The Effects of Maternal Western Style Diet Exposure on the Endocrine Pancreas of the Offspring

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CHAPTER I: INTRODUCTION

Adapted from Elsakr and Gannon, “Developmental programming of the pancreatic islet by *in utero* overnutrition”, *Trends in Developmental Biology*, 2017

General Background

Developmental Origins of Health and Disease

The Developmental Origins of Health and Disease (DOHaD) hypothesis postulates that the *in utero* environment influences postnatal health and plays a role in disease etiology. Early evidence for DOHaD was obtained from studies on humans born during the Dutch Hunger Winter, a period of famine in the Netherlands during World War II, and their siblings born outside this period of famine. Offspring born during this period of famine who were undernourished *in utero* during the first or second trimesters were more likely to develop obesity and metabolic disease in adulthood when compared with their siblings born before or after the famine [1]. Interestingly, the consequences of a poor *in utero* environment spanned multiple organ systems. In addition to being at an increased risk for metabolic and cardiovascular disease, these offspring also had significantly lower chances of being employed (likely due to decreased cognitive ability), increased incidence of obstructive airway disease, and increased incidence of microalbuminuria [2, 3].

In the subsequent decades following these observations, epidemiologist Dr. David Barker brought the DOHaD theory to prominence when he published a series of studies demonstrating that individuals born with low birthweight or in regions with high infant mortality (proxies for *in utero* undernutrition or stress) were at an increased risk of developing cardiovascular disease [4-6]. Interestingly, exposure to overnutrition *in utero* also results in an increased risk of metabolic disease later in life [7-9]. Like offspring born with low birthweight, high birthweight is also associated with increased body mass index (BMI) in adulthood [10], and offspring born to

overweight or obese mothers are more likely to be obese as children and into early adulthood [7]. Additionally, offspring of mothers who were obese before and during pregnancy developed insulin resistance and impaired glucose tolerance as adults at significantly higher rates independent of birthweight [11]. A series of studies investigating the relationship between maternal plasma glucose levels and offspring health outcomes has also demonstrated the negative consequences of exposure to overnutrition *in utero*. Offspring born to mothers with higher plasma glucose levels during pregnancy were more likely to have higher birth weight and a number of additional adverse outcomes, which were independent of maternal BMI [12].

It is important to note that maternal exposures extend beyond nutritional status alone, and include factors such as chemical exposure, infection, hormonal perturbations, or life stressors during pregnancy [13-17]. These other exposures can have significant effects on a variety of organ systems in the offspring as shown in Figure 1-1. For example, exposure to maternal psychosocial stress has been shown to lead to increased risk of insulin resistance in young adults independently of other major risk factors such as birthweight [17]. Young women who were exposed to maternal psychosocial stress also had decreased performance in a test of working memory [16]. The significant impact of a variety of *in utero* stressors on the offspring demonstrates the relevance of DOHaD and the importance of further investigation in this field.

Significance

The impact of these findings is magnified when considering the prevalence of overweight (BMI between 25 and 30) and obesity (BMI greater than 30) in the United States. As of 2016, the Centers for Disease Control and Prevention (CDC) estimates that over 70% of adults over the age of 20 are either overweight or obese [18]. While the prevalence of overweight has remained relatively unchanged over the past few decades, rates of obesity and extreme obesity (defined as BMI greater than or equal to 40) are increasing rapidly in both men and women [19]. While obesity rates are often higher in older populations, the increasing prevalence of obesity affects

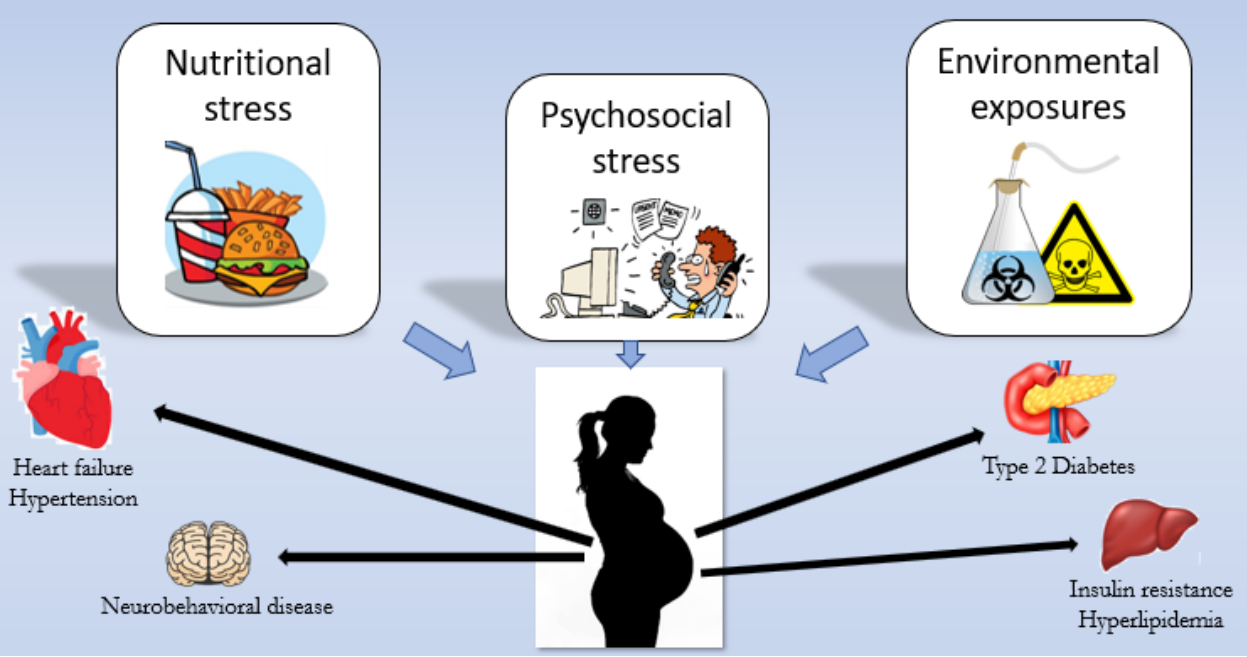


Figure 1-1: Various maternal stressors can have serious health consequences for the offspring. Nutritional, psychosocial, or environmental stressors can affect the function of multiple different organs in the offspring and lead to increased risk of disease.

reproductive age women to a significant extent. The most recent data from the Pregnancy Risk Assessment Monitoring System, a surveillance system of the CDC, indicate that nearly half of women in the United States are either overweight or obese at the time they become pregnant [20]. Thus, the consequences of maternal overnutrition on the developing fetus are likely to be realized in greater numbers in the coming decades. In order to appreciate how an *in utero* environment of overnutrition can lead to changes in pancreatic islet composition and function in the offspring (the focus of this thesis), it is necessary to highlight briefly the process of pancreatic and islet development.

Pancreas Development Primer

The following relies on a series of reviews [21-26] to which the reader is referred for a more in-depth analysis. Though the progression from progenitor to mature endocrine cells is very similar among different organisms, specific time points during mouse development are referred to, since most previous studies have utilized this model. For further reading on pancreas development in the human, two additional reviews on the topic may be of interest [27, 28].

During gastrulation at embryonic day 6.5 (e6.5) a migration inward of cells at or near the surface of the blastula forms a three-layered structure composed of ectoderm, mesoderm, and endoderm [22]. In the mouse, the embryo rotates from a lordotic position to a fetal position, leading to internalization of the endoderm layer to form a gut tube on e7.5 [25]. All endoderm-derived organs, including the pancreas, originate from this tube [21]. Signals from the overlying mesoderm, and other adjacent mesodermally-derived tissues, lead to patterning of the endodermal epithelium along the anterior-posterior axis as well as evagination of the dorsal and ventral pancreatic buds [24].

The basic cell lineage pathway and key developmental transcription factors leading to differentiated pancreatic endocrine cells are shown in Figure 1-2A. The transcription factor Pdx1 marks the pancreatic progenitor cells and is required for pancreas formation [23]. Pdx1 is

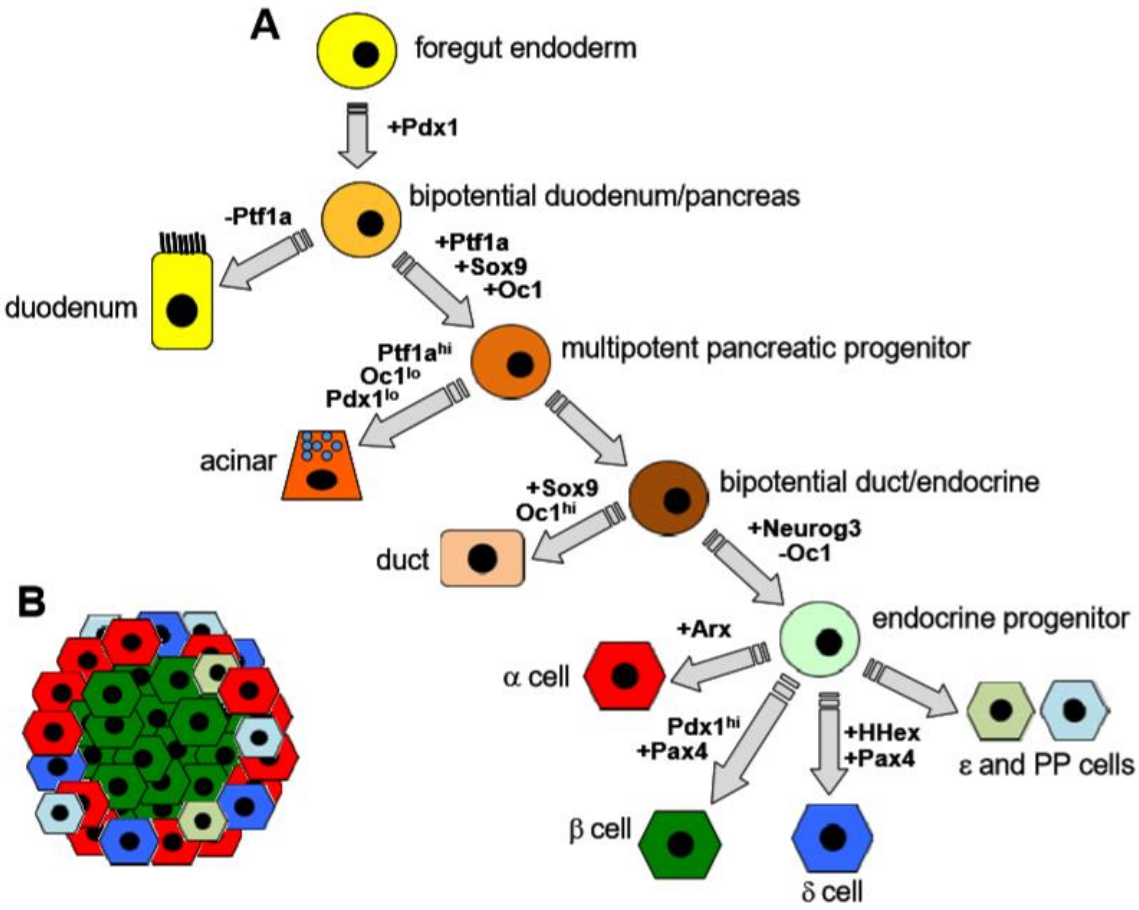


Figure 1-2. Schematic of pancreatic cell lineages and key lineage transcription factors. (A) Undifferentiated progenitor cell types are shown in circles. Differentiated cells are shown in other shapes. Key lineage-determining transcription factors up- or down-regulated at each stage are shown in bold. (B) Scheme of rodent islet showing insulin-producing β cells at the islet core and comprising the majority of the islet. All other endocrine cells are found at the islet periphery. Adapted from Elsakr and Gannon, *Trends in Developmental Biology*, 2017.

initially broadly expressed in the posterior foregut endoderm as early as embryonic day 8.5 (e8.5) in the mouse [26]. Shortly thereafter, the dorsal pancreatic bud evaginates from the dorsal foregut endoderm that lies between the putative stomach and duodenum domains. A region of the ventral foregut endoderm becomes the ventral pancreatic bud [24]. The dorsal bud first appears at e9.5. Co-expression of Pdx1 with the transcription factor Ptf1a defines the pancreatic progenitor cells and allows pancreas development to proceed [23]. The transcription factors Sox9 and Oc1 are also co-expressed with Ptf1a in a subset of Pdx1+ cells and are additional markers of pancreatic progenitor cells [26]. Pdx1+/Ptf1a+/Sox9+/Oc1+ multipotent pancreatic progenitors (MPCs) are highly proliferative and give rise to all the different pancreatic cell types (acinar cells, ducts, endocrine cells) [24]. The MPCs expand and rearrange to form a multilayered stratified epithelium. By e11.5 the two pancreatic buds have expanded into the surrounding mesenchyme, leading to the formation of a highly branched tree-like structure embedded in a loose mesenchyme [25].

The majority of endocrine cells in the mouse differentiate between e13.5 and e18.5 (with a peak at e15.5) in what is referred to as the secondary transition of pancreas differentiation (the "first wave" of differentiation produces a relatively lower number of primarily glucagon-positive cells that do not contribute to the adult pancreas [25]; it is unclear whether this first wave exists in humans). During this time, MPCs become lineage restricted to either Sox9+/Nkx6.1+ ductal-endocrine bipotent cells in the trunk of the tree-like structures, or Ptf1a+/Gata4+/Cpa+ cells in the tips that ultimately differentiate into acinar cells [24]. Key transcription factors required for endocrine differentiation are expressed at this stage. Neurogenin3 is the definitive marker of the endocrine progenitor population [23]. Bipotent endocrine/ductal cells are directed toward an endocrine fate by transient expression of this transcription factor, with expression peaking at e15.5 [29]. Pdx1 becomes down-regulated as the endocrine pancreas develops, but is ultimately expressed at high levels specifically in the β cell. Dynamic changes in Pdx1 expression are necessary for proper endocrine differentiation [24].

Committed endocrine cells delaminate from the ductal epithelium and aggregate to form nascent islets around e18.5 [22]. Differentiation of single-hormone positive cells (insulin, glucagon, somatostatin, PP, ghrelin) depends on specific combinations of transcription factor expression in gene regulatory networks that also repress alternate islet cell fates [29]. The transcription factor Pax4 plays a key role in the β cell gene regulatory network. *Pax4* mRNA is first detected at e9.5 and is transiently expressed in all endocrine progenitor cells during development [22]. It is downstream of Neurogenin3 and essential for appropriate initiation of β-cell differentiation [24]. Loss of Pax4 results in decreased expression of Pdx1 (which becomes restricted to the β cell) and insulin mRNA in β-cell precursors [22]. In the α cell, Arx plays a key role in differentiation. Arx expression begins at e9.5 and persists into mature α cells. Pax4 has an opposing role to Arx and helps to suppress the α-cell gene regulatory network in developing β cells [26]. In all species examined, the majority of hormone-expressing endocrine cells are β cells; however, the proportion of β cells and their localization within the islet can differ in different species [23, 30]. In mice, islets are ultimately composed of a core of insulin-producing β cells, which account for about 80% of all endocrine cells, surrounded by a mantle of the other endocrine cell types: glucagon-producing α cells, somatostatin-producing δ cells, pancreatic polypeptide-producing PP cells, and ghrelin-producing ε cells (Figure 1-2B). During the second wave of endocrine differentiation, endocrine cells increase in number mainly due to neogenesis from bipotent progenitors [22]. In both human and mouse, all the endocrine cell types are present at birth and mature endocrine cells aggregate into islet structures shortly after birth. Expansion of the endocrine population after birth is primarily due to proliferation rather than neogenesis [23].

Loss of β Cell Functionis a Critical Step in Developing Type 2 Diabetes (T2D)

The insulin-producing β cell plays an essential role in maintaining glucose homeostasis in healthy individuals by sensing plasma glucose levels and secreting appropriate levels of insulin [31]. Glucose enters the β cell through the facilitated glucose transporter GLUT2, and is then

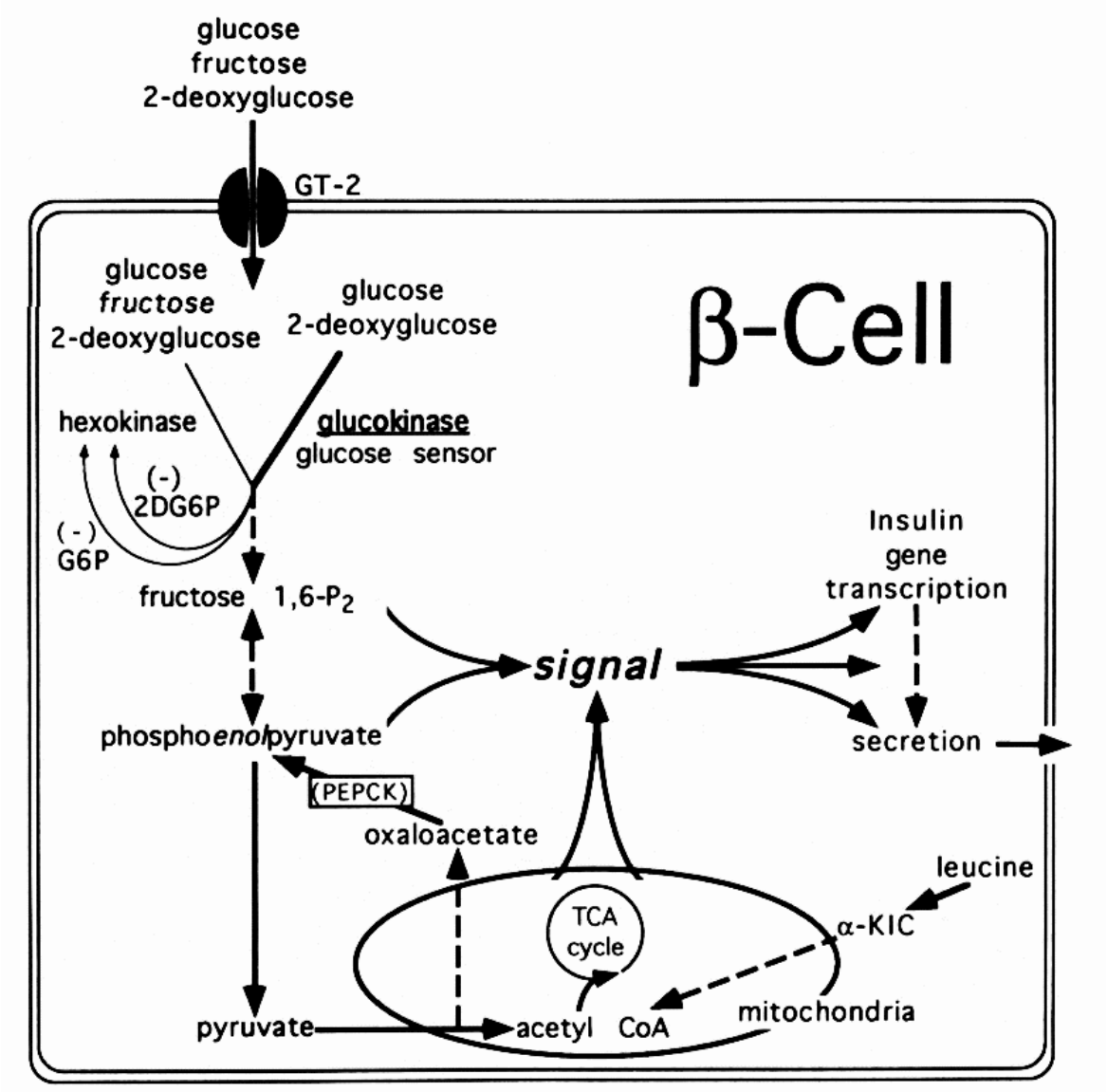


Figure 1-3: Glucose metabolism in the β cell. Glucose enters the cell through the transporter GLUT2 (GT-2). It is then phosphorylated by glucokinase in the rate-limiting step of glycolysis. Glycolysis produces pyruvate as an end product, which enters the TCA cycle to produce high energy electron carriers that will feed into the electron transport chain and ultimately generate ATP. From Michael German, *PNAS*, 1993.

phosphorylated by the enzyme glucokinase, which determines metabolic flux through glycolysis [31] as shown in Figure 1-3. The resulting product, glucose-6-phosphate, is further metabolized through glycolysis and the citric acid cycle, leading to the reduction of electron carriers NAD+ and NADP+. These electrons are then passed on to the electron transport chain and ultimately used to reduce oxygen. The flow of electrons is coupled to the pumping of hydrogen ions across the inner mitochondrial membrane as shown in Figure 1-4 [32]. Then, inner-membrane embedded ATP synthase couples the flow of hydrogen ions back down their concentration gradient to the production of ATP. An increase in the ratio of intracellular ATP to ADP results in closure of plasma membrane ATP-sensitive potassium channels [33, 34], causing membrane depolarization due to trapping of the positively-charged potassium ions within the cell. Depolarization leads to the opening of voltage-gated calcium channels and allows extracellular calcium to rush into the cell [33]. This then leads to further release of intracellular calcium stores within the β cell endoplasmic reticulum, which causes insulin granules to fuse to the plasma membrane and release insulin into the extracellular space [35]. Since islets are highly vascularized [36], insulin released by the β cell quickly enters the blood stream and acts on peripheral insulin-responsive tissues (liver, adipose, skeletal muscle), resulting in the uptake of glucose and the lowering of plasma glucose levels [37]. Studies in isolated mouse and human islets *ex vivo* revealed that following stimulation with glucose, insulin is released in two phases: an abrupt, short-lived early first phase followed by a more prolonged second phase of insulin release [38, 39]. This is shown schematically in Figure 1-5.

Human autopsy studies reveal that individuals with normal glucose homeostasis exhibit differences in total β-cell mass at birth and in adulthood (Figure 1-6). Though it is not possible to study β-cell mass longitudinally in humans, many factors can potentially lead to this individual variability. Fetal development represents a “critical window” where appropriate numbers of β cells are produced [40]. Early exposures during this period, such as *in utero* overnutrition or undernutrition, may influence eventual β-cell mass in adulthood. Indeed, the wide range of β-cell

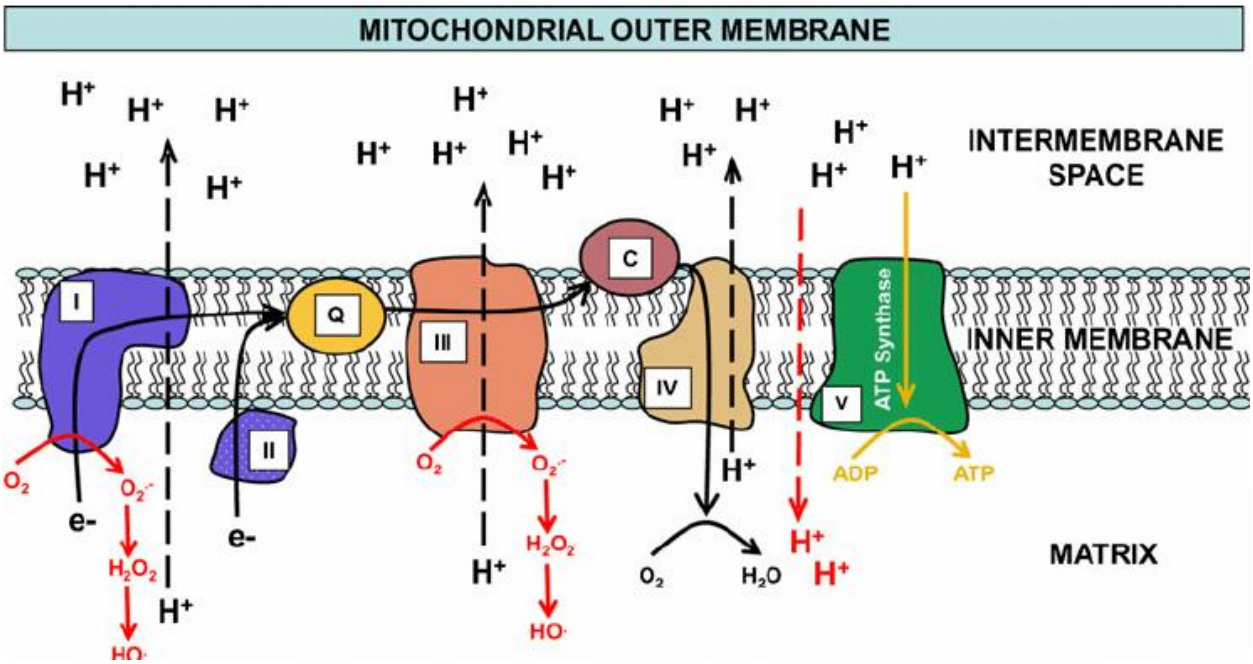


Figure 1-4: Electron flux through the electron transport chain. Within the inner membrane of the mitochondria, electrons are passed down an energy gradient through a serious of increasingly electronegative proteins. This exothermic process is coupled to the pumping of hydrogen ions across the inner membrane and into the intermembrane space. This creates a hydrogen ion gradient. Hydrogen ions flow down their concentration gradient into the mitochondrial matrix through ATP Synthase, which uses the energy released from this process to synthesize ATP. Adapted from Mcewen et al., *Neurotherapeutics*, 2011.

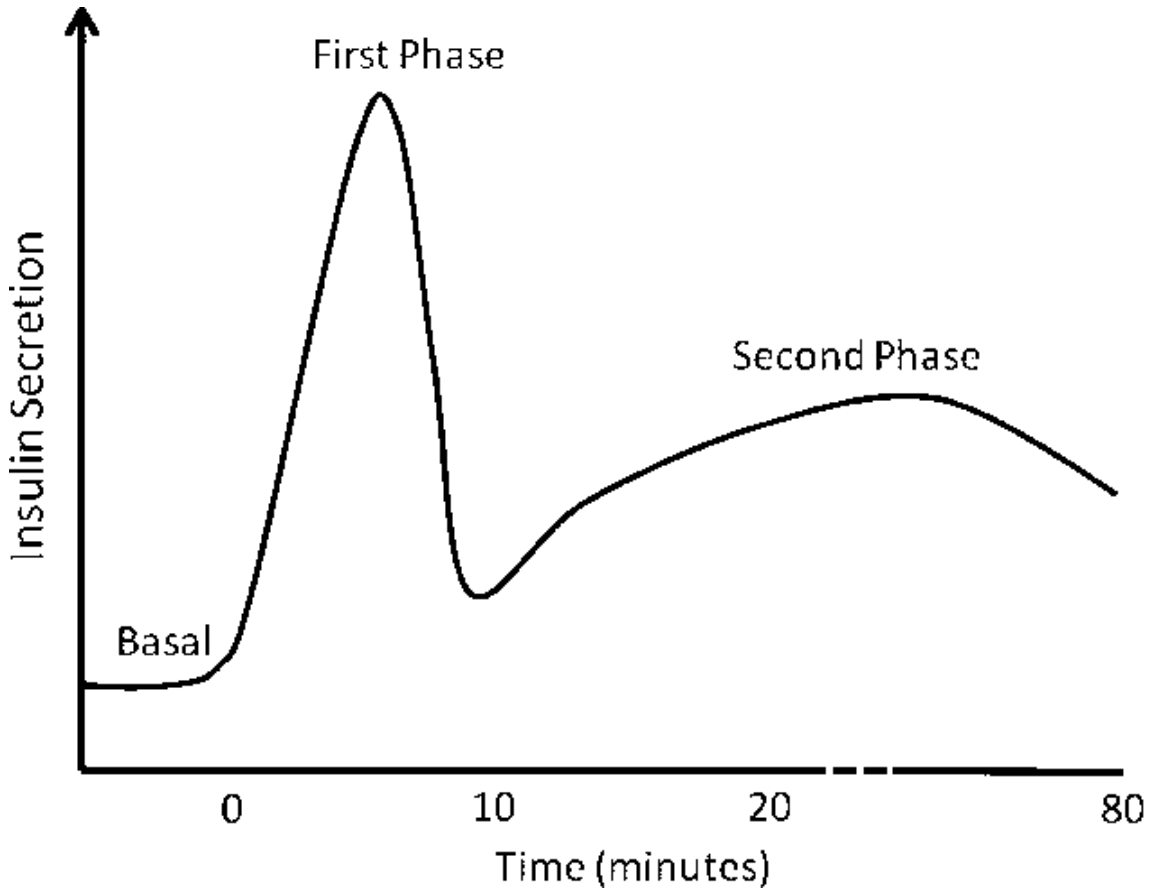


Figure 1-5: Biphasic insulin secretion by the islet. After stimulation with glucose at time = 0, the β cells respond by an abrupt, early phase of insulin secretion. This is then followed by a prolonged, sustained second phase of secretion. From Meloni et al, *Diabetes, Obesity, and Metabolism*, 2013

mass observed in healthy adults is already apparent in early childhood [41]. Because of the wide range of β-cell mass observed in healthy individuals, one can assume that there is a range of "normal" β-cell mass that can effectively maintain euglycemia under standard conditions. However, in both humans and mice, in the face of additional metabolic demand, such as obesity-induced insulin resistance, a higher level of functional β-cell mass is required to maintain euglycemia [42]. In the majority of individuals who are insulin resistant, β-cell compensation occurs, including an increase in insulin production and output per β cell, and an increase in β-cell mass via proliferation (Figure 1-6, dashed lines). However, in some individuals, often in the face of prolonged metabolic stress, β-cell compensation fails and there is a loss of functional β-cell mass leading to hyperglycemia and T2D (Figure 1-6, dotted lines) [32, 43].

Glucose homeostasis is assessed using a glucose tolerance test (GTT) (Figure 1-7A) in which fasting plasma glucose is measured and then a bolus of glucose is administered (orally or intraperitoneally/IP) followed by serial measurements of plasma glucose over a set time period. This test assesses the ability to clear glucose—a function of insulin secretion by the β cells and uptake by insulin-sensitive peripheral tissues (such as liver, muscle, and adipose). Plasma insulin levels can also be measured during the GTT (Figure 1-7B). Figure 1-7 depicts an illustrative GTT and plasma insulin profile showing typical results from a healthy individual (black line), someone with insulin resistance and impaired glucose tolerance (green line), and someone with diabetes (orange line). Obesity and insulin resistance are associated with increased glucose excursions and increased plasma insulin, with normal fasting and 2-hour post-prandial blood glucose levels (green lines). However, prolonged periods of insulin resistance place undue stress on the β cells [44], and in individuals with genetic susceptibility, can lead to fasting hyperglycemia and T2D (orange lines).

Insulin resistance is defined as an inability for a quantity of insulin to stimulate glucose uptake by the peripheral tissues [45]. A number of circulating factors, many of which are released

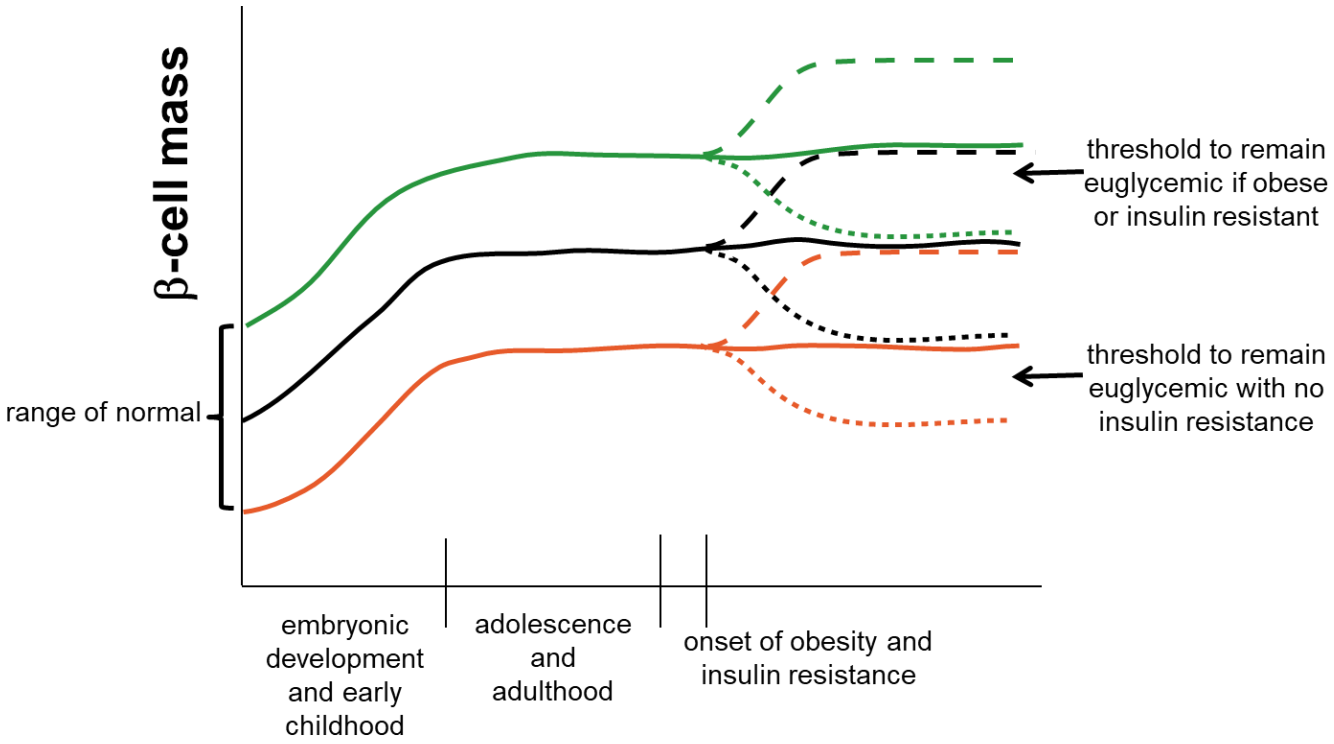


Figure 1-6. A model of the dynamics of β-cell mass under normal and impaired metabolic states. β-cell mass is established via neogenesis during embryogenesis and proliferation in early postnatal life, plateauing in childhood. Individuals are born with a range of β-cell mass that is considered "normal" in that it is sufficient to maintain euglycemia in the absence of metabolic stress. Average β-cell mass is shown in the black solid line, upper and lower ranges of normal are shown in green and orange solid lines, respectively. With the onset of obesity and insulin resistance, the normal response is an expansion in β-cell mass (by proliferation) shown in the dashed lines. Individuals starting with a lower level of β-cell mass may not reach the threshold required to maintain euglycemia in the setting of insulin resistance, leading to increased susceptibility for T2D. Alternatively, β-cell failure and/or death (dotted lines) can occur in the setting of obesity and insulin resistance, again leading to an increased susceptibility to T2D for individuals starting with fewer β-cells. Adapted from Elsakr and Gannon, *Trends in Developmental Biology*, 2017.

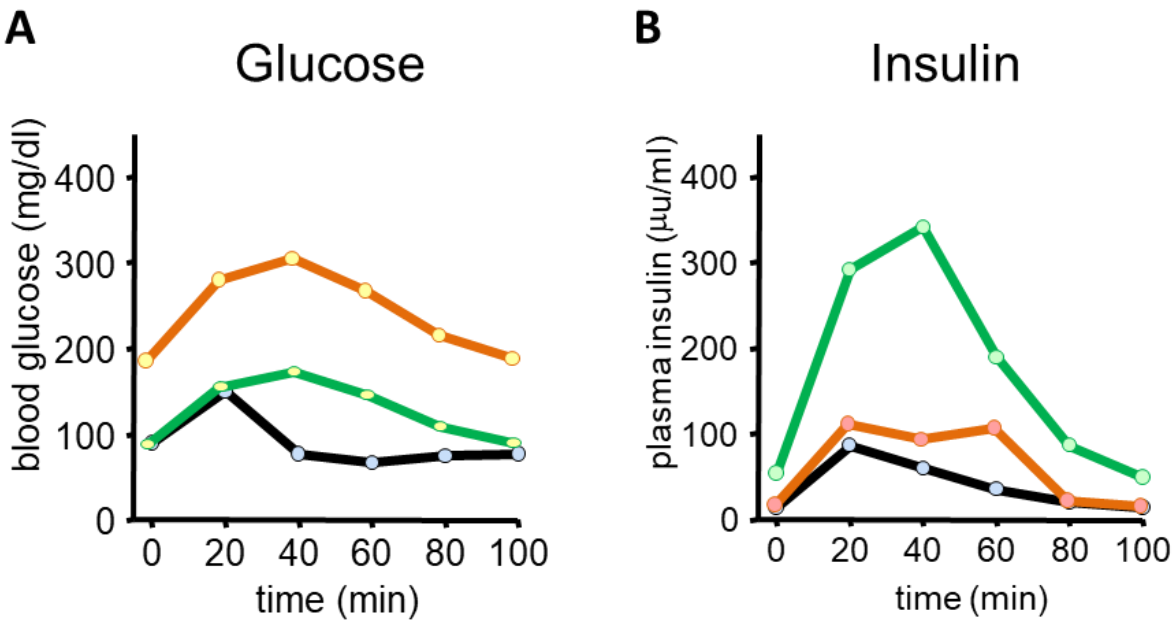


Figure 1-7. Correlation of glucose homeostasis with plasma insulin levels under normal and impaired metabolic states. (A) Representative intraperitoneal glucose tolerance test in which after an overnight fast, the subject is given a glucose bolus. Blood glucose is measured at fasting and at different time points after glucose administration over a two-hour period. Black line is the normal glucose excursion curve. Green line represents impaired glucose tolerance. Orange line represents diabetes. (B) Plasma insulin levels measured at the same time points as blood glucose in (A). Colored lines are same as in (A). Glucose intolerance is associated with hyperinsulinemia, while diabetes is associated with reduced plasma insulin in the face of elevated blood glucose. Adapted from Elsakr and Gannon, *Trends in Developmental Biology*, 2017.

from adipose tissue, can affect the sensitivity of the muscle and liver to insulin [46]. Specifically, both leptin and adiponectin are released from adipocytes and can increase insulin sensitivity [47]. Leptin is released in direct proportion to fat mass, while adiponectin is released in inverse proportion to fat mass [46]. Furthermore, in states of prolonged hyperleptinemia associated with obesity, leptin resistance can develop leading to a paradoxical decrease in insulin sensitivity as a result of higher leptin levels [47]. Additionally, in obesity there as an increase in the release of free fatty acids and adipokines such as tumor necrosis factor α and IL-6, which collectively reduce insulin sensitivity [46]. Finally, obese adipose tissue also releases chemoattractants, which leads to macrophage infiltration and ultimately results in additional tumor necrosis factor α release [48]. Together, these factors along with other genetic and environmental factors can lead to insulin resistance in obese individuals.

