poston, L., Eckert, J. J., Fleming, T. P., Duchen, M. R., &

McConnell, J. (2010). Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PloS One, 5*(4), e10074. doi:10.1371/journal.pone.0010074 [doi]

167. Taylor, P. D., McConnell, J., Khan, I. Y., Holemans, K., Lawrence, K. M., Asare-Anane, H., . . . Poston, L. (2005). Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 288*(1), R134-9. doi:10.1152/ajpregu.00355.2004 [doi]

168. Khan, I. Y., Taylor, P. D., Dekou, V., Seed, P. T., Lakasing, L., Graham, D., . . . Poston, L. (2003). Gender-linked hypertension in offspring of lard-fed pregnant rats. *Hypertension, 41*(1), 168-175.

169. Khan, I. Y., Dekou, V., Douglas, G., Jensen, R., Hanson, M. A., Poston, L., & Taylor, P. D. (2005). A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 288*(1), R127-33. doi:10.1152/ajpregu.00354.2004 [doi]

170. Traber, M. G., & Atkinson, J. (2007). Vitamin E, antioxidant and nothing more. *Free Radical Biology & Medicine, 43*(1), 4-15. doi:S0891-5849(07)00219-5 [pii]

171. Cedernerg J, Siman CM, Eriksson UJ. (2001). Combined treatment with vitamin E and vitamin C decreases oxidative stress and improves fetal outcome in experimental diabetic pregnancy. Pediatr Res, 49(6):755-62.

172. Viana, M., Aruoma, O. I., Herrera, E., & Bonet, B. (2000). Oxidative damage in pregnant

diabetic rats and their embryos. *Free Radical Biology & Medicine, 29*(11), 1115-1121. doi:S0891-5849(00)00397-X [pii]

173. Franco Mdo C, Akamine EH, Aparecide de Oliveria M, Fortes ZB, Tostes RC, Carvalho MH, Nigro D. (2003). Vitaminc C and E improve endothelial dysfunction in intrauterine-undernourished rats by decreasing vascular superoxide anion concentration. J Cardiovasc Pharmacol, 42(2):211-7.

174. Viana, M., Barbas, C., Castro, M., Herrera, E., & Bonet, B. (1999). Alpha-tocopherol concentration in fetal and maternal tissues of pregnant rats supplemented with alpha-tocopherol. *Annals of Nutrition & Metabolism, 43*(2), 107-112. doi:12774 [pii]

175. Viana, M., Castro, M., Barbas, C., Herrera, E., & Bonet, B. (2003). Effect of different doses of vitamin E on the incidence of malformations in pregnant diabetic rats. *Annals of Nutrition & Metabolism, 47*(1), 6-10. doi:68907 [doi]

176. Englund-Ogge, L., Brantsaeter, A. L., Haugen, M., Sengpiel, V., Khatibi, A., Myhre, R., . . . Jacobsson, B. (2012). Association between intake of artificially sweetened and sugar-sweetened beverages and preterm delivery: A large prospective cohort study. *The American Journal of Clinical Nutrition, 96*(3), 552-559. doi:10.3945/ajcn.111.031567 [doi]

177. Borgen, I., Aamodt, G., Harsem, N., Haugen, M., Meltzer, H. M., & Brantsaeter, A. L. (2012). Maternal sugar consumption and risk of preeclampsia in nulliparous norwegian women. *European Journal of Clinical Nutrition, 66*(8), 920-925. doi:10.1038/ejcn.2012.61 [doi]

178. Rawana S, Clark K, Zhong S, Buison A, Chackunkal S, Jenn KL. (1993). Low dose fructose

ingestion and lactation affects carbohydrate metabolism in rat dams and their offspring. J Nutr, 123(12):2158-65.

179. Rodriguez, L., Panadero, M. I., Rodrigo, S., Roglans, N., Otero, P., Alvarez-Millan, J. J., . . . Bocos, C. (2016). Liquid fructose in pregnancy exacerbates fructose-induced dyslipidemia in adult female offspring. *The Journal of Nutritional Biochemistry, 32*, 115-122. doi:10.1016/j.jnutbio.2016.02.013 [doi]

180. Saben, J. L., Asghar, Z., Rhee, J. S., Drury, A., Scheaffer, S., & Moley, K. H. (2016). Excess maternal fructose consumption increases fetal loss and impairs endometrial decidualization in mice. *Endocrinology, 157*(2), 956-968. doi:10.1210/en.2015-1618 [doi]

181. Tain, Y. L., Wu, K. L., Lee, W. C., Leu, S., & Chan, J. Y. (2015). Maternal fructose-intake-induced renal programming in adult male offspring. *The Journal of Nutritional Biochemistry, 26*(6), 642-650. doi:10.1016/j.jnutbio.2014.12.017 [doi]

182. Samuelsson, A. M., Matthews, P. A., Jansen, E., Taylor, P. D., & Poston, L. (2013). Sucrose feeding in mouse pregnancy leads to hypertension, and sex-linked obesity and insulin resistance in female offspring. *Frontiers in Physiology, 4*, 14. doi:10.3389/fphys.2013.00014 [doi]

183. Saad, A. F., Dickerson, J., Kechichian, T. B., Yin, H., Gamble, P., Salazar, A., . . . Costantine, M. M. (2016). High-fructose diet in pregnancy leads to fetal programming of hypertension, insulin resistance, and obesity in adult offspring. *American Journal of Obstetrics and Gynecology, 215*(3), 378.e1-378.e6. doi:10.1016/j.ajog.2016.03.038 [doi]

184. Clayton, Z. E., Vickers, M. H., Bernal, A., Yap, C., & Sloboda, D. M. (2015). Early life

exposure to fructose alters maternal, fetal and neonatal hepatic gene expression and leads to sex-dependent changes in lipid metabolism in rat offspring. *PloS One, 10*(11), e0141962. doi:10.1371/journal.pone.0141962 [doi]

185. Li, L., Xue, J., Li, H., Ding, J., Wang, Y., & Wang, X. (2015). Over-nutrient environment during both prenatal and postnatal development increases severity of islet injury, hyperglycemia, and metabolic disorders in the offspring. *Journal of Physiology and Biochemistry, 71*(3), 391-403. doi:10.1007/s13105-015-0419-7 [doi]

186. Sferruzzi-Perri, A. N., Vaughan, O. R., Haro, M., Cooper, W. N., Musial, B., Charalambous, M., . . . Fowden, A. L. (2013). An obesogenic diet during mouse pregnancy modifies maternal nutrient partitioning and the fetal growth trajectory. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 27*(10), 3928-3937. doi:10.1096/fj.13-234823 [doi]

187. Regnault, T. R., Gentili, S., Sarr, O., Toop, C. R., & Sloboda, D. M. (2013). Fructose, pregnancy and later life impacts. *Clinical and Experimental Pharmacology & Physiology, 40*(11), 824-837. doi:10.1111/1440-1681.12162 [doi]

188. Moffett-King, A. (2002). Natural killer cells and pregnancy. *Nature Reviews.Immunology, 2*(9), 656-663. doi:10.1038/nri886 [doi]

189. Rampersad, R., Cervar-Zivkovic, M., & Nelson, D. M. (2011; 2011). Development and anatomy of the human placenta. *The placenta* (pp. 17-26) Wiley-Blackwell. doi:10.1002/9781444393927.ch3

190. Huppertz, B. (2011; 2011). Vascular development in the placenta. *The placenta* (pp. 36-42)

Wiley-Blackwell. doi:10.1002/9781444393927.ch5

191. Parham, P. (2004). NK cells and trophoblasts: Partners in pregnancy. *The Journal of Experimental Medicine, 200*(8), 951-955. doi:jem.20041783 [pii]

192. Wang, Y., & Zhao, S. (2010). Vascular Biology of the placenta. doi:NBK53247 [bookaccession]

193. Xu, L., Kanasaki, K., Kitada, M., & Koya, D. (2012). Diabetic angiopathy and angiogenic defects. *Fibrogenesis & Tissue Repair, 5*(1), 13-1536-5-13. doi:10.1186/1755-1536-5-13 [doi]

194. Jansson, N., Pettersson, J., Haafiz, A., Ericsson, A., Palmberg, I., Tranberg, M., . . . Jansson, T. (2006). Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. *The Journal of Physiology, 576*(Pt 3), 935-946. doi:jphysiol.2006.116509 [pii]

195. Jardim, L. L., Rios, D. R., Perucci, L. O., de Sousa, L. P., Gomes, K. B., & Dusse, L. M. (2015). Is the imbalance between pro-angiogenic and anti-angiogenic factors associated with preeclampsia? *Clinica Chimica Acta; International Journal of Clinical Chemistry, 447*, 34-38. doi:10.1016/j.cca.2015.05.004 [doi]

196. Shore, V. H., Wang, T. H., Wang, C. L., Torry, R. J., Caudle, M. R., & Torry, D. S. (1997). Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. *Placenta, 18*(8), 657-665.

197. Andraweera, P. H., Dekker, G. A., & Roberts, C. T. (2012). The vascular endothelial growth factor family in adverse pregnancy outcomes. *Human Reproduction Update, 18*(4), 436-457.

doi:10.1093/humupd/dms011 [doi]

198. De Falco, S. (2012). The discovery of placenta growth factor and its biological activity. *Experimental & Molecular Medicine, 44*(1), 1-9. doi:10.3858/emm.2012.44.1.025 [doi]

199. Ahmed, A., Dunk, C., Ahmad, S., & Khaliq, A. (2000). Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble flt-1 by oxygen--a review. *Placenta, 21 Suppl A*, S16-24. doi:S0143400499905246 [pii]

200. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem, 267(36):26031-7.

201. Fischer, C., Mazzone, M., Jonckx, B., & Carmeliet, P. (2008). FLT1 and its ligands VEGFB and PlGF: Drug targets for anti-angiogenic therapy? *Nature Reviews.Cancer, 8*(12), 942-956. doi:10.1038/nrc2524 [doi]

202. Bellik, L., Vinci, M. C., Filippi, S., Ledda, F., & Parenti, A. (2005). Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. *British Journal of Pharmacology, 146*(4), 568-575. doi:0706347 [pii]

203. Park, J. E., Chen, H. H., Winer, J., Houck, K. A., & Ferrara, N. (1994). Placenta growth factor. potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to flt-1 but not to flk-1/KDR. *The Journal of Biological Chemistry, 269*(41), 25646-25654.

204. Xu, L., Cochran, D. M., Tong, R. T., Winkler, F., Kashiwagi, S., Jain, R. K., & Fukumura, D. (2006). Placenta growth factor overexpression inhibits tumor growth, angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. *Cancer Research, 66*(8), 3971-3977. doi:66/8/3971 [pii]

205. Gerber HP, Condorelli F, Park J, Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 1997;272:23659–23667.

206. Jackson, M. R., Carney, E. W., Lye, S. J., & Ritchie, J. W. (1994). Localization of two angiogenic growth factors (PDECGF and VEGF) in human placentae throughout gestation. *Placenta, 15*(4), 341-353.