The precise reasons why some insulin resistant individuals have β-cell failure and develop diabetes while others are able to compensate remains unclear. Likely, the etiology of diabetes can differ in different individuals. The final outcome is likely determined by a combination of the initial β-cell mass, the ability to increase insulin production and secretion, the extent of β-cell proliferation, and the susceptibility to β-cell dedifferentiation or death (Figure 1-6). An individual with β-cell mass at the upper end of normal (green line in Figure 51-6) has sufficient β-cell mass at baseline to maintain euglycemia even in the face of insulin resistance, provided there is no loss of β-cell mass. Conversely, an individual with β-cell mass at the lower end of normal (orange line) has sufficient β-cell mass to maintain euglycemia in the absence of metabolic stress, but in the face of insulin resistance the low initial β-cell mass is insufficient, even with a compensatory increase.

In summary, T2D develops when the supply of functional β-cell mass is insufficient to meet the metabolic demand for insulin. This can occur due to an initially low level of β-cell mass, or an inability to compensate for insulin resistance. As discussed in the previous section, the development of the endocrine pancreas (and resulting baseline level of β-cell mass) relies on

coordinated expression of specific transcription factors during critical developmental windows. Many of these transcription factors are also expressed in the mature β-cell and have important roles in β-cell function. As a result, exposure to overnutrition *in utero* may increase T2D susceptibility by affecting the initial mass of β cells produced during development, or altering the function of β cells such that they are unable to respond to metabolic stress.

Animal Models of Maternal Overnutrition

In the following sections I discuss the advantages and challenges of different animal models, the effects of exposure to overnutrition during distinct periods of development, the similarities and differences between and within model systems, and potential mechanisms and future directions in understanding how developmental alternations due to maternal diet exposure influence islet health and function later in life.

Rodent Models of Maternal Overnutrition

Rodent models are most frequently used to investigate the effects of *in utero* overnutrition on islet development and function. Rodents have several advantages that enable thorough, well-controlled experiments that would otherwise be difficult to conduct in large animal models. The advantages and disadvantages of both small and large animal models are discussed thoroughly in a 2009 review by McMullin and Mostyn [49], and the following is based on their review and my experience with both mouse and non-human primate models. Due to the short duration of gestation and lifespan, large litter size, and the relatively low cost of maintaining colonies, it is usually feasible to conduct rodent studies which are sufficiently powered. Additionally, it is easier to minimize genetic and environmental variability through inbred strains and uniform housing, it is feasible to monitor food intake, and multiple genetically modified models are available, enabling specific probing of pathways mediating the effects of maternal diet on the offspring and the ability

to sort out the role of and effects on specific cell types. Furthermore, dietary components can be easily manipulated to test the effects of specific macronutrients on the health of the offspring.

Conversely, significant challenges exist when interpreting and comparing results from different studies performed in rodent models due to differences in study design. Variables that must be taken into consideration include rat versus mouse, the strain of mouse used, the source of maternal overnutrition (e.g. high fat versus high fat/high sucrose diet, different commercial sources and percentage of calories from fat, etc.), timing of diet initiation relative to pregnancy, offspring post-weaning diet, and several other factors.

Multiple studies in rodents have investigated the effects of maternal high fat diet (HFD) or obesity during pregnancy and its effects on offspring islet development and function [50-60]. A summary of the effects of maternal overnutrition on offspring islets using rodent models is presented in Table A-1 (Appendix). The majority of these studies were conducted in rats and completed before the year 2010. As mentioned, comparisons between studies are often difficult due to differences in animal model, diet, and study design. However, three themes emerge when considering the body of results as a whole. First, overwhelmingly, maternal HFD results in significant changes in islet architecture or function, many of which could be predicted to increase susceptibility to T2D. For example, Graus-Nunes et al. reported that HFD-exposed male mice and their male offspring had increased islet mass and fasting hyperinsulinemia [55], which can lead to insulin resistance. Meanwhile, Wistar rats exposed to HFD had reduced β-cell mass at birth [52], which may predispose these animals to develop diabetes later in life (Figure 1-3). Second, while changes in endocrine cell mass or islet architecture vary, in the majority of studies there is some evidence of impaired islet function. This manifests as whole body impairments in glucose tolerance in the offspring or decreased insulin secretion from islets isolated from HFD- or obesity-exposed offspring when the islets were treated with high glucose or other insulin secretagogues. For example, Han et al. showed that mouse offspring exposed to maternal obesity initially have

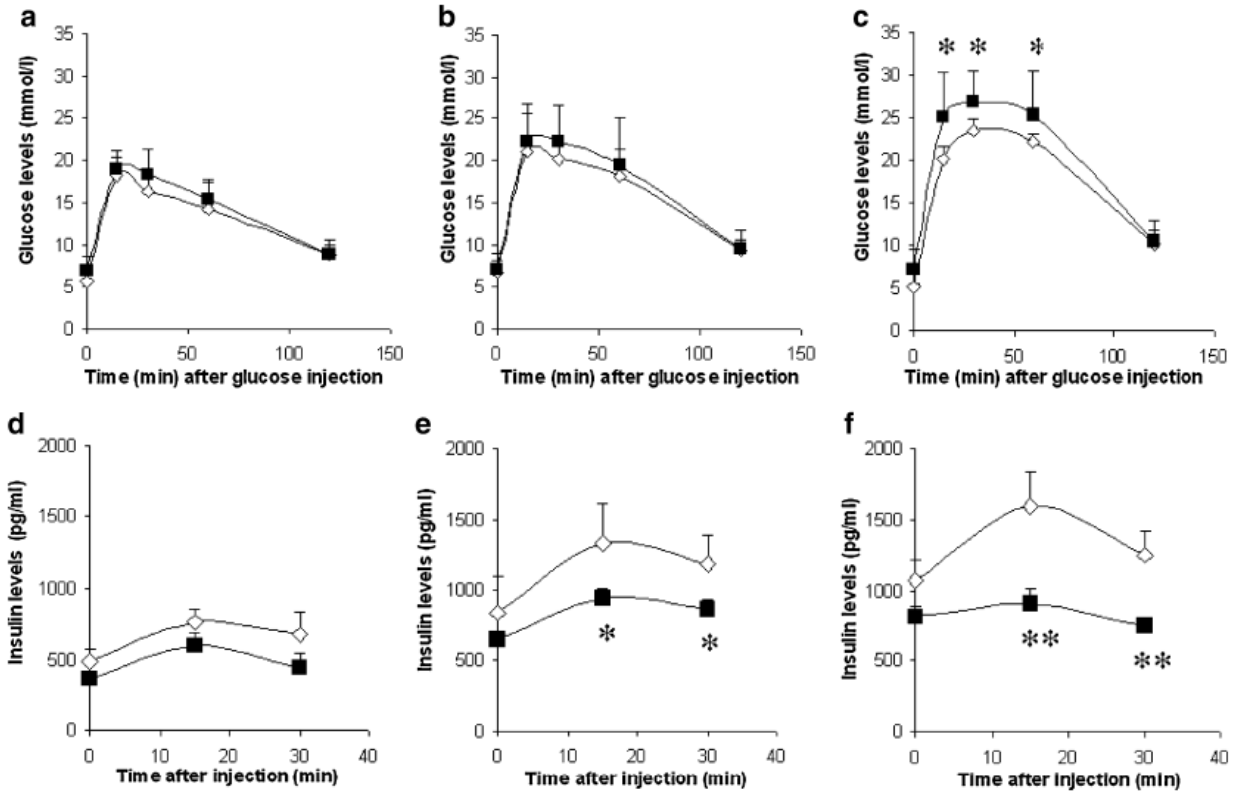


Figure 1-8: High fat diet impairs islet function in offspring from obese female mice. Glucose tolerance tests (A, B, C) and plasma insulin levels (D, E, F) from high fat diet-fed female offspring born to control (open diamonds) and obese (closed squares) dams. Offspring were analyzed at 20 (A, D), 30 (B, E), and 50 (C, F) weeks of age. Data are means ± SD. *p < 0.05, **p < 0.01 compared with control; n = 8–10. Adapted with permission from Han et. al. (2005). *Diabetologia* 48: 1810-1818.

normal glucose tolerance but demonstrate a progressive loss in β-cell function (i.e. insulin secretion) with age that ultimately results in impaired glucose tolerance in adulthood [57], as shown in Figure 1-8. Third, in the minority of studies that focused on sex differences, there were observable differences in islet function between males and females, although which sex was more severely affected depended on the study. There is some evidence in humans that adverse *in utero* exposures have differential effects on male and female offspring that can increase risk for metabolic disease. Specifically, male and female offspring born during the Dutch Hunger Winter had differential DNA methylation at metabolic gene loci [61]. Additionally, exposure to the chemical BPA *in utero* results in differential levels of the satiety hormone leptin in human males and females [62].

It is important to note that while some rodent studies show significant effects of maternal overnutrition alone on the offspring at a young age [50, 51, 55, 63], others have shown either no effect on the offspring or more subtle effects that only manifest with increased age or additional metabolic stress [57, 64]. For example, Han et al utilized a genetic model of maternal overnutrition—female mice on a C57Bl/6 background with the Agouti yellow mutation. Offspring were fed either a control or HFD (60% of calories from fat) starting at 15 weeks of age. Only females who were weaned to HFD had impaired glucose tolerance at 50 weeks of age, and not at earlier timepoints. Similarly, only female offspring of obese mothers had decreased insulin levels during glucose tolerance testing at 50 weeks of age, as well as at 30 weeks of age if the offspring had been on a HFD for the previous 15 weeks [57]. This study and others demonstrate that in some cases, the effects of maternal overnutrition may only manifest when offspring are exposed to additional metabolic stressors.

While rodent models have been beneficial in studying effects of maternal overnutrition on the offspring due to the advantages described above, there are also several disadvantages to these models [49] that warrant the addition of studies in larger animal models. For example, the large litter size leads to an *in utero* environment significantly different from that in humans. Space

sharing within the uterus results in differences in fetal access to nutrition depending on location within the uterus. Additionally, studies using multiple animals per litter per group when sample number is low may not be statistically sound. Most rodents are altricial—born at an underdeveloped state relative to larger animals or man. Development of the pancreas continues into postnatal life in rodents; islet neogenesis and significant endocrine cell proliferation occur post-natally in the mouse. Mouse diets, especially purified diets high in specific nutrients, are not representative of human diets, though cafeteria style diets partially mitigate this process.

The Zebrafish Model of Overnutrition During Development

The zebrafish may seem an unlikely model to study the effects of overnutrition during development, but it has a few unique advantages over other animal models. Zebrafish hatch at 3-4 days post fertilization (dpf) but the pancreas does not reach its mature shape and position until 6 dpf [65]. Besides the low cost and feasibility of maintaining sufficient numbers of fish, this developmental scheme allows incubation of zebrafish larvae in solutions with specific macronutrient composition. Thus, precise probing of specific macronutrients can determine which aspects of the diet affect the development of β cells. Additionally, since larvae are transparent and development proceeds externally, islet development can be monitored longitudinally in the same individual using fluorescently labeled transgenes (see Figure 1-9). These benefits are in addition to many of the same benefits in other small animal models discussed above (ease of genetic manipulation, large litter size, etc.).

A 2012 study by Maddison and Chen [66] investigated the effects of specific nutrient combinations on developing larvae at 5 dpf (the start of free feeding). At this stage, zebrafish possess a single islet with approximately 30 β cells, and are in the second wave of endocrine differentiation. At 5 dpf, unfed larvae had an average of 32 β cells in the islet. Experimental groups were provided hatchfry encapsulation (a zebrafish larval diet with 12% lipid), 20 mM (high) glucose, or 5% chicken egg yolk (a higher lipid diet at 26.5%). As shown in Figure 1-9C, HFD-

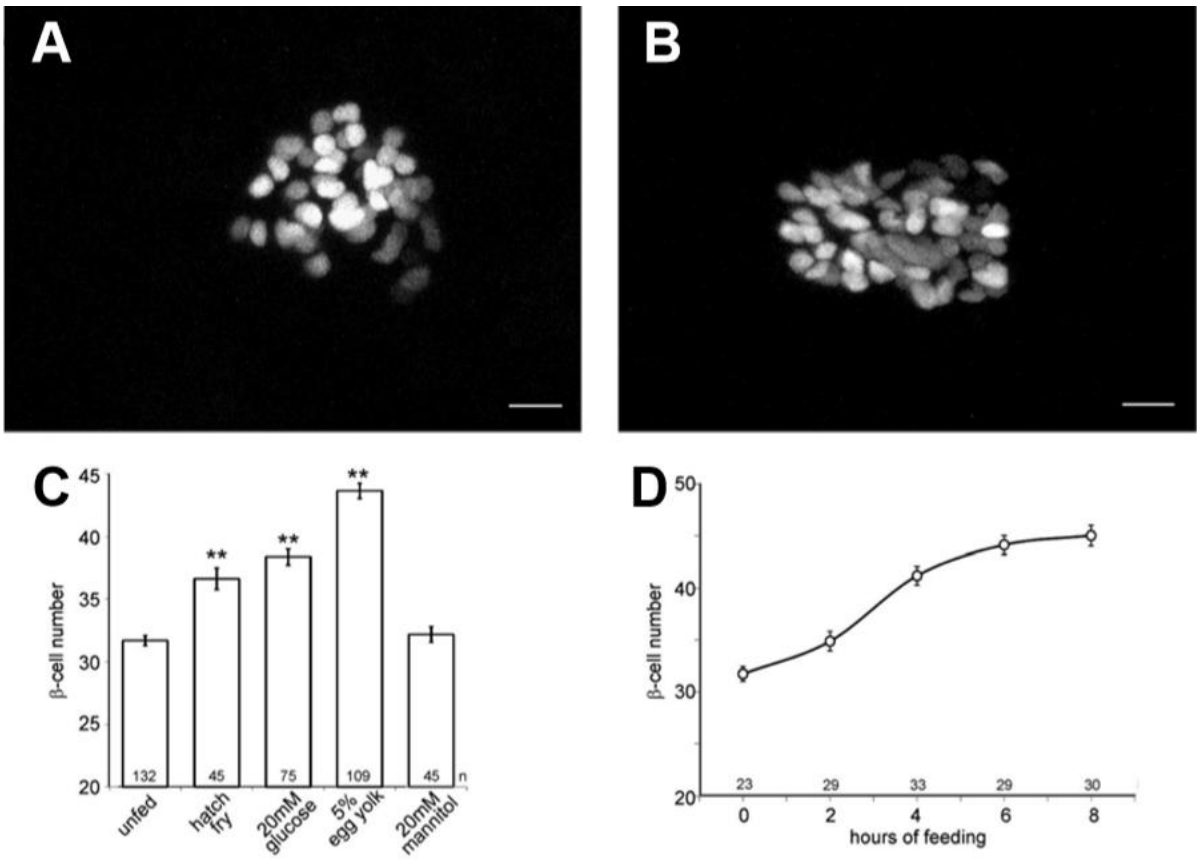


Figure 1-9: Exposure of zebrafish to overnutrition during islet development increases β-cell neogenesis. (A, B) Images of β cells expressing a nuclear mCherry transgene under control of the insulin promoter. Approximately 30 β-cells are observed in unfed larvae (A). This number increases after 8 h of culture in chicken egg yolk (B). Scale bars, 10 µm. (C) Effects of overnutrition on β-cell numbers in 6-dpf larvae. Mannitol changes the osmolarity similar to glucose. Bars indicate mean with SE (**ANOVA versus unfed, $P < 0.001$, Tukey HSD). (D) Time course of the increase in β-cell number within the first 8 h of culturing in 5% chicken egg yolk. n indicates the number of individual larvae in each sample group. Adapted from Maddison and Chen, *Diabetes,* 2012.

exposed fish had an average of 43.1 β cells after eight hours of incubation compared with 36.7 fed hatchfry. β cells were also larger in HFD-exposed fish (not shown). Interestingly, when specific components of this diet were administered individually (amino acids, intralipid, or glucose at low concentration), there was no increase in the number of β cells compared with unfed larvae, and only the three macronutrients combined resulted in β-cell numbers comparable with chicken egg yolk. Only with the administration of high concentrations of glucose were β-cell numbers increased, and still to a lesser extent than with chicken egg yolk, suggesting that overnutrition itself, rather than a specific macronutrient, was responsible for the phenotype observed. Another series of experiments demonstrated that the increase in β-cell number due to overnutrition was a result of increased differentiation from progenitors rather than proliferation of existing β cells, leading to the authors' conclusion that β-cell development was accelerated in this model. The long-term effects of these developmental alterations have not yet been examined in the fish.

Sheep As a Model of Maternal Overnutrition

A series of studies in sheep also demonstrated a similar response to overnutrition during development, with increases in β-cell in young offspring [67-69]. Ewes were fed either an obesogenic or control diet for 60 days before conception then throughout pregnancy and lactation. Fetuses analyzed in mid-gestation (day 75) were larger in the obese group, with a disproportionate increase in pancreatic weight. These animals also had higher plasma glucose and insulin levels and increased β-cell mass, suggesting that maternal obesity accelerated β-cell development in this model. Unlike zebrafish, however, this increase in β-cell mass was primarily due to increased proliferation. In late gestation (day 135), fetuses in the obese group had similar weights to control animals, and pancreatic weight and β-cell mass were now reduced, with increased β-cell apoptosis. At birth, obese-exposed sheep had elevated blood glucose and reduced insulin levels. The relative loss of β-cell mass due to increased apoptosis was likely due to chronic stress on the developing β cells from at least mid-gestation to the time of birth. As

adults, obese-exposed sheep were insulin resistant, glucose intolerant, and consumed more feed during a feeding challenge resulting in significant weight gain. This series of studies demonstrates that early increases in β-cell mass due to overnutrition during development, which would appear to be a beneficial compensation, may set the offspring up for failure later in life. Indeed, it has been proposed that β cells have a finite capacity for proliferation, and high levels of proliferation early in life can ultimately prohibit the expansion of β-cell mass later in life [70]. It would be interesting to see if this were true for the zebrafish model as well, despite the different etiologies of the additional β cells (neogenesis vs. proliferation).

Large animal models like the sheep have several distinct advantages when modelling maternal overnutrition [49]. These include fewer offspring per pregnancy—sheep give birth to between one and three live offspring. These offspring are born at a similar birthweight to humans. Unlike rodents, which are altricial species, large animal models have a fully developed hypothalamic-pituitary-adrenal axis before birth. However, there are also disadvantages specific to the sheep model—sheep have a ruminant GI system, breaking down plant products via fermentation to produce volatile fatty acids, which serve as a major energy source. This leads to important differences in metabolism between sheep and humans. Additionally, glucose tolerance tests are less meaningful in sheep since they rely more on fatty acids than glucose. Another large animal model, the non-human primate (NHP) has notable similarities with human pregnancy in terms of placentation, longer infant-dependent state, metabolism, and milk composition. Furthermore, islet structure and function have been well characterized in the NHP and are very similar to that of humans, especially compared with the mouse.

The Non-Human Primate Model of Maternal Overnutrition

NHP and Human Islets Have Similar Composition and Function

In humans, a phase of islet remodeling that occurs during development results in islets with a more complex structure than mouse islets. Initially, single endocrine cells can be found

within the epithelial cords as early as 6-9 weeks gestation [71]. Then, small clusters of α and/or β cells begin to appear and ultimately form mouse-like islets with β-cell cores by 12 weeks gestation [72]. Interestingly, these mouse-like islet structures then reorganize into homotypic groups of primarily a single type of endocrine cell. Finally, these smaller islet structures coalesce to form larger islets that contain additional endocrine cell types within the core of the islet by the third trimester, a process which continues postnatally [73, 74]. This is shown schematically and compared with mouse islet development in Figure 1-10. Importantly, the ratio of β:α cells in human islets is significantly lower than in mouse [75, 76]. While the clustering of endocrine cells types within the islet is likely not completely random [71], there is a greater proportion of non β-cell islet cells within the islet and in contact with β cells than in mouse islets. Together, these differences increase the degree of cell-to-cell contact among the different endocrine cell types in human compared with mouse, strongly suggesting a larger role of non-β islet cells in β-cell function specifically in human islets [71, 77, 78]. Additionally, unlike mouse islets, human islets do not display synchronous calcium oscillations in response to high glucose, and do demonstrate a calcium response to low glucose, further suggesting a more prominent role for the α cell in the human islet [76].

The differences in cell composition between rodent and primate islets are demonstrated in the immunohistochemically labelled islets in Figure 1-11A-C comparing NHP (specifically Japanese macaque), human, and mouse islets. Additionally, NHP and human islets have similar expression levels of key transcription factor genes (Figure 1-11E) and insulin secretory profiles in response to various stimuli [30]. Together, these structural and functional similarities between NHP and human islets indicate that the macaque islet may be a better model of human islet physiology than the mouse.

NHP, Human, and Rodent Pregnancy and Gestation

In addition to having similarities in islet composition and morphology, macaques are

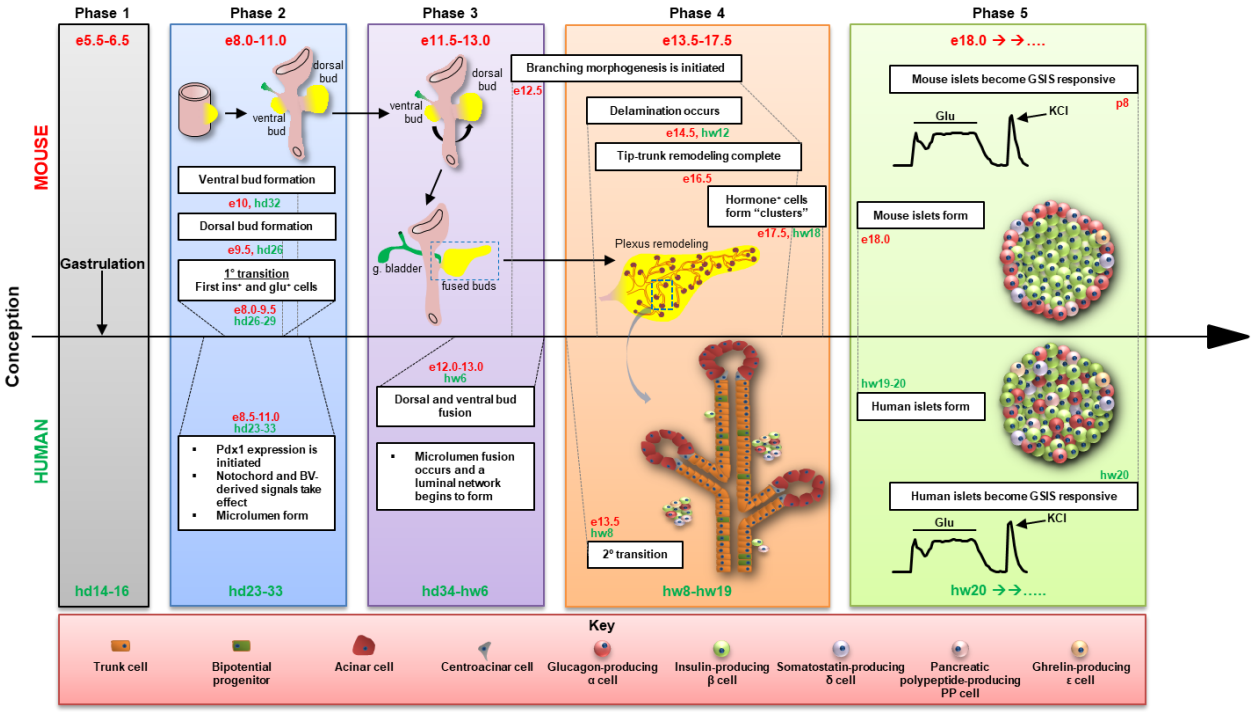


Figure 1-10: Comparison between mouse (top) and human (bottom) islet development. Though initial stages of pancreas and islet development are similar between species, human islets develop a more complex structure in Stage 5. Mouse-like islet structures reorganize into homotypic clusters of endocrine cells. These then coalesce to form larger islets with a high degree of cell-to-cell contact between the different islet cell types as shown above. This process begins in the third trimester but continues postnatally. Adapted from Andrei and Gannon "Embryonic Development of the Endocrine Pancreas" In: Transplantation, Bioengineering, and Regeneration of the Endocrine Pancreas: Volume 2, 2019.

similar to humans with regards to pregnancy and lactation. Macaques most commonly give birth to a single, live offspring. In contrast, rodents undergo a shorter gestation and give birth to multiple, altricial progeny [79]. While this is an attractive feature for biomedical research, increasing the number of replicates and decreasing animal housing and maintenance costs, it is a poor representation of human gestation. Conversely, the cost of maintaining NHP colonies and the length of gestation (in addition to time to reach sexual maturity, which is up to 4 years of age in this model) often make widespread use of this animal prohibitive.

With respect to the mother, female NHPs share many similarities with pregnant women [80]. NHPs demonstrate levels of hypothalamic and pituitary hormones throughout pregnancy that are distinct from mice and similar to humans [81]. Control of ovulation is also similar to humans and depends on factors like leukemia inhibiting factor which do not play a role in mouse ovulation [82]. NHP placental morphology and secretion of hormones, as well timing of the menstrual cycle and changes to the endometrium, closely resemble the human condition [83]. Both the similarities in NHP gestation and islet composition and function make this model ideal for studying the effects of maternal diet during pregnancy and lactation on the endocrine pancreas of the offspring. Taking advantage of this model, our collaborators have demonstrated significant effects of maternal overnutrition on offspring physiology in a variety of organ systems.

Maternal Western-Style Diet Has Significant Effects on Various Organ Systems in the NHP

Beginning in 2010, our collaborators have utilized a Japanese macaque model of maternal overnutrition to investigate the effects of a high-fat and high-sucrose Western-style Diet (WSD) on the offspring. In this model, female macaques are fed CTR or WSD for 2-7 years before pregnancy and throughout gestation and lactation. Offspring are analyzed as fetuses at gestational day (GD) 130, at 13 months of age, and at 3 years of age, or throughout life for behavioral studies. Offspring are weaned to a CTR or WSD at 8 months of age. This model is shared among multiple labs across the country, who study the effects of maternal WSD on

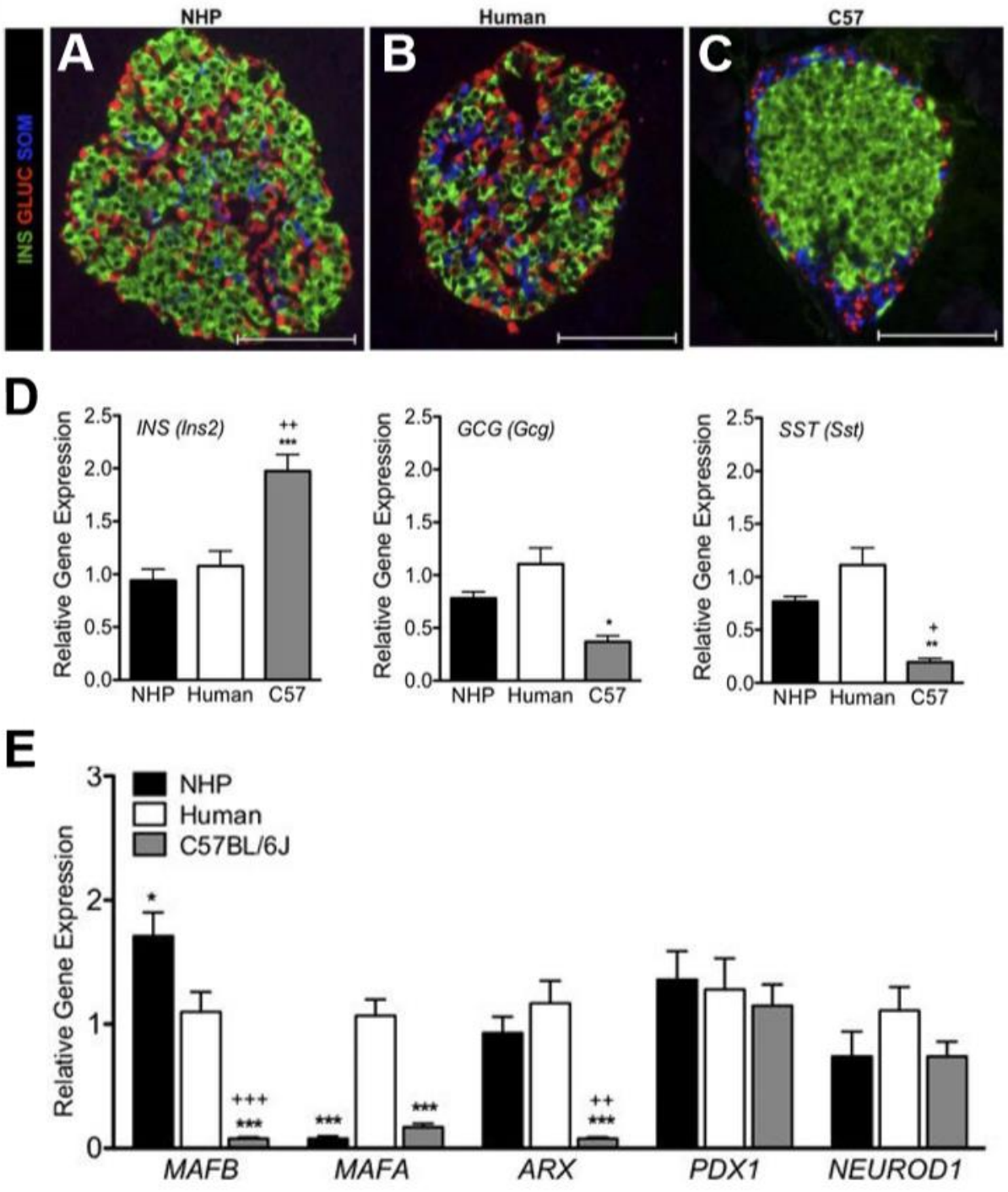


Figure 1-11: Differences in islet morphology and composition in different species. Non-human primate islets (A) are more similar in architecture to human islets (B). Both species have less defined mantle and core domains than mouse islets (C). In mice, β cells are restricted to the islet core while other cell types are found at the periphery. In non-human primates and humans, non-β cells are found at the periphery and internal to the islet. (D) β cells comprise a larger proportion of endocrine cells in mouse islets compared with the other two species. (E) Expression of islet transcription factors differs between species. *P < 0.05, **P < 0.01, and ***P < 0.001 mouse vs. human; + P < 0.05 and ++ P < 0.01, mouse vs. NHP. Adapted from Conrad et al. (2016). *American Journal of Physiology-Endocrinology and Metabolism* 310: E91-102.
different tissues in the macaque.

different tissues in the macaque.

Maternal overnutrition has resulted in consistent effects on the offspring in multiple organ systems [84-94]. In fetuses, maternal WSD consumption was associated with perturbations in the central serotonergic system; after birth, female infants exposed to WSD had increased anxiety responses to novel objects [92]. When given a choice of food, offspring exposed to maternal WSD preferred food that was higher in fat and sugar compared to offspring from lean CTR mothers; furthermore, these offspring demonstrated suppressed dopamine signaling, with fewer dopamine fibers and lover levels of dopamine receptor proteins [93].

Maternal WSD also led to signs of fatty liver disease along with epigenetic changes in NHP offspring. Livers of fetuses exposed to WSD had threefold increases in triglycerides, even when the mothers were lean (not all dams fed WSD become obese), and histologic correlates of fatty liver disease including evidence of hepatic oxidative stress when mothers were obese. The increased levels of triglycerides persisted to three months of age in the offspring and were associated with a two-fold increase in body fat [88]. Fetuses of obese, WSD-fed mothers also had increased expression levels of gluconeogenic enzymes and transcription factors. This was accompanied by hyperacetylation of histone H3 in the liver [91]. Also in fetal livers, expression levels of the circadian rhythm gene *Npas2* were altered as a result of WSD, which correlated with changes in histone modifications at the promotor region [90]. The expression and activity of lysine deacetylase and sensor of cellular metabolism SIRT1 was also altered by maternal WSD. In fetal livers, there was in increase in repressive histone marks at the SIRT1 locus and a concomitant decrease in expression levels of SIRT1, expression levels of known downstream effectors, *in vitro* deacetylase activity, and ability to form a complex with binding partner DBC1 [87]. When dams were switched from WSD to a healthy diet before pregnancy, fetal triglyceride levels improved, changes in SIRT1 complex formation was abrogated, and changes in gluconeogenic gene expression were partially, but not completely, reversed [87, 88]. This occurred independently of weight loss in the dams who switched diets [88]. An overview of maternal diet-induced changes

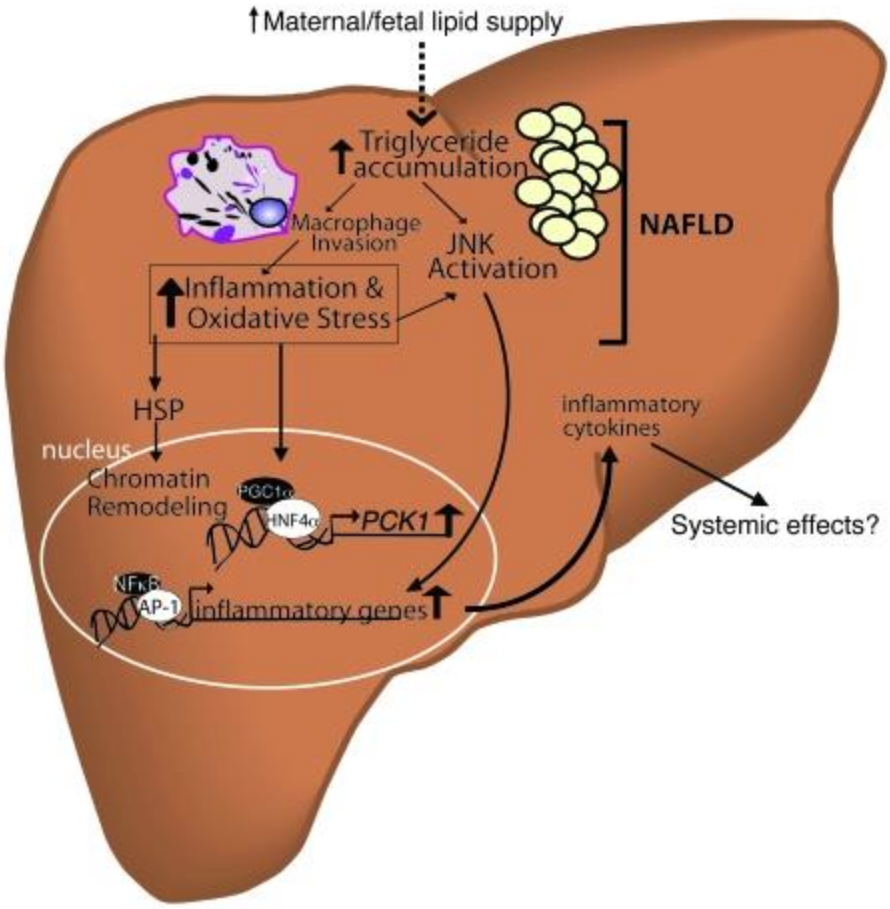


Figure 1-12: Maternal overnutrition induced changes to the fetal liver. An increase in the availability of lipids in the fetus results in excessive accumulation of triglycerides in the liver. This leads to the activation of inflammatory pathways, macrophage infiltration, and increased oxidative stress—a sign of non-alcoholic fatty liver disease. These changes are also associated with chromatin remodeling and altered expression of gluconeogenic genes. Adapted from McCurdy et al, *Journal of Clinical Investigation*, 2009.

in the liver is shown in Figure 1-12.

Fetuses exposed to WSD also had impairments in thyroid axis functions. Levels of free T_4 were significantly decreased with WSD exposure, while transcription of deiodinase genes were significantly disrupted in the hypothalamus, thyroid, and liver. Genes involved in the synthesis of thyroid hormone, such as thyroid releasing hormone in the hypothalamus, were also more lowly expressed. These changes were also associated with alternations in histone marks [89]. In addition to changes in the epigenome in these and other tissues, fetus exposed to WSD also had a modified metabolite profile [85].

In the intestine, maternal WSD led to an altered gut microbiome in the offspring, which was only partially reversed if offspring were weaned to CTR diet [84]. When these offspring were followed to 1- and 3-years of age, maternal WSD exposure led to persistent differences in the relative abundance of certain bacteria species, and partially mimicked the changes that occur with consumption of WSD alone [94]

Effects of Maternal Western-Style Diet on NHP Islets

Two studies by the Grove group [95, 96] using the Japanese Macaque investigated the effects of maternal WSD on offspring islets. Both studies utilized the model system that is explained above and fully described in the next chapter. In these studies, the authors used the terms WSD and HFD (high fat diet) interchangeably. I refer to the diet in the same manner the authors didfor the sake of consistency with the graphs in Figure 1-13. In all cases, HFD or WSD is the same high fat, high sucrose diet described above. Study groups are referred to by maternal and post-weaning diet, i.e. HFD/CTR animals were exposed to HFD *in utero* then weaned to CTR diet.

In fetuses exposed to HFD, α-cell mass was reduced, as well as pancreatic Insulin expression and GLUT2 expression when normalized to β-cell area. Due to the decreased α-cell mass, there was also an increase in the β:α cell ratio. After weaning, which occurs at 8 months of

age, both CTR/HFD and HFD/HFD offspring had increased islet mass (analyzed at 13 months of age). In CTR/HFD offspring this was due to an increase in islet number per unit area (suggesting increased neogenesis), whereas HFD/HFD offspring had larger islets (suggestive of β-cell proliferation). The latter group also had a persistently elevated β:α cell ratio (Figure 1-13A), which was likely due to a decrease in α-cell area. In a similar study design, Pound et al [96] found that fetuses exposed to WSD had reduced islet vascularization, indicating impaired vasculogenesis during development. Interestingly, if WSD-fed mothers were switched to CTR diet at the start of pregnancy, this developmental phenotype in the offspring was reversed (not shown), suggesting that acute exposure to WSD, and not the long-term metabolic milieu of the dam, is critical for islet vascularization. The reduction in islet vasculature observed in fetuses in response to *in utero* WSD-exposure was ameliorated at 13 months of age if offspring were weaned to CTR (Figure 1-13B). Weaning of CTR-exposed offspring to WSD increased islet vasculature at 13 months of age (Figure 1-13B), suggesting a normal compensatory response to increased metabolic demand. This increase in vasculature was absent in islets from WSD/WSD animals as shown in Figure 1-13B. Together, these studies suggest that defects in islet structure and function caused by maternal overnutrition may only manifest when animals consume an unhealthy diet after weaning. This observation is consistent with several of the rodent studies described in the previous section. Thus, it is important to study these offspring at a later time point after more prolonged WSD feeding. Additionally, the effects of maternal overnutrition and metabolic dysfunction due to WSD consumption may be prevented if mothers switch from a WSD to a CTR diet before pregnancy. Further studies are also needed to characterize the metabolic effects of WSD feeding in dams, as well as elucidate whether maternal metabolic phenotype or diet is the driving force behind the observed effects on the offspring.