207. Myatt, L., & Cui, X. (2004). Oxidative stress in the placenta. *Histochemistry and Cell Biology, 122*(4), 369-382. doi:10.1007/s00418-004-0677-x [doi]

208. Hutter, D., Kingdom, J., & Jaeggi, E. (2010). Causes and mechanisms of intrauterine hypoxia and its impact on the fetal cardiovascular system: A review. *International Journal of Pediatrics, 2010*, 401323. doi:10.1155/2010/401323 [doi]

209. Pereira, R. D., De Long, N. E., Wang, R. C., Yazdi, F. T., Holloway, A. C., & Raha, S. (2015). Angiogenesis in the placenta: The role of reactive oxygen species signaling. *BioMed Research International, 2015*, 814543. doi:10.1155/2015/814543 [doi]

210. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol, 16(9):4604-13.

211. Wu, F., Tian, F. J., & Lin, Y. (2015). Oxidative stress in placenta: Health and diseases. *BioMed Research International, 2015*, 293271. doi:10.1155/2015/293271 [doi]

212. Watson AL, Palmer ME, Jauniaux E, Burton GJ. (1997). Variations in expression of copper/zinc superoxide dismutase in villous trophoblast of the human placenta with gestational age. Placenta, 18(4):295-9.

213. James, J. L., Stone, P. R., & Chamley, L. W. (2006). The regulation of trophoblast differentiation by oxygen in the first trimester of pregnancy. *Human Reproduction Update, 12*(2), 137-144. doi:dmi043 [pii]

214. Jauniaux, E., Watson, A. L., Hempstock, J., Bao, Y. P., Skepper, J. N., & Burton, G. J. (2000). Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *The American Journal of Pathology, 157*(6), 2111-2122. doi:S0002-9440(10)64849-3 [pii]

215. Moncada S, Palmer RM, Higgs EA. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. Phamacol Rev, 43(2):109-42.

216. Radi R, Rodriguez M, Castro L, Telleri R. (1994). Inhibition of mitochondrial electron transport by peroxynitrite. Arch Biochem Biophys, 308(1):89-95.

217. Beckman JS, Ischiropoulos H,Zhu L, van der Woerd M, Smith C, Chen J, Harrison J, Martin JC, Tsai M. (1992). Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. Arch Biochem Biophys, 298(2):438-45.

218. Haddad IY, Pataki G, Hu P, Galliani C, Beckman JS, Matalon S. (1994). Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury.

94(6):2407-13.

219. Myatt L, Rosenfield RB, Eis AL, Brockman DE, Greer I, Lyall F. (1996). Nitrotyrosine residues in placenta. Evidence of peroxynitrite formation and action. 28(3):488-93.

220. Kossenjans, W., Eis, A., Sahay, R., Brockman, D., & Myatt, L. (2000). Role of peroxynitrite in altered fetal-placental vascular reactivity in diabetes or preeclampsia. *American Journal of Physiology.Heart and Circulatory Physiology, 278*(4), H1311-9.

221. Maisonneuve, E., Delvin, E., Edgard, A., Morin, L., Dube, J., Boucoiran, I., . . . Leduc, L. (2015). Oxidative conditions prevail in severe IUGR with vascular disease and doppler anomalies. *The Journal of Maternal-Fetal & Neonatal Medicine: The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, 28*(12), 1471-1475. doi:10.3109/14767058.2014.957670 [doi]

222. Zingg, J. M., Libinaki, R., Lai, C. Q., Meydani, M., Gianello, R., Ogru, E., & Azzi, A. (2010). Modulation of gene expression by alpha-tocopherol and alpha-tocopheryl phosphate in THP-1 monocytes. *Free Radical Biology & Medicine, 49*(12), 1989-2000. doi:10.1016/j.freeradbiomed.2010.09.034 [doi]

223. Zingg, J. M., Meydani, M., & Azzi, A. (2012). Alpha-tocopheryl phosphate--an activated form of vitamin E important for angiogenesis and vasculogenesis? *BioFactors (Oxford, England), 38*(1), 24-33. doi:10.1002/biof.198 [doi]

224. Munteanu, A., Taddei, M., Tamburini, I., Bergamini, E., Azzi, A., & Zingg, J. M. (2006). Antagonistic effects of oxidized low density lipoprotein and alpha-tocopherol on CD36

scavenger receptor expression in monocytes: Involvement of protein kinase B and peroxisome proliferator-activated receptor-gamma. *The Journal of Biological Chemistry, 281*(10), 6489-6497. doi:M508799200 [pii]

225. Wefers H, Sies H. (1988). The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. Eur J Biochem, 174(2):353-7.

226. Niki, E., & Noguchi, N. (2004). Dynamics of antioxidant action of vitamin E. *Accounts of Chemical Research, 37*(1), 45-51. doi:10.1021/ar030069m [doi]

227. Wang Y, Walsh SW, Kay HH. (1992). Placental lipid peroxides and thromboxane are increased and prostacyclin is decreased in women with preeclampsia. Am J Obstet Gynecol, 167(4 Pt 1):946-9.

228. Hubel CA, Roberts JM, Taylor RN, Musci TJ, Rogers GM, McL auglin MK. (1989). Lipid peroxidation in pregnancy: new perspectives on preeclampsia. Am J Obstet Gunecol, 161(4):1025-34.

229. Poranen AK, Ekblad U, Uotila P, Ahotupa M. (1996). Lipid peroxidation and antioxidants in normal and pre-eclamptic pregnancies. Placenta 17(7):401-5.

230. Poranen AK, Ekblad U, Uotila P, Ahotupa M. (1998). The effect of vitamin C and E on placental lipid peroxidation and antioxidative enzymes in perfused placenta. Acta Obstet Gynecol Scand, 77(4):372-6.

231. Brett, K. E., Ferraro, Z. M., Yockell-Lelievre, J., Gruslin, A., & Adamo, K. B. (2014). Maternal-fetal nutrient transport in pregnancy pathologies: The role of the placenta. *International Journal of Molecular Sciences, 15*(9), 16153-16185.

doi:10.3390/ijms150916153 [doi]

232. Sibley CP, Birdsey TJ, Brownbill P, Clarson LH, Doughty I, Glazier JD, Greenwood SL, Hughes J, Jansson T, Mylona P, Nelson DM, Powell. (1998). Mechanisms of maternofetal exchange across the human placenta. Biochem Soc Trans, 26(2):86-91.

233. Brown, K., Heller, D. S., Zamudio, S., & Illsley, N. P. (2011). Glucose transporter 3 (GLUT3) protein expression in human placenta across gestation. *Placenta, 32*(12), 1041-1049. doi:10.1016/j.placenta.2011.09.014 [doi]

234. Ericsson, A., Hamark, B., Powell, T. L., & Jansson, T. (2005). Glucose transporter isoform 4 is expressed in the syncytiotrophoblast of first trimester human placenta.*Human Reproduction (Oxford, England), 20*(2), 521-530. doi:deh596 [pii]

235. Quraishi, A. N., & Illsley, N. P. (1999). Transport of sugars across human placental membranes measured by light scattering. *Placenta, 20*(2-3), 167-174. doi:S0143-4004(98)90365-4 [pii]

236. Novakovic, B., Gordon, L., Robinson, W. P., Desoye, G., & Saffery, R. (2013). Glucose as a fetal nutrient: Dynamic regulation of several glucose transporter genes by DNA methylation in the human placenta across gestation. *The Journal of Nutritional Biochemistry, 24*(1), 282-288. doi:10.1016/j.jnutbio.2012.06.006 [doi]

237. Jansson, T. (2001). Amino acid transporters in the human placenta. *Pediatric Research, 49*(2), 141-147. doi:10.1203/00006450-200102000-00003 [doi]

238. Desforges, M., Mynett, K. J., Jones, R. L., Greenwood, S. L., Westwood, M., Sibley, C. P., & Glazier, J. D. (2009). The SNAT4 isoform of the system A amino acid transporter is

functional in human placental microvillous plasma membrane. *The Journal of Physiology, 587*(Pt 1), 61-72. doi:10.1113/jphysiol.2008.161331 [doi]

239. Jansson, T., & Powell, T. L. (2006). IFPA 2005 award in placentology lecture. human placental transport in altered fetal growth: Does the placenta function as a nutrient sensor? -- a review. *Placenta, 27 Suppl A*, S91-7. doi:S0143-4004(05)00310-3 [pii]

240. Verrey, F. (2003). System L: Heteromeric exchangers of large, neutral amino acids involved in directional transport. *Pflugers Archiv : European Journal of Physiology,445*(5), 529-533. doi:10.1007/s00424-002-0973-z [doi]

241. Cleal, J. K., Glazier, J. D., Ntani, G., Crozier, S. R., Day, P. E., Harvey, N. C., . . . Lewis, R. M. (2011). Facilitated transporters mediate net efflux of amino acids to the fetus across the basal membrane of the placental syncytiotrophoblast. *The Journal of Physiology, 589*(Pt 4), 987-997. doi:10.1113/jphysiol.2010.198549 [doi]

242. King, J. C. (2006). Maternal obesity, metabolism, and pregnancy outcomes. *Annual Review of Nutrition, 26*, 271-291. doi:10.1146/annurev.nutr.24.012003.132249 [doi]

243. Belkacemi, L., Lash, G. E., Macdonald-Goodfellow, S. K., Caldwell, J. D., & Graham, C. H. (2005). Inhibition of human trophoblast invasiveness by high glucose concentrations. *The Journal of Clinical Endocrinology and Metabolism, 90*(8), 4846-4851. doi:jc.2004-2242 [pii]

244. Furukawa, S., Hayashi, S., Usuda, K., Abe, M., Hagio, S., & Ogawa, I. (2011). Toxicological pathology in the rat placenta. *Journal of Toxicologic Pathology, 24*(2), 95-111. doi:10.1293/tox.24.95 [doi]

245. Malassine A, Cronier L. (2005). Involvement of gap junctions in placental functions and

development. Biochim Biophys Acta, 1719(1-2):117-24.

246. Jones, R. L., Stoikos, C., Findlay, J. K., & Salamonsen, L. A. (2006). TGF-beta superfamily expression and actions in the endometrium and placenta. *Reproduction (Cambridge, England), 132*(2), 217-232. doi:132/2/217 [pii]

247. Takata K, Hirano H. (1997). Mechanism of glucose transport across the human and rat placental barrier:a review. Microsc Res Tech, 15;38(1-2):145-52.