Maternal Diet Can Lead to Epigenetic Changes in the Offspring That Affect Gene Expression

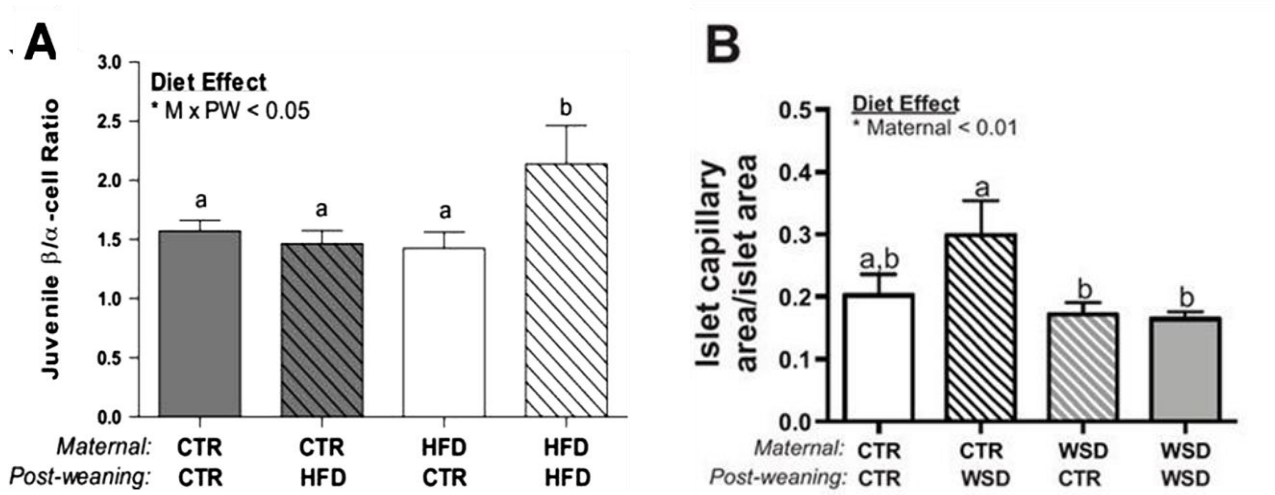


Figure 1-13: Effects of maternal HFD on offspring β:α cell ratio and islet vascularization at 13 months of age. In (A), offspring exposed to maternal HFD and weaned onto HFD had persistently elevated β:α cell ratio, consistent with the decreased α-cell mass in fetal offspring. In (B), post-weaning WSD results in increased islet capillary density, although this effect is lost if offspring were exposed to WSD *in utero*. Adapted from Comstock et al., *Molecular Metabolism*, 2013 and Pound et al., *AJP Endocrinology and Metabolism*, 2014.

Future studies are needed to help elucidate the mechanisms by which maternal overnutrition impairs offspring islet function. In rodent models, islets from offspring born to obese or HFD-fed mothers have decreased expression of genes involved in glucose metabolism and oxidative phosphorylation [51, 57]. Also, there is evidence of decreased insulin protein content in adult islets [57, 59, 60] and impaired insulin granule biosynthesis in β cells from adult offspring [59] exposed to maternal obesity during development. Changes in offspring gene expression due to *in utero* exposures are generally thought to be due to epigenetic mechanisms including DNA methylation (associated with gene repression) and histone marks associated with transcriptional activation (e.g. H3 Acetylation or H3K4 trimethylation) or repression (e.g. H3K9 dimethylation and H3K27 trimethylation). Support for this concept comes from mouse studies showing multi-generational effects of maternal HFD on offspring metabolism [55, 97] and mitochondrial function [98]. Additionally, paternal HFD often leads to impairments in offspring metabolism that can persist for multiple generations in mice [99]. In a rat model of maternal undernutrition, offspring born to protein-restricted dams had decreased activating and increased repressive histone marks at the locus for the transcriptional regulator *Hnf4α,* a T2D susceptibility gene [100]. These epigenetic changes resulted in decreased expression of *Hnf4α* due to weaker promotor-enhancer interaction.

In the NHP model, maternal WSD also led to epigenetic modifications in multiple tissues in the offspring. In fetuses of obese, WSD-fed mothers, hyperacetylation of histone H3 was observed globally in the liver [91]. At specific loci, there were epigenetic modifications associated with alterations in gene expression. For example, expression levels of *Npas2* were altered as a result of WSD in association with histone modifications at the promotor region [90]. Additionally, there was in increase in repressive histone marks at the SIRT1 locus associated with a decrease in expression [87]. Alterations in histone marks were also observed in the thyroid of maternal WSD-exposed fetuses [89].

In humans, male and female offspring born during the Dutch Hunger Winter had differential DNA methylation at the *IGF2* locus [61]. When this same cohort was analyzed further, persistent changes in DNA methylation were found and multiple other loci implicated in growth and metabolic disease [101]. Other maternal nutritional and environmental factors can also lead to epigenetic modifications in the offspring. Children born to mothers who used folic acid during pregnancy had increased DNA methylation at *IGF2* [102]. Interestingly, vast differences in DNA methylation were also observed from cord blood between pregnancies where conception occurred *in vitro* versus *in vivo* [103]. Together, these studies demonstrate that the gestational environment can affect epigenetic landscape in human offspring.

Further studies are needed to elucidate islet-specific epigenetic alterations in response to maternal overnutrition. While the precise mechanisms by which *in utero* nutritional changes lead to epigenetic modifications are largely unknown, diet is thought to influence DNA methylation or histone modifications in three ways: providing the substrates necessary for DNA or histone methylation, providing metabolic co-factors for DNA- or histone-modifying enzymes, and altering enzymes in the methionine cycle (ultimately changing the bioavailability of methyl groups) [104].

Goal of the Thesis

The adverse metabolic effects of maternal overnutrition during gestation for the developing fetus are becoming increasingly apparent. However, results of studies in mouse models can vary widely and the mechanism behind the observed offspring phenotypes is not known. As part of my thesis work, I sought to determine the effects of *in utero* overnutrition in a mouse model using HFD with a micronutrient matched control. I also sought to determine whether maternal HFD affected chromatin accessibility in the offspring, which could result in changes in gene expression. Additionally, many studies in rodent models do not accurately recapitulate the human condition due to differences in islet cell composition and function. Thus, I have utilized a NHP model of

maternal WSD feeling to determine the longer term effects of developmental exposure to this diet on offspring at three years of age. Specifically, I aimed to investigate the changes to offspring islet cell composition, islet function, and whole body metabolism induced by maternal WSD, and whether the effects of maternal WSD on one-year-old offspring persisted to three years of age. I also characterized the effects of WSD feeding on the glucose tolerance of female macaques during pregnancy. This type of analysis had not been performed in this model and is useful to put offspring phenotypes in context, which will be valuable to all who utilize this model in the future.

CHAPTER II: MATERIALS AND METHODS

Animal Care (NHP)

All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Oregon National Primate Research Center (ONPRC) and Oregon Health and Sciences University (OHSU) and were approved by the ONPRC IACUC. The ONPRC abides by the Animal Welfare Act and Regulations enforced by the USDA and the Public Health Service Policy on Humane Care and Use of Laboratory Animals in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Animal Housing and Diet (NHP)

Adult female Japanese macaques (*Macaca fuscata*), starting at four to seven years of age, were placed on either a control (CTR- Fiber Balanced Diet 5000; Purina Mills) or Western-style diet (WSD- TAD Diet no. 5LOP, Test Diet, Purina Mills) for a minimum of two years. The CTR diet is made up of 15% of calories from fat, whereas the WSD has approximately 36% of calories from fat. Animals in the WSD group also received calorically dense treats once per day as previously described [88].

All animals were housed in social environments comprising of several females to a male to facilitate optimal breeding. Females were sedated two to three times during pregnancy for fetal dating and third trimester measures. Pregnant females gave birth naturally in their social groups and most offspring began independently ingesting the maternal diet by four months of age but were allowed to stay with their mothers until weaning at between seven and nine months of age. Because of the length of the breeding season, multiple offspring groups are formed each year to keep offspring within the desired age range. Birth weight did not differ at one month of age

between male and female offspring exposed to either CTR or WSD diet (data not shown). Post weaning diet group was determined by the needs of the study and the maternal diet of the juveniles in each weaning group. Weaned offspring were placed in social groups with similarly aged offspring and one to two adult females. Offspring either remained on their maternal diet after weaning or were placed on the opposite diet to generate four groups based on maternal and offspring diet as previously described [84, 88, 105]. Islets and tissue samples utilized in the current study were limited based on the number of offspring of the correct age available during the study period.

Intravenous Glucose Tolerance Testing (NHP)

Intravenous glucose tolerance tests were performed on pregnant adult females during the early third trimester (gestational day ~123) and on offspring at approximately 36 months of age, just prior to necropsy, for a measurement of insulin sensitivity as previously described [88]. Briefly, animals were fasted overnight and sedated with Telazol (3–8 mg/kg IM Tiletamine HCl/Zolazepam HCl, Fort Dodge Animal Health, Fort Dodge, Iowa, USA). If needed, additional anesthesia was accomplished with Ketamine (3–10 mg/kg IM, Abbott Laboratories, North Chicago, Illinois, USA). Once sedated, animals received an IV glucose bolus (50% dextrose solution) at a dose of 0.6 g/kg via the saphenous vein. Baseline blood samples were obtained prior to the infusion and at 1, 3, 5, 10, 20, 40 and 60 min after infusion. Glucose was measured immediately using OneTouch Ultra Blood Glucose Monitor (LifeScan), and the remainder of the blood was kept in heparinized tubes on ice for insulin measurement. After centrifugation, samples were stored at –80°C until assayed. Insulin measurements were performed by the Endocrine Technologies Support Core (ETSC) at the ONPRC using a chemiluminescence-based automatic clinical platform (Roche Diagnostics Cobas e411, Indianapolis, IN, USA).

Percent Body Fat (NHP)

Total body fat mass, lean mass, percent body fat, bone mineral content (BMC), and bone mineral density (BMD) for each animal were measured using dual-energy X-ray absorptiometry (DEXA; Hologic QDR Discovery A; Hologic, Inc., Bedford, MA, USA). After an overnight fast, animals were sedated with 3–5 mg/kg Telazol (Tiletamine HCl/Zolazepam HCl, Fort Dodge Animal Health, Fort Dodge, Iowa, USA) and then supplemented with small doses of Ketamine (5–10 mg/kg, Abbott Laboratories, North Chicago, Illinois, USA) if needed to maintain sedation during the procedure and positioned prone on the bed of the scanner. Total body scans were performed on each animal. QDR software (Hologic) was used to calculate body composition, BMC, and BMD.

Isolation of Pancreatic Islets (NHP)

Primates were sedated with 15-20 mg/kg ketamine and humanely euthanized with sodium pentobarbital followed by exsanguination. Pancreata were excised from surrounding tissues and placed in ice-cold phosphate-buffered saline (PBS, Sigma-Aldrich) prior to islet isolations. For proliferation and cell death assays, as well as for the first batch of RNA-Sequencing, pancreata were shipped on ice to Dr. Rita Bottino at Allegheny Health Center in Pennsylvania for islet isolation. For all other *ex vivo* assays, islets were isolated at OHSU. Pancreata were removed from PBS and inflated with collagenase P (0.5 mg/mL, 80 mL total volume, Sigma-Aldrich) via cannulation of the pancreatic duct using 18-20-gauge catheters. Once inflated, each pancreas was divided into 12 sections and digested for 27-30 minutes at 37ºC in a collagenase solution. Using a histopaque gradient (Sigma-Aldrich), islets were collected at the interphase between histopaque and medium. Islets were cultured overnight in supplemented RMPI 1640 media (Sigma-Aldrich) at 37ºC and 5% CO_2. Islets were shipped overnight to Vanderbilt University Medical Center in media at room temperature for *ex vivo* proliferation, cytokine, and oxygen consumption assays. Islets were shipped frozen in Trizol reagent (ThermoFisher) for RNA-Sequencing.

Glucose Stimulated Insulin Secretion (GSIS) Assay (NHP)

After overnight recovery, islets were transferred into prepared columns and placed in a perifusion system (PERI-4.2, Biorep Technologies, Miami Lakes, FL) maintained at 37°C. Islets were pre-incubated in Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 4.0 mM glucose for one hour at a flow rate of 100 μL/minute. After pre-incubation, islets underwent 4x15-minute washes in 4.0 or 16.7 mM glucose-supplemented KRBH for a total of 60 minutes, with collections every three minutes. Collections were stored at 4°C and processed the same day. All perifusion GSIS assays were done in triplicate. Insulin was assayed using a Human Insulin ELISA kit (ALPCO, Salem, NH) and normalized to the number of islets.

Tissue Processing (NHP)

The pancreas was divided into 10 pieces from head to tail, with one being head and 10 being tail as follows: 1-4 head, 5-8 body, and 9-10 tail. Every other piece was used for fresh frozen or for paraffin/OCT embedding. The pancreas was weighed and then sectioned. For paraffin embedding, tissue was placed in a cassette and submerged in 10% zinc formalin for 48 hours at room temperature, then transferred to 70% ethanol at 4°C until embedded. For frozen sections, tissue was fixed in 4% paraformaldehyde (PFA) for three hours, then washed four times and equilibrated overnight in 30% sucrose/10 mM PBS at 4°C. Tissue was embedded in OCT in a cryomold (VWR, cat. # 25608-916) and frozen at -80°C. For electron microscopy (EM), a portion of the tail of the pancreas was minced, placed in a fixation solution composed of 2.5% paraformaldehyde/2.5% glutaraldehyde in 0.1M cacodylate (Electron Microscopy Sciences), and shipped overnight to Vanderbilt University Medical Center.

β-Cell Mass (NHP and Mice)

Paraffin embedded pancreata were sectioned at 5 μm. Six to nine slides (2-3 each from the head, body, and tail of the pancreas) were immunolabelled in NHPs. In mice, slides taken every 250 μm throughout the pancreas were immunolabelled. Slides were labelled for insulin with a guinea pig anti-insulin primary antibody (Dako; 1:500), and a peroxidase-conjugated anti-guinea pig secondary antibody (Jackson ImmunoResearch; 1:400). β cells were visualized after incubation with DAB (Vector Laboratories), and the remaining tissue was counter-stained with eosin. Slides were imaged using a Scanscope CS bright field microscope (Aperio Technologies). An Aperio-based classifier was created using sample images which could accurately quantify DAB+ area and total tissue area, as previously described [106]. Distinct classifiers were created and optimized for NHP and mouse analyses. β-cell mass was calculated by multiplying the insulin-positive fraction (total DAB-positive area/total tissue area) by the weight of the pancreas.

α-Cell Mass (NHP and Mice)

Slides were immunolabelled, stained, imaged, and quantified as above with the following exceptions. Glucagon was labelled using a mouse anti-glucagon primary antibody (Millipore; 1:400) and an HRP-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch; 1:400).

β-Cell Size (NHP)

Paraffin embedded sections were immunolabelled for insulin as above and a Cy2-congugated anti-guinea pig secondary antibody (Jackson ImmunoResearch; 1:400), then stained with DAPI. Slides were scanned using a Scanscope FL microscope (Aperio Technologies). Insulin-positive area was annotated on the resulting images. An Aperio-based algorithm calculated total annotated area and the number of nuclei within the annotated area, which was then used to calculate average β-cell size in μm^2. Over 12,000 cells were included per sample.

Islet Vascularization (NHP)

Paraffin embedded sections were immunolabelled for insulin and glucagon as above, and sheep anti-PECAM-1 (R&D Systems; 1:25). Cy2-congugated anti-guinea pig and anti-mouse secondary antibodies (Jackson ImmunoResearch; 1:400) were used to label insulin- and glucagon-positive cells and a Cy3-congugated secondary antibody was used to label PECAM-1-postive cells (Jackson ImmunoResearch; 1:400). PECAM-1-positive area was measured from at least 150 islets per sample from at least three slides >150 μm apart. Proportion of islet vascularization was determined by dividing PECAM-1-positive area by combined insulin- and glucagon-positive area.

α- and β-Cell Proliferation and Ratio (NHP)

OCT-embedded frozen (CTR/CTR and WSD/CTR groups) and paraffin embedded (CTR/WSD and WSD/WSD groups) slides were immunolabelled for insulin and glucagon as above, and Ki67 using rabbit anti-Ki67 (Abcam; 1:500), followed by Cy5-conjugated anti guinea pig, Cy3-conjugated anti-mouse, and Cy2-conjugated anti-rabbit secondary antibodies. At least 10,000 endocrine cells were counted for each sample. For CTR/CTR and WSD/CTR groups samples, at least three slides >150 μm apart from the body of the pancreas were analyzed. For CTR/WSD and WSD/WSD samples, at least three slides were analyzed from the head, body, and tail of the pancreas. The percentage of proliferating cells was determined by dividing the number of Ki67+/hormone+ cells by the total number of hormone+ cells (either insulin+ or glucagon+ cells). β:α cell ratio was determined by dividing the number of insulin+ cells by the number of glucagon+ cells.

Somatostatin and Pancreatic Polypeptide (PP) Immunolabeling (NHP)

Slides were incubated with guinea pig anti-insulin and mouse anti-glucagon antibodies as above, plus goat anti-somatostatin (1:250) and rabbit anti-PP (1:500). This was followed by

incubation with Cy5 anti-guinea pig, Cy5 anti-mouse, Cy3 anti-rabbit, and Cy2 anti-goat (all 1:400). Percent somatostatin+ and PP+ cells were quantified by dividing respective cell numbers by the total number of islet cells. Between two and five thousand cells were counted per sample.

Electron Microscopy (NHP)

Samples were prepared for imaging by the Vanderbilt University Cell Imaging Shared Resource. Images were acquired on a Philips/FEI T-12 transmission electron microscope. For insulin granule density and maturity, individual granules were manually counted and binned based on electron density. For mitochondrial density, mitochondria within β cells were annotated and area was quantified using ImageJ. Granule density and mitochondrial density were normalized to β-cell area. At least 1,500 μm^2 of β-cell area was analyzed per sample.

Gene Expression Analysis (NHP)

Tissue Processing for RNA Acquisition

NHP islets frozen in Trizol were allowed to thaw over ice, and lysed by vortexing. RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in water and concentration and integrity were assessed using a ND-1000 Spectrophotometer (NanoDrop).

RNA-Sequencing

mRNA enrichment and cDNA library preparation were performed utilizing the stranded mRNA (polyA-selected) library preparation kit at the Vanderbilt University VANTAGE core. Libraries were pair-end sequenced to 150 bp on the Illumina NovaSeq 6000 targeting an average of 50M reads per sample. DeSeq2 was used to determine differentially expressed genes using an FDR cutoff of 0.05 and $\log_2$-fold change of 2.

Oxygen Consumption Assay (NHP)

Oxygen Consumption was assessed using the Seahorse XFe96 instrument (Agilent Technologies). On the day prior to the assay, a cell adhesive solution was prepared by combining 50 µl of Cell-Tak (Corning) with 700 µl of filter-sterilized sodium bicarbonate. Wells of a XFe96 spheroid microplate (Agilent Technologies) were coated with 20 µl of cell adhesive solution and incubated at 37°C for one hour. Wells were then washed with 400 µl of 37°C sterile water, allowed to air dry for 40 minutes, and then covered and stored overnight at 4°C. Also on the day prior to the assay, the sensor cartridge and utility plate were removed from an XFe96 Extracellular Flux Assay Kit (Agilent Technologies). The sensor cartridge was hydrated overnight in a 37°C, non-CO_2 incubator overnight. 30 mL of XF Calibrant (Agilent Technologies) was incubated at 37°C overnight.

On the day of the assay, Seahorse media was assembled by supplementing a minimal-DMEM Seahorse base media (Agilent Technologies) with 0.5 mL each of 1 mM sodium pyruvate and 2 mM glutamine as well as 0.496 mL of 200 mg/ml glucose. The sensor cartridge was removed from the incubator and the water in the utility plate was replaced with the pre-warmed calibrant and incubated at 37°C. Next, the wells of the cell adhesive-coated microplate were loaded with 175 µl of assembled medium. Isolated islets—15 per well—were then added to non-corner wells of the microplate as previously described [107]. The microplate was then covered and placed in a 37°C incubator. The first three ports of sensor cartridge were loaded with 45 µM oligomycin, 10 µM FCCP, and 25 µM rotenone/antimycin A. Concentrations of oligomycin and rotenone/antimycin A were based on the literature [108], and the concentration of FCCP was titrated to produce maximal islet respiration. A Wave assay (Agilent Technologies) was programed for a 30-minute baseline period, 42-minute oligomycin period, 30-minute FCCP period, and 30-minute rotenone/antimycin A period as previously described [107].

Ex Vivo Proliferation and Cell Death Assays (NHP)

Isolated NHP islets were cultured overnight in a 96-well plate in proliferation media: DMEM (+1 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate), 10% horse serum, 100 U/ml penicillin, and 100 ug/ml streptomycin. Islets were then cultured for four days in proliferation media containing 0.1 mM EGTA and one of the following treatments: vehicle (PBS), 30 nM DG-041, 10 nM CAY10598, or a combination of 30 nM DG-041 and 10 nM CAY10598 as described in [109]. Each treatment was applied to two wells, and each well contained 40 islets. To determine β-cell proliferation, islets were cytospun onto charged slides and immunolabeled for insulin, Ki67, and DAPI as described above.

For cytokine-induced cell death assays, islets were cultured overnight as above in cytokine media: DMEM (+1 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate), 10% fetal bovine serum, 100 U/ml penicillin, and 100 ug/ml streptomycin. On the following day islets were treated with a cytokine cocktail including tumor necrosis factor (TNF)-α (20 ng/mL; Sigma–Aldrich), interleukin (IL)-1β (5 ng/mL; Sigma–Aldrich), and interferon (IFN)-γ (10 ng/mL; Sigma–Aldrich) for 48 hours. After treatment, islets were cytospun onto charged slides. β-cell death was measured using the ApoAlert DNA fragmentation kit (Clontech) according to the manufacturer's instructions. Dispersed cells were co-immunolabeled with guinea pig anti-insulin (1:400; Dako) and Cy3-conjugated anti-guinea pig (1:400; Jackson ImmunoResearch) and nuclei were stained with DAPI (1 μg/mL). The proportion of β cells undergoing apoptosis was quantified by manually counting TUNEL-positive nuclei.

Transgenic and Wild Type Mice

mIns1-H2B-mCherry mice are described in [110]. Male transgenic mice were on a C57Bl/6N background, and were mated with C57Bl/6J WT females. All experimental animals (offspring) were an equal mix of C57Bl/6J and C57Bl/6N. Mice were housed in a 12 hour light/dark cycle and given *ad libitum* access to food. *All* experiments involving mice were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center.

Genotyping (Mice)

Genotyping was performed using tail or ear punch DNA and the primer sets listed in Table 2-1.

High Fat and Control Diet (Mice)

8-week-old female mice were place on high fat diet (HFD; 58% kcal from fat; TestDiet 58Y1) or control diet (CTR; 11% kcal from fat; TestDiet 58R0) for 5 weeks pre-pregnancy and throughout gestation and lactation. Offspring were weaned to HFD or CTR from 3 weeks of age until sacrifice at 4- or 12-weeks of age. For diet reversal experiments, female mice were placed on HFD from 8-13 weeks of age then switched to CTR pre-pregnancy. Body weight was measured each week after diet initiation in both dams and offspring.

Intraperitoneal (IP) Glucose Tolerance Testing (Mice)

Mice were fasted for 16 hours and then glucose was administered by intraperitoneal injection in mice sedated with isoflurane for less than 5 minutes. For pregnant female mice, a 60 mg dose of dextrose (as a 20% solution in PBS) was administered. For 4-week-old offspring, 22 mg of dextrose was administered. For 12-week-old offspring, 40 mg of dextrose was administered. Blood glucose was measured using a glucometer (Accu-Check) before injection and at 15, 30, 60, 90, and 120 minute time points. Four-week-old mice were sacrificed for tissue harvesting immediately following the GTT. Twelve-week-old mice were sacrificed two days after the GTT.

Tissue Dissection, Preparation, and Histology (Mice)

Mice were sacrificed by cervical dislocation, and pancreata were extracted and fixed for 4 hours in 4% PFA, cleared in xylenes, and embedded in paraffin. Tissue blocks were sectioned at

Allele	Forward Primer	Reverse Primer
mIns1-H2B-mCherry	GTAACCCCCAGCCCTTAGTG	GCGCATGAACTCCTTGATGA
Internal Control	CTAGGCCACAGAATTGAAAGATCT	GTAGGTGGAAATTCTAGCATCATCC

Table 2-1: Genotyping primers

5 μm onto charged slides. Slides were deparaffinized in xylenes and rehydrated in water for all downstream analyses. After immunolabelling, fluorescent and bright field images were captured using a Scanscope FL microscope (Aperio Technologies) or a Scancope CS bright field microscope (Aperio Technologies), respectively.

Assessment of Chromatin State (Mice)

Islets from mIns1-H2B-mCherry transgenic mice were isolated as previously described by the Vanderbilt Islet Procurement and Analysis Core [109]. Islets were dispersed with Accutase (Fischer Scientific) and single cells were filtered through a 100 μm filter. Live β cells were sorted by Fluorescence Activated Cell Sorting (FACS) in collaboration with the Vanderbilt Flow Cytometry Shared Resource. DAPI was used as a viability marker, and gating was established using single-color controls (DAPI only, mCherry only). Open chromatin was assessed by the Assay for Transposase Accessible Chromatin (ATAC) by Sequencing as previous described [111]. Briefly, live β cells were centrifuged, lysed, and incubated with a Tn5 transposase to add adapter sequences to regions of open chromatin. DNA was isolated using a Zymo DNA Clean and Concentrator kit, and transposase-accessible DNA was amplified and sent to the VANTAGE core at Vanderbilt for sequencing.

GPR 155 Overexpression and Immunofluorescence in INS-1 Cells

INS-1 832/13 cells [112] were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 10mM HEPES, 2 mM L-glutamine, 1mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol. Cells were transfected using Lipofectamine 2000 (Thermo Fisher) with a GPR155 overexpression vector (GenScript). This vector contained a GPR155-GFP fusion protein driven by the CMV promotor and an Ampicillin resistance gene. Transfected cells were selected using hygromycin in the culture media.

For immunofluerescence experiments, cells were grown until confluence on glass coverslips and fixed in 4% PFA. A rabbit GPR155 primary antibody (1:50; Proteintech) was used for labelling along with guinea pig anti-insulin (1:500; Dako) for co-labelling experiments. Coverslips were incubated with primary antibodies overnight, washed in PBS, and incubated with Cy3 anti-rabbit (1:400; Jackson ImmunoResearch) with or without Cy2 anti-guinea pig (1:400; Jackson ImmunoResearch) overnight. Coverslips were imaged using a LSM 710 META Inverted confocal microscope.

GPR155 Immunolabelling in Tissue Sections

Paraffin slides of mouse or NHP pancreata were processed as described above. Primary antibodies included rabbit GPR155 (1:50; Proteintech), plus guinea pig anti-insulin (1:500; Dako) and mouse anti-glucagon (1:400; Millipore) for co-labelling experiments. Secondary antibodies included Cy5 anti-mouse (1:400; Jackson ImmunoResearch), Cy3 anti-guinea pig (1:400; Jackson ImmunoResearch), and Cy2 anti-rabbit (1:400; Jackson ImmunoResearch).

Statistics (NHP and Mice)

Data were analyzed utilizing GraphPad Prism Software (Graphpad Software, Inc., La Jolla, CA, USA) for calculating glucose and insulin area under the curve from zero. For immunohistochemical analyses, two-way ANOVA was performed to test for effects of maternal diet, offspring diet, or interaction. For direct comparisons of continuous measures between two groups, unpaired t-test was used for normally-distributed measures and the Wilcoxon rank-sum test was used for non-parametric measures. For comparisons of categorical measures, the Pearson chi-squared test was used. For comparison of continuous measures among all four diet groups, two-way ANOVA and Tukey's multiple comparisons test were used to determine statistical significance (data are expressed as mean +/- SD). For GSIS assay, a two-way ANOVA

followed by Sidak post-hoc test with multiple comparisons was used to evaluate differences between treatment and time (data are expressed as mean +/- SEM).

Multivariate Analyses for Maternal-Offspring Correlations (NHP)

Linear regression was used to evaluate whether maternal factors including glucose area under the curve (GAUC) during pregnancy glucose tolerance test (GTT), diet history (years dam was on WSD), age, and parity were independently associated with offspring metabolic health, with and without adjustment for other factors. 26-36 animals were included in each analysis. Linear regression was also used to calculate coefficients and 95% confidence intervals (CIs) to determine whether maternal WSD was associated with impaired glucose tolerance in offspring, stratified by offspring diet (N = 11-21 per group). Maternal parity and GAUC were selected as *a priori* confounders based on the literature and were included separately in adjusted models to avoid model inflation. The sandwich estimate of variance is used to obtain robust variance that accounts for correlation among siblings [113, 114]. All statistical analyses were performed at a 2-sided significance level of 0.05 using STATA 14.2 (StataCorp, Texas, USA).

Multivariate Analyses for Maternal Phenotyping (NHP)

Maternal characteristics were compared by diet status using the Wilcoxon rank-sum test for continuous measures and the Pearson chi-squared test for categorical measures. Linear regression was used to calculate beta coefficients and 95% confidence intervals (CIs) to determine whether WSD was associated with impaired glucose tolerance. Age, parity, and pre-pregnancy weight were selected as *a priori* confounders based on the literature. In the primary analysis, we did not adjust for pre-pregnancy GTT, gestational weight gain, body fat percent, triglyceride, LDL, or Leptin levels since they may mediate the effect of WSD on third-trimester GAUC and IAUC. The sandwich estimate of variance is used to obtain robust variance that accounts for correlation among pregnancies from the same mother. Two sensitivity analyses were

also performed. First, the relationship between years on WSD and third-trimester measures was examined since this relationship may vary based on time on WSD. Second, the analysis was repeated with only first pregnancies after diet initiation since the number of pregnancies on WSD may influence GAUC and IAUC. All statistical analyses were performed at a 2-sided significance level of 0.05 using STATA 14.2 (StataCorp, Texas, USA).

CHAPTER III: THE EFFECTS OF MATERNAL WESTERN-STYLE DIET ON MATERNAL AND OFFSPRING GLUCOSE TOLERANCE IN THE NON-HUMAN PRIMATE

Introduction

The deleterious effects of maternal diabetes during pregnancy on the offspring are well documented in humans [115-121]. Offspring exposed to maternal diabetes *in utero* are at an increased risk for obesity and Type 2 diabetes (T2D). This increased risk may be mediated by increased fetal growth due to high levels of leptin or insulin [115]. A separate study showed that 9.5 year old offspring exposed to T2D *in utero* had increased skin folds, higher blood glucose and insulin levels, decreased insulin sensitivity as measured by HOMA-IR, and higher blood pressure [118]. Impaired glucose tolerance in the offspring is also a long-term consequence of maternal diabetes. Offspring born to diabetic mothers have increased insulin secretion *in utero*, which correlates with impaired glucose tolerance in childhood independent of obesity in the offspring [120].

While the increased risk of metabolic disease in the offspring could be due to genetic factors, several studies suggest a major role for a deleterious *in utero* environment above that of genetic factors [116-118]. For example, offspring who were born to mothers with T2D during pregnancy are at a significantly increased risk of developing diabetes compared to siblings born when the same mother did not have diabetes [116]. The risk of T2D in the offspring is similar when the mother has T1D or T2D [115]. Additionally, mothers with T2D transmit risk of obesity, T2D, and impaired glucose tolerance to their offspring at significantly higher rates than fathers with T2D [117].

Maternal factors other than diabetes can also lead to a poor *in utero* environment. Fetuses of obese mothers have increases in body fat, insulin resistance, and umbilical cord leptin levels, with a significant correlation between maternal BMI and fetal insulin resistance [121]. Interestingly, even increases in maternal glucose levels below the threshold of clinical diabetes are deleterious

to the offspring. There is a strong, continuous association with maternal blood glucose levels during pregnancy and increased body weight and cord blood C-peptide levels at birth [122]. When offspring were 7 years old, maternal glucose tolerance was directly correlated with offspring glucose tolerance independent of BMI before pregnancy, childhood obesity, or being born large for gestational age [123]. Together, these studies suggest an important role for the gestational environment in programming offspring metabolic disease. Maternal blood glucose, even below diabetic levels, plays an important role in the future health of the offspring.

In the following chapters a NHP model of maternal overnutrition is utilized to investigate the effects of maternal WSD on offspring islets. As discussed in Chapter I, this model is highly related to humans in terms of gestation and islet physiology. Thus, this model is useful in better understanding the relationships between maternal diet, maternal metabolism, and increased risk of diabetes in the offspring. In this model, female Japanese macaques are fed CTR or WSD before and during pregnancy, and offspring are weaned to either CTR or WSD. In order to better understand how WSD affects the gestational environment and the health of the offspring, the effects of this diet on maternal glucose and insulin levels during pregnancy are characterized in this chapter. Furthermore, the effects of maternal glucose and insulin levels on offspring glucose metabolism are investigated.

Results

WSD Increases Body Weight, Body Fat Percentage, and Insulin Levels in Female NHPs Before Pregnancy

In order to investigate the effects of WSD on maternal metabolism during pregnancy, glucose and insulin levels from a third trimester glucose tolerance test (GTT) were assessed as primary outcomes. Specifically, the glucose area under the curve (GAUC) and insulin area under the curve (IAUC) during the glucose tolerance test were measured. The following parameters were identified as potential confounders and were adjusted for accordingly: parity, age, and pre-

pregnancy weight. The following parameters were identified as potential mediators on the causal pathway between WSD and changes in GAUC or IAUC compared with controls, if differences were observed: pre-pregnancy glucose tolerance, gestational weight gain, body fat percentage, LDL/triglyceride levels, and leptin levels. This is shown schematically in Figure 3-1.

A total of 254 pregnancies from 95 dams were included in this analysis, after excluding aborted pregnancies and pregnancies from which no glucose tolerance data was available (Figure 3-2). Maternal age ranged from 5 to 17 years. Among all pregnancies (regardless of diet) maternal GAUC ranged from 5479 to 15996 arbitrary units (AU) with a median of 9132 prior to pregnancy and ranged from 3969 to 11336 AU with a median of 6818 during pregnancy. Maternal IAUC ranged from 249 to 26312 arbitrary units (AU) with a median of 4866 prior to pregnancy and ranged from 801 to 78741 AU with a median of 6634 during pregnancy. Compared to dams consuming CTR, dams consuming WSD were more likely to have significantly higher weight (11 vs. 9 kg), body fat percentage (32% vs. 21%), and IAUC (6270 vs 3312 AU) prior to pregnancy. Dams on WSD were also more likely to have significantly higher weight (11 vs. 10 kg) and leptin levels (78 vs. 58 ng/ml) during pregnancy. Other characteristics did not differ by diet status (Table 3-1).

WSD Consumption is Not Associated with Impaired Glucose Tolerance During Pregnancy

After adjusting for confounders of age, parity, and pre-pregnancy weight, dams consuming WSD were more likely to have lower GAUC (coefficient -678, 95% CI -1072, -283), when compared to dams consuming CTR. Third-trimester IAUC was not significantly different among WSD- and CTR-fed dams (coefficient 934, 95% CI -1804, 3671, Table 3-2). When stratified by years of exposure to WSD, dams exposed for 1.1-2.0 years and 2.1-3.0 years were more likely to have lower GAUC than controls (beta coefficient: -1008, 95% CI -1585, -430; beta coefficient: -833, 95% CI: -1393, -272). Third trimester IAUC was not significantly different by diet status (Table 3-3).

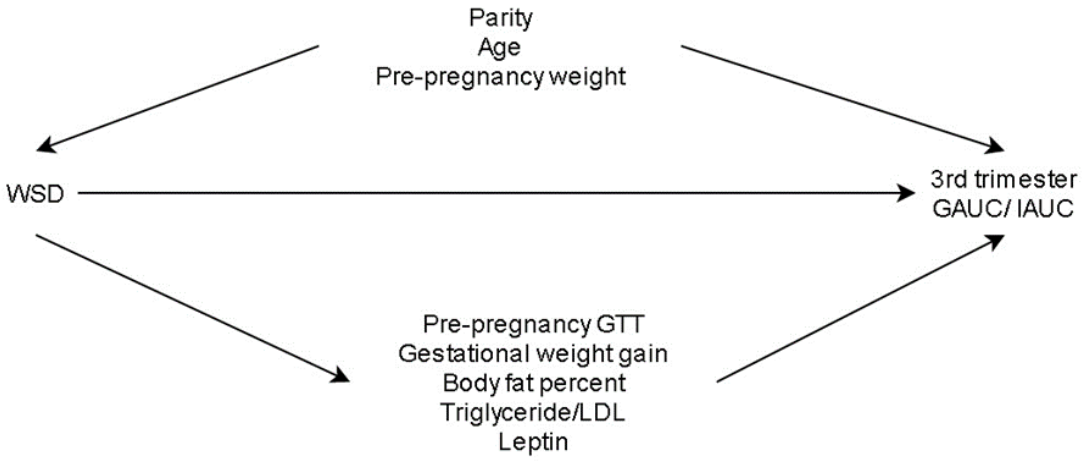


Figure 3-1: Directed Acyclic Graph (DAG) representing the relationship among covariates with WSD and third-trimester glucose tolerance. Confounders (top) are characteristics potentially associated with both the exposure and the outcome. Potential mediators (bottom) may lie on the cause pathway between the exposure and the outcome.