248. Picut, C. A., Swanson, C. L., Parker, R. F., Scully, K. L., & Parker, G. A. (2009). The metrial gland in the rat and its similarities to granular cell tumors. *Toxicologic Pathology, 37*(4), 474-480. doi:10.1177/0192623309335632 [doi]

249. Shin, B. C., Fujikura, K., Suzuki, T., Tanaka, S., & Takata, K. (1997). Glucose transporter GLUT3 in the rat placental barrier: A possible machinery for the transplacental transfer of glucose. *Endocrinology, 138*(9), 3997-4004. doi:10.1210/endo.138.9.5369 [doi]

250. Liang, C., DeCourcy, K., & Prater, M. R. (2010). High-saturated-fat diet induces gestational diabetes and placental vasculopathy in C57BL/6 mice. *Metabolism: Clinical and Experimental, 59*(7), 943-950. doi:10.1016/j.metabol.2009.10.015 [doi]

251. Kim, D. W., Young, S. L., Grattan, D. R., & Jasoni, C. L. (2014). Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse. *Biology of Reproduction, 90*(6), 130. doi:10.1095/biolreprod.113.117259 [doi]

252. Hayes, E. K., Lechowicz, A., Petrik, J. J., Storozhuk, Y., Paez-Parent, S., Dai, Q., . . . Raha, S. (2012). Adverse fetal and neonatal outcomes associated with a life-long high fat diet: Role

of altered development of the placental vasculature. *PloS One, 7*(3), e33370. doi:10.1371/journal.pone.0033370 [doi]

253. Furuya, M., Ishida, J., Inaba, S., Kasuya, Y., Kimura, S., Nemori, R., & Fukamizu, A. (2008). Impaired placental neovascularization in mice with pregnancy-associated hypertension. *Laboratory Investigation; a Journal of Technical Methods and Pathology, 88*(4), 416-429. doi:10.1038/labinvest.2008.7 [doi]

254. Li, H. P., Chen, X., & Li, M. Q. (2013). Gestational diabetes induces chronic hypoxia stress and excessive inflammatory response in murine placenta. *International Journal of Clinical and Experimental Pathology, 6*(4), 650-659.

255. Roberts VH, Smith J, McLea SA, Heizer AB, Richardson JL, Myatt L. (2009). Effect of increasing maternal body mass index on oxidative and nitrative stress in the human placenta. Placenta, 30(2):169-75.

256. Saad, M. I., Abdelkhalek, T. M., Haiba, M. M., Saleh, M. M., Hanafi, M. Y., Tawfik, S. H., & Kamel, M. A. (2016). Maternal obesity and malnourishment exacerbate perinatal oxidative stress resulting in diabetogenic programming in F1 offspring. *Journal of Endocrinological Investigation, 39*(6), 643-655. doi:10.1007/s40618-015-0413-5 [doi]

257. Lin, Y., Zhuo, Y., Fang, Z. F., Che, L. Q., & Wu, D. (2012). Effect of maternal dietary energy types on placenta nutrient transporter gene expressions and intrauterine fetal growth in rats. *Nutrition (Burbank, Los Angeles County, Calif.), 28*(10), 1037-1043. doi:10.1016/j.nut.2012.01.002 [doi]

258. Jones, H. N., Woollett, L. A., Barbour, N., Prasad, P. D., Powell, T. L., & Jansson, T. (2009).

High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 23*(1), 271-278. doi:10.1096/fj.08-116889 [doi]

259. Cisse, O., Fajardy, I., Dickes-Coopman, A., Moitrot, E., Montel, V., Deloof, S., . . . Laborie, C. (2013). Mild gestational hyperglycemia in rat induces fetal overgrowth and modulates placental growth factors and nutrient transporters expression. *PloS One, 8*(5), e64251. doi:10.1371/journal.pone.0064251 [doi]

260. Strakovsky, R. S., Zhou, D., & Pan, Y. X. (2010). A low-protein diet during gestation in rats activates the placental mammalian amino acid response pathway and programs the growth capacity of offspring. *The Journal of Nutrition, 140*(12), 2116-2120. doi:10.3945/jn.110.127803 [doi]

261. O'Tierney-Ginn, P., Roberts, V., Gillingham, M., Walker, J., Glazebrook, P. A., Thornburg, K. L., . . . Frias, A. E. (2015). Influence of high fat diet and resveratrol supplementation on placental fatty acid uptake in the japanese macaque. *Placenta, 36*(8), 903-910. doi:10.1016/j.placenta.2015.06.002 [doi]

262. Brett, K. E., Ferraro, Z. M., Holcik, M., & Adamo, K. B. (2015). Prenatal physical activity and diet composition affect the expression of nutrient transporters and mTOR signaling molecules in the human placenta. *Placenta, 36*(2), 204-212. doi:10.1016/j.placenta.2014.11.015 [doi]

263. Gadd, T. S., Aitken, R. P., Wallace, J. M., & Wathes, D. C. (2000). Effect of a high maternal

dietary intake during mid-gestation on components of the utero-placental insulin-like growth factor (IGF) system in adolescent sheep with retarded placental development. *Journal of Reproduction and Fertility, 118*(2), 407-416.

264. O'Tierney-Ginn, P., Roberts, V., Gillingham, M., Walker, J., Glazebrook, P. A., Thornburg, K. L., . . . Frias, A. E. (2015). Influence of high fat diet and resveratrol supplementation on placental fatty acid uptake in the japanese macaque. *Placenta, 36*(8), 903-910. doi:10.1016/j.placenta.2015.06.002 [doi]

265. Daltveit, A. K., Tollanes, M. C., Pihlstrom, H., & Irgens, L. M. (2008). Cesarean delivery and subsequent pregnancies. *Obstetrics and Gynecology, 111*(6), 1327-1334. doi:10.1097/AOG.0b013e3181744110 [doi]

266. Sibai BM, Gordon T, Thom E, Cartis SN, Klebanoff M, McNelis D, Paul RH. (1995). Risk factor for preeclampsia in healthy nulliparous women: a prospective multicenter study. The National Institute of Child Health and Human Development Network of Maternal0Fetal Medicine Units. Am J Obstet Gynecol. 172(2 Pt 1):642-8.

267. Trinder P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann clin Biochem 6(24).

268. Arvidsson-Lenner R, Asp N, Axelsen M, Bryngelsson S, Haapa E, Jarvi A, Karlstrom B, Raben A, Sohlstrom A, Thorsdottir I and Vessby B. (2004). Glycaemic index, Relevance for health, dietary recommendations and food labelling. Scand J of Nutr, 48(2).

269. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. (1985). Measurement of protein using bicinchoninic

acid. Anal Biochem, 150(1):76-85.

270. Winer, J., Jung, C. K., Shackel, I., & Williams, P. M. (1999). Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Analytical Biochemistry, 270*(1), 41-49. doi:S0003-2697(99)94085-X [pii]

271. Introduction to Real-Time Quantitative PCR (qPCR), SABiosciences, A QIAGEN Company.

272. Bustin SA (2003). A-Z of Quantitative PCR-Quantification strategies in real-time RT-PCR.

273. Singh J. e of multiple housekeeping genes for normalization of quantitative RT-PCR Data.

274. Dheda K, Huggett JE, Chang JS, Kim LU, Bustin SA, Johnson MA, Rook GA, Zumla A. (2005). The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal Biochem, 344(1):141-3.

275. Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications, 313*(4), 856-862. doi:S0006291X03025646 [pii]

276. Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--excel-based tool using pair-wise correlations. *Biotechnology Letters, 26*(6), 509-515.

277. Andersen CL, Jensen JL, Orntoft TF. 2004. Normalization of real-time quantitative reverse

transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer research 64:5245-5250.

278. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology, 3*(7), RESEARCH0034.

279. Silver, N., Best, S., Jiang, J., & Thein, S. L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology, 7*, 33. doi:1471-2199-7-33 [pii]

280. Xie, F., Xiao, P., Chen, D., Xu, L., & Zhang, B. (2012). miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Molecular Biology,* doi:10.1007/s11103-012-9885-2 [doi]

281. Akamine, R., Yamamoto, T., Watanabe, M., Yamazaki, N., Kataoka, M., Ishikawa, M., . . . Shinohara, Y. (2007). Usefulness of the 5' region of the cDNA encoding acidic ribosomal phosphoprotein P0 conserved among rats, mice, and humans as a standard probe for gene expression analysis in different tissues and animal species. Journal of Biochemical and Biophysical Methods, 70(3), 481-486. doi:S0165-022X(06)00215-6 [pii]

282. Kasimanickam, R. K., Kasimanickam, V. R., Rodriguez, J. S., Pelzer, K. D., Sponenberg, P. D., & Thatcher, C. D. (2010). Tocopherol induced angiogenesis in placental vascular network in late pregnant ewes.Reproductive Biology and Endocrinology : RB&E, 8, 86-7827-8-86.

doi:10.1186/1477-7827-8-86 [doi]

283. Kramer, J. K., Blais, L., Fouchard, R. C., Melnyk, R. A., & Kallury, K. M. (1997). A rapid method for the determination of vitamin E forms in tissues and diet by high-performance liquid chromatography using a normal-phase diol column. Lipids, 32(3), 323-330.

284. Blake CJ. (2007). Status of methology for the determination of fat-soluble vitamins in foods, dietary supplements, and vitamin premixes. J AOAC Int, 90(4):897-910.

285. Caminos, J. E., Nogueiras, R., Gallego, R., Bravo, S., Tovar, S., Garcia-Caballero, T., . . . Dieguez, C. (2005). Expression and regulation of adiponectin and receptor in human and rat placenta. *The Journal of Clinical Endocrinology and Metabolism, 90*(7), 4276-4286. doi:jc.2004-0930 [pii]

286. Silva, J. F., Ocarino, N. M., & Serakides, R. (2015). Placental angiogenic and hormonal factors are affected by thyroid hormones in rats. Pathology, Research and Practice, 211(3), 226-234. doi:10.1016/j.prp.2014.11.003 [doi]

287. Birot, O. J., Koulmann, N., Peinnequin, A., & Bigard, X. A. (2003). Exercise-induced expression of vascular endothelial growth factor mRNA in rat skeletal muscle is dependent on fibre type. The Journal of Physiology, 552(Pt 1), 213-221. doi:10.1113/jphysiol.2003.043026 [doi]

288. Shen, X. H., Tang, Q. Y., Huang, J., & Cai, W. (2010). Vitamin E regulates adipocytokine expression in a rat model of dietary-induced obesity. *Experimental Biology and Medicine (Maywood, N.J.), 235*(1), 47-51. doi:10.1258/ebm.2009.009122 [doi]

289. Mark, P. J., Lewis, J. L., Jones, M. L., Keelan, J. A., & Waddell, B. J. (2013). The

inflammatory state of the rat placenta increases in late gestation and is further enhanced by glucocorticoids in the labyrinth zone. Placenta, 34(7), 559-566. doi:10.1016/j.placenta.2013.04.006 [doi]

290. Simmons, D. G., Fortier, A. L., & Cross, J. C. (2007). Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Developmental Biology, 304(2), 567-578. doi:S0012-1606(07)00023-1 [pii]

291. Mills, J. L., Jovanovic, L., Knopp, R., Aarons, J., Conley, M., Park, E., . . . Metzger, B. (1998). Physiological reduction in fasting plasma glucose concentration in the first trimester of normal pregnancy: The diabetes in early pregnancy study. Metabolism: Clinical and Experimental, 47(9), 1140-1144. doi:S0026-0495(98)90290-6 [pii]

292. Coan, P. M., Vaughan, O. R., McCarthy, J., Mactier, C., Burton, G. J., Constancia, M., & Fowden, A. L. (2011). Dietary composition programmes placental phenotype in mice. The Journal of Physiology, 589(Pt 14), 3659-3670. doi:10.1113/jphysiol.2011.208629 [doi]