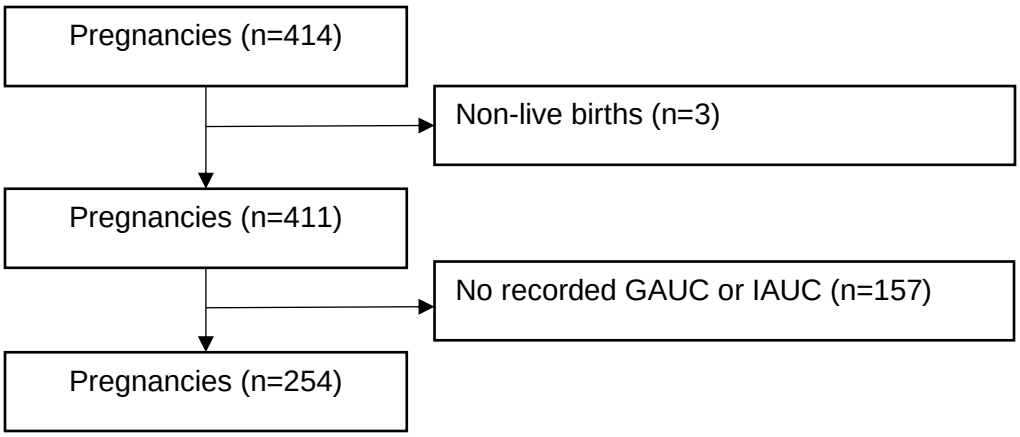


Figure 3-2: Flowchart of study subject exclusion criteria.

Characteristics	CTR (N=116)		WSD (N=138)		P-value[2]
	n	% or median (IQR) [1]	n	% or median (IQR) [1]	
Age, years	116	10 (7-13)	138	9 (8-11)	0.39
Parity[3]					0.12
0	20	17	12	9	
1	19	16	23	17	
≥2	77	66	103	75	
Pre-pregnancy measures					
Weight (kg)	116	9 (8-11)	138	11 (9-13)	<**0.01**
Body fat percentage	91	21 (12-28)	75	32 (26-39)	<**0.01**
GAUC	93	9480 (8089-10169)	120	9006 (8104-10312)	0.56
IAUC	91	3312 (2338- 5786)	121	6270 (4189-9389)	<**0.01**
Third-trimester measures					
Weight (kg)	116	10 (9-11)	137	11 (9-13)	**<0.01**
Leptin (ng/ml)	91	58 (28-88)	92	78 (53-163)	**<0.01**
LDL	34	41 (34-47)	32	37 (29-46)	0.26
Triglycerides	34	62 (54-83)	33	72 (59-108)	0.10
Gestational weight gain	116	1 (0-1)	137	1 (0-1)	0.39

Table 3-1: Distribution of maternal characteristics stratified by diet (n=254).
Abbreviations: WSD, western-style diet; CTR, control diet; IQR, interquartile range; AU, arbitrary units; GAUC, glucose tolerance test area under the curve; IAUC, insulin area under the curve; LDL, low-density lipoproteins.
[1] Median and IQR are reported for continuous variables due to small sample size and skewness; percent is reported for parity.
[2] From Wilcoxon rank-sum test for continuous measures; Pearson chi-squared test for parity.
[3] Parity refers to number of live births prior to study pregnancy.

	Crude			Adjusted[1]		
	n[2]	Coefficient	95% CI	n[2]	Coefficient	95% CI
GAUC	252	**-424.19**	**-814.84, -33.54**	252	**-677.50**	**-1071.65, -283.35**
IAUC	245	**4410.69**	**1306.35, 7515.04**	245	933.59	-1803.57, 3670.74

Table 3-2: Association between WSD and third-trimester GAUC and IAUC (n=254).
Abbreviations: WSD, western-style diet; CI, confidence interval; GAUC, glucose tolerance test area under the curve; IAUC, insulin area under the curve.
[1] Adjusting for age, parity, and pre-pregnancy weight
[2] Sample size differs from 254 due to missing GAUC or IAUC measurements

Years on WSD		**Crude**		**Adjusted**[1]	
	n[2]	Coefficient	95% CI	Coefficient	95% CI
GAUC					
No WSD	115	Reference	Reference	Reference	Reference
0.1-1.0	23	-138.77	-690.88, 413.35	-453.56	-969.20, 62.09
1.1-2.0	29	**-1220.76**	**-1818.03, -623.50**	**-1007.81**	**-1585.19, -430.43**
2.1-3.0	23	**-744.44**	**-1299.43, -189.44**	**-832.54**	**-1392.84, -272.24**
3.1-4.0	28	-301.56	-991.03, 387.91	-571.11	-1143.26, 1.03
5.0+	34	177.81	-295.60, 651.22	-407.80	-954.87, 139.28
IAUC					
No WSD	113	Reference	Reference	Reference	Reference
0.1-1.0	23	**6549.30**	**1539.90, 11558.69**	3548.41	-707.87, 7804.69
1.1-2.0	24	1950.72	-2018.65, 5920.09	2004.01	-959.46, 4967.48
2.1-3.0	22	300.54	-2845.39, 3446.46	-2432.07	-5426.73, 562.60
3.1-4.0	28	4169.79	-1681.32, 10020.90	-161.38	-4504.25, 4181.49
5.0+	35	**7468.42**	**2547.22, 12389.62**	908.87	-5002.24, 6819.98

Table 3-3: Association of years on WSD with third-trimester GAUC and IAUC (n=254).
Abbreviations: WSD, western-style diet; CI, confidence interval; GAUC, glucose tolerance test area under the curve; IAUC, insulin area under the curve.
[1] Adjusting for age, parity, and pre-pregnancy weight
[2] Sample size differs from 254 due to missing GAUC or IAUC measurements

When the analysis was restricted to first study pregnancies (n=95), WSD was associated with a decrease in third-trimester GAUC (-943.21, 95% CI -1383.21, -503.22) and an increase in third-trimester IAUC (beta coefficient: 2566.27, 95% CI 536.14, 4596.40, Table 3-4).

Time on WSD is Positively Correlated with GAUC

The tables above demonstrate that WSD consumption is not associated with worsened (increased) GAUC, and in several cases is associated with improved GAUC. This is sometimes, but not always, associated with increased IAUC. In order to better understand the populations of macaques consuming WSD and CTR diet, we first investigated whether there might be an interaction between pregnancy number and WSD. For example, WSD could have less of an effect on early pregnancies and a more severe effect on later pregnancies. However, in both groups, neither pre-pregnancy or pregnancy GAUC significantly differed between early pregnancies and later pregnancies as shown in Figure 3-3. Interestingly, GAUC tended to be lower during pregnancy than before pregnancy in both diet groups.

At least two possibilities could explain why WSD may be associated with improved GAUC. First, the diet itself may be beneficial to glucose tolerance. Second, the population of macaques consuming WSD may be inherently different than the population consuming CTR, either due to genetic factors or other factors not considered in our analysis. To address these possibilities, data from dams who had had multiple pregnancies on different diets was utilized. In the initial analysis, pregnancies on CTR diet where the dam had previously been fed WSD were excluded, but below this data is shown to address the possibilities above. If WSD is indeed beneficial, it is expected that GAUC would decrease when an animal was switched from CTR to WSD, or increase when an animal was switched from WSD to CTR. This trend was observed in some but not all cases (Figure 3-4). Ultimately, a definitive conclusion cannot be made due to the limitation in the number of animals who switched diets.

Another approach to determine the independent effects of WSD is to test whether there

	Crude			Adjusted[1]		
	n[2]	Coefficient	95% CI	n[2]	Coefficient	95% CI
GAUC	95	**-778.84**	**-1204.24, -353.44**	95	**-943.21**	**-1383.21, -503.22**
IAUC	89	**4958.28**	**2469.49, 7447.07**	89	**2566.27**	**536.14, 4596.40**

Table 3-4: Association between WSD and third-trimester GAUC and IAUC among first study pregnancies (n=95).
Abbreviations: WSD, western-style diet; CI, confidence interval; GAUC, glucose tolerance test area under the curve; IAUC, insulin area under the curve.
[1] Adjusting for age, parity, and pre-pregnancy weight
[2] Sample size differs from 95 due to missing IAUC measurement

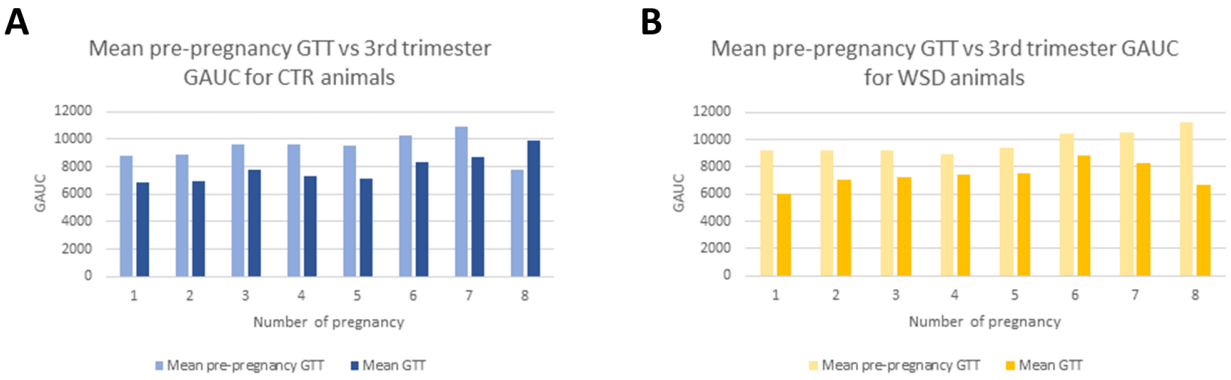
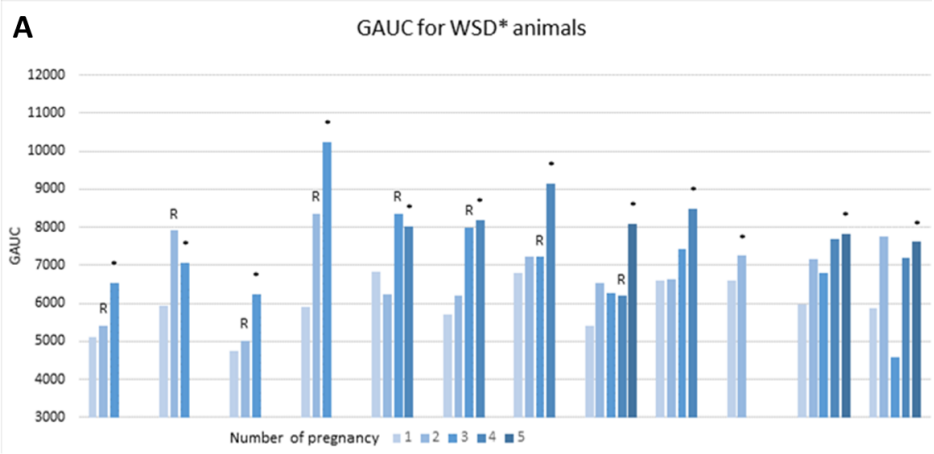
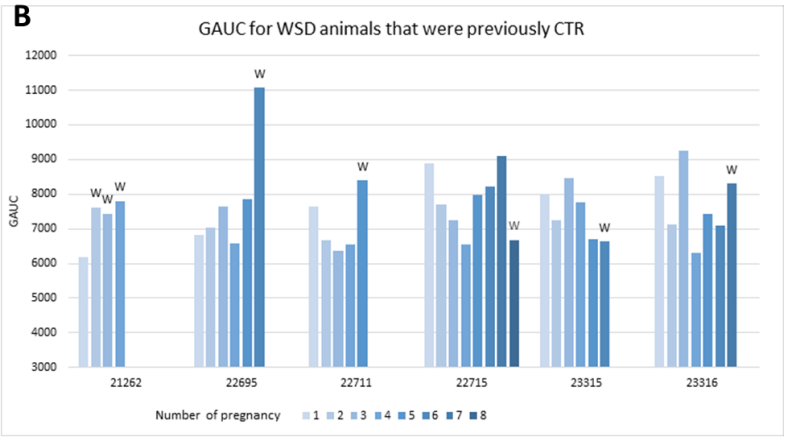


Figure 3-3: The relationship between maternal GAUC and number of pregnancies. Lighter bars indicate pre-pregnancy GAUC before the pregnancy in question. Dark bars indicate pregnancy GAUC. CTR group is shown in (A), WSD group is shown in (B). Pre-pregnancy and pregnancy GAUC did not significantly increase with increasing pregnancy number.





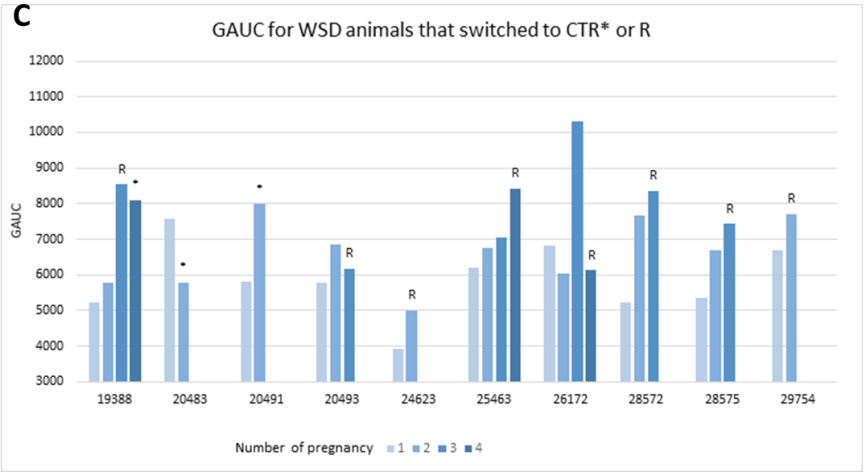


Figure 3-4: GAUC in dams who switched diets. Each cluster of bars represents GAUCs in different pregnancies for the same animal. Bars with no label represent pregnancies where animals were fed WSD. Bars labelled "R" represent "Reversal" pregnancies—the first pregnancy after switching from WSD to CTR. Bars marked with an asterisk indicate pregnancies where the animal was placed back on WSD (A) or CTR (C). In (A), switching from WSD to CTR generally either worsened or had no effect on GAUC, while WSD after Reversal tended to lead to the highest GAUC values. In (B), switching from CTR to WSD was deleterious in 3 out of 6 cases, although GAUC either improved or remained similar in the other 3 cases. In (C), GAUC increased when switching from WSD to CTR in most cases, but stayed similar or decreased in a few cases.

is a correlation between months on WSD and GAUC, only considering animals within the WSD group. As shown in Figure 3-5, we observed a weak but significant correlation ($r^2 = 0.16$; $p < 0.01$) between GAUC and months on WSD.

Identification of Maternal Factors that Correlate with Offspring Glucose Metabolism

In order to identify maternal factors that may negatively affect the *in utero* environment and result in poor metabolic health in the offspring, maternal factors including age, parity, and glucose tolerance were examined for correlations with offspring phenotypes. In these analyses, only maternal-offspring pairs from which offspring tissue samples were available were considered. Maternal GAUC did not correlate with offspring β-cell mass regardless of maternal or offspring diet (Figure 3-6A). However, offspring GAUC was significantly correlated with maternal GAUC (Figure 3-6B) as well as maternal parity (Figure 3-6C). Offspring GAUC was not significantly correlated with maternal age (Figure 3-6D). Other correlations including maternal body fat percentage vs offspring GAUC or β-cell mass, maternal IAUC vs offspring or maternal GAUC, maternal years on WSD vs maternal or offspring GAUC, and offspring β-cell mass vs offspring GAUC were not significant (data not shown).

Maternal GAUC is Independently Associated with Offspring GAUC

To identify independent risk factors associated with offspring metabolic health, multivariate regression analyses were performed adjusting for maternal factors including age, parity, and GAUC during pregnancy. Characteristics of maternal/offspring pairs included in the analysis are shown in Table 3-5. Maternal GAUC was significantly correlated with offspring GAUC (Figure 3-6B). However, this significance was no longer observed when adjusting for age or parity (Table 3-6A). Maternal parity was also correlated with offspring GAUC (Figure 3-6C). While this correlation was significant when adjusting for age, it was no longer significant when adjusting for maternal GAUC (Table 3-6B). Maternal age was not significantly correlated with offspring GAUC

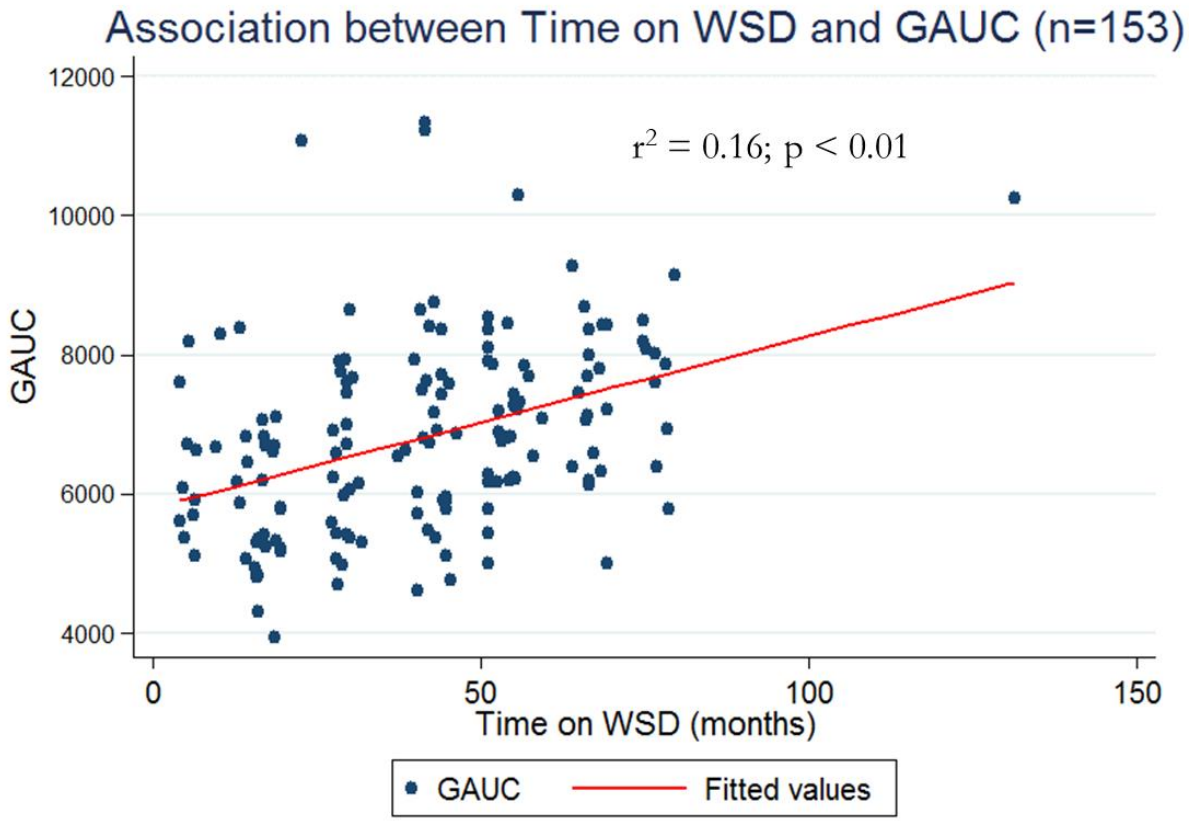


Figure 3-5: Relationship between time on WSD and GAUC. Time on WSD was significantly associated with increased GAUC. For every month increase in time on WSD, GAUC increases by 24.69 units (95% CI: 15.50, 33.87, p<0.01). n = 153.

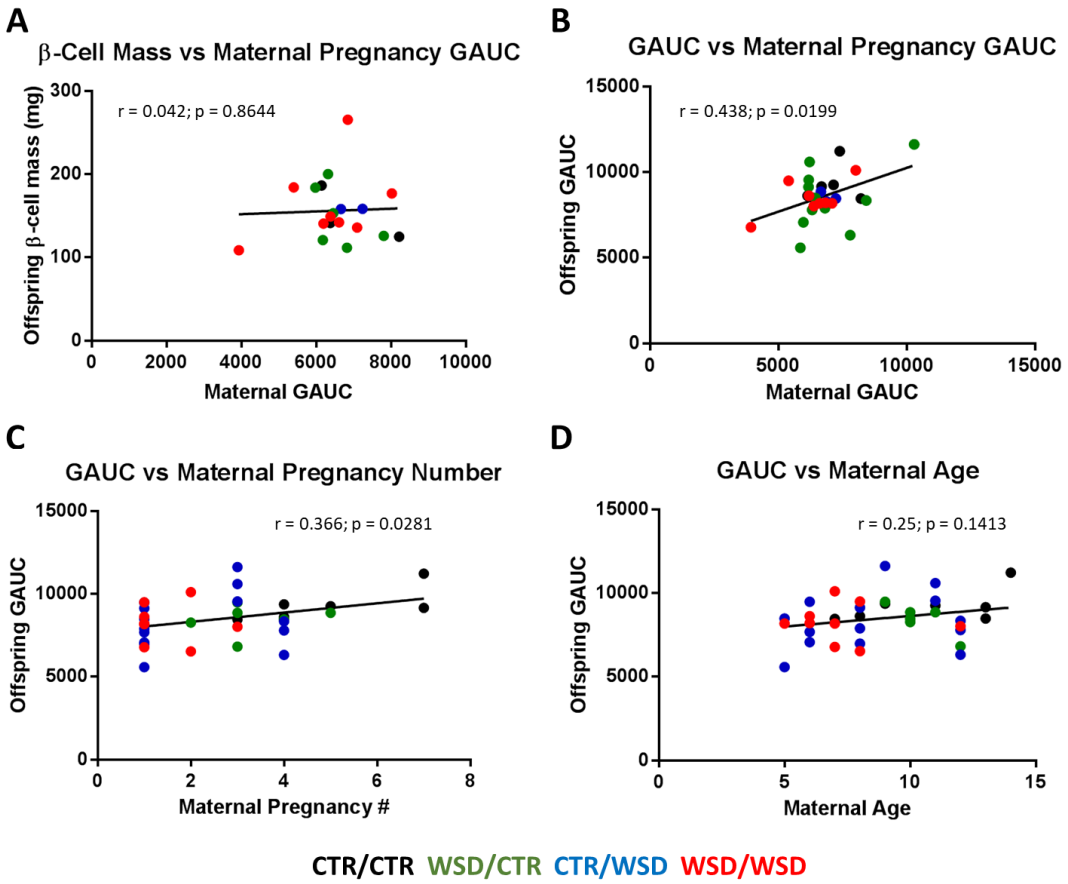


Figure 3-6: Correlations between maternal characteristics and offspring phenotypes. Maternal GAUC pregnancy was not correlated with β-cell mass in the offspring (A). Increases in both maternal glucose tolerance (B) and parity (C) were correlated with increasing GAUC in the offspring; p = 0.02 and p = 0.03, respectively. Maternal age trended toward correlation with impaired offspring GAUC, but this was not significant (D); p = 0.14. n = 26-36.

A

	No.[1]	CTR/CTR (n=8)		WSD/CTR (n=14)		P-value[3]
		No.	% or median (IQR)[2]	No.	% or median (IQR)	
Maternal characteristics						
Age, years	22	8	10 (8-13)	14	8 (6-11)	0.12
Parity[4]						**0.01**
0	8	0	0	8	57	
≥1	14	8	100	6	43	
Body fat percentage	22	8	20 (10-26)	14	23 (13-35)	0.25
GAUC	18	7	7153 (6381-7655)	11	6321 (6181-7807)	0.39
Offspring characteristics						
Sex						0.38
Male	11	3	38	8	57	
Female	11	5	62	6	43	
Fasting insulin	21	7	3 (2-4)	14	5 (3-7)	0.16
α-cell mass, mg	12	5	109 (80-121)	7	59 (48-69)	0.06
β-cell mass, mg	12	4	164 (133-188)	8	139 (116-192)	0.61

Table continued on next page.

B

	No.[1]	CTR/WSD (n=6)		WSD/WSD (n=9)		P-value[3]
		No.	% or median (IQR)[2]	No.	% or median (IQR)	
Maternal characteristics						
Age, years	15	6	10 (10-11)	9	7 (6-8)	**0.01**
Parity[4]						**0.01**
0	6	0	0	6	67	
≥1	9	6	100	3	33	
Body fat percentage	15	6	15 (12-20)	9	13 (10-15)	0.36
GAUC	10	3	6894 (6667-7242)	8	6502 (5802-6974)	0.22
Offspring characteristics						
Sex						0.83
Male	8	3	43	5	57	
Female	7	3	57	4	43	
Fasting insulin	15	6	16 (7-35)	9	11 (5-14)	0.19
α-cell mass, mg	10	5	100 (56-107)	5	64 (54-74)	0.25
β-cell mass, mg	14	5	158 (158-251)	9	149 (141-184)	0.84

Table 3-5: Distribution of maternal and offspring characteristics, stratified by offspring diet (N=37). CTR offspring in (A), WSD in (B).
Abbreviations: No., number; IQR, interquartile range; WSD, western-style diet; CTR, control diet; GAUC, glucose area under the curve.
[1] Numbers may not add up to total number of offspring on CTR (n=22) or WSD (n=15) due to missing data.
[2] Median and IQR are reported for continuous variables due to small sample size and skewness; percent is reported for categorical variables.
[3] P-value from Wilcoxon rank-sum test.
[4] Parity refers to number of pregnancies prior to study pregnancy.

A

Influence of covariates on the relationship between maternal GAUC and offspring GAUC (N=36)[1]

Covariates in model	No.[2]	Coefficient	95% CI	P-value
Maternal GAUC only	28	0.51	0.07, 0.95	**0.02**
Maternal GAUC, age	28	0.45	-0.01, 0.92	0.05
Maternal GAUC, parity	28	0.42	-0.07, 0.90	0.09
Maternal GAUC, parity, age	28	0.43	-0.08, 0.93	0.09

B

Influence of covariates on the relationship between parity and offspring GAUC (N=36)[1]

Covariates in model	No.[2]	Coefficient	95% CI	P-value
Parity only	36	280.36	59.62, 501.09	**0.02**
Parity, age	36	386.41	57.35, 715.46	**0.02**
Parity, maternal GAUC	28	184.56	-69.22, 438.34	0.15
Parity, age, maternal GAUC	28	111.30	-286.34, 508.94	0.57

Table 3-6: Maternal factors associated with offspring GAUC
Abbreviations: No., number; CI, confidence interval; GAUC, glucose tolerance test area under the curve.
[1] One offspring missing GAUC value.
[2] Eight offspring missing maternal GAUC value.

(Figure 3-6D), regardless of adjustment for other maternal factors (data not shown).

Crude and adjusted models comparing the effect of maternal diet on offspring GAUC were stratified by offspring diet (Table 3-7). Although maternal diet was not statistically associated with offspring GAUC, some patterns deserve note. Among offspring placed on CTR at weaning (Table 3-7A), maternal WSD diet appeared to be associated with a slight improvement in offspring GAUC (Table 3-7A); however, this was not statistically significant (p=0.12). The point estimate further shifted towards the null when adjusting for parity (coefficient went from -897 to +33) or maternal GAUC (coefficient went from -897 to -778), suggesting that maternal WSD is unlikely to improve offspring metabolic health. Among offspring weaned onto WSD (Table 3-7B), the direction of estimate shifted towards increased GAUC as a result of maternal WSD when adjusting for parity (coefficient went from -219 to -76) or maternal GAUC (coefficient went from -219 to +231). Thus, as hypothesized, it is likely that offspring exposed to maternal WSD would have impaired glucose tolerance, on either post-weaning diet, though we were underpowered to observe this effect.

Discussion

This NHP model of maternal overnutrition is highly related to humans, both in terms of islet composition and function as well as in gestation. Several studies in humans have suggested that a poor gestational environment is deleterious to the metabolic health of the offspring, even in the absence of overt diabetes. As in humans, NHPs consuming an unhealthy may or may not gain weight, and do not all gain the same amount of weight. Additionally, many female NHPs do not show impaired glucose tolerance on a WSD, both before and during pregnancy. In the specific population analyzed, WSD appeared to be associated with slightly improved GAUC. When dams were fed CTR and WSD in different pregnancies, no clear pattern emerged, although some dams had worsened GAUC on CTR diet. In animals who were fed WSD after reversal, GAUC tended to be significantly elevated. This may be partially due to the

A

Covariates in model	No.[2]	GAUC of offspring on CTR (n=21)		
		Coefficient	95% CI	P-value
Diet only	21	-897.39	-2038.54, 243.75	0.12
Diet, parity	21	33.29	-1858.65, 1925.24	0.97
Diet, maternal GAUC	17	-777.90	-2151.12, 595.32	0.25

B

Covariates in model	No.[2]	GAUC of offspring on WSD (n=15)		
		Coefficient	95% CI	P-value
Diet only	15	-219.22	-1565.04, 1126.59	0.73
Diet, parity	15	-75.63	-1962.25, 1810.98	0.93
Diet, maternal GAUC	11	231.48	-686.32, 1149.28	0.58

Table 3-7: Influence of covariates on the relationship between maternal WSD and offspring GAUC, stratified by offspring diet (N=36)[1]
Abbreviations: No., number; WSD, western-style diet; CTR, control diet; CI, confidence interval; GAUC, glucose tolerance test area under the curve.
[1] One offspring on CTR is missing GAUC value.
[2] Four offspring on CTR and four offspring on WSD are missing maternal GAUC value.

negative effects of metabolic cycling [124]. One possibility for the similar or slightly improved GAUC in dams fed WSD is that in our populations, inherent differences in the two groups existed. Supporting this hypothesis is the significant correlation between time on WSD and GAUC, suggesting that consuming WSD is indeed deleterious to maternal metabolic health.

A subset of dams in this analysis, as well as their offspring, were used to determine relationships between maternal factors and offspring metabolic health. One limitation of these analyses was that a small proportion of mothers (n=7) gave birth more than once during the study period and thus multiple offspring from the same dam are included in the study. Given the small number of animals in this study, robust variance was used to account for correlation among siblings rather than performing a sensitivity analysis excluding subsequent pregnancies. In this genetically variable animal model, heterogeneity existed among dams in terms of age, parity, glucose tolerance, and response to glucose administration during a GTT. This heterogeneity could be viewed as additional limitations to the study design; however, it is more similar to the human situation and has allowed us to probe for independent maternal factors that affect offspring metabolic health.

This data shows that increased maternal GAUC is a potential predictor of elevated offspring GAUC. It should be noted that for completion, all dam/offspring pairs in our cohort were retained in this analysis, despite the spread in sample values. All GTTs used in the study were determined to be valid and true values based on a clear response to glucose stimulation. Thus, there was no scientific basis for discarding any of the values. The robustness of these observations was significant but did rely on a limited number of dams, limiting generalization to other experimental settings and populations. However, despite these limitations, it is worth noting that several studies in humans also demonstrate the impact of worsening maternal glucose tolerance on the offspring. A study on offspring of 1,049 Pima Indian women found that offspring of women with impaired glucose tolerance (but not gestational diabetes) during pregnancy were more likely to weigh more and have impaired glucose tolerance [125]. Another study in humans

showed that increasing maternal glucose levels (even in the absence of overt diabetes) during glucose tolerance tests in pregnancy correlate with neonatal adiposity [12]. When this same cohort of offspring was assessed seven years later, increases in maternal glucose levels--both fasting and during glucose tolerance testing--were significantly associated with offspring glucose tolerance as assessed by area under the curve, independent of childhood obesity or being born large for gestation age [123]. Indeed, a currently ongoing randomized clinical trial aims to improve maternal glucose tolerance during pregnancy in order to improve health outcomes in both mother and offspring [126].

In addition to changes in the *in utero* environment, the relationship between maternal and offspring GAUC may be due to genetic factors. Indeed, previous work in the Japanese macaque model identified three single nucleotide polymorphisms associated with weight stability and insulin sensitivity in the dams [127]. These genomic variants that affect maternal GAUC may have been passed to the offspring that show strong correlations between maternal and offspring GAUC.

Maternal parity was also a significant predictor of worsened offspring glucose tolerance. A study in humans found that neither maternal age nor parity was associated with increased offspring adiposity, but that offspring adiposity was independently associated with maternal BMI [128]. However, a larger study conducted in The Netherlands showed that multiparity was associated with maternal obesity, which was in turn associated with offspring childhood obesity [129]. Thus, the consequences of maternal parity on the offspring in humans remains somewhat unclear. Another human study showed an association between maternal age (above 35 or below 25) and poor metabolic health in the offspring [130]. In this study, there was no statistically significant association between maternal age and offspring metabolic health.

In conclusion, maternal parity and GAUC during pregnancy are potential independent predictors of worsened glucose tolerance in the offspring. These data suggest that the *in utero* and early postnatal environment produced by elevated maternal GAUC impacts the metabolic health of juvenile offspring.

CHAPER IV: MATERNAL WESTERN-STYLE DIET AFFECTS OFFSPRING ISLET COMPOSITION IN THE NON-HUMAN PRIMATE

Adapted from Elsakr et al., “Maternal Western-style diet affects offspring islet composition and function in a non-human primate model of maternal over-nutrition”, *Molecular Metabolism,* 2019

Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis states that the gestational and immediate postnatal environment influences long-term offspring health and plays a role in adult disease etiology. Early evidence for the DOHaD hypothesis stemmed from epidemiological studies on offspring conceived during the Dutch Hunger Winter--a period of famine in the Netherlands during World War II where birth registries were maintained and records of rations were kept [131]. Children of the women who were exposed to famine during pregnancy were more likely to be obese as adults than their siblings born outside the period of famine [1, 131].

In addition to studies in humans evaluating the effects of *in utero* exposure to undernutrition on offspring [1, 2], it is clear that maternal obesity also leads to an increased risk of metabolic diseases in the offspring, such as obesity and/or T2D [8, 9, 131, 132]. In the US, it is estimated that over 50% of women are overweight or obese at the start of pregnancy [20, 133]. Findings from human cohort studies have been replicated with high fat diet feeding in animal models, and collectively suggest that a calorically dense diet, in both the presence and absence of maternal obesity, results in an unfavorable *in utero* metabolic environment rendering risk of offspring obesity, glucose intolerance, and diabetes. Thus, future generations could face the consequences of maternal caloric dense diets and obesity, highlighting the need to understand the mechanisms by which maternal diet and metabolic lead to increased risk of T2D.

Studies in rodent models have described structural and functional changes to the pancreatic islets of offspring exposed to maternal obesity or high fat diet *in utero* [50-60]. In general, exposure to overnutrition leads to alterations in endocrine cell mass and diminished islet function, although outcomes vary from study to study and appear to be dependent on the specific obesity model and mouse strain used [133]. These parameters can result in vastly different offspring phenotypes in response to exposure to overnutrition, from impaired glucose tolerance persisting to the second generation [55], to a complete absence of islet structural or functional changes [64]. The discrepancies in rodent models highlight the need for animal models that more closely mimic human physiology to better understand how exposure to maternal overnutrition might affect human islet development and postnatal function, as well as increased risk for T2D.

Pregnancy in the Japanese macaque (*Macaca fuscata*) more closely approximates human gestation than rodent and other litter models of gestational exposure. Dams typically give birth to a single offspring at a time, and macaque colonies are genetically heterogeneous. Islets from the macaque are well characterized and are similar to human islets in terms of structure, insulin secretion during perifusion, and expression of endocrine hormones and key transcription factors [30]. Here, an established macaque model of maternal high fat, calorically dense diet is utilized to investigate the effects of maternal overnutrition on the offspring [105]. Female macaques were fed control diet (CTR) or Western-style diet (WSD) before and during pregnancy and through lactation. Offspring were weaned onto CTR or WSD, resulting in four different maternal/offspring diet groups: CTR/CTR, WSD/CTR, CTR/WSD, and WSD/WSD. As in human populations, maternal obesity and glucose tolerance varied within the same diet group, and thus one can independently analyze the effects of maternal metabolic phenotype as well as diet composition on the offspring.

In previous studies using this model, offspring of WSD-fed mothers had increased liver triglycertides and, when mothers were obese, histologic evidence of nonalcoholic fatty liver disease [88]. Additionally, maternal WSD exposure resulted in gut dysbiosis in the offspring, which

was only partially corrected when offspring were weaned to a CTR diet [84]. Fetuses exposed to WSD had normal β-cell mass but reduced α-cell mass at late gestation [95]. At 13 months of age (five months post-weaning) offspring exposed to maternal WSD and weaned onto WSD (WSD/WSD) had an elevated β:α cell ratio compared with all other groups (CTR/CTR, WSD/CTR, CTR/WSD). Thirteen-month-old offspring exposed to maternal WSD also had impairments in islet vascular expansion in response to post-weaning WSD [96].

Here, the longer-term consequences of maternal and postnatal WSD exposure on offspring islet morphology and function are assessed. Specifically, the effects of maternal WSD when offspring are fed a healthy diet for 2.5 years post-weaning are investigated in the following experiments.

Results

Reductions in α-cell mass persist in three-year-old offspring exposed to maternal WSD

In previous studies using this model, α-cell mass was reduced in fetuses exposed to WSD. Thus, α-cell mass was quantified (Figure 4-1A, 4-1B) to test whether this phenotype persisted in offspring at three years of age. α-cell mass was indeed significantly reduced by nearly 50% as a result of maternal WSD (Figure 4-1C). Interestingly, a few animals had severely reduced, but detectable, α-cell mass. Offspring α-cell mass did not correlate with maternal metabolic health during pregnancy as assessed by intravenous glucose tolerance testing (data not shown). β-cell mass was unaffected by maternal diet in both WSD/CTR and WSD/WSD offspring (Figure 4-1D). There was no difference between male and female offspring in α-cell or β-cell mass outcomes in response to maternal diet.