293. Montonen, J., Jarvinen, R., Knekt, P., Heliovaara, M., & Reunanen, A. (2007). Consumption of sweetened beverages and intakes of fructose and glucose predict type 2 diabetes occurrence. The Journal of Nutrition, 137(6), 1447-1454. doi:137/6/1447 [pii]

294. de Koning, L., Malik, V. S., Rimm, E. B., Willett, W. C., & Hu, F. B. (2011). Sugar-sweetened and artificially sweetened beverage consumption and risk of type 2 diabetes in men. The American Journal of Clinical Nutrition, 93(6), 1321-1327. doi:10.3945/ajcn.110.007922 [doi]

295. Schulze, M. B., Manson, J. E., Ludwig, D. S., Colditz, G. A., Stampfer, M. J., Willett, W. C.,

& Hu, F. B. (2004). Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. Jama, 292(8), 927-934. doi:10.1001/jama.292.8.927 [doi]

296. Dunn, G. A., & Bale, T. L. (2011). Maternal high-fat diet effects on third-generation female body size via the paternal lineage. Endocrinology, 152(6), 2228-2236. doi:10.1210/en.2010-1461 [doi]

297. Tillman, E. J., Morgan, D. A., Rahmouni, K., & Swoap, S. J. (2014). Three months of high-fructose feeding fails to induce excessive weight gain or leptin resistance in mice. PloS One, 9(9), e107206. doi:10.1371/journal.pone.0107206 [doi]

298. Sievenpiper, J. L., de Souza, R. J., Mirrahimi, A., Yu, M. E., Carleton, A. J., Beyene, J., . . . Jenkins, D. J. (2012). Effect of fructose on body weight in controlled feeding trials: A systematic review and meta-analysis. Annals of Internal Medicine, 156(4), 291-304. doi:10.7326/0003-4819-156-4-201202210-00007 [doi]

299. Livesey, G., & Taylor, R. (2008). Fructose consumption and consequences for glycation, plasma triacylglycerol, and body weight: Meta-analyses and meta-regression models of intervention studies. The American Journal of Clinical Nutrition, 88(5), 1419-1437. doi:88/5/1419 [pii]

300. Martinez, F. J., Rizza, R. A., & Romero, J. C. (1994). High-fructose feeding elicits insulin resistance, hyperinsulinism, and hypertension in normal mongrel dogs.*Hypertension, 23*(4), 456-463.

301. Doherty, D. A., Magann, E. F., Francis, J., Morrison, J. C., & Newnham, J. P. (2006).

Pre-pregnancy body mass index and pregnancy outcomes. *International Journal of Gynaecology and Obstetrics: The Official Organ of the International Federation of Gynaecology and Obstetrics, 95*(3), 242-247. doi:S0020-7292(06)00350-X [pii]

302. Davenport, M. H., Ruchat, S. M., Giroux, I., Sopper, M. M., & Mottola, M. F. (2013). Timing of excessive pregnancy-related weight gain and offspring adiposity at birth. *Obstetrics and Gynecology, 122*(2 Pt 1), 255-261. doi:10.1097/AOG.0b013e31829a3b86 [doi]

303. Vickers, M. H., Clayton, Z. E., Yap, C., & Sloboda, D. M. (2011). Maternal fructose intake during pregnancy and lactation alters placental growth and leads to sex-specific changes in fetal and neonatal endocrine function. Endocrinology, 152(4), 1378-1387. doi:10.1210/en.2010-1093 [doi]

304. Brunst, K. J., Wright, R. O., DiGioia, K., Enlow, M. B., Fernandez, H., Wright, R. J., & Kannan, S. (2014). Racial/ethnic and sociodemographic factors associated with micronutrient intakes and inadequacies among pregnant women in an urban US population. *Public Health Nutrition, 17*(9), 1960-1970. doi:10.1017/S1368980013003224 [doi]

305. Shamim, A. A., Kabir, A., Merrill, R. D., Ali, H., Rashid, M., Schulze, K., . . . Christian, P. (2013). Plasma zinc, vitamin B(12) and alpha-tocopherol are positively and plasma gamma-tocopherol is negatively associated with hb concentration in early pregnancy in north-west bangladesh. Public Health Nutrition, 16(8), 1354-1361. doi:10.1017/S1368980013000475 [doi]

306. Virkamaki, A., Korsheninnikova, E., Seppala-Lindroos, A., Vehkavaara, S., Goto, T.,

Halavaara, J., . . . Yki-Jarvinen, H. (2001). Intramyocellular lipid is associated with resistance to in vivo insulin actions on glucose uptake, antilipolysis, and early insulin signaling pathways in human skeletal muscle. Diabetes, 50(10), 2337-2343.

307. Zhang, Z. Y., Zeng, J. J., Kjaergaard, M., Guan, N., Raun, K., Nilsson, C., & Wang, M. W. (2011). Effects of a maternal diet supplemented with chocolate and fructose beverage during gestation and lactation on rat dams and their offspring. Clinical and Experimental Pharmacology & Physiology, 38(9), 613-622. doi:10.1111/j.1440-1681.2011.05568.x [doi]

308. Buresova, M., Zidek, V., Musilova, A., Simakova, M., Fucikova, A., Bila, V., . . . Pravenec, M. (2006). Genetic relationship between placental and fetal weights and markers of the metabolic syndrome in rat recombinant inbred strains. *Physiological Genomics, 26*(3), 226-231. doi:26/3/226 [pii]

309. Jansson, T., Wennergren, M., & Illsley, N. P. (1993). Glucose transporter protein expression in human placenta throughout gestation and in intrauterine growth retardation. The Journal of Clinical Endocrinology and Metabolism, 77(6), 1554-1562. doi:10.1210/jcem.77.6.8263141 [doi]

310. Ganguly, A., Collis, L., & Devaskar, S. U. (2012). Placental glucose and amino acid transport in calorie-restricted wild-type and Glut3 null heterozygous mice. Endocrinology, 153(8), 3995-4007. doi:10.1210/en.2011-1973 [doi]

311. Boileau, P., Mrejen, C., Girard, J., & Hauguel-de Mouzon, S. (1995). Overexpression of GLUT3 placental glucose transporter in diabetic rats. The Journal of Clinical Investigation, 96(1), 309-317. doi:10.1172/JCI118036 [doi]

312. Ericsson, A., Saljo, K., Sjostrand, E., Jansson, N., Prasad, P. D., Powell, T. L., & Jansson, T. (2007). Brief hyperglycaemia in the early pregnant rat increases fetal weight at term by stimulating placental growth and affecting placental nutrient transport. The Journal of Physiology, 581(Pt 3), 1323-1332. doi:jphysiol.2007.131185 [pii]

313. Ogura, K., Sakata, M., Yamaguchi, M., Kurachi, H., & Murata, Y. (1999). High concentration of glucose decreases glucose transporter-1 expression in mouse placenta in vitro and in vivo. The Journal of Endocrinology, 160(3), 443-452. doi:JOE02951 [pii]

314. Das, U. G., Sadiq, H. F., Soares, M. J., Hay, W. W.,Jr, & Devaskar, S. U. (1998). Time-dependent physiological regulation of rodent and ovine placental glucose transporter (GLUT-1) protein. The American Journal of Physiology, 274(2 Pt 2), R339-47.

315. Hahn T, Barth S, Weiss U, Mosgoeller W, Desoye G. (1998). Sustained hyperglycemia in vitro down-regulates the GLUT1 glucose transport system of cultured human term placental trophoblast: a mechanism to protect fetal development? FASEB J 12(12):1221-31.

316. Hahn, T., Hahn, D., Blaschitz, A., Korgun, E. T., Desoye, G., & Dohr, G. (2000). Hyperglycaemia-induced subcellular redistribution of GLUT1 glucose transporters in cultured human term placental trophoblast cells. Diabetologia, 43(2), 173-180. doi:10.1007/s001250050026 [doi]

317. Wang, G., Bonkovsky, H. L., de Lemos, A., & Burczynski, F. J. (2015). Recent insights into the biological functions of liver fatty acid binding protein 1. Journal of Lipid Research, 56(12), 2238-2247. doi:10.1194/jlr.R056705 [doi]

318. Watters, J. L., Satia, J. A., da Costa, K. A., Boysen, G., Collins, L. B., Morrow, J. D., . . .

Swenberg, J. A. (2009). Comparison of three oxidative stress biomarkers in a sample of healthy adults. Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals, 14(8), 587-595. doi:10.3109/13547500903183954 [doi]

319. Ho, E., Karimi Galougahi, K., Liu, C. C., Bhindi, R., & Figtree, G. A. (2013). Biological markers of oxidative stress: Applications to cardiovascular research and practice. Redox Biology, 1, 483-491. doi:10.1016/j.redox.2013.07.006 [doi]

320. Hogg N, Darley-Usmar VM, Vilson MT, Moncada S. (1993). The oxidation of alpha-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide. FEBS Lett, 326(1-3):199-203.

321. Kim, Y. W., & Byzova, T. V. (2014). Oxidative stress in angiogenesis and vascular disease. Blood, 123(5), 625-631. doi:10.1182/blood-2013-09-512749 [doi]

322. Chung, A. S., & Ferrara, N. (2011). Developmental and pathological angiogenesis. Annual Review of Cell and Developmental Biology, 27, 563-584. doi:10.1146/annurev-cellbio-092910-154002 [doi]

323. Ushio-Fukai, M., & Alexander, R. W. (2004). Reactive oxygen species as mediators of angiogenesis signaling: Role of NAD(P)H oxidase. Molecular and Cellular Biochemistry, 264(1-2), 85-97.

324. Arbiser, J. L., Petros, J., Klafter, R., Govindajaran, B., McLaughlin, E. R., Brown, L. F., . . . Lambeth, J. D. (2002). Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proceedings of the National Academy of Sciences of the United States of America, 99(2), 715-720. doi:10.1073/pnas.022630199 [doi]

325. Birukova, A. A., Lee, S., Starosta, V., Wu, T., Ho, T., Kim, J., . . . Birukov, K. G. (2012). A role for VEGFR2 activation in endothelial responses caused by barrier disruptive OxPAPC concentrations. PloS One, 7(1), e30957. doi:10.1371/journal.pone.0030957 [doi]

326. Armitage, J. A., Taylor, P. D., & Poston, L. (2005). Experimental models of developmental programming: Consequences of exposure to an energy rich diet during development. The Journal of Physiology, 565(Pt 1), 3-8. doi:jphysiol.2004.079756 [pii]

327. Castelhano-Carlos, M. J., & Baumans, V. (2009). The impact of light, noise, cage cleaning and in-house transport on welfare and stress of laboratory rats. Laboratory Animals, 43(4), 311-327. doi:10.1258/la.2009.0080098 [doi]

328. Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews.Genetics, 13(4), 227-232. doi:10.1038/nrg3185 [doi]

329. Rumbold, A., Ota, E., Hori, H., Miyazaki, C., & Crowther, C.A. (2015). Vitamin E supplementation in pregnancy. Cochrane Database Syst Rev, 7;(9):CD004069. doi:10.1002/14651858 [doi]

330. Whitley, G.S.J. & Cartwright, J.E. (2009). Trophoblast-mediated spiral artery remodeling: a role for apoptosis. J Anat, 215(1): 21-26. Doi: 10.1111/j.1469-7580.2008.01039 [doi]

331. Boeldt, D.S., Bird, I.M. (2017). Vasculaar adaptation in pregnancy and endothelial dysfunction in preeclampsia. *The journal of Endocrinology*, 232(1): R27-R44, Epub 2016 Oct 11.

332. Shaw, G.M., Quach, T., Nelson V., Carmichael, S.L, Schaffer, D.M., Selvin S. & Yang W.

(2003). Neural tube defects associated with maternal periconceptional dietary intake of simple sugars and glycemic index. Am J Clin Nutr, 78(5):972-8.

333. Grundt, J.H., Nakling, J, Eide, G.E. & Markestad T. (2012). Possible relation between maternal consumption of added sugar and sugar-sweetened beverages and birth weight – time trends in a population. BMC Public Health, 12:901. Doi: 10.1186/1471-2458-12-901 [doi]

334. Rodrigo, S., Rodriguez, L., Otero, P., Ranadero, M.I., Garcia, A., Barbas, C., Roglans, N., Ramos, S., Goya, L., Laguna, J.C., Alvarez-Millan, J.J. & Bocos, C. (2016). Fructose during pregnancy provokes fetal oxidative stress: The key role of the placental heme oxygenase-1. Mol Nutr Food Res, doi: 10.1002/mnfr.201600193.

335. Asghar, Z.A., Thompson, A., Chi, M., Cusumano, A., Scheaffr, S., Al-Hammadi, N., Saben, J.L., Moley, K.H. (2016). Maternal fructose drives placental uric acid production leading to adverse fetal outcomes. Sci Rep, 29(6):25091. Doi:10.1038/srep25091 [doi]

336. Saben, J.L., Asghar, Z., Rhee, J.S., Drury, A., Scheaffer, S. & Moley, K.H. (2016). Excess maternal fructose consumption increases fetal loss and impairs endometrial decidualization in mice. Endocrinology, 157(2):956-68. Doi: 10.1210/en.2015-1618 [doi]

337. Reynolds, C.M., Vickers, M.H., Harrison, C.J., Segovia, S.A. & Gray, C. (2015). Maternal high fat and/or salt consumption induces sex-specific inflammatory and nutrient transport in the rat placenta. Physiol Rep, 3(5). Doi:10.14814/phy2.12399.

338. Gaccioli, F., Lager, S., Powell, T.L. & Jansson, T. (2015). Placental transport in response to altered maternal nutrition. J Dev Orig Health Dis, 4(2):101-15. Doi:10.1017/S2040174412000529 [doi]

339. Magnusson, A.L., Waterman, I.J., Wennergren, M., Jansson, T. & Powell, T.L. (2004). Triglyceride hydrolase activities and expression of fatty acid binding proteins in the human placenta in pregnancies complicated by intrauterine growth restriction and diabetes. *The Journal of Clinical Endocrinology & Metabolism*, 89:9, 4607-4614.

340. Scifres, C.M., Chen, B., Nelson D.M. & Sadovsky, Y. (2011). Fatty acid binding protein 4 regulates intracellular lipid accumulation in human trophoblasts. J Clin Endocrinol Metab 96:E1083-E1091.

341. Zhu, M.J., Ma, Y, Long, N.M, Du, M. & Ford, S.P. (2010). Maternal obesity markedly increases placental fatty acid transporer expression and fetal blood triglycerides at mid gestation in the ewe. Am J Physiol Regul Intergr Comp Physiol. 299:R1224-R1231.

342. Daltveit, A.K., Tollanes, M.C., Pihlstrom, H., Irgens, L.M. (2008). Cesarean delivery and subsequent pregnancies. Obstet Gynecol, 111(6):1327-34. Doi:10.1097/AOG.0b013e3181744110 [doi]

343. Sibai, B.M., Gordon, T., Thom, E., Caritis, S.N, Klebanoff, M., McNelis, D. & Paul, R.H. (1995). Risk factors for preeclampsia in healthy nulliparous women: a prospective multicenter study. The national Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Unites. Am J Obstet Gynecol, 172(2):642-8.

344. Evans, H.M. & Bishop, K.S. (1922). On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science, 8;56(1458):650-1.

345. Fawzi, W.W., Msamanga, G.I., Spiegelman, D., Urassa, E.J., McGrath, N, Mwakagile, D., Antelman, G., Mbise, R., Herrera, G., Kapiga, S., Willett, W. & Hunter, D.J. (1998).

Randomised trial of effects of vitamin supplements on pregnancy outcomes and T cell counts in HIV-1-infected women in Tanzania. 16;351(9114):1477-82.

346. Kawai, K., Kupka, R., Mugusi, F., Aboud, S., Okuma, J., Villamor, E., Spiegelman, D. & Fawzi, W.W. (2010). A randomized trial to determine the optimal dosage of multivitamin supplements to reduce adverse pregnancy outcomes among HIV-infected women in Tanzania. Am J Clin Nutr, 91(2):391-7. Doi:10.3945/ajcn.2009.28483 [doi]

347. Niki, E. & Traber, M.G. (2012). A history of vitamin E. Ann Nutr Metab, 61(3):207-12. Doi:10.1159/000343106 [doi]

348. Bagul, P., Middela, H., Matapally, S., Radiya, R., Bastia, T., Reddy, M., Chakravarty, S., Banerjee, S. (2012). Attenuation of insulin resistance, metabolic syndrome and hepatic oxidative stress by resveratrol in fructose-fed rats. Pharmacol Res, 66(3):260-268.

349. Mock, K., Lateef, S., Benedito, Vagner. And Tou, J.C. (2017) High-fructose corn syrup-55 consumption alters hepatic lipid metabolism and promotes triglyceride accumulation. Journal of Nutritional Biochemistry, 39:32-39.

350. Goulopoulou, S., and Davidge, S. (2015) Molecular mechanisms of maternal vascular dysfunction in preeclampsia. Trends Mol Med, 21(2):88-97.

351. Matthews, D.R., Hosker, J.P., Rudenski, A.S., et al. (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia, 28:412-419.

352. Nagaretani, H., Nakamura, T., Funahashi, T., et al. (2001) Visceral fat is a major contributor for multiple risk factor clustering in Japanese men with impaired glucose tolerance. Diabetes

Care, 24(12):2127-2133.

Appendix 1. A summary of studies regarding fructose intake and the level of oxidative stress

Study	Year	Reference	Model	Age of animal /Body weight	Treatment
Delbosc et al.	2005	66	Sprague–Dawley rats	8-week-old	Control=tap water; Fructose=60% (w/v) Fructose solution
Faure et al.	2006	69	Male Wistar rats	Weaning	Control=61% (w/w) carbohydrate (no fructose) with of Zn, Se, vitamin E (each 50IU/Kg); Fructose=58% (w/w) of the carbohydrate as fructose; Supplemented group=fructose diet with supplemented Zn, Se, vitamin E (each 500 IU/kg)
Zagrodzki et al.	2007	68	Male Wistar rats	54 – 61days of age	Control=standard diet; Fructose=standard diet with the extra amount of fructose (310g/kg)
Bagul et al.	2012	348	Male Sprague–Dawley rats	Not mentioned	Control=65% (w/w) corn starch diet; Fructose=65% (w/w) fructose diet
Zhou et al.	2012	67	Male Wistar rats	180 – 200g	Control=regular diet; Fructose=diet containing 60% (w/w) fructose
Lozano et al.	2016	59	Male Wistar rats	8-week-old	Control=standard diet; Fructose=standard diet with 25% (w/v) fructose solution
Fisher-Wellman et al.	2013	65	Healthy normal weight men (BMI 24.9±4.0kg/m^2)	27.3±7.0 -year-old	High-fat diet=100% of total calorie from whipping cream; high-carbohydrate diet=100% of total calorie as carbohydrate from sugar; high-protein diet=100% of total calorie as protein from protein powder; combination of the three diet=33% of each high-fat, high-carbohydrate and high-protein diet based on calories

Appendix 1. A summary of studies regarding fructose intake and the level of oxidative stress (continued)

Study	Year	Reference	Treatment duration	Analytical items	Maternal diet effects
Delbosc et al.	2005	66	42 days	TBARS and AOPP in plasma, superoxide anion in cell culture	↑TBARS and ↑AOPP in plasma, ↑Superoxide anion in cell culture in the fructose group
Faure et al.	2006	69	6 weeks	GSH, GSSG (total glutathione) and TBARS in blood, plasma tocopherols	↓GSH, ↓GSSG, ↑TBARS in blood in the fructose group; ↑GSH, ↓GSSG, ↑GSH/GSSG and no change in TBARS in the supplemented group
Zagrodzki et al.	2007	68	9 weeks	SOD in RBC, MDA and FRAP in plasma, Tissue activity of GPX1 and TR	No change in liver GPX activity, ↑SOD activity in RBC, x MDA in plasma, x FRAP in plasma in the fructose group
Bagul et al.	2012	348	8 weeks	Nitric oxide in serum, TBARS, GSH, SOD, CAT and GPX in the liver	Serum nitric oxide, ↑Conjugated dienes level, ↑TBARS, no change in Catalase, ↓SOD activity, ↓Vitamin C, x GSH in the fructose group
Zhou et al.	2012	67	>4 weeks	Plasma nitrite	↑Plasma nitrite in the fructose group
Lozano et al.	2016	59	(from 10 weeks of age) 2 or 8 months	TBARS in plasma, DHE and TBARS in the liver	Fructose diet for 2mths=↑Plasma TBARS but not significant, ↑Liver TBARS; fructose diet for 8mths= no change in oxidative stress, no change in SOD activity, no change in catalase activity, no change in TBARS in the liver, x Plasma TBARS
Fisher-Wellman et al.	2013	65	6hours-postprandial observation	Blood MDA, hydrogen peroxide and nitrate	No changes in Blood MDA, hydrogen peroxide and nitrate in the fructose group

Zn=zinc, TBARS=thiobarbituric acid reactive substances, AOPP=advanced oxidation protein products, GSH=glutathione, GSSG=Glutathione disulfide, SOD=superoxide dismutase, RBC=red blood cell, MDA=malondialdehyde, FRAP=ferric reducing ability of plasma, GPX=glutathione peroxidase, TR=thioredoxin reductase, CAT=catalase, DHE=dihydroethidium

Appendix 2. A summary of studies regarding vitamin E intake and the level of oxidative stress