Like human islets, non-human primate (NHP) islets show heterogeneity in the proportions of the different endocrine cell types (Figure 4-2A), and lack the distinct mantle/core architecture observed in rodent islets, where β cells localize to the core of the islet and α cells are located at the periphery [30]. WSD-exposed fetuses had a greater than 50% increase in β:α cell ratio due to

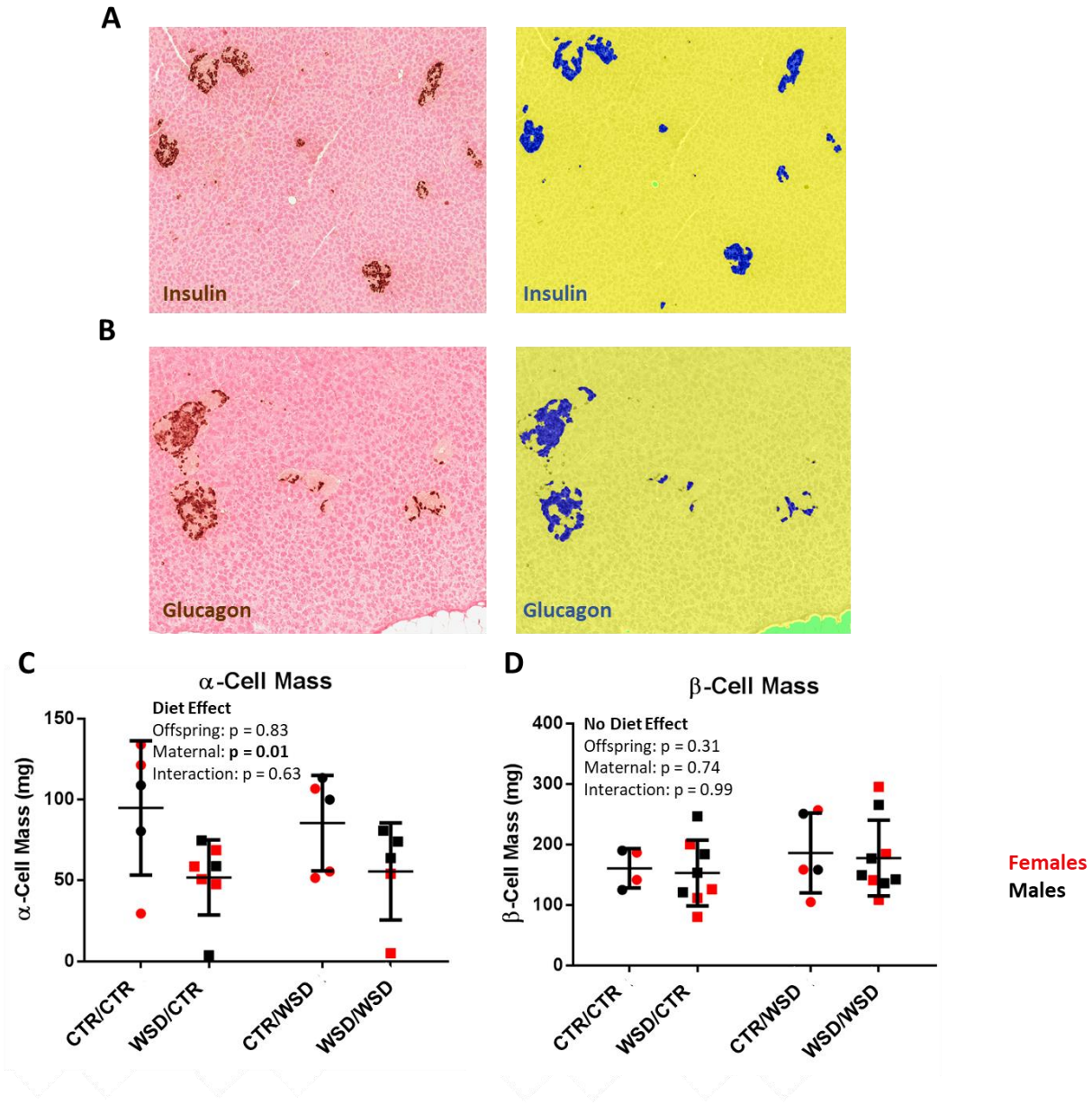


Figure 4-1: α- and β-cell mass as assessed by immunohistochemistry. Pancreas sections were immunolabelled for either (A) insulin (brown) or (B) glucagon (brown) and counterstained with eosin (pink). Hormone-positive area was measured using a customized Aperio-based algorithm. Algorithm markup images are shown on the right in (A) and (B); 20X magnification, scale bar = 300 um. Hormone-positive tissue is marked in blue, hormone-negative tissue is in yellow, and the glass slide is green. (C) α-cell mass is significantly reduced as a result of maternal WSD; p = 0.01. (D) β-cell mass was unaffected by maternal or offspring diet. Groups are named by maternal diet/offspring diet. WSD = Western-Style Diet, CTR = Control Diet. n = 4-9 per group. Two-way ANOVA.

a 50% decrease in α-cell mass, and 13-month-old offspring in the WSD/WSD group also had an increased β:α cell ratio (from ~1.5 to 2.1) and decreased α-cell number [95]. At three years of age, we observed a significant increase in β:α cell ratio as a result of both maternal and offspring WSD (Figure 4-2B), with the WSD/WSD group having nearly double the β:α cell ratio of CTR/CTR animals. The proportion of both δ and PP cells was not affected by maternal or offspring diet as shown in Figure 4-2C and Figure 4-2D, respectively.

WSD/WSD Animals Tend To Have Reduced Endocrine Cell Proliferation

Next, mechanisms of endocrine cell expansion, namely hypertrophy and proliferation, were analyzed to test whether they were affected by maternal diet and could explain the reduction in α-cell mass in WSD/CTR offspring. There was no evidence for β-cell hypertrophy, as β-cell size was not affected by maternal diet (Figure 4-3A). In general, proliferation of both α and β cells was extremely low (<0.6%) in samples from all four diet groups (Figure 4-3B, 4-3C). Interestingly, offspring WSD led to a significant decrease in α-cell proliferation (Figure 4-3C). Exposure to maternal WSD had no significant effect on proliferation of either cell type when offspring were weaned onto CTR diet or WSD. Thus, the persistent effects of *in utero* and early postnatal WSD exposure on α-cell mass cannot be explained by differences in α-cell proliferation at this time point.

Maternal WSD Has No Lasting Effects on Islet Vascularization

Pancreatic islets are highly vascularized [134], and the proportion of the islet that is vascularized increases in response to insulin resistance [135]. Indeed, in 13-month-old offspring, post-weaning exposure to WSD resulted in increased islet vascularization in CTR/WSD offspring relative to CTR/CTR animals [96]. This compensatory increase was absent in WSD/WSD offspring, suggesting that exposure to maternal WSD impairs the islet vascular expansion that should occur in response to WSD. We quantified islet vascularization at three years of age by

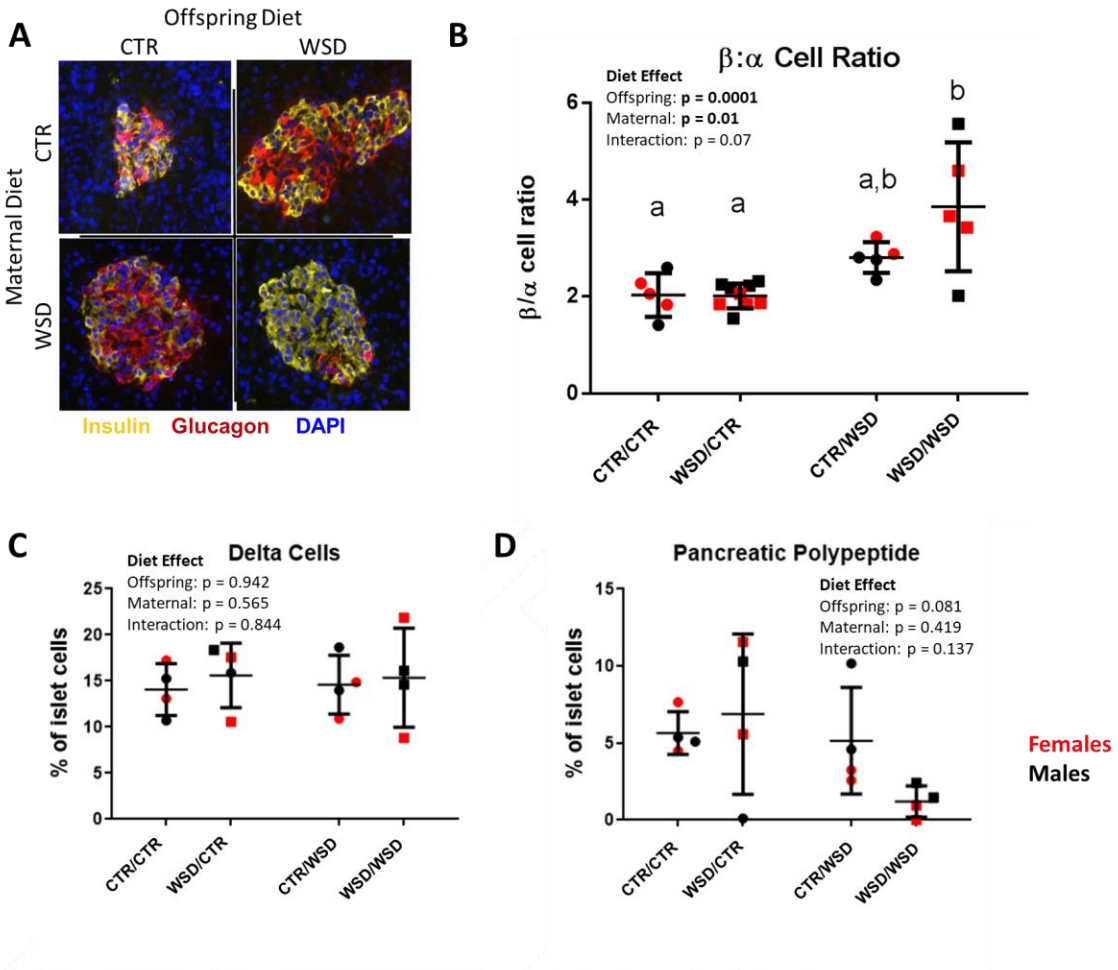


Figure 4-2: Maternal diet affects offspring islet cell composition. Non-human primate islets display a heterogeneous distribution of β and α cells (A); 20X magnification, scale bar = 100 um. (B) β:α cell ratio was increased as a result of maternal diet; p = 0.01. WSD/WSD offspring had significantly greater β:α cell ratio than CTR/CTR (p = 0.0017) and WSD/CTR (p = 0.0004) offspring. Delta Cells (C) and PP cells (D) were not affected by maternal or offspring diet, two way ANOVA. n = 4-9 per group.

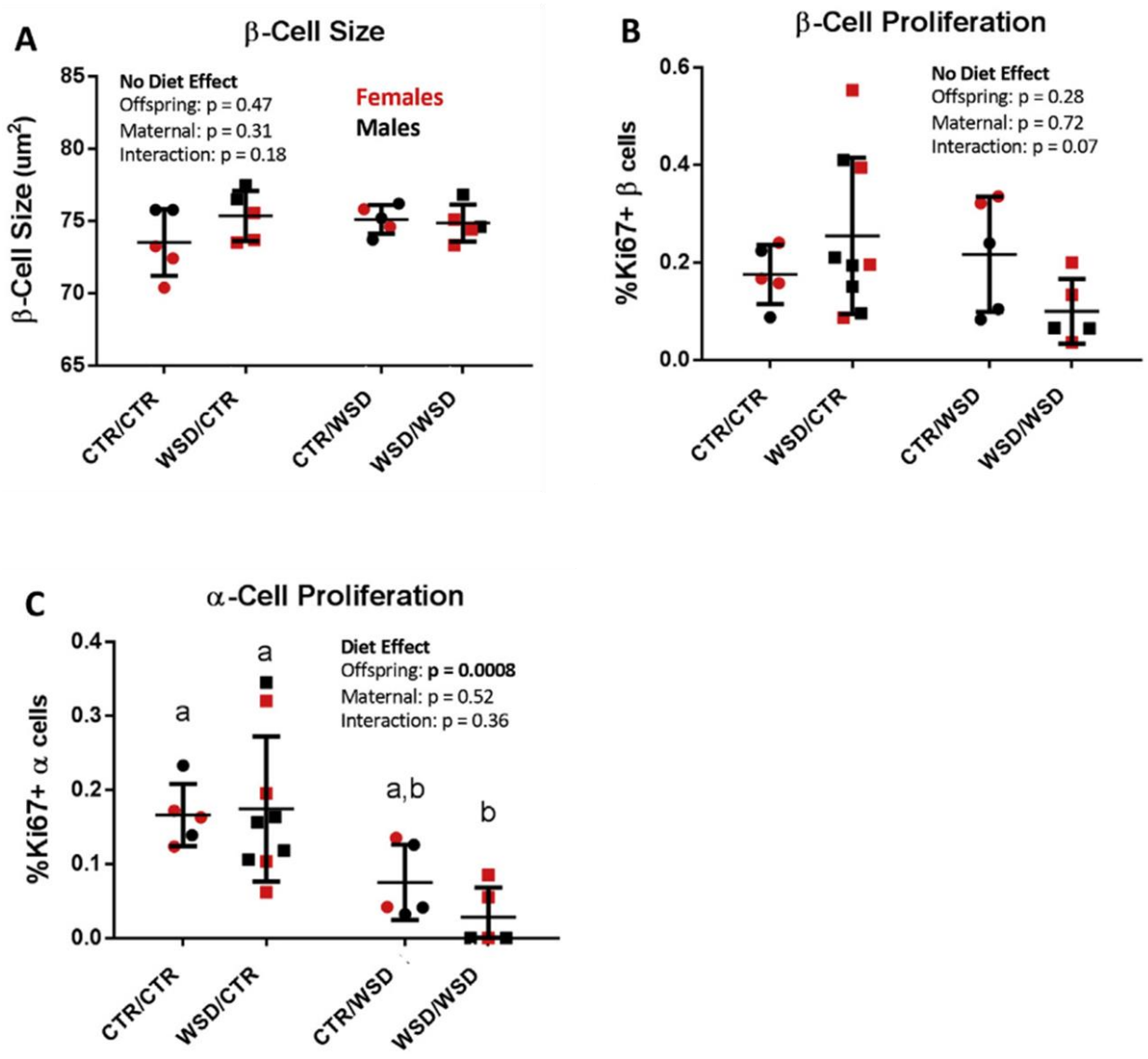


Figure 4-3: α-cell proliferation was significantly reduced by offspring WSD. (A) β-cell size was not affected by maternal or offspring WSD. (B) β-cell proliferation trended toward reduction as a result of interaction between maternal and offspring WSD; p = 0.07, two way ANOVA. (C) α-cell proliferation was reduced as a result of offspring WSD (p = 0.0008), but not affected by maternal diet. WSD/WSD had significantly less α-cell proliferation than CTR/CTR (p = 0.03) and WSD/CTR (p = 0.01) offspring. n = 5-9 per group.

utilizing the endothelial cell marker PECAM-1 (Figure 4-4A). Overall, islets were highly vascularized as expected. However, the CTR/WSD group no longer had elevated vascularization compared to WSD/WSD animals (Figure 4-4B). Thus, previous differences in islet vascularization due to maternal WSD are no longer present at three years of age.

Maternal WSD Does Not Affect Offspring Glucose Tolerance

Although long-term effects of maternal WSD exposure on islet morphology and vascularization are subtle, offspring islet cell function could still be compromised due to maternal diet exposure without obvious changes in whole islet morphology. Thus, the effects of maternal diet on whole body glucose metabolism were investigated using intravenous glucose tolerance tests (GTTs) conducted before necropsy. Figure 5A shows the glucose area under the curve (GAUC) for the offspring GTTs. We found that offspring GAUC was not affected by either maternal or post-weaning diet. However, regardless of maternal diet, offspring weaned onto WSD had higher insulin area under the curve (IAUC) and higher insulin-to-glucose ratio by about 1.5-fold (Figures 5B and 5C), consistent with WSD-induced insulin resistance.

Discussion

In this study, persistent effects of maternal WSD on offspring islet function were identified in an animal model closely related to humans. Consistent with the phenotype observed in this model at earlier time points [95], α-cell mass is reduced at three years of age as a result of maternal WSD exposure. The reduction in α-cell mass may be due to impairments in differentiation during embryogenesis as a result of maternal diet. Indeed, fetuses exposed to WSD had decreases in the expression of transcription factors important for α-cell differentiation [94]. Interestingly, this deficit in α-cell mass was not overcome even after 2.5 years on a healthy diet. The long-term functional consequences of α-cell loss remain unclear. Mice exposed to a high fat diet for 12 weeks also show reduced α-cell mass [128]. However, in mice, near complete ablation

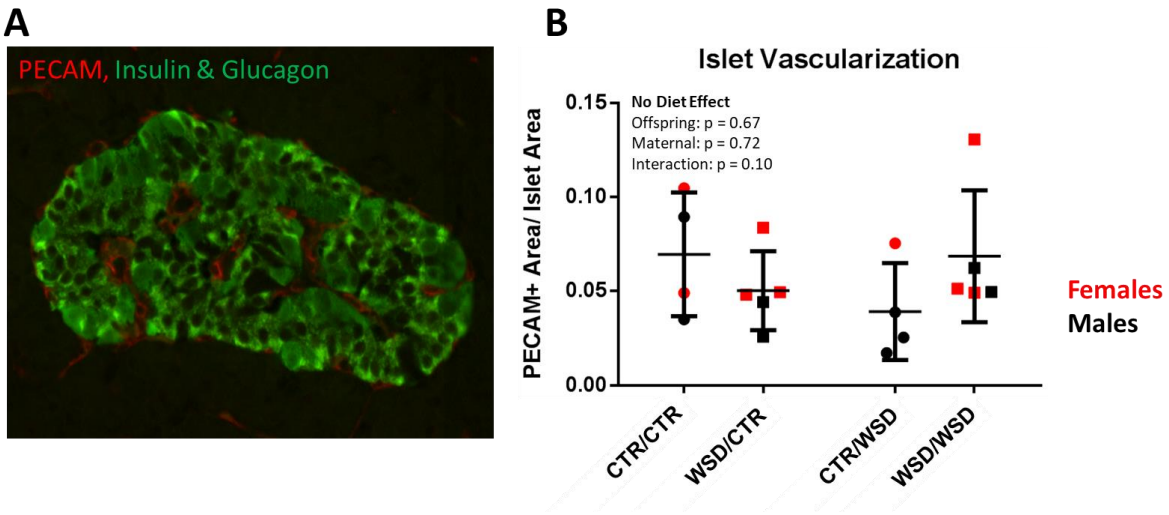


Figure 4-4: Islet vascularization in 3-year-old offspring. (A) NHP islets are well vascularized, as labelled with the endothelial marker PECAM-1 (A; green = insulin + glucagon); 20X magnification, scale bar = 100 um. At three years of age, there were no differences in islet vascularization among the different diet groups (B); two-way ANOVA. n = 4-5 per group.

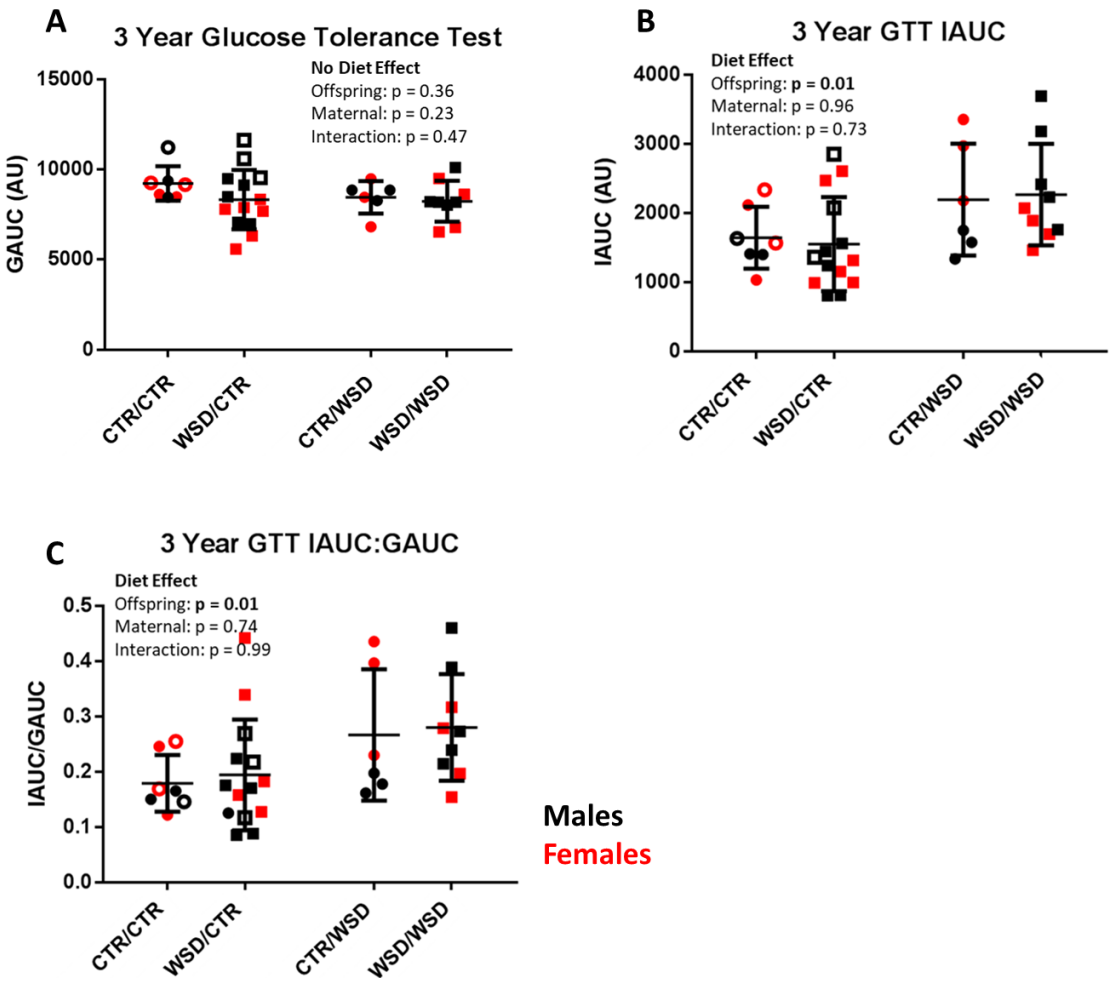


Figure 4-5: Effects of maternal and offspring diet on glucose tolerance. Glucose area under the curve (GAUC) was not affected by maternal or post-weaning diet (A). (B) Insulin area under the curve (IAUC) during glucose tolerance tests was higher in post-weaning WSD animals (p = 0.012) but not affected by maternal diet. (C) The ratio of insulin to blood glucose was similarly elevated by offspring WSD (p = 0.0143), but unaffected by maternal diet. Two way ANOVA. n = 6-14 per group. Open symbols in A-C represent data from animals used for the perifusions in Chapter V.

of α cells had no observable effects on insulin secretion and glucose tolerance [129]. Additionally, small β-cell-only pseudoislets formed from dispersed and sorted mouse islets display near-normal glucose stimulated insulin secretion [130]. However, nearly 50 years ago, rat pancreata were shown to release more insulin in response to glucose when glucagon was present [131]. This effect has more recently been observed in mice, where IV glucose plus glucagon led to higher insulin secretion in vivo than IV glucose alone [132]. In addition to glucagon, α cells also produce insulin secretagogues such as ghrelin [133] and GLP-1 [134] which act in a paracrine manner to stimulate insulin secretion. However, we observe increased insulin secretion in GSIS in islets from WSD/CTR offspring despite reduced α-cell mass.

Importantly, studies investigating the impact of maternal diet and metabolic health on islet cell mass are currently not possible in humans with available imaging techniques, highlighting the significance of the NHP model. Future studies in this model can elucidate the physiological consequences of decreased α-cell mass in a species more closely related to humans. Both NHP and human islets demonstrate a higher degree of intermingling between the different endocrine cell types, compared to rodents, with α cells making up a larger proportion of the islet in primates [30]. This suggests that paracrine interactions between α and β cells may be even more important in these species than in rodents.

Islet vascularization can increase in response to insulin resistance [127], and is also increased in T2D [135]. Islet vasculature was increased in CTR/WSD animals relative to WSD/WSD at 13 months of age [95]. The increase in vascularization at that time point was likely a response to acute exposure to WSD, while this response was absent in WSD/WSD animals, potentially due to prior exposure to the WSD. We observed that at three years of age, CTR/WSD animals no longer displayed an increase in islet vascularization (Figure 4-4), perhaps due to compensatory changes that re-establish homeostasis. Further studies are needed to determine whether vascular compensation in WSD/WSD offspring is impaired in other models of metabolic stress such as pregnancy.

Together, these results demonstrate that longer-term effects of maternal WSD exposure on offspring islet composition and morphology are evident. WSD-exposed animals continue to display reduced α-cell mass at three years of age, while previous effects of WSD-exposure on islet vascularization are no longer present. Importantly, the impact of maternal WSD feeding on the offspring cannot be offset with weaning onto a control diet.

CHAPTER V: MATERNAL WESTERN-STYLE DIET AFFECTS OFFSPRING ISLET FUNCTION IN NON-HUMAN PRIMATES

Introduction

Studies in both human and rodent models suggest that a gestational environment of overnutrition can compromise islet function in the offspring. In humans, increased maternal blood glucose levels during pregnancy are significantly correlated with increased cord-blood C-peptide levels, indicating that a gestational environment of overnutrition can alter fetal islet function [122]. While increased secretion of insulin/C-peptide can be viewed as an enhancement in islet function, a similar study demonstrated that increased insulin secretion *in utero* predicts impaired glucose tolerance in childhood independent of obesity [120]. Indeed, a significant correlation exists between maternal glucose tolerance and offspring glucose tolerance at seven years of age, independent of childhood obesity [123]. This suggests a role for intrinsic islet dysfunction, and not simply insulin resistance, in the impairment of glucose tolerance in these offspring.

In mice exposed to HFD *in utero*, islet insulin content was decreased in males, but increased in females at six weeks of age [60]. In a genetic model of maternal obesity, mouse offspring fed a HFD had increased islet insulin content if they were born to control dams, but not obese dams [57]. Another study in mice showed that HFD-exposed offspring had fasting hyperinsulinemia, a phenotype which persisted to the second generation [55]. In rats, fetal islets exposed to HFD *in utero* had high insulin secretion when stimulated with high glucose or insulin secretagogues *ex vivo* [58].

Collectively, these data suggest that a gestational environment of maternal overnutrition can affect islet function independently of other phenotypes such as insulin resistance. The NHP model is a valuable tool to study this effect. This model allows for the isolation of pancreatic islets in offspring exposed to CTR or WSD *in utero*, enabling the study of maternal diet-induced effects on islets that are functionally similar to human islets.

Results

Islets Exposed to WSD *In Utero* Secrete More Insulin in Response to High Glucose

Islets from two cohorts of three-year-old NHP offspring were isolated for perifusion and *ex vivo* assays. These islets came from CTR/CTR and WSD/CTR groups, and thus belonged to offspring who had consumed a healthy diet for nearly 2.5 years before necropsy. As shown in Figure 5-1A, WSD/CTR islets secreted significantly more insulin in response to high glucose than CTR/CTR islets. Due to limitations in sample availability, the WSD/CTR group contained only males. However, when only males were compared between the two diet groups WSD/CTR islets still showed increased insulin secretion, suggesting an effect of maternal diet rather than offspring sex (Figure 5-2B). In addition to the islets from three-year-old offspring, islets from one-year-old offspring were also isolated for perifusion experiments. These islets came from different diet groups, CTR/WSD and WSD/WSD. However, even at an earlier age and in a separate cohort, maternal WSD led to increased glucose-stimulated insulin secretion in offspring islets (Figure 5-1C). Four possibilities were identified to explain the mechanism behind the increased insulin secretion: an increase in mitochondrial function, an increase in mitochondrial density, increased insulin production or turnover, and changes in islet gene expression. Each of these possibilities were tested in turn.

WSD/CTR Islets Show Similar Oxygen Consumption Profiles to CTR/CTR Islets

Historically, it has been challenging to assess oxygen consumption in spheroid clusters of cells due to the large number of cells required to perform the assay. A recent publication demonstrated the use of a newly designed, specialized 96-well microplate to reliably measure oxygen consumption in small numbers of human and mouse islets [108]. This technique was adapted for use with primate islets with modifications made to key steps to enhance the

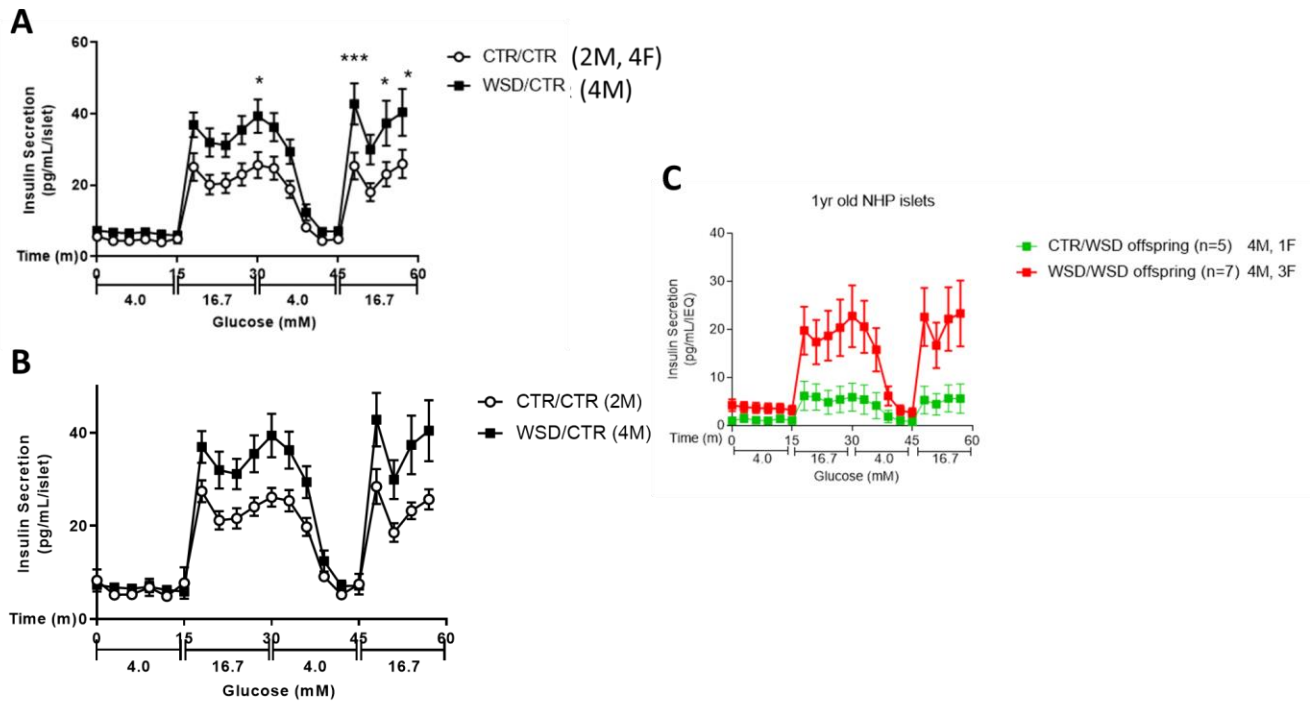


Figure 5-1: *Ex vivo* islet perifusion. (A) At three years of age, WSD/CTR islets secreted higher insulin in response to glucose (maternal diet effect p = 0.026, two-way repeated measures ANOVA). This effect persisted when analyzing males only (B). At one year of age, WSD/WSD islets secreted more insulin in response to glucose (C), though this did not reach statistical significance (p=0.068, two-way repeated measures ANOVA).

reproducibility of the assay [107]. Using this technique, it is possible to probe the following aspects of mitochondrial function: basal respiration--islet oxygen consumption at baseline; ATP production--oxygen consumption that is used for the production of ATP; coupling efficiency—the proportion of basal respiration used for ATP production; non-mitochondrial oxygen consumption; maximal respiration; spare respiratory capacity--the difference between maximal and basal oxygen consumption; and proton leak--mitochondrial oxygen consumption not used for the production of ATP, which can be elevated in the presence of uncoupling proteins whose expression is induced by oxidative stress. This is shown schematically in Figures 5-2A and 5-3A.

Since WSD/CTR islets had similar basal insulin secretion, but elevated GSIS, it was hypothesized that spare capacity would be increased in the WSD/CTR group. However, no changes in mitochondrial function due to maternal WSD were detected in any of the parameters tested (Figure 5-2). Oxygen consumption assays were also performed on one-year-old CTR/WSD and WSD/WSD samples (Figure 5-3). Similarly, no changes were detected at this time point.

WSD/CTR Islets Show No Increase in Mitochondrial Density

To test the possibility that increased insulin secretion in three-year-old WSD was due to an increase in mitochondrial density, electron microscopy (EM) was utilized to image β cells from CTR/CTR and WSD/CTR samples. This allowed for the quantification of mitochondrial area in the β cells. Using ImageJ, each mitochondrian was circled (Figure 5-4A), and mitochondrial density was calculated by dividing mitochondrial area by β-cell area. As shown in Figure 5-4B, maternal WSD did not lead to an increase in offspring mitochondrial area. In fact, if anything, it appears that β cells from offspring exposed to early life WSD have reduced mitochondrial area, although a larger cohort would need to be examined to definitely determine this. Thus, the enhanced insulin secretion cannot be explained by an increase in mitochondrial function or mitochondrial density.

Insulin Granule Density And Maturity Are Similar Between Diet Groups

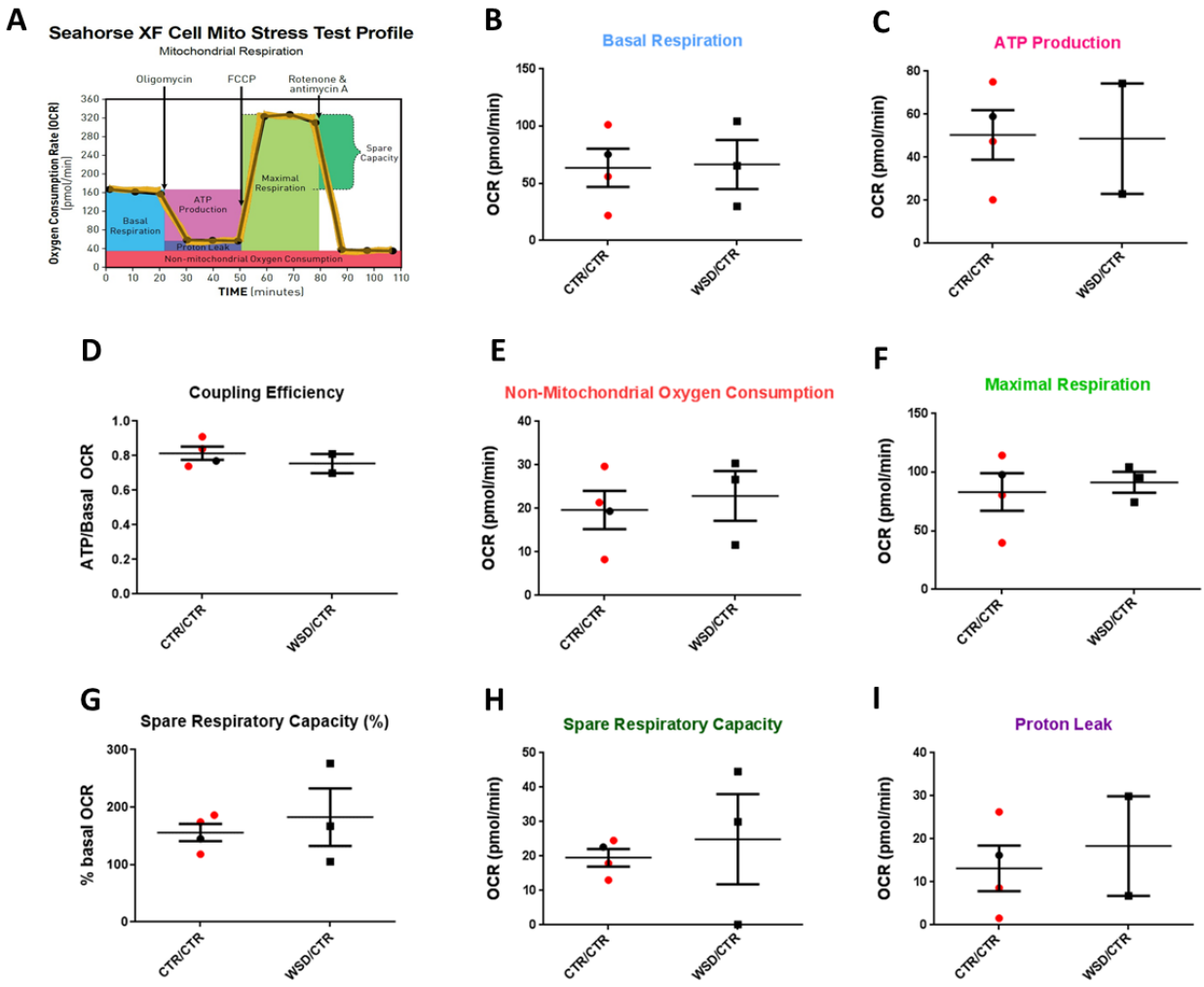


Figure 5-2: Oxygen consumption assay in three-year-old islets. Various aspects of islet mitochondrial function were pharmacologically probed in CTR/CTR and WSD/CTR islets and analyzed using Seahorse. No significant differences in any panel; $P > 0.05$, Student's *t* test.

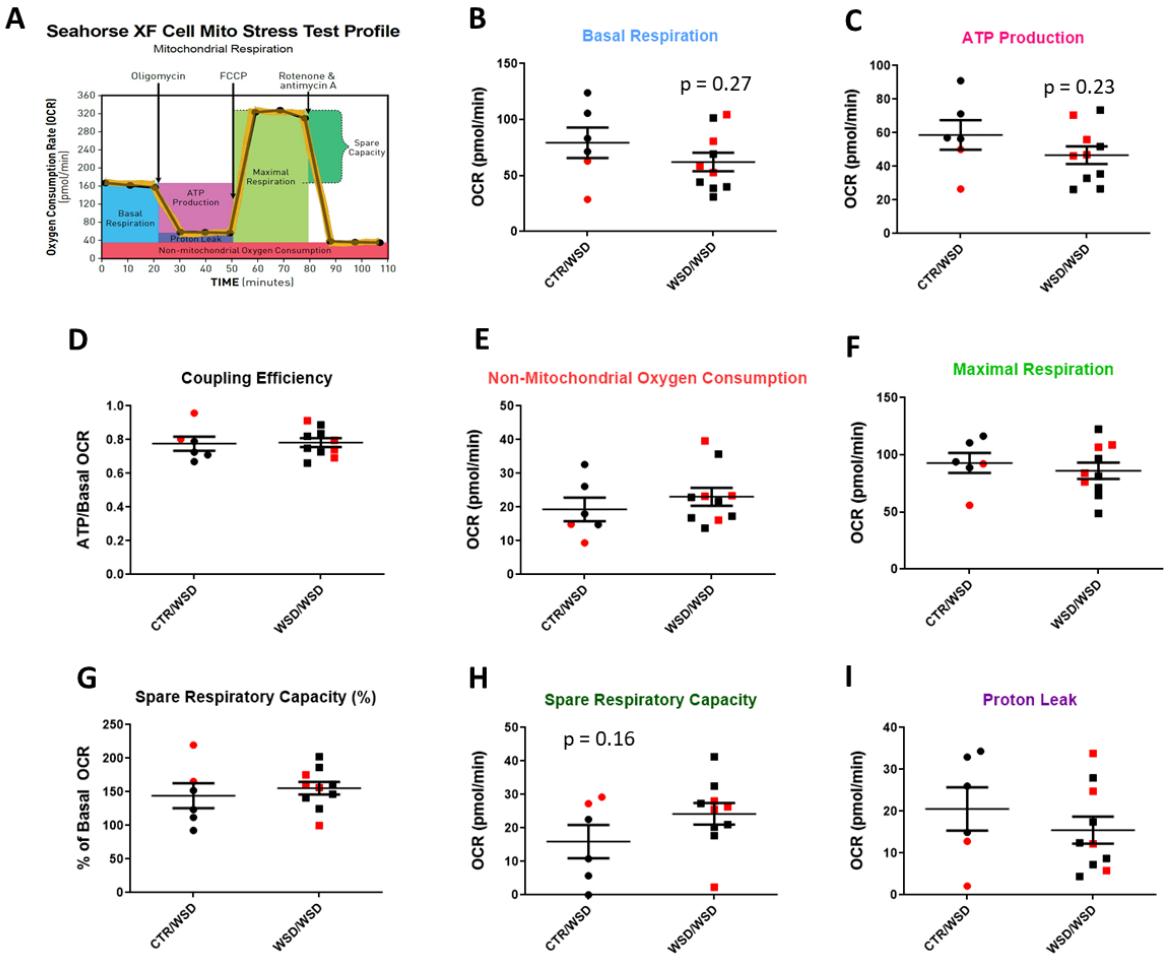


Figure 5-3: Oxygen consumption assay in one-year-old islets. No differences were observed between CTR/WSD and WSD/WSD islets in any panels. P > 0.05, Student's *t* test.

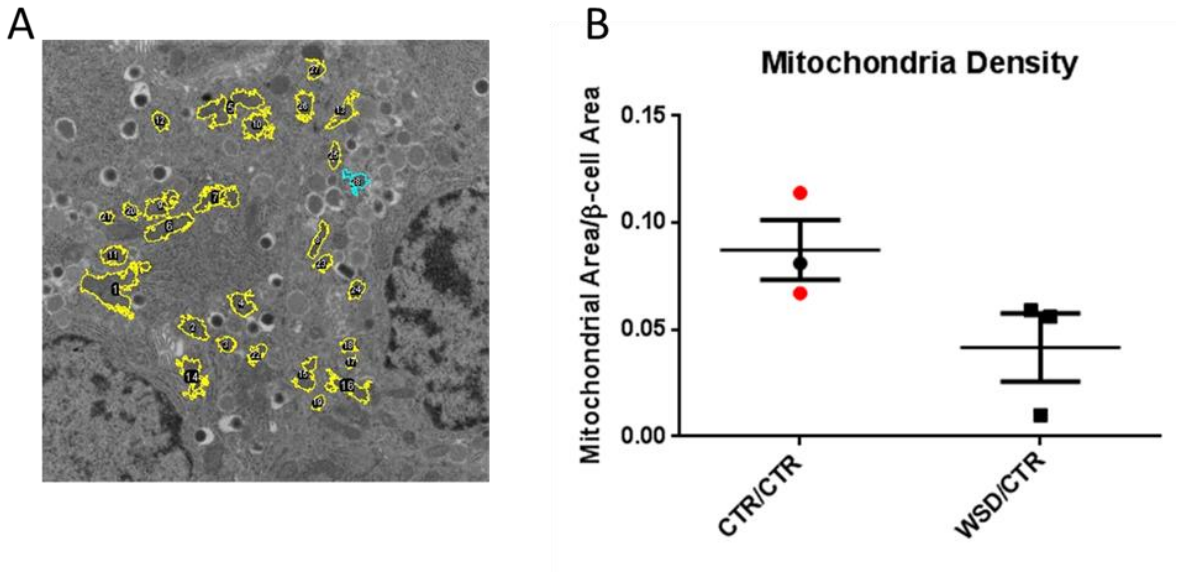


Figure 5-4: Mitochondrial density as quantified by EM. p = 0.10, Student's *t* test. Males are shown in black, females in red.

Increased insulin secretion could also be due to an increase in the production of insulin by the β cell. Additionally, in situations of enhanced insulin secretion, β cells must produce insulin and package it into vesicles at an increased rate. This higher turnover of insulin can result in a decreased ratio of mature-to-immature insulin granules observed in the β cell by EM. Both insulin granule density and maturity from CTR/CTR and WSD/CTR islets were analyzed by EM. While the sample size is too small to make definitive conclusions, no obvious differences in granule density (Figure 5-5B) were observed. Additionally, no decrease in the ratio of mature to immature granules (Figure 5-5C) was observed in the WSD/CTR group.

Maternal WSD Leads to A Few Significant Changes in Islet Gene Expression

Finally, increased insulin secretion in WSD/CTR islets at three years of age may be due persistent maternal diet-induced changes in gene expression. RNA-Sequencing was performed on whole islets from two distinct cohorts of CTR/CTR and WSD/CTR offspring. In both cases, maternal WSD led to changes in gene expression that could affect β-cell function, proliferation, or insulin secretion (Figure 5-6). However, these differentially expressed genes did not overlap between the two cohorts. In the first cohort, G Protein-Coupled Receptor 155 (*GPR155*) was up-regulated as a result of maternal WSD. GPR155 is an orphan G-protein coupled receptor that is associated with diabetes in a quantitative trait locus but has no known function in the islet [136]. Three genes were down-regulated: Interferon Kappa (*IFNK*), Inhibin Subunit Beta A (*INHBA*), and 5-Hydroxytryptamine Receptor 3A (*HTR3A*). These genes have been shown to play roles in β-cell inflammation, proliferation, and insulin secretion, respectively. In the second cohort, Opioid Related Nociceptin Receptor 1 (*OPRL1*) and Regenerating Family Member 1 Alpha (*REG1A*) were up-regulated as a result of maternal WSD. REG1A is secreted by the exocrine pancreas, stimulates β-cell proliferation, is expressed in human diabetic islets and correlates with diabetes duration [137].

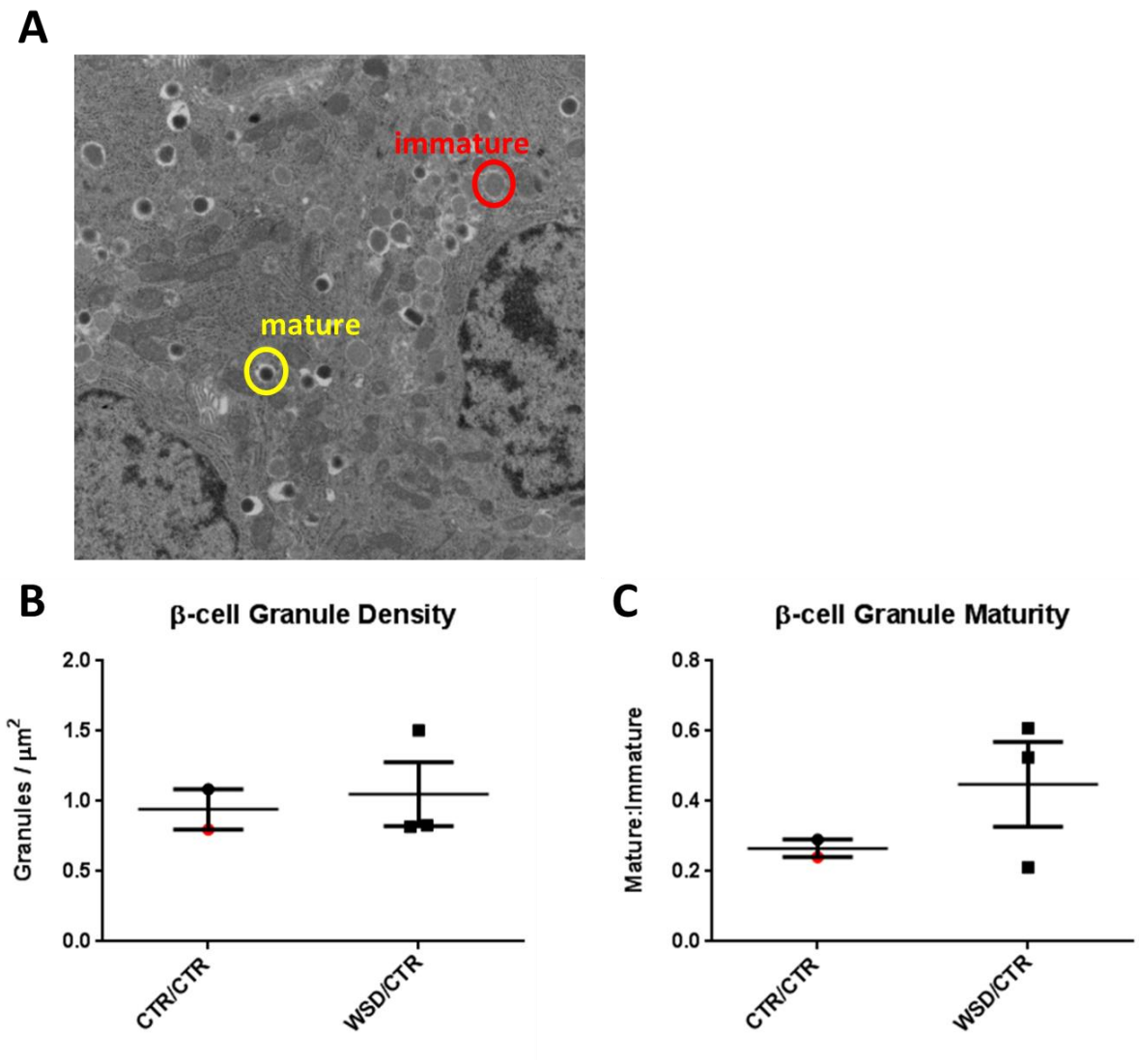


Figure 5-5: β-cell granule density and maturity as quantified by EM. Mature granules appear darker (more dense) than immature granules on EM as shown in yellow in (A). Insulin granules were counted manually and normalized to β-cell area (B). The ratio of mature to immature granules was calculated as a measure of granule maturity (C). Males are shown in black, females in red.

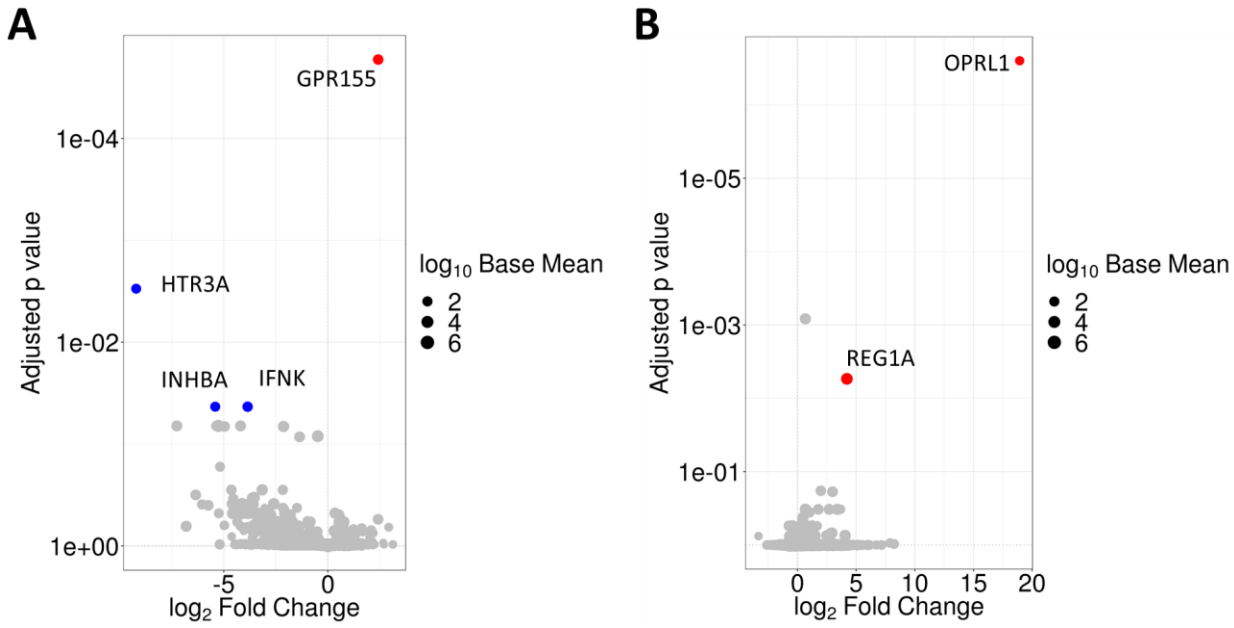


Figure 5-6: Maternal WSD-induced islet gene expression changes in two cohorts of three-year-old NHP offspring. RNA integrity scores were high for each sample.

GPR155 Is Detectable in INS-1 Cells by Antibody Labelling

Because *GPR155* was overexpressed in WSD/CTR islets and is implicated in diabetes, its expression pattern and function in the β cell was further investigated. INS-1 832/13 cells, a rat insulinoma-based β-cell line that is strongly responsive to glucose stimulation [112] were obtained and cultured. A commercially available antibody was used to label GPR155 in INS-1 cells. INS-1 cells showed significant antibody labelling for GPR155 (Figure 5-7).

Transfected, Overexpressing Cells Label Strongly for GPR155

In order to probe the function of GPR155 in INS1 cells, a custom-designed overexpression plasmid from GenScript was obtained and used to stably transfect INS-1 cells. In addition to GPR155, this plasmid also expresses GFP and a hygromycin resistance gene. The plasmid was amplified in bacteria, purified, and then used to transfect cells using Lipofectamine. The transfection protocol was optimized through several rounds of trial and error. Transfection efficiency was approximately 10 percent.

Transfected and non-transfected cells were labelled for GPR155. GFP-positive transfected cells labelled much more intensely for GPR155 than neighboring non-transfected cells (Figure 5-8). This strongly suggested that the antibody was indeed recognizing GPR155 and confirmed successful transfection and overexpression.

GPR155 Co-Localizes With Insulin in INS-1 Cells

In over-expressing cells, GPR155 localized to the perinuclear region. Thus, it was hypothesized that GPR155 may co-localize with insulin. To test for co-localization, non-transfected INS-1 cells were immunolabelled for insulin and GPR155 and stained for actin (cell perimeter) and DAPI as shown in Figure 5-9. GPR155 co-localized with insulin granules to a striking degree.

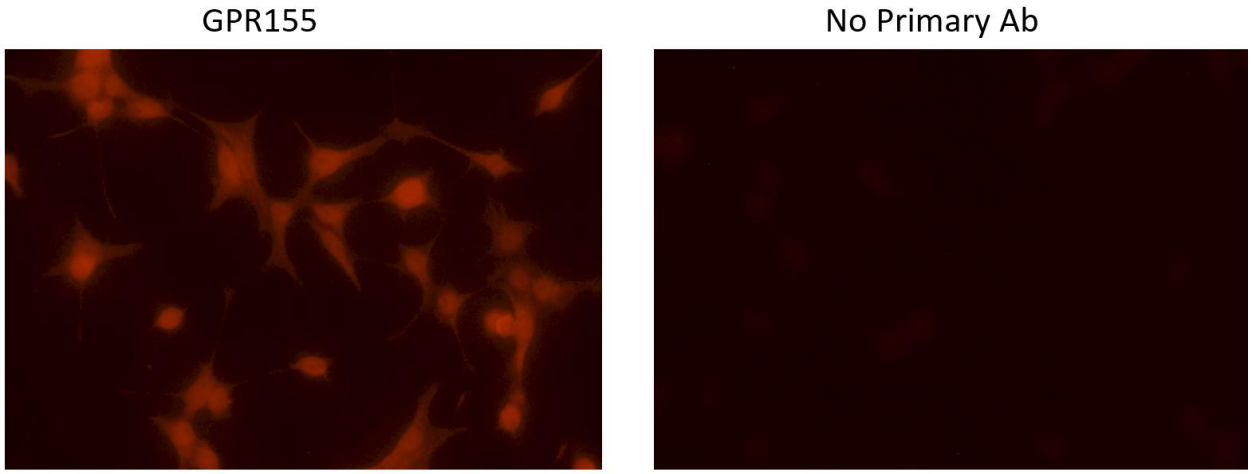


Figure 5-7: GPR155 is detected in INS-1 cells by antibody labelling.

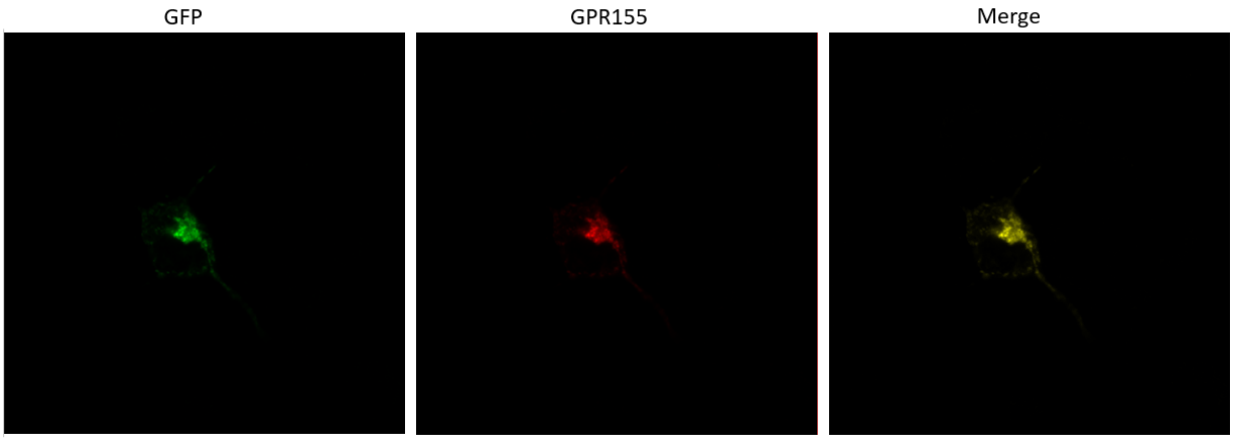


Figure 5-8: Transfected cells show strong GPR155 labelling. Left panel—GFP-positive transfected cell. Middle panel—transfected cell showing strong perinuclear GPR155 labelling. Neighboring, non-transfected cells were also positive for GPR155 but are not visible in this image so that transfected signal is not over-saturated. Right panel—merged image.

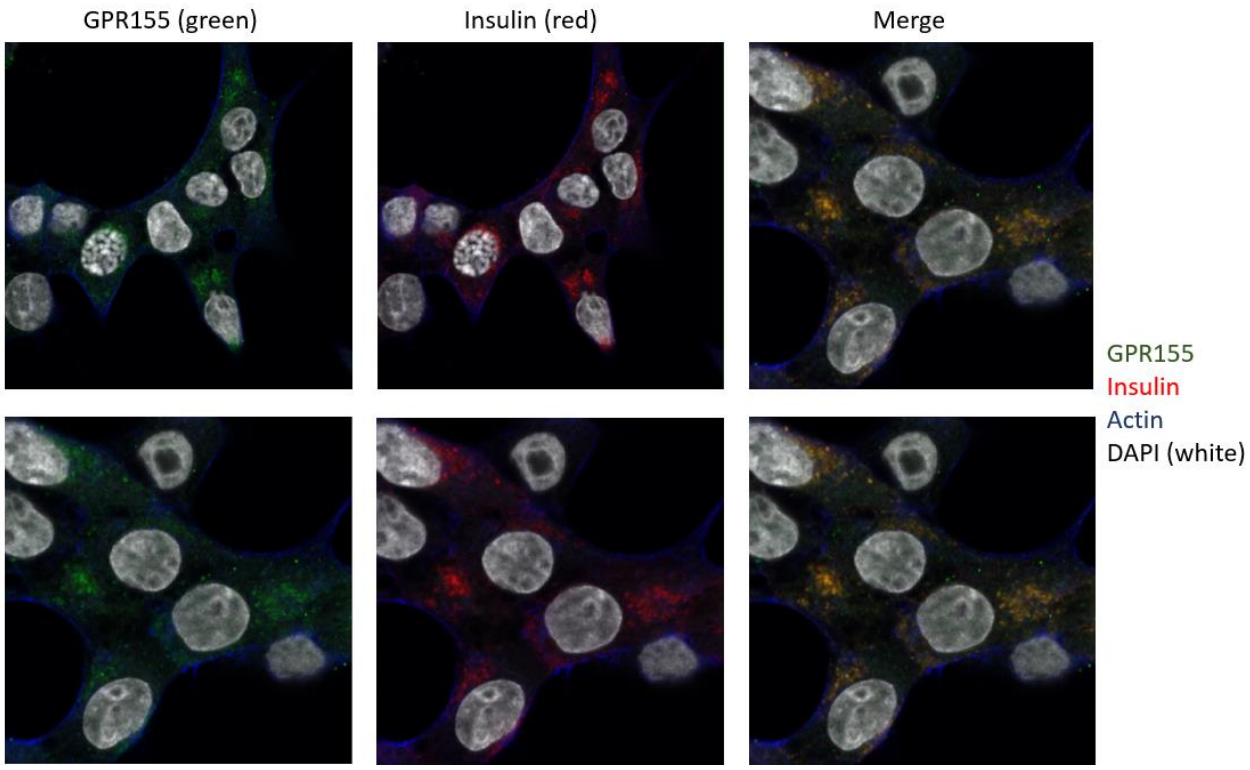


Figure 5-9: GPR155 co-localizes with insulin in INS-1 cells. Two rows show two different examples of GPR155 (left column), insulin (middle column), and the co-localization of the two (right column).

GPR155 Is Detectable in NHP But Not Mouse Islets, Is Specifically Expressed in β Cells, and Protein Levels Do Not Appear to be Regulated by Diet

Next, the protein expression of GPR155 in both mouse and NHP pancreatic sections was investigated. Slides from two NHPs and two mice were labeled for GPR155, testing two different methods of antigen retrieval. Both species had consumed CTR diet. Significant GPR155 labelling was only detected in NHP islets, but not in mouse islets (Figure 5-10).

To determine whether expression of GPR155 in the islet was cell-type specific, NHP pancreatic sections were labelled for GPR155, insulin, and glucagon. Interestingly, all GPR155-positive cells were also positive for insulin, but not glucagon, as shown in Figure 5-11. This suggests that GPR155 is specifically expressed in islet β cells.

To examine whether maternal or offspring WSD also affected GPR155 expression at the protein level, slides were immunolabelled for insulin and GPR155. Based on preliminary data, no differences in GPR155 intensity or total area were observed due to maternal or offspring diet (Figure 5-12). Though differences in transcript levels were observed based on maternal diet (Figure 5-6), mRNA levels varied among samples within each group and the effect size was small, which may explain the lack of diet effects on protein levels of GPR155.

***Ex Vivo* Cytokine and Proliferation Assays**

The above data demonstrate that while maternal diet does not appear to have long-term consequences on offspring glucose tolerance (Chapter IV), isolated islets from WSD/CTR animals at three years of age have increased glucose-stimulated insulin secretion *ex vivo,* which is associated with persistent changes in gene expression. This suggests an intrinsic difference in islet function between these groups that may only manifest under certain conditions. To further investigate this possibility, *ex vivo* cytokine-induced cell death and proliferation assays were conducted using CTR/CTR and WSD/CTR islets. These assays represent physiologic conditions that islets may face throughout life, such as surviving in states of high inflammation and

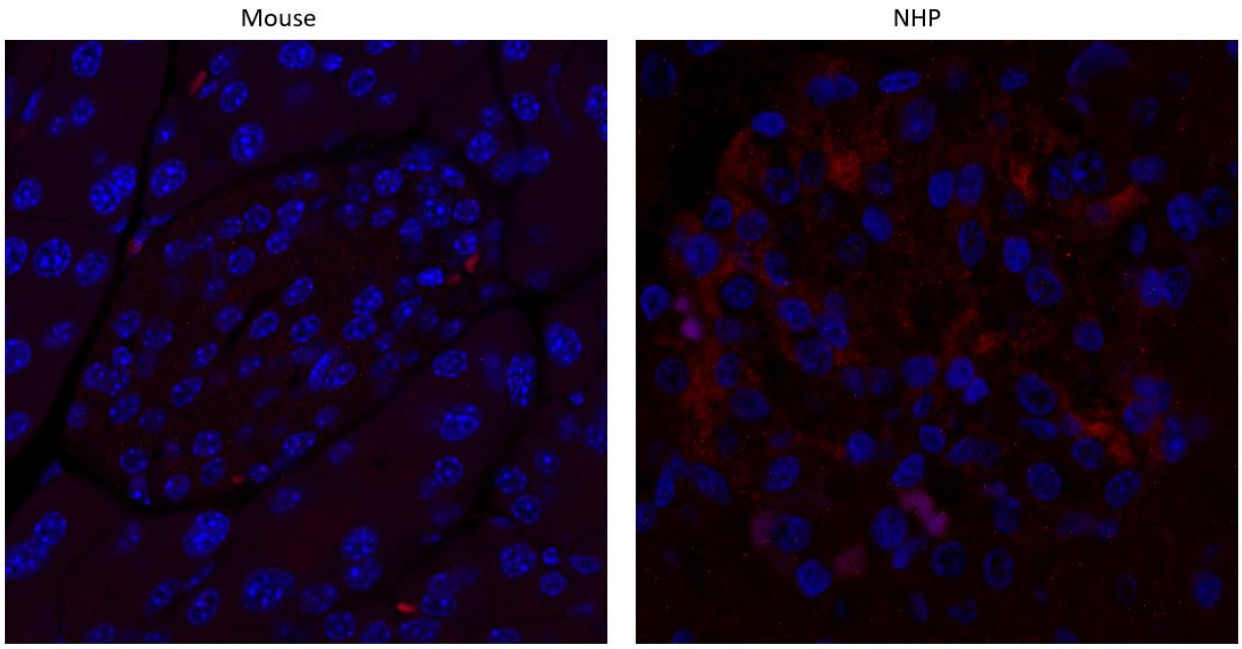


Figure 5-10: GPR155 is only detected in NHP islets (right panel) and not in mouse islets (left panel)

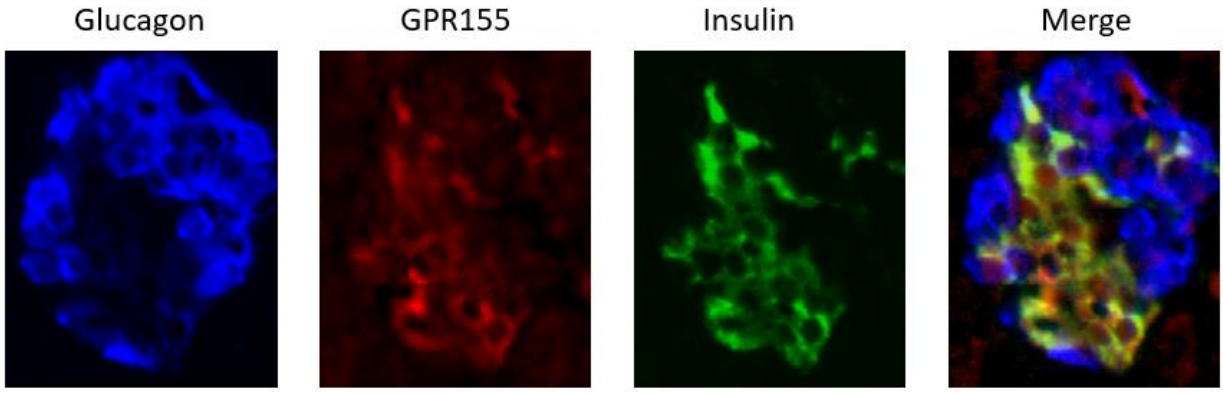


Figure 5-11: GPR155 co-localizes with insulin, but not glucagon, in NHP islets.

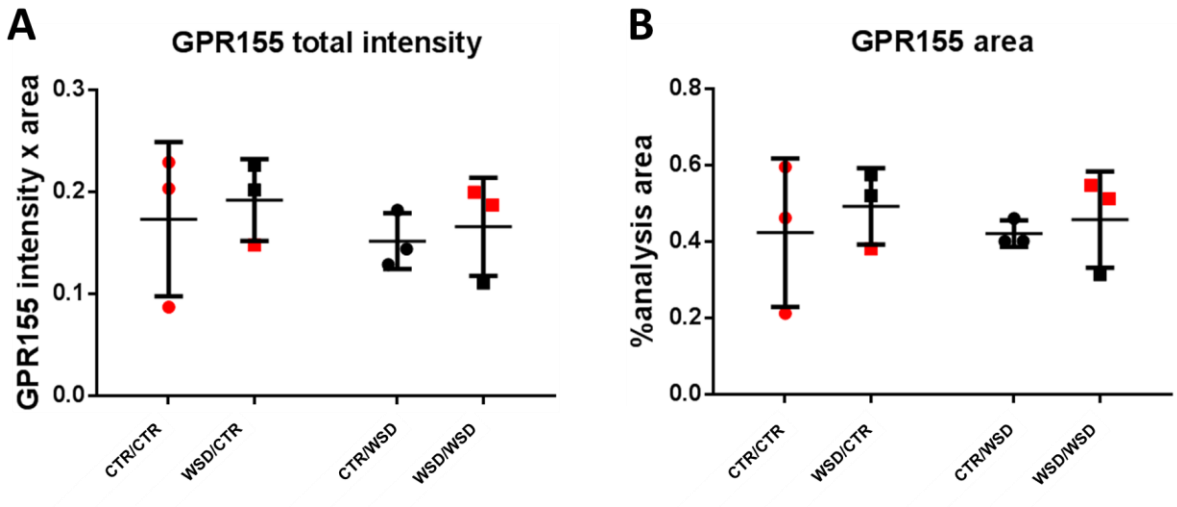


Figure 5-12: GPR155 total intensity and area. GPR155 intensity (A) and total area (B) did not appear differ by maternal or offspring diet, based on preliminary data. Red points indicate tissue sections from females; black points indicate males.

responding to proliferative cues during times of metabolic stress. In proliferation assays, islets were treated with the known mouse and human β-cell mitogens DG-041 (an inhibitor of the prostaglandin receptor EP3) and/or Sulprostone (an agonist of the prostaglandin receptor EP4) to stimulate proliferation [109] (Figure 5-13A). However, unlike mouse and human islets, NHP islets did not survive well in culture, and ultimately the proliferation assay was not feasible (Figure 5-13B). These assays were attempted with a separate batch of islets after optimizing isolation and shipping protocols with collaborators at OHSU. However, islet health in culture remained poor and the assays were not successful (Figure 5-13C, 5-13D).

Discussion

The results above demonstrate that maternal WSD has significant, persistent effects on offspring islet function. Islets exposed to WSD *in utero* secreted more insulin in response to glucose at one and three years of age. This effect was persistent across separate cohorts, and is likely not due to a sex imbalance between the two groups. Given that *in vivo,* there were no differences in plasma insulin levels or glucose tolerance due to maternal WSD, this difference likely represents an islet-intrinsic phenomenon that may only manifest under certain conditions.

While hypersecretion of insulin in response to glucose may appear to be beneficial, it is also associated with poor health. Islets from deceased donors who were overweight secrete more insulin in response to glucose [138]. Though NHP offspring exposed to WSD appear metabolically normal at one and three years of age, their islets behave similarly to those of overweight adult humans. While not evident *in vivo*, this difference in islet function may manifest under conditions of metabolic stress or increased age. Indeed, studies in rodent models have shown that, in some cases, advanced age and continued HFD feeding is necessary to elicit a phenotype as a result of maternal overnutrition [57].

To investigate the mechanism behind increased insulin secretion *ex vivo,* mitochondrial function was assessed using an oxygen consumption assay, while mitochondrial area, insulin

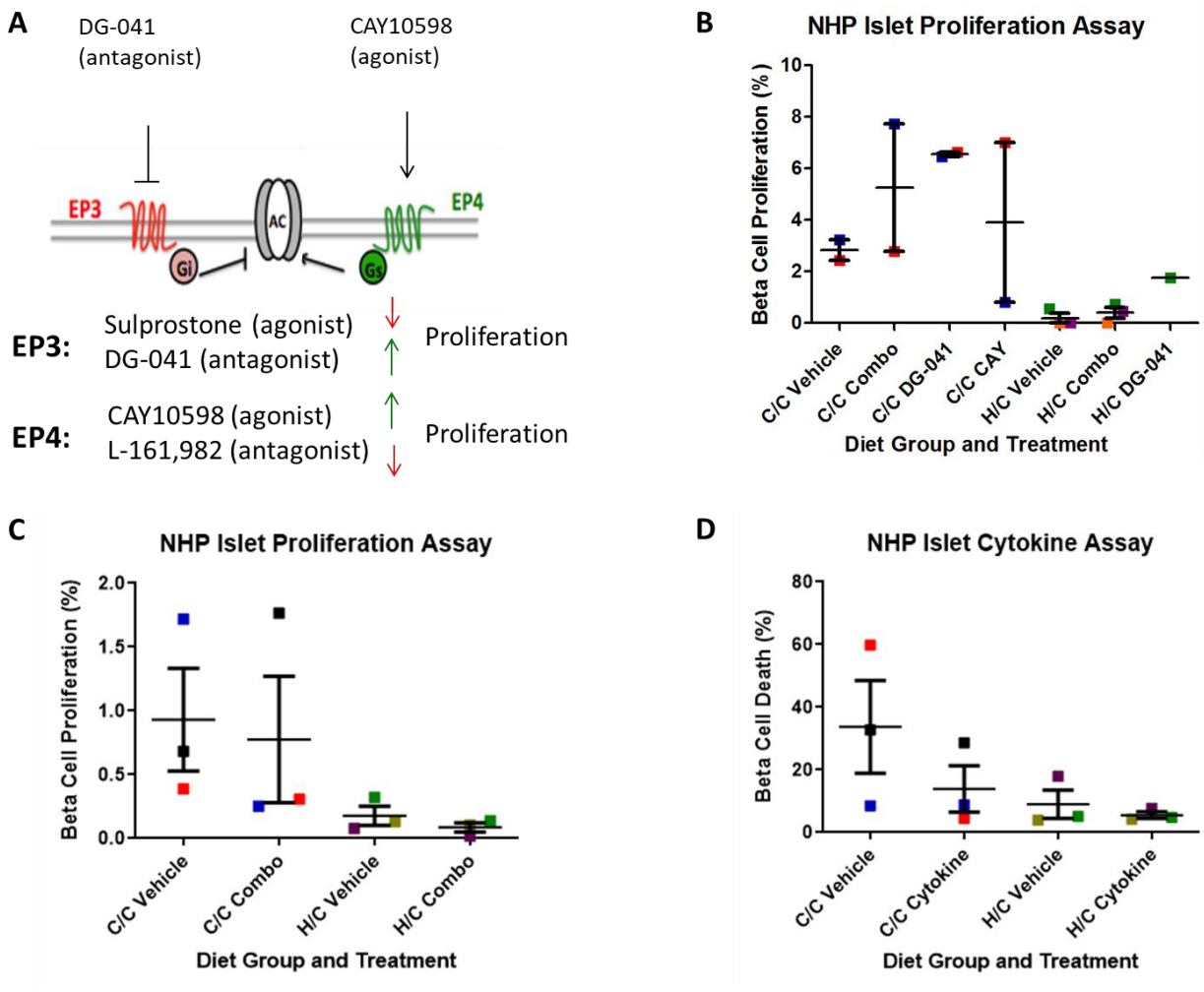


Figure 5-13: *Ex vivo* proliferation and cytokine assays. (A) For proliferation assays, islets were treated with the EP3 antagonist DG-041, the EP4 agonist CAY10598, or a combination of both drugs to stimulate proliferation. The EP3 antagonist DG-041 and EP4 agonist CAY10598 stimulate β-cell proliferation by increasing cAMP. (B) Ex vivo islet proliferation assay. Points of the same color represent individual animals. The orange and purple points represent animals where the number of remaining β cells at the end of the assay were significantly fewer than in other groups due to poor islet health and technical difficulties with the assay, which may be the cause of the 0% proliferation in those animals. Proliferation (C) and cell death assays (D) were also performed in a separate batch of islets in which islet isolation and shipping procedures were optimized. However, the majority of islets did not survive in culture during the assay.

granule density, and insulin granule maturity were quantified using EM. No differences were detected in insulin granule density, insulin granule maturity, mitochondrial density, or mitochondrial function that could explain the enhanced GSIS in WSD/CTR animals. It was hypothesized that maternal WSD would decrease the proportion of mature granules. However, this group tended to have an increase in mature granules, though this was not statistically significant. Similarly, though WSD/CTR islets secreted more insulin in response to high glucose, this group showed a non-significant trend toward reduced mitochondrial density. Interestingly, fetal NHP exposed to WSD have reduced mitochondrial content in skeletal muscle, but increased mitochondrial complex activity [139]. Perhaps a similar phenomenon could also explain the enhanced insulin secretion in these islets. However, additional samples are needed before definitive conclusions can be made about mitochondrial density or function at 3 years of age.

A few persistent changes in islet gene expression due to early life exposure to maternal WSD were observed in two cohorts of islets isolated from three year old offspring. While both cohorts demonstrated gene expression changes that could affect islet function, the specific gene expression changes differed between cohorts. Thus, further study is needed to fully elucidate the mechanism of enhanced GSIS.

The investigation of changes in islet gene expressed paved the way to a series of experiments investigating the localization and potential function of GPR155. Using INS-1 cells, GPR155 was found to co-localize with insulin. Based on its localization, it is possible that GPR155 plays some role in insulin processing and secretion, and this would be an interesting avenue for future study. GPR155 protein was only detected in NHP islets, specifically in β cells, and absent from mouse islets. It was, however, detected in a rat β cell line. Further study is needed to investigate whether GPR155 is also expressed in human β cells, as well as its function in the islet.

Despite the challenges of the primate model, which include low sample availability and high individual variability, consistent effects of exposure to maternal WSD on offspring islets are apparent. Specifically, maternal WSD results in reduced α-cell mass (Chapter IV) and increased

glucose-stimulated insulin secretion (this chapter). These effects are observed despite the offspring having consumed a healthy diet for nearly 2.5 years after weaning to a control diet, demonstrating the persistent changes induced in the islet by maternal overnutrition.