Study	Year	Reference	Model	Age of animal/ Body weight	Vitamin E supplementation
Cederberg et al.	2001	171	Female Sprague Dawley rats injecting STZ	250g	Commercial pellet with vitamin E and C supplementation: 1) 0.5% (w/w) vitamin E + 1% (w/w) vitamin C 2) 2% (w/w) vitamin E + 4% (w/w) vitamin C
Viana et al.	1999	174	Female Wistar rats	200 – 220g	Control diet with 150mg/day of α-tocopherol administration by gavage on GD18 and 19
Viana et al.	2000	172	Female Wistar rats injecting STZ	190 – 220g	Control diet with 150mg/day of vitamin E administration by gavage
Viana et al.	2003	175	Female Wistar rats injecting STZ	190 – 220g	Control diet with 25, 50, 100, 150, 500 mg/day of vitamin E by gavage
Franco et al.	2003	173	Female Wistar rats	9 – 11 weeks	Dams: Control=22% protein, 43.5% carbohydrates, 4.2% fat (w/w); nutritionally restricted group fed 50% of the *ab libitum* diet; offspring at 14weeks of age=offspring of nutritionally restricted dams were vehicle treated, vitamin C (ascorbic acid 150mg/kg/day for 15 days by gavage) or vitamin E (350mg/kg α-tocopherol/day for 15 days by gavage) treated
Cederberg et al.	2001	171	During pregnancy	α-tocopherol, ascorbic acid, TBARS and protein carbonylation in plasma and liver	↓resorptions and ↓TBARS in diabetic dams in the supplementation group at GD20; ↓malformations ↓resorptions; ↑α-tocopherol and ↑ascorbic acid in the supplementation group

STZ=streptozotocin, ROS=reactive oxygen species, TBARS=thiobarbituric acid reactive substances, EB=ethidium bromide

Appendix 2. A summary of studies regarding vitamin E intake and the level of oxidative stress (continued)

Study	Year	Reference	Treatment duration	Analytical items	Results
Viana et al.	1999	174	During pregnancy	α-tocopherol in plasma and liver	↑α-tocopherol in plasma and the liver in the supplementation group; a positive correlation of α-tocopherol concentration between maternal plasma and fetal plasma, maternal plasma and the placenta, fetal liver and maternal plasma, and fetal plasma and placenta
Viana et al.	2000	172	During pregnancy	Vitamin E in plasma, TBARS, DNA isolation and protein carbonyls in the liver	↑vitamin E concentration in plasma in the vitamin E supplementation group vs. control group and diabetes without the supplementation; no change in TBARS in plasma among groups; ↓rate of malformations and reabsorptions in diabetic rats with supplementation vs. diabetic rats; ↓TBARS in the liver in diabetic rats fed supplementation vs. diabetic rats
Viana et al.	2003	175	GD0 – GD11.5	Tocopherols in plasma	A positive correlation between vitamin E supplementation and vitamin E concentrations in plasma
Franco et al.	2003	173	Dams=during pregnancy; offspring= from 14 weeks of age for 15 days	Systolic blood pressure, vascular reactivity, ROS in mesenteric arteriolar wall	↑SBP in vehicle-treated offspring vs. rats treated with vitamin E or C; ↓% change in diameter of arterioles in vehicle treated offspring vs. offspring treated with vitamin C or E; ↑EB in vehicle treated offspring vs. offspring treated with vitamin C or E

Appendix 3. A summary of studies regarding the effects of fructose intake on metabolic and physiologic profiles during pregnancy

Study	Year	Reference	Model	Age of animal/ Body weight	Treatment
Rawana et al.	1994	171	Female Sprague-Dawley rats	Not mentioned	Control=regular chow *ad libitum*; fructose=regular chow *ad libitum* with a 10% (w/v) fructose solution; glucose=regular chow *ad libitum* with a 10% (w/v) glucose water
Alzamendi et al.	2012	162	Female Sprague-Dawley rats	Not mentioned	Control=regular chow *ad libitum* with tap water; fructose=regular chow *ad libitum* with a 10% (w/v) fructose water
Fuete-Martin et al.	2012	172	Female Wistar rats	Postnatal day 21 to postnatal day 65 or 80 day of age	Control=regular chow *ad libitum*; Sucrose=regular chow *ad libitum* with a 33% (w/v) sucrose solution
Malo et al.	2012	175	Female Sprague Dawley rats	9 weeks of age	Dams=standard chow or 50% of the *ad libitum* food; Offspring=standard chow or fructose-rich diet (60% fructose as total calories)
Rodriguez et al.	2013	173	Female Sprague-Dawley rats	200 – 220g	Control=regular chow *ad libitum*; fructose=regular chow *ad libitum* with a 10% (w/v) fructose solution; glucose=regular chow *ad libitum* with a 10% (w/v) glucose solution
Samuelsson et al.	2013	171	Female C57Bl/6J mice	Not mentioned	Control=standard chow diet (7% simple sugars, 3% fat, 50% polysaccharide, 15% protein (w/w)); sucrose=standard chow diet with sweetened condense milk (55% simple sugars, 10% fat, 9% protein (w/w))
Clayton et al.	2015	174	Virgin Wistar rats	Not mentioned	Control=55% carbohydrate, 30% fat, 15% protein (w/w); Fructose=control diet with 3.5g fructose/kg fat-free mass (+35% energy requirement)

Appendix 3. A summary of studies regarding the effects of fructose intake on metabolic and physiologic profiles during pregnancy (continued)

Study	Year	Reference	Model	Age of animal/ Body weight	Treatment
Lineker et al.	2015	163	Female Wistar rats	Not mentioned	Control=standard chow diet; fructose=standard chow diet with a 10% fructose solution
Li et al.	2015	185	Female C57BL/6J mice	4-week-old	Control (NC)=a standard normal chow diet (10% fat, 20% protein, 70% carbohydrate (w/w)); high fat diet group (HFFD)= 60% fat, 20% protein, 20% carbohydrate (w/w) with a 10% (w/v) fructose solution; prenatal/postnatal diet=NC or HFFD (Final groups were NC/NC, NC/HFFD, HFFD/NC, HFFD/HFFD); After weaning (at 3-weeks-old), NC/NC and HFFD/NC female offspring still fed on NC while NC/HFFD and HFFD/HFFD female offspring fed on HFFD
Tain et al.	2015	181	Virgin Sprague-Dawley rat	12 to 16-week-old	Control=regular chow *ad libitum*; fructose=regular chow ad libitum with a fructose (60% diet by weight)
Rodriguez et al.	2016	179	Female Sprague-Dawley rats	Dam=200 – 400 g; offspring= 240 days of age	Dams=a standard diet (control group, C), a 10% (w/v) fructose solution (a fructose-supplemented group, F) or a 10% (w/v) glucose solution (a glucose-supplemented group, G); offspring=a 10% (w/v) of fructose (F); final groups were C/F, F/F and G/F
Saad et al.	2016	183	Female C57BL/6J mice	Not mentioned	Control=regular chow *ad libitum* with tap water; fructose=regular chow *ad libitum* with a 10% (w/v) fructose solution
Saben et al.	2016	180	Female C57Bl/6 mice	6-week-old	Control=28.5% protein, 58% complex carbohydrate, 13.5% fat (w/w); fructose=66.8% fructose, 20.2% protein, 12.9% fat (w/w)

Appendix 3. A summary of studies regarding the effects of fructose intake on metabolic and physiologic profiles during pregnancy (continued)

Study	Year	Reference	Treatment duration	Analytical items	Results
Rawana et al.	1994	171	Before and during pregnancy; euthanized at GD19	Plasma glucose and insulin, liver TG and glycogen	↑plasma glucose, insulin, liver glycogen in fructose-fed rats vs. control (but not higher than glucose-fed rats); ↑liver weight in fructose-fed rats vs. glucose-fed rats and rats fed tap water on GD19; ↑total fat relative to body weight in fructose-fed rats vs. glucose- and tap water-fed rats at GD19; ↓body weight of newborn and weaned rats in the fructose group and glucose group vs. control; ↓plasma glucose, ↑plasma insulin in weaned pups (but not in newborns) of fructose-fed rats vs. glucose- or tap water-fed rats
Alzamendi et al.	2012	162	During pregnancy	OGTT on GD14; Placental structure (H&E) at late pregnancy (GD20)	↑energy intake in rats fed fructose vs. control at GD14 and GD20; ↑AUC of insulin (no change in AUC of glucose) in rats fed fructose vs. control; ↓vessel area (%) in rats fed fructose vs. control; ↑the fetal:placental weight ratio in the fructose group vs. control on GD20
Fuete-Martin et al.	2012	172	Postnatal day 21 to postnatal day 65 or 80 days of age; euthanized at 80 days of age	OGTT on postnatal day 73; corticosterone, ghrelin, leptin, insulin, adiponectin, cholesterol, TG, FFA, glycerol and total protein in serum	↑serum leptin, adiponectin, % acylated ghrelin, FFA in rats fed sucrose solution

TG=triglycerides, OGTT=oral glucose tolerance test, H&E=hematoxylin and eosin, FFA=free fatty acid, AUC=area under the curve

Appendix 3. A summary of studies regarding the effects of fructose intake on metabolic and physiologic profiles during pregnancy (continued)

Study	Year	Reference	Treatment duration	Analytical items	Results
Malo et al.	2012	175	Dams= GD4 – delivery; Offspring=1mth of age – 6mths of age	Plasma cholesterol, TG, blood glucose, MRI	Male offspring=↑plasma TG, fasting insulin, HOMA-IR in litters fed fructose solution; ↓plasma LDL cholesterol in litters fed fructose solution vs. from control dams and diet-restricted dams; female offspring=↑total cholesterol, TG, HDL cholesterol and fasting insulin, ↓fasting glucose in the fructose group vs. from control and diet-restricted dams; ↑lipid content in retroperitoneal adipose tissue and intra-abdominal adipose tissue in male offspring (not in female offspring) fed fructose
Rodriguez et al.	2013	173	During pregnancy; euthanized on GD21	Plasma glucose, FFA, cholesterol, TG, insulin, leptin and adiponectin	↑plasma TG, hepatic TG, FAS and ACC in fructose-fed rats vs. control; ↑hepatic SREBP1 in the fructose group vs. control and glucose group
Samuelsson et al.	2013	171	Prior to pregnancy for 6 weeks, throughout pregnancy and lactation	Arterial pressure and heart rate were assessed at 3 months of age	Dams=↑body weight, calorie intake in rats fed sucrose rich diet; Offspring=↑body weight of female offspring, not in male, at weaning; ↑calorie intake of male and female offspring of sucrose-fed dams at weaning; ↑body weight, fat pad weight, heart weight, liver weight, muscle weight in female offspring, not in male offspring, of sucrose-fed dams at 3 months of age; ↓muscle weight and kidney weight in female offspring, not in male offspring; (continued next page)

HOMA=homeostatic model assessment, IR=insulin resistance, LDL=low-density lipoprotein, TG=triglycerides, FFA=free fatty acid, ACC=acetyl-CoA carboxylase, SREBP1=sterol regulatory element-binding protein 1

Appendix 3. A summary of studies regarding the effects of fructose intake on metabolic and physiologic profiles during pregnancy (continued)