CHAPTER VI: THE EFFECTS OF MATERNAL HIGH FAT DIET ON ISLET FUNCTION AND WHOLE BODY METOBLISM IN MOUSE OFFSPRING

Introduction

The NHP models offers great insight into human gestation, metabolism, and islet function. However, several key limitations are inherent to this model. First, sample availability is low and sporadic. Because mating occurs naturally in social groups, it is difficult to predict offspring sample numbers in advice. In addition to this, dams are utilized for multiple pregnancies in order to maximize offspring sample size. However, this introduces additional variables that could affect the *in utero* environment such as advanced maternal age and increased parity. NHPs are housed outdoors in large corrals. In this arrangement, it is possible to consume other food besides the provided diet, such as insects and small vertebrates which are a part of the macaque diet in the wild. The difference in diet between CTR and WSD groups in the current study is thus less well-controlled than a mouse study and may also be less distinct.

While mouse models of maternal overnutrition are less similar to the human condition, they offer several unique advantages. The most significant of these is the ability to control the study design. Both dams and offspring can be age matched between diet groups. Micronutrient-matched control diets can be used to specifically test the effects of increased fat in the diet. Diet composition can be tested to determine which type of high fat diet produces a metabolic phenotype in dams. Additionally, mice give birth to litters that are consistently 7-8 or more in size. This makes appropriate sample sizes in each group more feasible, and eliminates the need to use a single dam in multiple pregnancy. Mice can also be genetically manipulated to label distinct cell populations or induce obesity, if needed.

In the following experiments, a mouse model of maternal HFD-feeding was used to investigate the effects of *in utero* overnutrition on islet composition and whole body metabolism in the offspring. The transgenic mIns1-H2B-mCherry mouse was utilized so that β cells could be

isolated to probe for cell-specific effects of maternal HFD. This mouse model has β cells that express the fluorescent mCherry protein driven by the mouse *Insulin 1* promotor, which can be used for cell sorting, specifically in all β cells while not affecting glucose tolerance in male or female transgenic mice [110]. In a study design similar to that used in the NHP model, female mice were fed HFD or micro-nutrient matched CTR diet for five weeks beginning at eight weeks of age. Mice were then mated and maintained on the diet throughout pregnancy and lactation. Offspring were weaned to either CTR or HFD to generate four diet groups. Offspring were analyzed just before reaching sexual maturity at 4 weeks of age, and in adulthood at 12 weeks of age.

Results

60% HFD Leads to Significant Weight Gain and Impaired Glucose Tolerance in Female Mice

Two different high fat diets were examined to determine which diet would produce significant weight gain and glucose intolerance in female mice. When 8-week-old mice were fed HFD with 45% of calories from fat, no weight gain or glucose intolerance was observed after five weeks on the diet (Figure 6-1A, 6-1B). HFD with 60% of calories from fat did induce significant weight gain above the CTR group, as well as impaired glucose tolerance before pregnancy. Thus, 60% HFD was used in all further experiments. When HFD-fed females became pregnant, glucose tolerance was no longer different than CTR animals, likely due to pregnancy-induced physiological insulin resistance in the CTR group (Figure 6-1C).

Maternal HFD Leads to Increased Weight at Weaning Which Normalizes by Four Weeks of Age

Offspring exposed to maternal HFD were heavier at weaning (Figure 6-2A). However, by four weeks of age, weight was similar among all four diet groups (Figure 6-2B). As mice aged, post-weaning HFD resulted in increased body weight, however maternal HFD did not affect body

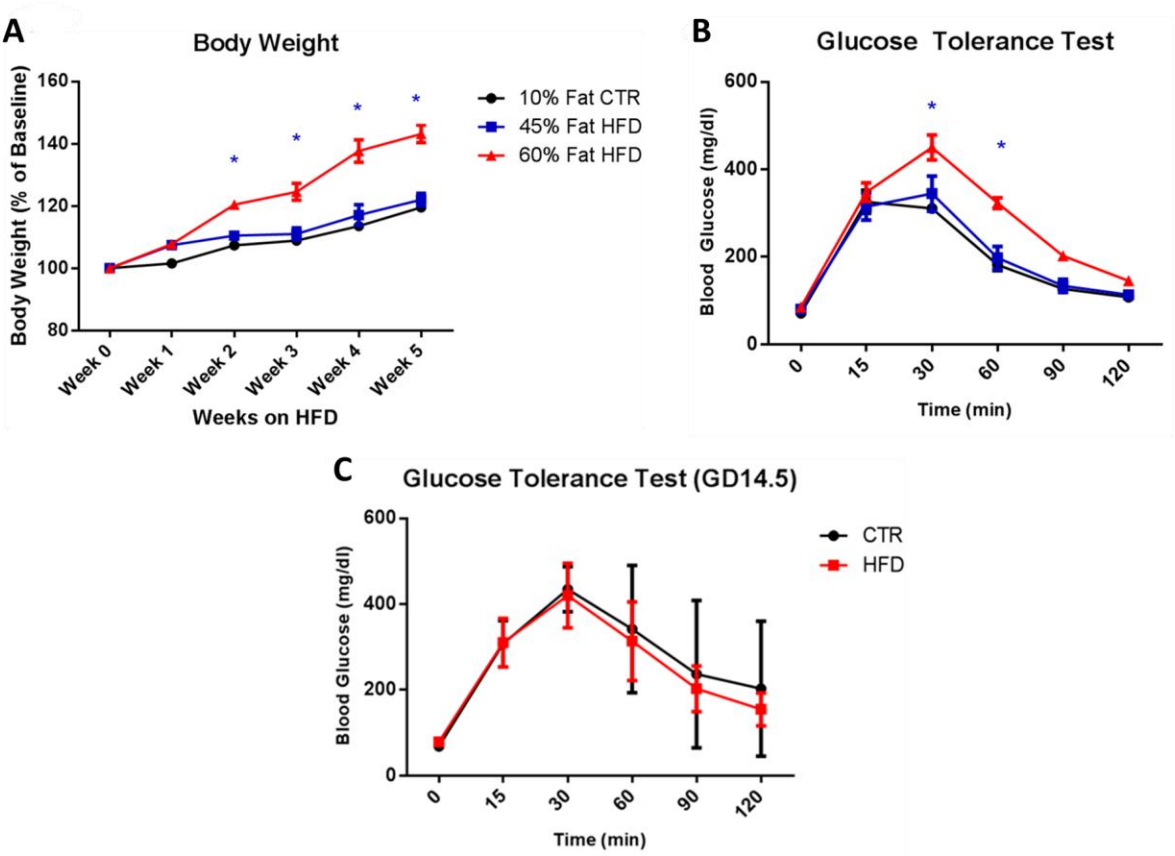


Figure 6-1: 60% HFD induces weight gain and glucose intolerance before pregnancy in female mice. (A). HFD with 60% calories from fat, but not 45%, results in significant weight gain. N=5. (B) HFD with 60% calories from fat, but not 45%, results in increased blood glucose levels during GTT. N=5. (C) No difference in glucose tolerance during pregnancy between CTR and 60% HFD females. N=9 (CTR), N=16 (HFD). *p<0.05, repeated measures ANOVA.

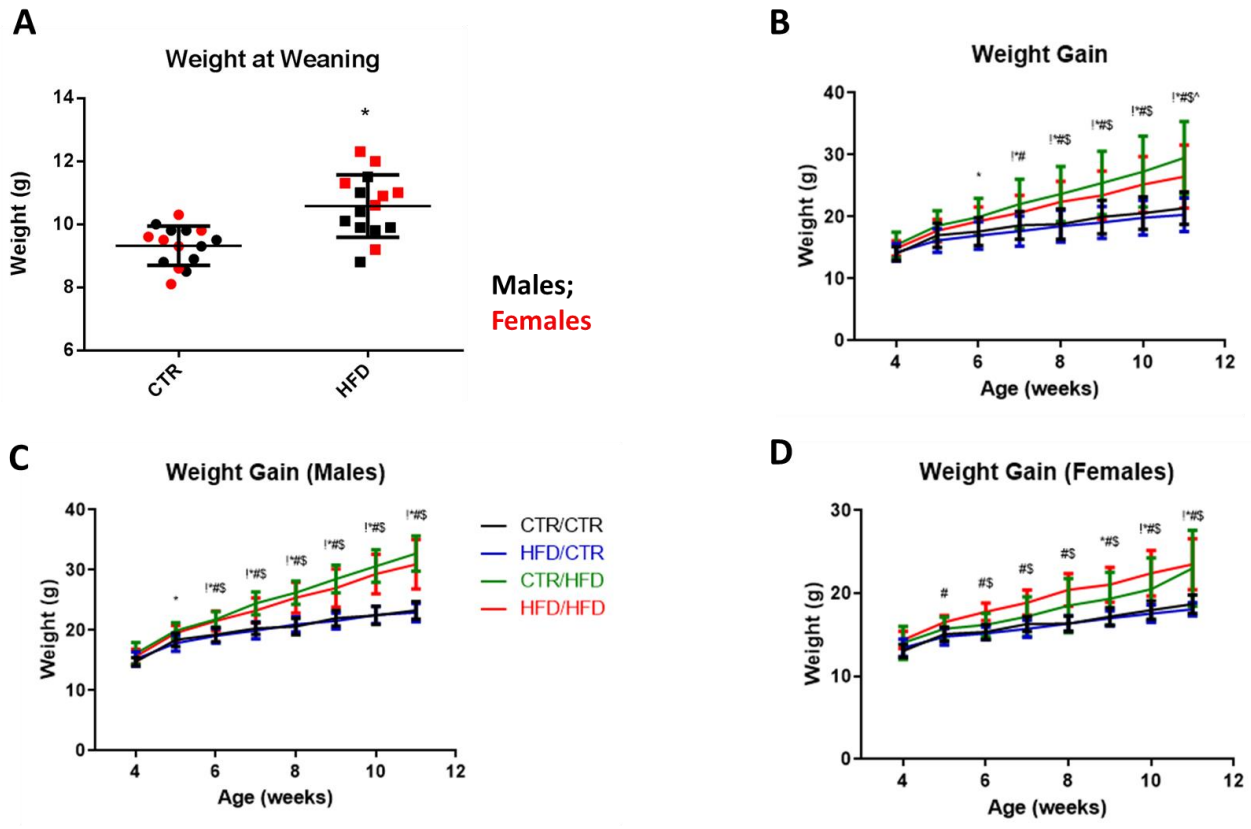


Figure 6-2: Maternal HFD results in increased weight at weaning. (A) Offspring exposed to HFD are heavier at weaning. P<0.05, student t test. (B) Post-weaning HFD resulted in significant weight gain. CTR/HFD mice were significantly heavier than HFD/HFD mice at 11 weeks of age. However, maternal diet had no effect on offspring weight at any time point when males (C) or females (D) were analyzed separately. N = 15-26 per group (5-14 females and 8-12 males). Two way repeated measures ANOVA. For (B)-(D), * HFD/CTR vs CTR/HFD; ! CTR/CTR vs CTR/HFD; # HFD/CTR vs HFD/HFD; $ CTR/CTR vs HFD/HFD; ^ CTR/HFD vs HFD/HFD.

weight in the offspring through 10 weeks of age. At 11 weeks, HFD/HFD animals weighed less than CTR/HFD animals (Figure 6-2B). However, when groups were stratified by offspring sex, maternal diet did not affect weight in either males or females (Figure 6-2C, 6-2D).

Maternal HFD Does Not Affect Offspring Glucose Tolerance

Offspring glucose tolerance was assessed both before sexual maturity at four weeks of age, as well as in adulthood at 12 weeks of age. At both ages, maternal HFD had no effect on offspring glucose tolerance (Figure 6-3). Though both sexes were included in each group, sex was not perfectly balanced. In 12-week-old offspring, data was stratified by offspring sex in Figure 6-3C and 6-3D. When stratified by sex, maternal diet still had no effect on offspring glucose tolerance.

As a measure of glycemia throughout the GTT, area under the curve was calculated for combined, male, and female GTTs at 12 weeks of age (Figure 6-4). While post-weaning HFD feeding led to the expected increase in offspring GAUC both in combined data and when stratified by sex, maternal HFD did not have a significant effect on offspring glucose tolerance.

Maternal HFD Does Not Affect Offspring α- or β-Cell Mass

Because underlying changes in endocrine cell mass may precede changes in whole body metabolism, α- and β-cell mass were analyzed. In the NHP, maternal WSD led to a decrease in α-cell mass with no changes in whole body glucose metabolism. In mice at 12 weeks of age, maternal diet had no effect on α-cell mass (Figure 6-5A). Similarly, maternal diet did not affect β-cell mass at either time point (Figure 6-5B, 6-5C). When offspring were fed HFD after weaning, the expected increase in β-cell mass to compensate for metabolic stress was observed in 12-week-old offspring (Figure 6-5C). Thus, HFD does elicit a physiological response in this model, but the effects of maternal diet on offspring islet composition and function are not apparent at these ages.

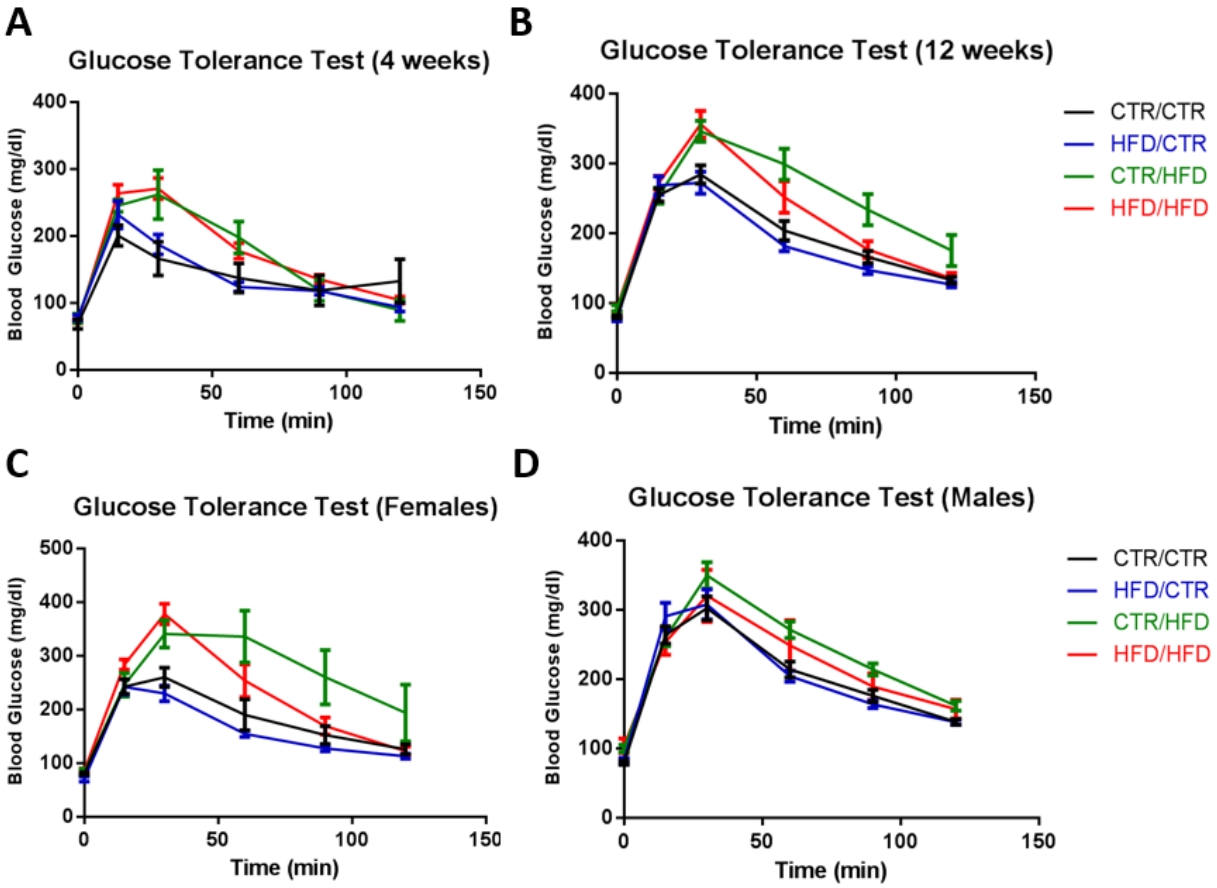


Figure 6-3: Glucose tolerance tests at 4 and 12 weeks of age. Maternal diet had no effect (HFD/CTR was not different than CTR/CTR; HFD/HFD not different than CTR/HFD) on glucose tolerance at 4 weeks of age. N = 4-9 per group. Maternal diet also had no effect on glucose tolerance at 12 weeks of age (B). N = 16-26. This was also the case when females (C) and males (D) were analyzed separately. N = 9-11 for females and 6-15 for males. Two-way repeated measures ANOVA

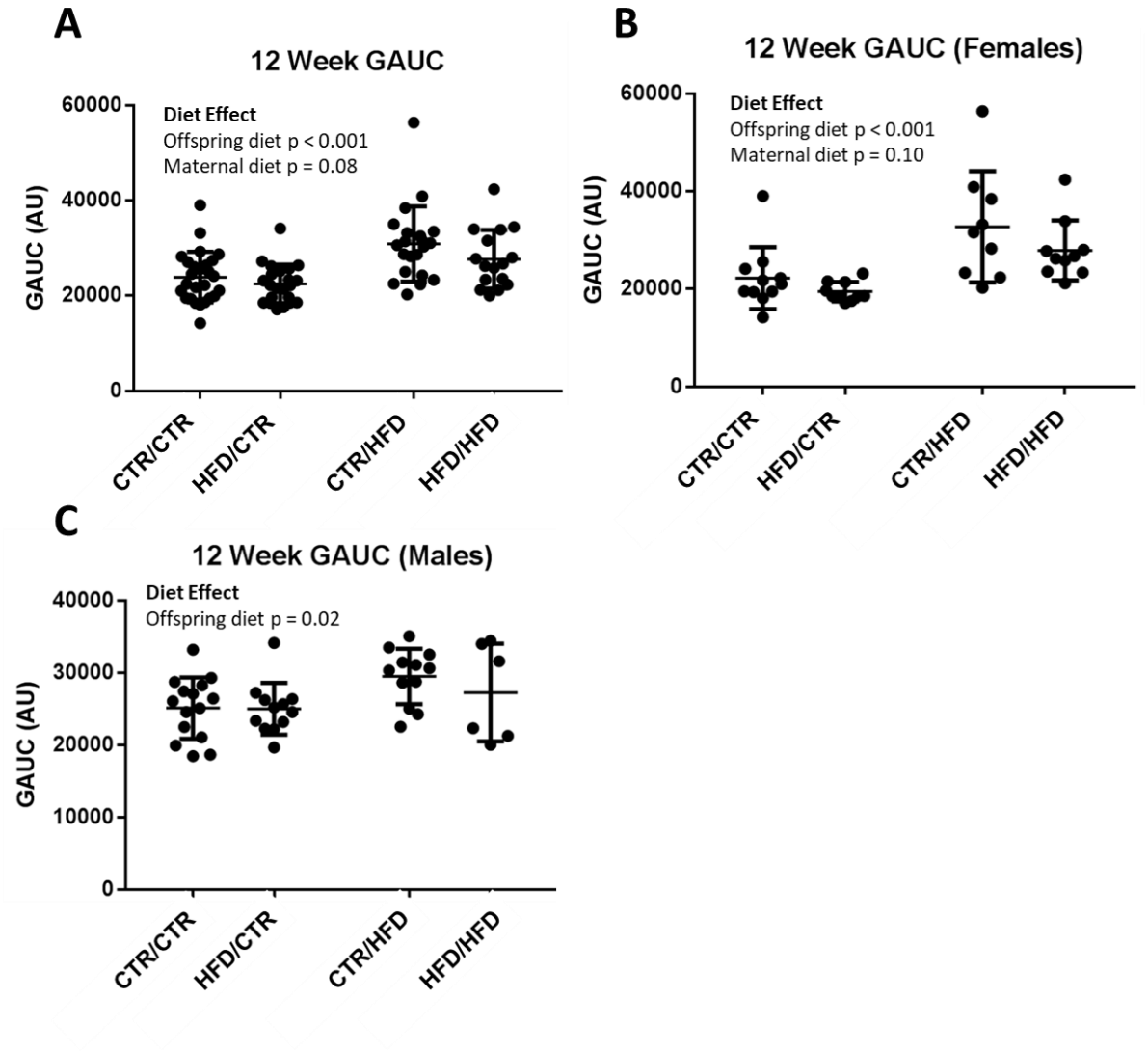


Figure 6-4: Glucose area under the curve during glucose tolerance testing. Glucose area under the curve was assessed at 12 weeks of age in all samples (A), only females (B), and only males (C). While offspring HFD increased GAUC, maternal diet did not significantly affect offspring glucose tolerance ($p>0.05$, two-way ANOVA).

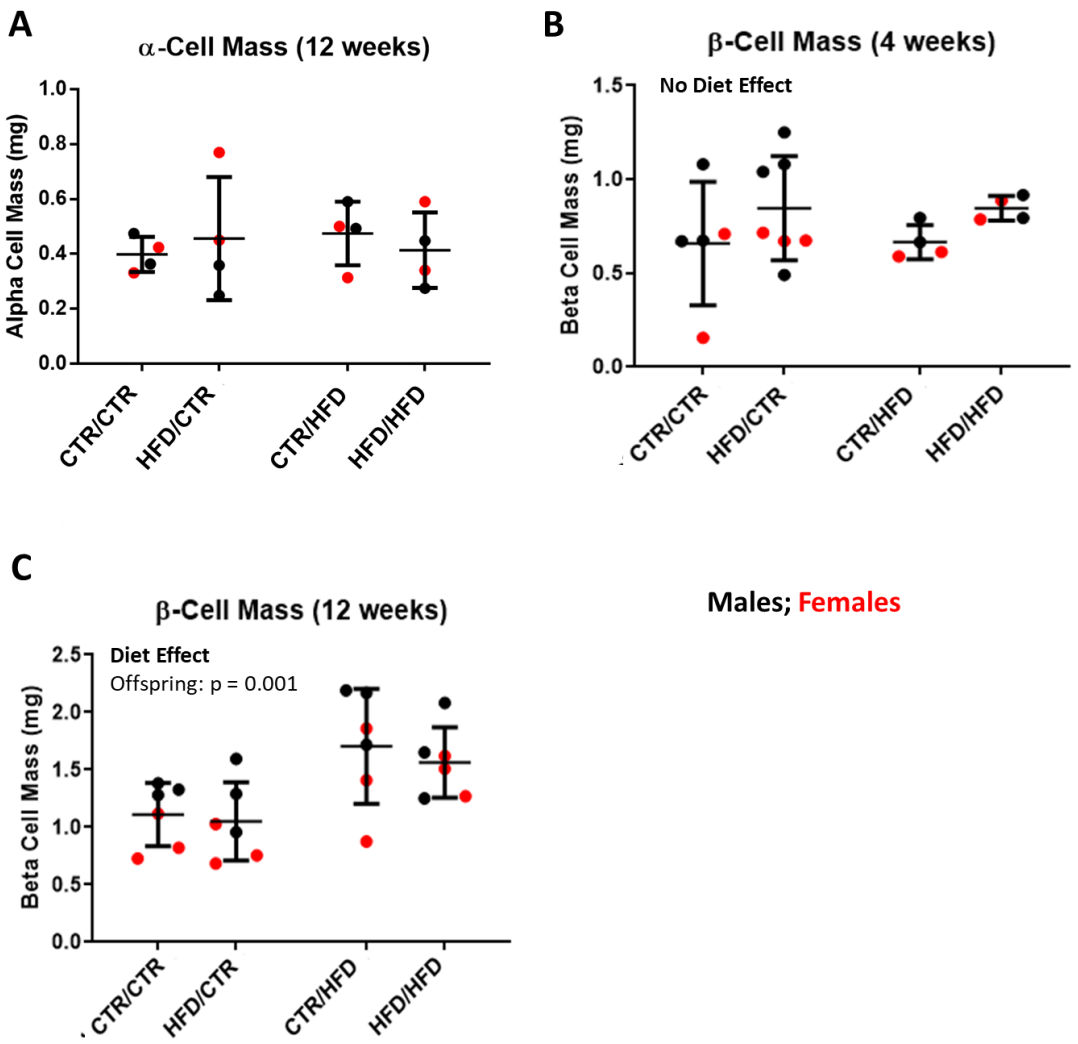


Figure 6-5: Maternal HFD does not affect α- or β-cell mass. α-cell mass was similar across all four diet groups (A). β-cell mass was similar between diet groups at four weeks (B), but elevated due to offspring HFD at 12 weeks (C). No differences were observed as a result of maternal diet (two-way ANOVA, $p>0.05$).

Investigation of Maternal HFD-Induced Chromatin State Changes

The effects of maternal diet on offspring physiology are thought to be mediated by epigenetic changes [97-100]. The mIns1-H2B-mCherry model allows for the sorting of β cells to probe for maternal diet-induced epigenetic changes specifically in β cells. Despite the lack of an overt phenotype in the offspring in this mouse model of maternal overnutrition, changes in chromatin state may exist that could alter gene expression under certain conditions not tested here. To assess chromatin state changes in 12-week-old mouse offspring, the Assay for Transposase Accessible Chromatin by Sequencing (ATAC-Seq) was utilized. This assay was successfully performed on cultured white blood cells, as indicated by the prototypical banding pattern observed on gel electrophoresis (Figure 6-6). ATAC-Seq was performed on a total of 10 different mouse samples. However, due to challenges with cell yield and viability, only four samples yielded DNA of sufficient quality for sequencing. Given that these four samples represented three different diet groups, further analyses could not be performed.

Discussion

In this specific mouse model of maternal overnutrition, no offspring phenotype was observed. While some mouse studies have demonstrated significant and persistent effects of maternal overnutrition on the offspring [55], others have shown either no effect [64] or effects only after prolonged HFD-feeding and increased age in the offspring [57]. In the current studies, mice were fed either HFD or a micronutrient matched CTR diet. A subset of offspring were weaned to HFD, but the latest age assessed was 12 weeks. Thus, it is possible that early life exposure to maternal overnutrition did induce changes to islet gene regulation and function in the offspring that may only manifest with additional stressors such as increased age or pregnancy.

A key advantage of the mouse model is the ability to alter the study design. Taking advantage of this, some mice were fed HFD, but reversed to CTR at the start of pregnancy. Study designs such as this are useful in elucidating whether maternal diet *per se* or maternal metabolic

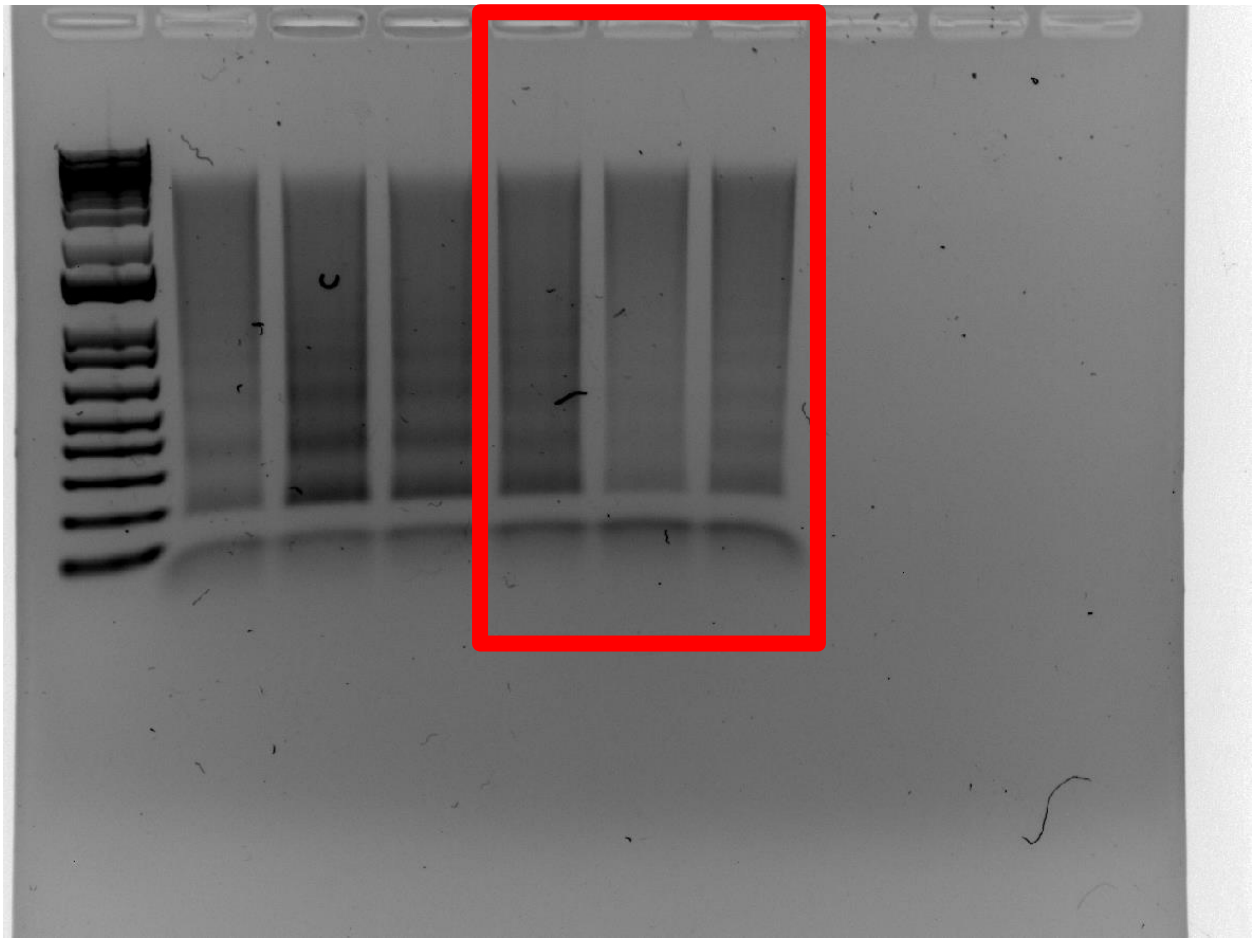


Figure 6-6: Prototypical banding pattern after DNA amplification in ATAC-Seq. Red box represents ATAC assays performed on cultured white blood cells as part of the current thesis project. This is compared with the banding pattern of an individual highly experienced in this tehcnique who performed the assay in the first three lanes.

phenotype is more important in influencing offspring health. However, since no phenotype was observed as a result of maternal HFD, this avenue was not further pursued.

This mouse model also allowed the sorting of β cells. This is useful to probe maternal diet induced changes to parameters such as β-cell gene expression and chromatin state. Unfortunately, DNA isolated from ATAC experiments was of insufficient quality in the majority of cases. To address this issue, samples can be pooled to increase the starting material, or different ATAC protocols can be utilized in future studies.

Though no phenotype was observed at four and 12 weeks of age, these experiments are in line with a body of evidence in rodents, as well as in the NHP model, that the effects of maternal diet on the offspring may only manifest under certain metabolically stressful conditions. Thus, even in this model, phenotypes may be observed if offspring are further stressed.

CHAPTER VII: SUMMARY AND FUTURE DIRECTIONS

The effects of maternal overnutrition on offspring health and disease risk are becoming increasingly apparent. In humans, maternal obesity during pregnancy is associated with increased offspring adiposity and future development of metabolic syndrome [140-142]. Importantly, the deleterious effects of maternal obesity can be present independently of maternal diabetes. Indeed, even elevated maternal glycemia below diabetic levels has been shown to lead to childhood obesity and impaired glucose tolerance [12, 122]. As rates of obesity continue to rise in the United States, future generations will face the consequences of *in utero* overnutrition. Thus, it is becoming extremely important to understand how increased risk of disease is transferred to the offspring. In human studies, it is difficult to distinguish whether diet, maternal metabolic phenotype, or both are important in transmitting disease risk to the offspring.

In this thesis, an animal model closely related to humans was used to investigate the effects of a WSD on the offspring. To do this, both isolated islets and tissue samples were processed for a number of assays including perifusion, RNA-Sequencing, cell death and proliferation assays, oxygen consumption assays, electron microscopy, and immunohistochemistry. This allowed for a thorough analysis of the effects of maternal WSD on offspring islet cell composition and function. In addition to the samples available for tissue analysis, a full database of maternal and offspring metabolic parameters was available for all primates in the entire colony at OHSU. Using these data, work as part of this thesis fully characterized the effect of WSD on female macaques during pregnancy. Understanding how this diet affects dams is critical to elucidating the link between a poor gestational environment and offspring health. This information is essential not only to the work in this thesis, but also to all groups utilizing this shared resource to study the effects of WSD on different tissues and organ systems in the offspring. Indeed, using only a small subset of maternal/offspring pairs, an

independent group showed significant association between maternal metabolic parameters and offspring health [127].

In the full cohort of female NHPs, WSD was associated with increases in weight, body fat percentage, and IAUC before pregnancy. Though the primary outcomes of this study were GAUC and IAUC during pregnancy, it is important to understand the effects of this diet in the non-pregnant state. The increase in body weight and body fat percentage associated with WSD indicates that the increased fat and cholesterol content of the diet had the predicted effect. Furthermore, increased insulin levels in the presence of obesity and normal glucose levels suggests that these animals were insulin resistant. During pregnancy, WSD-fed dams had higher leptin levels and higher weight than CTR dams.

It was hypothesized that WSD would lead to elevated GAUC during pregnancy. However, instead, we found that WSD was associated with a reduction in GAUC and an increase in IAUC. After adjustment for confounders of age, parity, and pre-pregnancy weight (a measure of baseline metabolic status), WSD was still associated with a decrease in GAUC but the increase in IAUC was now no longer present. A few possible factors may account for this effect. First, perhaps in this specific model, with this specific diet, WSD is indeed beneficial to glucose tolerance during pregnancy. Though the WSD is higher in saturated fat, cholesterol, and sucrose, it is possible that this diet may be better meeting the nutritional needs of the NHP dams during pregnancy. Before pregnancy, there was no effect of diet on GAUC, while during pregnancy WSD was associated with improved GAUC. Perhaps importantly, neither CTR- nor WSD-fed dams gained significant amounts of weight during pregnancy, and many animals actually lost weight while pregnant. This suggests the possibility that neither diet is fully providing the caloric needs required during gestation. In this scenario, the more calorically dense diet being consumed by the WSD group, combined with their increased body fat percentage pre-pregnancy, may lead to improved metabolic health if the energy demands of pregnancy are closer to being met in the WSD group.

To explore the possibility that WSD in beneficial during pregnancy, dams who had multiple

pregnancies on different diets throughout the study were investigated. In many cases, switching from CTR to WSD did result in increased GAUC. However, in some cases switching diets had no effect on GAUC. When WSD-fed dams were switched to CTR, then switched back to WSD, GAUC tended to be even higher than in any previous pregnancies. This suggests that diet cycling may be more detrimental than remaining on any individual diet, a phenomenon that has been demonstrated in other models [124]. However, in order to make a definitive conclusion about whether or not WSD improved GAUC, a prospective study appropriately powered would be needed.

Another possible explanation of decreased GAUC during pregnancy in the WSD group is that the CTR and WSD populations are inherently different in a manner not accounted for when adjusting for differences in age, parity and weight. In this view, WSD is still detrimental to the glucose tolerance of the dam, and thus GAUC should increase when animals are fed WSD. Consistent with this hypothesis, when analyzing only those animals on WSD, a significant positive correlation was found between time on WSD and GAUC during pregnancy. Consistent with pre-pregnancy data, this suggests that WSD does indeed lead to metabolic changes in female macaques which could create an *in utero* environment of overnutrition.

It was also hypothesized that age and parity would increase GAUC. However, when comparing GAUC across pregnancies in the CTR and WSD, GAUC in latter pregnancies was similar to that in early pregnancies. In correlations with offspring phenotype, maternal age was not correlated with offspring GAUC and there was a weak but significant correlation between maternal parity and offspring GAUC. However, there was a significant correlation between maternal GAUC and offspring GAUC. After adjustment for confounders, the association between parity and offspring GAUC was no longer significant ($p=0.57$). The association between maternal and offspring GAUC was not statistically significant after adjustment for confounders ($p=0.09$) but trended toward a positive association. In humans, parity does not appear to be associated with

offspring obesity, or is associated through effects on maternal BMI, which independently leads to increased childhood adiposity [128, 129].

Together, data from this model and in humans suggest that maternal metabolic phenotype may be more important for offspring health than diet itself. This has important implications for future research, especially in the NHP model where the effects of WSD are not completely clear. Like humans, NHPs display varying levels of adiposity and metabolic health on the same diet. Additionally, animals are housed outdoors and have access to any additional sources of food in their environment. For example, insects and seeds are both part of the Japanese macaque diet in nature [143], and could be consumed by animals in both CTR and WSD groups. Since food intake is not monitored, the amount of intake from experimental diets versus other sources cannot be measured. In future studies, one approach would be to keep all animals on a similar diet and stratify by metabolic phenotype. This eliminates a confounding variable and isolates the effects of metabolic phenotype on the offspring. Indeed, other studies in this model have stratified WSD-fed dams into lean and obese groups [139]. However, this approach is only feasible with a sufficiently large sample size, which was lacking in our offspring analysis.

In addition to larger sample sizes, controlling for several other factors would improve the NHP model as a model of maternal overnutrition. Because this is a limited resource, females were used for multiple pregnancies. Though in some analyses parity was adjusted for, ideally all offspring analyzed would have been the result of a first pregnancy, eliminating any effects of subsequent pregnancies on offspring phenotype. Similarly, ideally all dams should be the same age at diet initiation and during pregnancy, with a similar duration of WSD feeding leading up to pregnancy. Additionally, a controlled environment where food intake is monitored and all caloric intake is from the experimental and control diets in both dams and offspring would eliminate confounding effects of consuming other food such as insects. The WSD group was also allowed access to calorically dense treats. A more consistent diet, along with a micronutrient matched CTR diet would be a better controlled experimental design. Finally, the ability to analyze offspring

in adulthood and after prolonged WSD feeding may reveal phenotypes not observed before puberty at three years of age. Despite these limitations to the model, the analyses in this thesis demonstrated that GAUC worsens with prolonged WSD feeding and that maternal glucose tolerance during pregnancy remains a predictor of offspring glucose tolerance.