Study	Year	Reference	Treatment duration	Analytical items	Results
Samuelsson et al.	2013	171	Prior to pregnancy for 6 weeks, throughout pregnancy and lactation	Arterial pressure and heart rate were assessed at 3 months of age	↑serum inulin and leptin in female, not in male, offspring of sucrose-fed dams at 3 months of age; ↑systolic and diastolic arterial pressure, heart rate, activity in male offspring (changes were more pronounced in male offspring vs. female offspring) of sucrose-fed dams at 3 months; ↑renal noradrenaline in male and female offspring of sucrose-fed dams at 3 months
Clayton et al.	2015	174	GD0 – GD21, postnatal day (P) 2 or postnatal day 10	Plasma NEFA, liver glycogen and TG, hepatic GLUT5, PEPCK, SREBP1c and PPAR-α	Dams=↑hepatic GLUT5, ↓hepatic PEPCK in the fructose group at GD21 and P10, ↓hepatic glycogen in the fructose group only at P10 not GD21, no change in hepatic TG in the fructose group at both GD21 and P10, ↑hepatic SREBP1c in the fructose group at GD21, ↑PPAR-α in the fructose group at P10; offspring= ↑GLUT5 in the liver of female offspring at GD21, ↓liver glycogen in male offspring but ↓ in females, ↑liver TG at P10 (not GD21)
Lineker et al.	2015	163	During pregnancy; euthanized at GD20	Plasma metabolites; maternal vascular responses (assed in uterine and mesenteric arteries at GD20)	↑ retroperitoneal fat pad weight, caloric intake, fluid intake, blood glucose, insulin, TG in rats fed fructose on GD20; ↓food intake in fructose group on GD20, x litter size, fetal weight, placental weight, placental:fetal ratio, fetal glucose, fetal insulin in rats fed fructose on GD20;

GLUT (glucose transporter), PEPCK=phosphoenolpyruvate carboxykinase, NEFA=non-esterified fatty acid, PPAR=peroxisome proliferator-activated receptor

Appendix 3. A summary of studies regarding the effects of fructose intake on metabolic and physiologic profiles during pregnancy (continued)

Study	Year	Reference	Treatment duration	Analytical items	Results
Li et al.	2015	185	Dams=before mating for 10 weeks and during pregnancy and lactation (euthanized at 17-week-old); offspring=3-week-old to 13-week-old	Insulin signaling test, energy expenditure; immunohistochemistry of the pancreas; insulin secretion; insulin, GLUT2, PGC-1 alpha, GCK, G6Pase in the liver	Dams=↑body weight, fat content, blood glucose, plasma insulin, blood glucose during OGTT in HFFD; ↑body weight, fat content, liver weight, liver TG, plasma FFA and plasma TG, plasma leptin, food intake, blood glucose and plasma insulin during OGTT and β-cell mass in NC/HFFD and HFFD/HFFD; ↑hepatic mRNA expressions of insulin, PGC-1a and G6Pase in HFFD/HFFD; ↓O_2 consumption and CO_2 production, % of total islets and hepatic mRNA expression of GLUT2 and GCK in NC/HFFD and HFFD/HFFD
Tain et al.	2015	181	During pregnancy and lactation	blood pressure	↑both systolic and diastolic arterial pressure in rats fed fructose
Saben et al.	2016	179	6 weeks	Serum TG, NEFA, cholesterol, uric acid, glucose, insulin	↑serum glucose concentration in high fructose-fed rats vs. control; no change in TG, NEFA, cholesterol, uric acid, glucose, insulin in serum; ↓placental weight in fructose group vs. control
Rodriguez et al.	2016	183	During pregnancy	Glucose, insulin, TG, NEFA, cholesterol, adiponectin in plasma, mRNA expression of IRS1, PEPCK, PPAR, UCP, ACC and FAS in the liver	No change in body weight, plasma glucose, insulin, TG, NEFA, cholesterol among dams; ↑IRS1 in the liver of C/F and F/F vs. G/F; ↑liver weight of C/F, F/F and G/F vs. C/C; ↑adiponectin in plasma of C/F and F/F vs. C/C; ↑plasma TG of F/F than C/C, but not different between C/C vs. C/F and G/F; ↑NEFA in plasma of F/F vs. C/F and G/F, but not different between F/F vs. C/C; (continued next page)

OGTT=oral glucose tolerance test, GLUT=glucose transporter, PGC-1=PPARγ Coactivator-1, GCK=glucokinase, G6Pase=glucose-6-phosphatase complex, TG=triglycerides, NEFA=non-esterified fatty acid, PEPCK=phosphoenolpyruvate carboxykinase, PPAR=peroxisome proliferator-activated receptor, UCP=uncoupling protein, ACC=acetyl-CoA carboxylase, FAS=fatty acid synthase

Appendix 3. A summary of studies regarding the effects of fructose intake on metabolic and physiologic profiles during pregnancy (continued)

Study	Year	Reference	Treatment duration	Analytical items	Results
Rodriguez et al.	2016	183	During pregnancy	Glucose, insulin, TG, NEFA, cholesterol, adiponectin in plasma, mRNA expression of IRS1, PEPCK, PPAR, UCP, ACC and FAS in the liver	↑liver TG of F/F vs. C/F, but not different between F/F vs. C/C and G/F; ↓mRNA expression of PEPCK in the liver of C/F, F/F, G/F vs. C/C; ↓hepatic PPAR alpha of C/F and F/F vs. C/C; ↑hepatic ACC gene of F/F vs. C/C, but no differences between C/F vs. G/F; x hepatic FAS among groups
Saad et al.	2016	180	During pregnancy; euthanized at GD18 – 21	Blood pressure; serum fasting glucose, TG, cholesterol, insulin; leptin and adiponectin in mouse sera	Differences were found only in female mice fed fructose solution vs. female fed tap water (no change between male mice fed fructose solution vs. male mice fed tap water): ↑weight, HOMA-IR, VAT, liver fat, leptin, and insulin AUC, and ↓adiponectin in female mice fed fructose solution vs. female fed tap water; ↑mean arterial pressure, serum glucose concentration during OGTT in both 12-month-old female and male offspring at 12 months of age vs. control group

HOMA=homeostatic model assessment, IR=insulin resistance, OGTT=oral glucose tolerance test, PPAR=peroxisome proliferator-activated receptor, ACC=acetyl-CoA carboxylase, FAS=fatty acid synthase, VAT=visceral adipose tissue

Appendix 4. A summary of studies regarding maternal undernutrition and placental development

Study	Year	Reference	Model	Diet	Treatment duration	sample collection
Yiallourides et al.	2009	170	Ewe	A calorie restricted diet (50% of the requirement for weight maintenance) or a control diet (100% of the requirement for weight maintenance)	GD66 to 110	GD110
Gnanalingham et al.	2007	171	Ewe	A calorie restricted diet (60% of requirement) or a high amount of a complete diet (150% of energy requirement)	GD28 to 80	GD80 or GD140
Osgerby et al.	2004	174	Ewe	A restricted diet (70% of their maintenance requirements during pregnancy) or a control diet (100% of their maintenance requirements during pregnancy)	GD26 to GD45, 90, or 135	GD45, 90, or 135
Rutland et al.	2007	160	Mouse	A low-protein diet (9% (w/w) protein) or a control diet (18% (w/w) protein)	GD0.5 to either GD14.5 or 18.5	GD14.5 or 18.5
Luther et al.	2007	161	Ewe	A restricted diet (L; 70% of their maintenance requirements during pregnancy) or a control diet (C; 100% of their maintenance requirements during pregnancy); switched diet on GD90	Throughout pregnancy	GD90 or GD130
McMullen et al.	2005	184	Ewe	a diet-restricted or control groups	Throughout pregnancy	GD90 or 135
Coan et al.	2010	175	Mouse	An 80% of the *ad libitum* diet or a 100% *ad libitum* diet	Throughout pregnancy	GD16 or 19
Strakovsky et al.	2010	186	Rat	An *ad libitum* diet (control, 18% (w/w) protein) or a low protein diet (9% (w/w) protein)	Throughout pregnancy	GD22-23
Malandro et al.	1996	187	Rat	A control diet (19.3% (w/w) protein) or a low protein diet (4.6% (w/w) protein)	Throughout pregnancy	GD20
Sen et al.	2012	190	Ewe	A calorie restricted diet (50% of daily requirements) or control diet (100% of daily requirements)	GD30 to 80	GD140
Bertram et al.	2001	191	Rat	A low-protein diet (9% (w/w) protein) or a control diet (18% (w/w) protein)	Throughout pregnancy	GD14 or 20

Appendix 4. A summary of studies regarding maternal undernutrition and placental development (continued)

Study	Year	Reference	Analytical items	Methods	Maternal diet effects
Yiallourides et al.	2009	170	Proliferating cell, IGF-2	Immunohistochemistry, PCR	↓ cell proliferation in the placenta, ↓ IGF-2 expression at GD110
Gnanalingham et al.	2007	171	Proliferating cell	Immunohistochemistry	↓ cell proliferation in the placenta at GD140
Osgerby et al.	2004	174	The localization of IGFBP -3, -5, and -6 mRNA expressions	ISH	↓ IGFBP-3, -5 and -6 expression between GD45 and 90
Rutland et al.	2007	160	The number of giant cells, spongiotrophoblast cells and labyrinth cells	Immunohistochemistry	No change in the number of giant cells and spongiotrophoblast cells present, no change in the volume of labyrinth tissue at GD14.5 and 18.5
Luther et al.	2007	161	Cellular proliferation, total cotyledon area, vessel density (%), vessel number density, vessel surface density, the level of vascular endothelial cell adhesion molecules; the mRNA expression of angiopoietin 1, VEGF-A and FLT1	Immunohistochemistry, histology, RT-PCR	No change in the proliferation rate (%) of placentome cells, ↑ capillary surface density in the L group, ↓ area density (%) of caruncel capillary and surface density by the L, ↓ area density (%) of caruncle capillary in the placenta of L-C ewes at both GD90 and 130; no change in % of blood vessels, ↓ the length of maternal blood vessels by the L, no change in the length of fetal blood vessels, fetal blood vessel diameter, and maternal blood vessel diameter at both GD14.5 and 18.5; ↑ VEGF-A at GD130
McMullen et al.	2005	184	mRNA expression of VEGF; placental weight	Real-time PCR	↓ the level of VEGF mRNA expression at GD90; ↓ placental weight at GD90, no change in placental weight at GD135

PCR=polymerase chain reaction, IGF=Insulin-like growth factor, IGFBP=IGF-binding proteins, VAGFA=vascular endothelial growth factor A, FLT-1=Fms-like tyrosine kinase receptor-1

Appendix 4. A summary of studies regarding maternal undernutrition and placental development (continued)

Study	Year	Reference	Analytical items	Methods	Maternal diet effects
Coan et al.	2010	175	The mRNA expression of GLUT1, GLUT3, SNAT2, SNAT4; placental weight	Real-time PCR	↓ mRNA expression of GLUT1, x GLUT3, SNAT2 and SNAT4 at GD16 ↑ the mRNA expression of GLUT1 and SNAT2, ↓ the mRNA expression of SNAT4 at GD19; ↓ the absolute volume of the placenta at GD16 and 19
Strakovsky et al.	2010	186	The mRNA expression of SNAT2	Real-time PCR	↑ mRNA expression of SNAT2 at GD22 – 23
Malandro et al.	1996	187	The protein expression of system y+, system XAG	Northern blots	↓ mRNA expression of system y+ and system XAG at GD 20
Sen et al.	2012	190	The number and density of cotyledons, weights of the placenta and cotyledons	Histology	x the number and density of cotyledons, ↓ average weights of the placenta and cotyledons at GD140
Bertram et al.	2001	191	The protein expression of 11β-HSD1 and 11β-HSD2 isoforms	Northern blots	↓ 11βHSD2 expression, x the expression of 11βHSD1 at both GD14 and 20

GLUT=glucose transporter, SNAT=Sodium-coupled neutral amino acid transporter, 11β-HSD=11β-hydroxysteroid dehydrogenase, PCR=polymerase chain reaction

Appendix 5. A summary of studies regarding maternal overnutrition and placental development

Study	Year	Reference	Model	Diet	Treatment duration	sample collection
Kim et al.	2014	251	Mouse	A control diet (10% of total energy intake as fat) or a high-fat diet (45% of total energy intake as fat)	(For 8 weeks) beginning at four weeks of age	GD15.5 or 17.5
Liang et al.	2010	250	Mouse	A high-fat intake (60% of total energy intake as fat) or a control diet (5% of total energy intake as fat)	One month before conception and during pregnancy	GD19
King et al.	2013	208	Mouse	A high-fat diet (58% of total energy intake as fat) or a control-diet (10.5% of total energy intake as fat)	(For 15 weeks) Beginning at five weeks of age and during pregnancy	GD14.5 or 18.5
Gadd et al.	2000	263	Ewe	A high amount of a complete diet (H, 300g/day which is considered to achieve rapid maternal growth) or a moderate amount of a complete diet (M, 50g/day which is considered to achieve normal maternal growth)	During pregnancy at GD52, a half of ewes in each group had their dietary intake switched, yielding HH, MM, HM and MH groups	GD104
Hayes et al.	2012	252	Rat	A high-fat diet (a diet containing 45% of total energy intake from fat) or a control diet (a diet containing 16% of total energy intake from fat)	3 to 19 weeks of age	GD15
Li et al.	2013	254	Mouse	A high-fat diet (40% of total energy intake as fat) or a control diet (14% of total energy intake as fat)	(For 14 weeks) Before and during pregnancy	GD18.5
Jone et al.	2009	258	Mouse	A high-fat diet (32% of total energy intake as fat), or a control diet (11% of total energy intake as fat)	(For 8 weeks) beginning at 8 weeks of age – the end of pregnancy	GD18.5

Appendix 5. A summary of studies regarding maternal overnutrition and placental development (continued)

Study	Year	Reference	Model	Diet	Treatment duration	sample collection
Sferruzzi-Perri et al.	2013	186	Rat	An obesogenic diet (containing 30% of total energy intake as fat and 36% of total energy intake as simple sugar) or a control diet (containing 11% of total energy intake as fat and 7% of total energy intake as simple sugar)	GD1 to either GD16 and 19	GD16 or 19
Lin et al.	2012	257	Rat	A diet containing high fat/low fiber (HL; 25% (w/w) fat/2.29% (w/w) fiber), high fat/high fiber (HH; 25% (w/w) fat/10.94% (w/w) fiber), low fat/low fiber (LL; 5% (w/w) fat/2.29% (w/w) fiber), or low fat/high fiber (LH; 5% (w/w) fat/10.94% (w/w) fiber)	(For 4 weeks) before pregnancy to GD13.5 and 17.5.	GD13.5 or 17.5
O'Tierney-Ginn et al.	2015	264	Japanese macaques	A control chow diet (15% of total energy intake as fat) or a high-fat diet (35% of total energy intake as fat)	(For 4 – 7 years) before and during pregnancy	GD130

Appendix 5. A summary of studies regarding maternal overnutrition and placental development (continued)

Study	Year	Reference	Analytical items	Methods	Maternal diet effects
Kim et al.	2014	251	Proliferation, apoptosis; placental layers, placental weight	Histology	↓the proliferation rate of cytotrophoblasts in the LZ of the placenta at both GD15.5 and 17.5; ↓in thickness of LZ, ↑decidua at both GD15.5 and 17.5, ↑placental weight at GD15.5
Liang et al.	2010	250	The number of cellular necrosis, trophoblast cells	Histology	↑cellular necrosis and decreased the type of trophoblast cells in labyrinth in the placenta at GD19, ↑density of fibrinonecrosis and cellular fragmentations in endothelium in the LZ at GD19
King et al.	2013	208	The mRNA expression of IGF-2 and IGF2 receptor	Real-time PCR	↑the expression of IGF-2 and the IGF-2 receptor in the placenta of male pups at GD14.5
Gadd et al.	2000	263	The localization of IGF-1, IGF-2, and IGFBP1-5 mRNA expression; placental weight	ISH	No change IGF-2 expression at GD104; ↑the expression of IGFBP-1, ↓that of IGFBP-3 in the endometrial glands of the placenta at GD104; ↓placental weight at GD104
Hayes et al.	2012	252	The expression of CD31	Immunohistochemistry	↑the level of CD31, ↓the expression of SMA by 30% at GD15
Li et al.	2013	254	The mRNA and protein expressions of HIF-1α, VEGF-A and CD31	Real-time PCR, immunohistochemistry	↑mRNA expression of both HIF-1α, VEGF-A, and CD31 in the placenta at GD18.5
Jone et al.	2009	258	The protein expression of GLUT1, GLUT3, SNAT2, SNAT4	Western blotting	↑GLUT1 at GD18.5, -GLUT3 at GD 18.5, ↑SNAT2 at GD18.5, no change in SNAT4 at GD18.5

IGF=Insulin-like growth factor, IGFBP=IGF-binding proteins, HIF-1α=hypoxia inducible factor-1α, CD31=cluster of differentiation 31, VEGF=vascular endothelial growth factor, GLUT=glucose transporter, SNAT=sodium-coupled neutral amino acid transporter, FATP=fatty acid transport protein, FAS=fatty acid synthase, ISH=*in situ* hybridization, PCR=polymerase chain reaction, LZ=labyrinth zone, SMA=smooth muscle actin

Appendix 5. A summary of studies regarding maternal overnutrition and placental development (continued)

Study	Year	Reference	Analytical items	Methods	Maternal diet effects
Sferruzzi-Perri et al.	2013	186	The mRNA expression of GLUT1, GLUT3, SNAT1, SNAT2, SNAT4, FATP, FAS; placental layers, placental weight	Real-time PCR; histology	No change in GLUT1 at both GD16 and 19, ↑GLUT3 at GD16, but not GD19, x SNAT1 at GD16, ↑SNAT2 at GD 16, no change in SNAT4 at GD16, no change in SNAT2 at GD19, no change in SNAT4 at GD19, no change in SNAT1 at GD19, no change in FATP at GD16, ↑FATP at GD19, ↓FAS at GD16, no change in FAS at GD19; ↓ deciduas, no change in JZ and LZ at GD16, ↓LZ, no change in JZ, no change in deciduas at GD19, ↓placental weight at GD16 & 19
Lin et al.	2012	257	The mRNA expression of GLUT1, GLUT3, SNAT2, SNAT4	Real-time PCR	↓GLUT1 at both GD13.5 and 17.5, x GLUT3 at GD13.5 but↑GLUT3 at 17.5, xSNAT2 at GD13.5, ↓SNAT4 at GD13.5, xSNAT2 at GD17.5, xSNAT4 at GD17.5
O'Tierney-Ginn et al.	2015	264	The mRNA expression of FATP1, FATP2, FATP4, FATP6, FABP3, FABP4, FABP5, FABPpm; placental weight	Real-time PCR, immunoblotting	x mRNA expression of FATP1, FATP2, FATP6, FABP3, FABP4, FABP5 at GD130, ↓mRNA expression of FATP4, FABPpm at GD130, x protein expression of FATP4, FABPpm at GD130; x placental weight at GD130

GLUT=glucose transporter), SNAT=sodium-coupled neutral amino acid transporter, FATP=fatty acid transport protein, FAS=fatty acid synthase, FABP=fatty acid binding protein, FABPpm=plasma membrane FABP, PCR=polymerase chain reaction

Appendix 6. Laboratory Rodent Diet (Purina 5001)

Nutrient		Nutrient	
Protein (%)	25.0	Fat (ether extract) (%)	5.0
Arginine (%)	1.57	Fat (acid hydrolysis) (%)	6.4
Cystine (%)	0.39	Cholesterol (ppm)	209
Glycine (%)	1.28	Linoleic acid (%)	1.05
Histidine (%)	0.62	Linolenic acid (%)	0.09
Isoleucine (%)	1.06	Arachidonic acid (%)	0.02
Leucine (%)	1.89	Omega-3 fatty acid (%)	0.30
Lysine (%)	1.48	Total saturated fatty acid (%)	1.48
Methionine (%)	0.59	Total monounsaturated fatty acid (%)	1.62
Phenylalanine (%)	1.11	Fiber (Crude) (%)	5.3
Tyrosine (%)	0.77	Neutral detergent fiber (%)	16.7
Threonine (%)	0.97	Acid detergent fiber (%)	6.9
Tryptophan (%)	0.28	Nitrogen-Free extract (%)	47.5
Valine (%)	1.16	Starch (%)	21.0
Serine (%)	1.18	Glucose (%)	0.19
Aspartic acid (%)	2.81	Fructose (%)	0.27
Glutamic acid (%)	4.74	Sucrose (%)	3.83
Alanine (%)	1.44	Lactose (%)	2.01
Proline (%)	1.47	Total digestible nutrients (%)	73.8
Taurine (%)	0.03		

Appendix 6. Laboratory Rodent Diet (Purina 5001) (continued)

Nutrient		Nutrient	
Ash (%)	7.0	Vitamin K (ppm)	1.3
Calcium (%)	0.95	Thiamin Hydrochloride (ppm)	16
Phosphorus (%)	0.70	Riboflavin (ppm)	4.7
Phosphorus (non-phytate) (%)	0.42	Niacin (ppm)	120
Potassium (%)	1.28	Pantothenic acid (ppm)	24
Magnesium (%)	0.23	Choline chloride (ppm)	2250
Sulfar (%)	0.36	Folic acid (ppm)	7.1
Sodium (%)	0.39	Pyridoxine (ppm)	6.0
Chloride (%)	0.64	Biotin (ppm)	0.30
Fluorine (ppm)	15	B12 (mcg/kg)	51
Iron (ppm)	240	Vitamin A (IU/gm)	15
Zinc (ppm)	85	Vitamin D (IU/gm)	4.6
Manganese (ppm)	75	Vitamin E (IU/kg)	42
Copper (ppm)	15	Ascorbic acid (mg/gm)	-
Cobalt (ppm)	0.91	**Calories provided by:**	
Iodine (ppm)	0.99	**Protein (%)**	**29.829**
Chromium (ppm)	0.01	**Fat (ether extract) (%)**	**13.427**
Selenium (ppm)	0.41	**Carbohydrates (%)**	**56.744**
Carotene (ppm)	2.3	**Calories in 1g pellet**	**4.09**