These observations have important implications for human pregnancy. Rather than focusing on restricting or increasing consumption of specific macronutrients, interventions may be more effective if they focus specifically on improving metabolic phenotype regardless of the diet used to achieve this goal. This approach is currently being pursued in a clinical trial recruiting 1800 females across three nations [126]. In this study, a nutritional drink fortified with micronutrients that have been shown to both increase cellular glucose uptake and correct vitamin deficiencies that lead to maternal dysglycemia is being used to lower maternal blood glucose levels during pregnancy. It will be interesting to see how this intervention affects maternal and offspring metabolic parameters, which will both be analyzed.

While maternal metabolic phenotype may be more important than diet alone, the WSD model has still provided a relevant model of maternal overnutrition, as evidenced by increased weight and adiposity in dams and signs of poor health in multiple organ systems in the offspring [87, 88, 94, 105, 127, 144]. In the islet, maternal WSD results in consistent changes across cohorts and at multiple time points [95, 144]. Fetal offspring exposed to WSD had reduced α-cell mass. In three-year-old samples assessed as part of this project, maternal WSD led to a significant reduction in α-cell mass. At one year of age, WSD/WSD animals had in increased β:α cell ratio, a phenotype also observed in this project at three years of age. Finally, utilizing islets from both one-year-old and three-year-old offspring, maternal WSD was associated with increased glucose-stimulated insulin secretion at both ages, and decreased mitochondrial density in three-year-old offspring.

While many parameters measured in the islet were not affected by maternal diet, the phenotypes observed were striking and consistent. In a model with high individual variability

where samples were collected in groups across multiple seasons, clear and consistent effects of maternal diet are still observed. Islets from offspring of WSD-fed mothers behave similarly to islets from obese humans [138]. Hyper-secretion of insulin under certain circumstances could predispose offspring to developing insulin resistance. Islets exposed to WSD also had reduced α-cell mass. The consequences of α-cell loss in the NHP and human are still not completely understood. While α-cell have been shown to be dispensable for islet function and glucose tolerance in mice [145], the more heterogeneous architecture of human and NHP islets suggests a more important role for non-β cell endocrine cells within the islet. It has been shown in human β cells that both contact with other β cells as well as non-β endocrine cells is important for insulin secretion [78]. Additionally, it has been recognized for decades that glucagon can enhance insulin secretion and that α-cells produce insulin secretagogues [146-148]. In the *ex vivo* perifusion assay, maternal WSD led to enhanced insulin secretion. However, an α-cell deficit may predispose these offspring to insufficient insulin secretion with increased age or the development of insulin resistance. It would be interesting to test the effects of altering the proportion of α cells in human pseudoislets that are transplanted into mice to determine the consequences of α cell loss *in vivo.*

In order to investigate the mechanism behind increased glucose-stimulated insulin secretion, mitochondrial density and function were assessed. In β cells, maternal WSD appeared to cause a reduction in mitochondrial density, although this did not reach significance due to the low sample number. Interestingly, WSD-exposed offspring also had a reduction in mitochondria in skeletal muscle [139], suggesting that exposure to maternal over-nutrition may lead to decreased mitochondrial mass in several tissues. In future studies, a more thorough investigation would be useful to test whether a reduction in mitochondria occurs in multiple tissues as a common result of exposure to maternal WSD. Indeed, studies in other animal models have suggested that poor maternal nutrition can affect mitochondrial mass and function in multiple tissues in the offspring. A study in rats fed HFD showed that offspring had decreased

mitochondrial DNA in the kidney and aorta [149]. Another study in rats showed that offspring of calorically-restricted dams had alternations in the expression of regulators of mitochondrial biogenesis in the islet [150]. In an intra-uterine growth restriction model in rats, a gestational environment of undernutrition ultimately led to reductions in islet mitochondrial DNA content in the offspring [151]. Thus, it is possible that in the NHP model, maternal WSD leads to decreases in mitochondrial mass in multiple tissues due to maternal diet-induced reductions in regulators of mitochondrial biogenesis at key time points during early life.

To test whether maternal WSD exposure leads to long-term changes in offspring islet gene expression, RNA-Sequencing was performed in two separate cohorts of CTR/CTR and WSD/CTR islets at three years of age. While gene expression changes were not consistent between the two cohorts, maternal WSD did lead to differences in expression in genes important in islet function in both experiments. Additionally, *GPR155*, a protein with no known role in the islet, was up-regulated by maternal WSD in one cohort. This gene presents an interesting candidate for future study, as it is associated with T2D in mice and preliminary data as part of this thesis work strongly suggests that it co-localizes with insulin granules in the β cell. Future studies using knockout mice and siRNA in cell lines may uncover the role of this protein in the islet.

Changes in islet gene expression due to maternal diet may be due to epigenetic effects. In a rat model of maternal undernutrition due to uteroplacental insufficiency, which limits the supply of nutrients to the fetus, offspring islets had decreases in histone H3 lysine 4 methylation and increases in histone H3 lysine 9 methylation at the Pdx1 locus. These repressive histone marks were associated with a reduction in Pdx1 expression which could be reversed by inhibiting histone deacetylation pharmacologically. The reduction in Pdx1 expression ultimately contributed to the development of T2D in these offspring [152]. In this same model, maternal overnutrition led to changes in DNA methylation at multiple loci in offspring islets, several of which were associated with changes in gene expression [153]. In another model of maternal undernutrition, there was a reduction in the expression of Hnf4a in offspring islets. This was associated with increased

repressive histone marks and an enhancer region and weakened promotor-enhancer interaction [100]. Interestingly, paternal HFD can also affect offspring islet gene expression in a mouse model [154]. This suggests that diet can lead to epigenetic changes in gametes which are then passed down to offspring. In the case of maternal WSD, changes in offspring gene expression may be due to epigenetic modifications directly inherited from the mother or induced by the *in utero* environment. Indeed, maternal WSD has been shown to lead to epigenetic changes in other tissues in the NHP. Exposure to maternal WSD led to increased histone 3 acetylation in the liver [91] and altered expression of circadian rhythm genes associated with repressive histone marks [86]. Also in the liver, there was in increase in repressive histone marks at the SIRT1 locus and a concomitant decrease in expression levels of SIRT1, as well as a reduction in its deacetylase activity [87]. Thus, future studies are needed to further investigate whether maternal WSD leads to epigenetic changes in the islet. Two approaches could address this question. First, ATAC-Seq can be used to asses global changes in chromatin state within the islet. The ATAC-Seq approach is unbiased toward histone modifications versus DNA methylation, and can serve as a starting point to identify loci to further investigate. Another approach is to use chromatin immunoprecipitation to investigate histone modifications in genes whose expression affected by maternal WSD. These studies would help elucidate how maternal diet can affect islet function in this model.

Importantly, work done as part of this thesis represents a key component of a broader effort to investigate the effects of WSD on multiple tissues in the offspring. As shown in the previous chapters, maternal WSD leads to significant changes in islet cell composition and function. Maternal WSD also leads to many striking phenotypes in other tissues as highlighted in Figure 7-1. In the central nervous system, maternal WSD was associated with perturbations in the serotonergic system of fetuses. Maternal WSD-exposed offspring had increased anxiety responses to novel objects after birth [92], an increased preference for foods high in fat and sugar, and suppressed dopamine signaling [93]. In the liver, maternal WSD led to signs of fatty liver

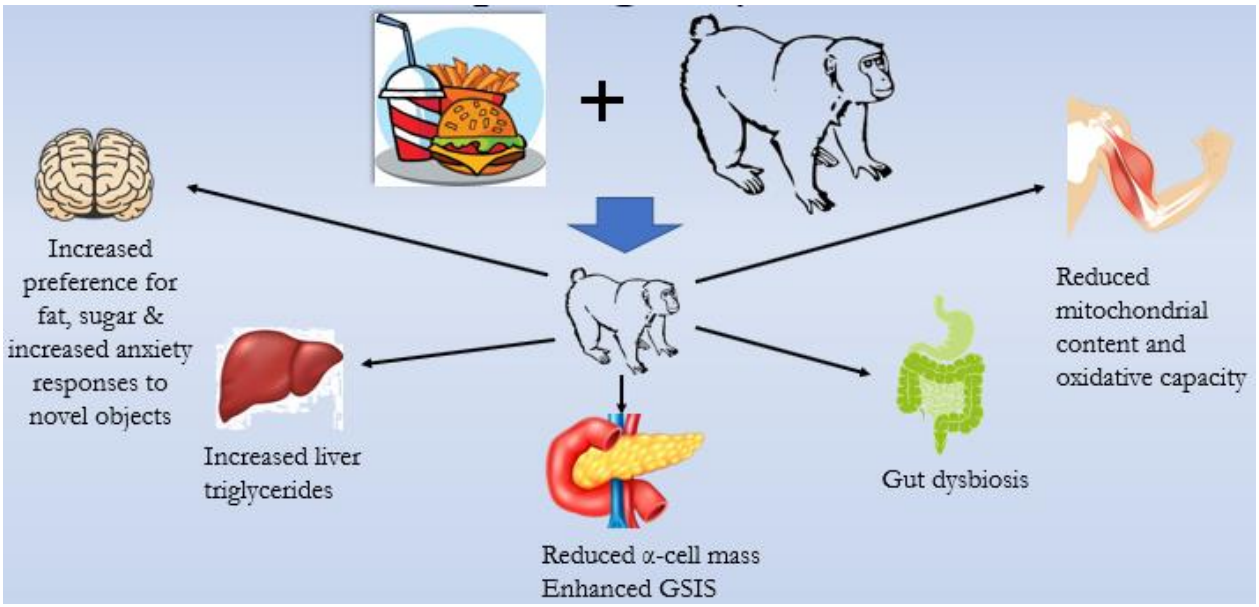


Figure 7-1: Maternal Western-style (WSD) diet has significant effects on multiple organ systems. In addition to the studies described in this thesis, multiple groups utilizing this model have shown that maternal WSD can affect offspring behavior, liver triglyceride levels, gut microbiome, and muscle oxidative capacity. Together these results demonstrate the widespread deleterious effects of maternal WSD on the offspring.

disease with increases in liver triglycerides and histologic correlates of fatty liver disease such evidence of hepatic oxidative stress, in addition to the epigenetic changes described above. The increased levels of triglycerides persisted to three months of age in the offspring and were associated with a two-fold increase in body fat [88]. In the intestine, maternal WSD led to an altered gut microbiome in the offspring, some of which were irreversible even if offspring were weaned to CTR diet [84]. Maternal WSD also led to deficits in thyroid axis function, with reduced levels of free T_4 and disrupted transcription of deiodinase genes and genes involved in thyroid hormone synthesis. These changes were also associated with alternations in histone marks [89].

The scope of deleterious effects of maternal WSD in the offspring warrants further investigation in this model. In the islet, several lines of study would be interesting to pursue. Specifically, the mechanism behind increased glucose-stimulated insulin secretion and the consequence of reduced α-cell mass should be investigated. Though mitochondrial function was probed pharmacologically in oxygen consumption assays, it would be interesting to test whether WSD-exposed islets show increases in oxygen consumption when incubated in high glucose. This would directly test whether mitochondrial function is enhanced in the same conditions as the glucose-stimulated insulin secretion assay. It would also be interesting to test whether there are changes in islet calcium signaling that could explain enhanced insulin secretion. Since α-cell mass is reduced as a result of maternal WSD, perhaps there are compensatory changes to maintain glucagon secretion. Using electron microscopy, α-cell glucagon granule density and docking to the plasma membrane can be quantified. Additionally, perifusion studies that include measurement of glucagon secretion can be performed to assess α-cell function.

As part of this thesis, a mouse model of maternal overnutrition was utilized in parallel with the NHP model. In this model, maternal HFD led to no changes in glucose tolerance and endocrine cell mass in the offspring at the ages investigated. As in other studies in rodent models, additional metabolic stress through prolonged HFD feeding or advanced age could reveal the effects of maternal diet. Additionally, offspring may be “primed” for poor metabolic health in the

future despite the absence of an overt phenotype in early adulthood. To investigate this possibility, ATAC-Seq experiments were conducted to assess differences in chromatin state due to maternal HFD. Despite extensive effort to optimize the isolation and sorting of β cells, the ATAC sequencing was unsuccessful in a majority of cases. However, this is an important area to pursue, as it is well recognized in multiple animal models and humans that poor maternal nutrition leads to epigenetic changes in the offspring [13, 49, 61, 85, 100, 104, 119]. In order to successfully perform the ATAC-Seq protocol, the number of β cells could be increased by pooling islets from mice of the same diet group. Additionally, there are variations of the ATAC-Seq protocol [155] that may be more successful in this specific system than the protocol utilized [111] as part of this thesis. Future studies can thus assess whether maternal HFD primes the offspring for poor metabolic health, even in the absence of a phenotype at the time point investigated.

In addition to investigating epigenetic changes in the mouse as a result of maternal HFD, future studies in this model can further stress the offspring in order to reveal a maternal phenotype. While previous studies in rodent models have utilized prolonged HFD feeding and increased age to stress the offspring, an additional stressor that could be utilized is pregnancy. Female offspring in the four diet groups investigated in this thesis could be mated after reaching sexual maturity, and glucose tolerance can be assessed during pregnancy. Perhaps the combination of HFD feeding post-weaning and the metabolic stress of pregnancy would reveal an effect of maternal overnutrition on these female offspring. This approach has the advantage of adding a metabolic stressor without the need to house and maintain mice for prolonged periods of time. Another alternative approach to the mouse model would be to initiate HFD feeding earlier in the dams, perhaps before reaching sexual maturity. The additional metabolic stress of going through puberty while on HFD would likely lead to a more deleterious *in utero* environment during gestation and lead to observable phenotypes in the offspring.

In conclusion, this work has expanded and added to the DOHaD field by demonstrating the following:

1. Maternal glycemia during pregnancy negatively affects offspring metabolic health in a model closely related to humans. This is consistent with increasing evidence in humans that maternal glycemia is important for the metabolic health of the offspring. Additionally, data from this thesis suggests that in terms of glucose tolerance, maternal diet itself does not lead to impairments in offspring glucose tolerance in the same way as maternal metabolic status.
2. The effects of WSD on islet cell composition are clear and consistent, although neither the cause nor the physiological consequences are not well understood. These studies showed consistent effects of WSD on three-year-old offspring as those observed in one-year-old offspring in an independent laboratory.
3. Maternal WSD affects offspring islet insulin secretion *ex vivo.* This occurs at one and three years of age, and when offspring are fed WSD or CTR post-weaning. This work suggests that an unhealthy diet during pregnancy leads to changes intrinsic to the islet and independent of effects on whole body insulin sensitivity.
4. The effects of maternal overnutrition are manifest under specific circumstances and vary between animal models. Maternal HFD led to no phenotype in four- and 12-week old mouse offspring, consistent with rodent studies showing that effects of maternal overnutrition may only manifest after prolonged metabolic stress in the offspring. NHP islet insulin secretion *ex vivo* was affected by maternal diet, but plasma insulin levels in these offspring were not affected by maternal diet. Together, these results demonstrate that an unhealthy maternal diet leads to deficits in offspring metabolic and islet health in a context-specific manner.

REFERENCES

1. Ravelli, G.P., Z.A. Stein, and M.W. Susser, *Obesity in young men after famine exposure in utero and early infancy.* N Engl J Med, 1976. **295**(7): p. 349-53.
2. Scholte, R.S., G.J. van den Berg, and M. Lindeboom, *Long-run effects of gestation during the Dutch Hunger Winter famine on labor market and hospitalization outcomes.* J Health Econ, 2015. **39**: p. 17-30.
3. Painter, R.C., T.J. Roseboom, and O.P. Bleker, *Prenatal exposure to the Dutch famine and disease in later life: an overview.* Reprod Toxicol, 2005. **20**(3): p. 345-52.
4. Barker, D.J., *Maternal nutrition and cardiovascular disease.* Nutr Health, 1993. **9**(2): p. 99-106.
5. Barker, D.J. and C. Osmond, *Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales.* Lancet, 1986. **1**(8489): p. 1077-81.
6. Barker, D.J., et al., *Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease.* BMJ, 1989. **298**(6673): p. 564-7.
7. Drake, A.J. and R.M. Reynolds, *Impact of maternal obesity on offspring obesity and cardiometabolic disease risk.* Reproduction, 2010. **140**(3): p. 387-98.
8. Freinkel, N., *Banting Lecture 1980. Of pregnancy and progeny.* Diabetes, 1980. **29**(12): p. 1023-35.
9. PM, C. and E. HM, *Review article: The short- and long-term implications of maternal obesity on the mother and her offspring.* BJOG: An International Journal of Obstetrics & Gynaecology, 2006. **113**(10): p. 1126-1133.
10. Oken, E. and M.W. Gillman, *Fetal origins of obesity.* Obes Res, 2003. **11**(4): p. 496-506.
11. Mingrone, G., et al., *Influence of maternal obesity on insulin sensitivity and secretion in offspring.* Diabetes Care, 2008. **31**(9): p. 1872-6.
12. *Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study.* Associations With Neonatal Anthropometrics, 2009. **58**(2): p. 453-459.
13. Wadhwa, P.D., et al., *Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms.* Semin Reprod Med, 2009. **27**(5): p. 358-68.
14. Mandy, M. and M. Nyirenda, *Developmental Origins of Health and Disease: the relevance to developing nations.* Int Health, 2018. **10**(2): p. 66-70.
15. Entringer, S., C. Buss, and P.D. Wadhwa, *Prenatal stress, development, health and disease risk: A psychobiological perspective-2015 Curt Richter Award Paper.* Psychoneuroendocrinology, 2015. **62**: p. 366-75.
16. Entringer, S., et al., *Prenatal psychosocial stress exposure is associated with subsequent working memory performance in young women.* Behav Neurosci, 2009. **123**(4): p. 886-93.
17. Entringer, S., et al., *Prenatal psychosocial stress exposure is associated with insulin resistance in young adults.* Am J Obstet Gynecol, 2008. **199**(5): p. 498.e1-7.
18. Prevention, C.f.D.C.a. *Overweight and Obesity*. 2016; Available from: https://www.cdc.gov/nchs/fastats/obesity-overweight.htm.
19. Diseases, N.I.o.D.a.D.a.K., *Overweight & Obesity Statistics*.
20. System, P.R.A.M.
21. Mastracci, T.L. and L. Sussel, *The Endocrine Pancreas: insights into development, differentiation and diabetes.* Wiley Interdiscip Rev Membr Transp Signal, 2012. **1**(5): p. 609-28.
22. O'Dowd, J.F. and C.J. Stocker, *Endocrine pancreatic development: impact of obesity and diet.* Front Physiol, 2013. **4**.
23. Romer, A.I. and L. Sussel, *Pancreatic Islet Cell Development and Regeneration.* Curr Opin Endocrinol Diabetes Obes, 2015. **22**(4): p. 255-64.

24. Pan, F.C. and C. Wright, *Pancreas organogenesis: From bud to plexus to gland.* Developmental Dynamics, 2011. **240**(3): p. 530-565.
25. Gittes, G.K., *Developmental biology of the pancreas: A comprehensive review.* Developmental Biology, 2009. **326**(1): p. 4-35.
26. Bastidas-Ponce, A., et al., *Cellular and molecular mechanisms coordinating pancreas development.* Development, 2017. **144**(16): p. 2873-2888.
27. Jennings, R.E., et al., *Development of the human pancreas from foregut to endocrine commitment.* Diabetes, 2013. **62**(10): p. 3514-22.
28. Piper K, B.S., Turnpenny LW, Cameron IT, Ball SC, Wilson DI, Hanley NA, *Beta cell differentiation during early human pancreas development.* J Endocinrology, 2004. **181**(1): p. 11-23.
29. Bechard, M.E., et al., *Precommitment low-level Neurog3 expression defines a long-lived mitotic endocrine-biased progenitor pool that drives production of endocrine-committed cells.* Genes Dev, 2016. **30**(16): p. 1852-65.
30. Conrad, E., et al., *The MAFB transcription factor impacts islet alpha-cell function in rodents and represents a unique signature of primate islet beta-cells.* Am J Physiol Endocrinol Metab, 2016. **310**(1): p. E91-e102.
31. Rorsman, P. and M. Braun, *Regulation of Insulin Secretion in Human Pancreatic Islets.* Annual Review of Physiology, 2013. **75**(1): p. 155-179.
32. McEwen, M., et al., *Targeting Mitochondrial Function for the Treatment of Acute Spinal Cord Injury.* Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics, 2011. **8**: p. 168-79.
33. Rorsman, P., et al., *Electrophysiology of pancreatic β-cells in intact mouse islets of Langerhans.* Progress in Biophysics and Molecular Biology, 2011. **107**(2): p. 224-235.
34. Kulkarni, R.N., *The islet β-cell.* The International Journal of Biochemistry & Cell Biology, 2004. **36**(3): p. 365-371.
35. Marchetti, P., et al., *An overview of pancreatic beta-cell defects in human type 2 diabetes: Implications for treatment.* Regulatory Peptides, 2008. **146**(1): p. 4-11.
36. Brissova, M., et al., *Pancreatic Islet Production of Vascular Endothelial Growth Factor-A Is Essential for Islet Vascularization, Revascularization, and Function.* Diabetes, 2006. **55**(11): p. 2974-2985.
37. Ruud, J., S.M. Steculorum, and J.C. Brüning, *Neuronal control of peripheral insulin sensitivity and glucose metabolism.* Nature Communications, 2017. **8**(1): p. 15259.
38. Del Prato, S., P. Marchetti, and R.C. Bonadonna, *Phasic Insulin Release and Metabolic Regulation in Type 2 Diabetes.* Diabetes, 2002. **51**(suppl 1): p. S109-S116.
39. Meloni, A.R., et al., *GLP-1 receptor activated insulin secretion from pancreatic beta-cells: mechanism and glucose dependence.* Diabetes Obes Metab, 2013. **15**(1): p. 15-27.
40. Bouwens, L. and I. Rooman, *Regulation of Pancreatic Beta-Cell Mass.* Physiological Reviews, 2005. **85**(4): p. 1255-1270.
41. Matveyenko, A.V. and P.C. Butler, *Relationship between beta-cell mass and diabetes onset.* Diabetes Obes Metab, 2008. **10 Suppl 4**(0 4): p. 23-31.
42. Asghar, Z., et al., *Insulin resistance causes increased beta-cell mass but defective glucose-stimulated insulin secretion in a murine model of type 2 diabetes.* Diabetologia, 2006. **49**(1): p. 90-9.
43. Rahier, J., et al., *Pancreatic beta-cell mass in European subjects with type 2 diabetes.* Diabetes Obes Metab, 2008. **10 Suppl 4**: p. 32-42.
44. Fonseca, S.G., J. Gromada, and F. Urano, *Endoplasmic reticulum stress and pancreatic beta cell death.* Trends Endocrinol Metab, 2011. **22**(7): p. 266-74.

45. Lebovitz, H.E., *Insulin resistance: definition and consequences.* Exp Clin Endocrinol Diabetes, 2001. **109 Suppl 2**: p. S135-48.
46. Beale, E.G., *Insulin signaling and insulin resistance.* J Investig Med, 2013. **61**(1): p. 11-4.
47. Ahima, R.S. and M.A. Lazar, *Adipokines and the peripheral and neural control of energy balance.* Mol Endocrinol, 2008. **22**(5): p. 1023-31.
48. Olefsky, J.M. and C.K. Glass, *Macrophages, inflammation, and insulin resistance.* Annu Rev Physiol, 2010. **72**: p. 219-46.
49. McMullen, S. and A. Mostyn, *Animal models for the study of the developmental origins of health and disease.* Proc Nutr Soc, 2009. **68**(3): p. 306-20.
50. Cerf, M.E. and J. Louw, *Islet cell response to high fat programming in neonate, weanling and adolescent Wistar rats.* Jop, 2014. **15**(3): p. 228-36.
51. Cerf, M.E., et al., *Hyperglycaemia and reduced glucokinase expression in weanling offspring from dams maintained on a high-fat diet.* Br J Nutr, 2006. **95**(2): p. 391-6.
52. Cerf, M.E., et al., *Islet cell response in the neonatal rat after exposure to a high-fat diet during pregnancy.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(5): p. R1122-8.
53. Dyrskog, S.E., S. Gregersen, and K. Hermansen, *High-fat feeding during gestation and nursing period have differential effects on the insulin secretory capacity in offspring from normal Wistar rats.* Rev Diabet Stud, 2005. **2**(3): p. 136-45.
54. Elena Zambrano, T.S.-L., Lizbeth Calzada, Carlos A Ibanez, Carmen A Mendoza-Rodriguez, Angelica Morales, Sumiko Morimoto, *Decreased basal insulin secretion from pancreatic islets of pups in a rat model of maternal obesity.* Journal of Endocrinology, 2016(231): p. 49-57.
55. Graus-Nunes, F., et al., *Pregestational maternal obesity impairs endocrine pancreas in male F1 and F2 progeny.* Nutrition, 2015. **31**(2): p. 380-7.
56. Guo, F. and K.L. Jen, *High-fat feeding during pregnancy and lactation affects offspring metabolism in rats.* Physiol Behav, 1995. **57**(4): p. 681-6.
57. Han, J., et al., *Long-term effect of maternal obesity on pancreatic beta cells of offspring: reduced beta cell adaptation to high glucose and high-fat diet challenges in adult female mouse offspring.* Diabetologia, 2005. **48**(9): p. 1810-8.
58. Srinivasan, M., et al., *Maternal high-fat diet consumption results in fetal malprogramming predisposing to the onset of metabolic syndrome-like phenotype in adulthood.* Am J Physiol Endocrinol Metab, 2006. **291**(4): p. E792-9.
59. Taylor, P.D., et al., *Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(1): p. R134-9.
60. Yokomizo H, I.T., Sonoda N, Sakaki Y, Maeda Y, Inoue T, Hirata E, Takei R, Ikeda N, Fujii M, Fukuda K, Sasaki H, Takayanagi R, *Maternal high-fat diet induces insulin resistance and deterioration of pancreatic beta-cell function in adult offspring with sex differences in mice.* Am J Physiol Endocrinol Metab, 2014. **306**(10): p. E1163-65.
61. Tobi, E.W., et al., *DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific.* Hum Mol Genet, 2009. **18**(21): p. 4046-53.
62. Kautzky-Willer, A., J. Harreiter, and G. Pacini, *Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus.* Endocr Rev, 2016. **37**(3): p. 278-316.
63. Cerf, M.E., et al., *Compromised beta-cell development and beta-cell dysfunction in weanling offspring from dams maintained on a high-fat diet during gestation.* Pancreas, 2007. **34**(3): p. 347-53.
64. Platt, K.M., R.J. Charnigo, and K.J. Pearson, *Adult offspring of high-fat diet-fed dams can have normal glucose tolerance and body composition.* J Dev Orig Health Dis, 2014. **5**(3): p. 229-39.

65. Gnugge, L., D. Meyer, and W. Driever, *Pancreas development in zebrafish.* Methods Cell Biol, 2004. **76**: p. 531-51.
66. Maddison, L.A. and W. Chen, *Nutrient Excess Stimulates β-Cell Neogenesis in Zebrafish.* Diabetes, 2012. **61**(10): p. 2517-24.
67. Ford, S.P., et al., *Maternal obesity accelerates fetal pancreatic β-cell but not α-cell development in sheep: prenatal consequences.* Am J Physiol Regul Integr Comp Physiol, 2009. **297**(3): p. R835-43.
68. Long, N.M., et al., *Maternal obesity and increased nutrient intake before and during gestation in the ewe results in altered growth, adiposity, and glucose tolerance in adult offspring1.* Journal of Animal Science, 2010. **88**(11): p. 3546-3553.
69. Zhang, L., et al., *Maternal obesity in the ewe results in reduced fetal pancreatic β-cell numbers in late gestation, and decreased circulating insulin concentration at term.* Domest Anim Endocrinol, 2011. **40**(1): p. 30-9.
70. Kropp, P.A., et al., *Cooperative function of Pdx1 and Oc1 in multipotent pancreatic progenitors impacts postnatal islet maturation and adaptability.* Am J Physiol Endocrinol Metab, 2018. **314**(4): p. E308-e321.
71. Nair, G. and M. Hebrok, *Islet formation in mice and men: lessons for the generation of functional insulin-producing β-cells from human pluripotent stem cells.* Curr Opin Genet Dev, 2015. **32**: p. 171-80.
72. Jeon, J., et al., *Endocrine cell clustering during human pancreas development.* J Histochem Cytochem, 2009. **57**(9): p. 811-24.
73. Bocian-Sobkowska, J., et al., *Polyhormonal aspect of the endocrine cells of the human fetal pancreas.* Histochem Cell Biol, 1999. **112**(2): p. 147-53.
74. Robb, P., *The development of the islets of Langerhans in the human foetus.* Q J Exp Physiol Cogn Med Sci, 1961. **46**: p. 335-43.
75. Brissova, M., et al., *Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy.* J Histochem Cytochem, 2005. **53**(9): p. 1087-97.
76. Cabrera, O., et al., *The unique cytoarchitecture of human pancreatic islets has implications for islet cell function.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2334-9.
77. Huypens, P., et al., *Glucagon receptors on human islet cells contribute to glucose competence of insulin release.* Diabetologia, 2000. **43**(8): p. 1012-9.
78. Wojtusciszyn, A., et al., *Insulin secretion from human beta cells is heterogeneous and dependent on cell-to-cell contacts.* Diabetologia, 2008. **51**(10): p. 1843-52.
79. Carter, A.M., *Animal Models of Human Placentation – A Review.* Placenta, 2007. **28**: p. S41-S47.
80. Phillips, K.A., et al., *Why primate models matter.* Am J Primatol, 2014. **76**(9): p. 801-27.
81. Plant, T.M., *A comparison of the neuroendocrine mechanisms underlying the initiation of the preovulatory LH surge in the human, Old World monkey and rodent.* Front Neuroendocrinol, 2012. **33**(2): p. 160-8.
82. Murphy, M.J., et al., *Leukemia Inhibitory Factor Is Necessary for Ovulation in Female Rhesus Macaques.* Endocrinology, 2016. **157**(11): p. 4378-4387.
83. Golos, T.G., et al., *On the role of placental Major Histocompatibility Complex and decidual leukocytes in implantation and pregnancy success using non-human primate models.* Int J Dev Biol, 2010. **54**(2-3): p. 431-43.
84. Ma, J., et al., *High-fat maternal diet during pregnancy persistently alters the offspring microbiome in a primate model.* Nat Commun, 2014. **5**: p. 3889.
85. Cox, J., et al., *A maternal high-fat diet is accompanied by alterations in the fetal primate metabolome.* American Journal of Obstetrics and Gynecology, 2009. **201**(3): p. 281.e1-281.e9.

86. Suter, M., et al., *Epigenomics: maternal high-fat diet exposure in utero disrupts peripheral circadian gene expression in nonhuman primates.* The FASEB Journal, 2010. **25**(2): p. 714-726.
87. Suter, M.A., et al., *A maternal high-fat diet modulates fetal SIRT1 histone and protein deacetylase activity in nonhuman primates.* The FASEB Journal, 2012. **26**(12): p. 5106-5114.
88. McCurdy, C.E., et al., *Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates.* J Clin Invest, 2009. **119**(2): p. 323-35.
89. Suter, M.A., et al., *Maternal High-Fat Diet Modulates the Fetal Thyroid Axis and Thyroid Gene Expression in a Nonhuman Primate Model.* Molecular Endocrinology, 2012. **26**(12): p. 2071-2080.
90. Suter, M.A., et al., *Postweaning exposure to a high-fat diet is associated with alterations to the hepatic histone code in Japanese macaques.* Pediatric Research, 2013. **74**(3): p. 252-258.
91. Kjersti, M.A.-T., et al., *Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome.* Journal of Molecular Endocrinology, 2008. **41**(2): p. 91-102.
92. Sullivan, E.L., et al., *Chronic Consumption of a High-Fat Diet during Pregnancy Causes Perturbations in the Serotonergic System and Increased Anxiety-Like Behavior in Nonhuman Primate Offspring.* The Journal of Neuroscience, 2010. **30**(10): p. 3826-3830.
93. Rivera, H.M., et al., *Maternal high-fat diet and obesity impact palatable food intake and dopamine signaling in nonhuman primate offspring.* Obesity (Silver Spring), 2015. **23**(11): p. 2157-64.
94. Prince, A.L., et al., *The development and ecology of the Japanese macaque gut microbiome from weaning to early adolescence in association with diet.* American Journal of Primatology, 2019. **81**(10-11): p. e22980.
95. Comstock, S.M., et al., *High-fat diet consumption during pregnancy and the early post-natal period leads to decreased alpha cell plasticity in the nonhuman primate.* Mol Metab, 2012. **2**(1): p. 10-22.
96. Pound, L.D., S.M. Comstock, and K.L. Grove, *Consumption of a Western-style diet during pregnancy impairs offspring islet vascularization in a Japanese macaque model.* Am J Physiol Endocrinol Metab, 2014. **307**(1): p. E115-23.
97. Huang, Y.H., et al., *Maternal high-fat diet impairs glucose metabolism, β-cell function and proliferation in the second generation of offspring rats.* Nutr Metab (Lond), 2017. **14**.
98. Saben, J.L., et al., *Maternal Metabolic Syndrome Programs Mitochondrial Dysfunction via Germline Changes across Three Generations.* Cell Reports. **16**(1): p. 1-8.
99. Cropley, J.E., et al., *Male-lineage transmission of an acquired metabolic phenotype induced by grand-paternal obesity.* Mol Metab, 2016. **5**(8): p. 699-708.
100. Sandovici, I., et al., *Maternal diet and aging alter the epigenetic control of a promoter–enhancer interaction at the Hnf4a gene in rat pancreatic islets.* Proceedings of the National Academy of Sciences, 2011. **108**(13): p. 5449-5454.
101. Tobi, E.W., et al., *DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific.* Hum Mol Genet, 2009. **18**(21): p. 4046-53.
102. Steegers-Theunissen, R.P., et al., *Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child.* PLoS One, 2009. **4**(11): p. e7845.
103. Katari, S., et al., *DNA methylation and gene expression differences in children conceived in vitro or in vivo.* Hum Mol Genet, 2009. **18**(20): p. 3769-78.
104. Jimenez-Chillaron, J.C., et al., *The role of nutrition on epigenetic modifications and their implications on health.* Biochimie, 2012. **94**(11): p. 2242-63.

105. Friedman, J.E., *Developmental Programming of Obesity and Diabetes in Mouse, Monkey, and Man in 2018: Where Are We Headed?* Diabetes, 2018. **67**(11): p. 2137-2151.
106. Golson, M.L., W.S. Bush, and M. Brissova, *Automated quantification of pancreatic beta-cell mass.* Am J Physiol Endocrinol Metab, 2014. **306**(12): p. E1460-7.
107. Elsakr, J.M., et al., *Analysis of Non-Human Primate Pancreatic Islet Oxygen Consumption.* JoVE, 2019(154): p. e60696.
108. Taddeo, E.P., et al., *Individual islet respirometry reveals functional diversity within the islet population of mice and human donors.* Molecular Metabolism, 2018. **16**: p. 150-159.
109. Carboneau, B.A., et al., *Opposing effects of prostaglandin E(2) receptors EP3 and EP4 on mouse and human β-cell survival and proliferation.* Mol Metab, 2017. **6**(6): p. 548-559.
110. Benner, C., et al., *The transcriptional landscape of mouse beta cells compared to human beta cells reveals notable species differences in long non-coding RNA and protein-coding gene expression.* BMC Genomics, 2014. **15**: p. 620.
111. Corces, M.R., et al., *An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues.* Nature Methods, 2017. **14**(10): p. 959-962.
112. Hohmeier, H.E., et al., *Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion.* Diabetes, 2000. **49**(3): p. 424-430.
113. Williams, R.L., *A note on robust variance estimation for cluster-correlated data.* Biometrics, 2000. **56**(2): p. 645-6.
114. Rogers, W., *Regression standard errors in clustered samples.* Stata Technical Bulletin, 1993(13): p. 19-23.
115. Dabelea, D., *The predisposition to obesity and diabetes in offspring of diabetic mothers.* Diabetes Care, 2007. **30 Suppl 2**: p. S169-74.
116. Dabelea, D., et al., *Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships.* Diabetes, 2000. **49**(12): p. 2208-11.
117. Dabelea, D. and D.J. Pettitt, *Intrauterine diabetic environment confers risks for type 2 diabetes mellitus and obesity in the offspring, in addition to genetic susceptibility.* J Pediatr Endocrinol Metab, 2001. **14**(8): p. 1085-91.
118. Krishnaveni, G.V., et al., *Intrauterine exposure to maternal diabetes is associated with higher adiposity and insulin resistance and clustering of cardiovascular risk markers in Indian children.* Diabetes Care, 2010. **33**(2): p. 402-4.
119. Nuyt, A.M. and M. Szyf, *Developmental programming through epigenetic changes.* Circ Res, 2007. **100**(4): p. 452-5.
120. Silverman, B.L., et al., *Impaired glucose tolerance in adolescent offspring of diabetic mothers. Relationship to fetal hyperinsulinism.* Diabetes Care, 1995. **18**(5): p. 611-7.
121. Catalano, P.M., et al., *Fetuses of obese mothers develop insulin resistance in utero.* Diabetes Care, 2009. **32**(6): p. 1076-80.
122. Metzger, B.E., et al., *Hyperglycemia and adverse pregnancy outcomes.* N Engl J Med, 2008. **358**(19): p. 1991-2002.
123. Tam, W.H., et al., *In Utero Exposure to Maternal Hyperglycemia Increases Childhood Cardiometabolic Risk in Offspring.* Diabetes Care, 2017. **40**(5): p. 679-686.
124. Cottam, M.A., et al., *Links between Immunologic Memory and Metabolic Cycling.* J Immunol, 2018. **200**(11): p. 3681-3689.
125. Pettitt, D.J., et al., *Gestational Diabetes Mellitus and Impaired Glucose Tolerance During Pregnancy: Long-Term Effects on Obesity and Glucose Tolerance in the Offspring.* Diabetes, 1985. **34**(Supplement 2): p. 119-122.

126. Godfrey, K.M., et al., *Nutritional Intervention Preconception and During Pregnancy to Maintain Healthy Glucose Metabolism and Offspring Health ("NiPPeR"): study protocol for a randomised controlled trial.* Trials, 2017. **18**(1): p. 131.
127. Harris, R.A., et al., *Genomic Variants Associated with Resistance to High Fat Diet Induced Obesity in a Primate Model.* Sci Rep, 2016. **6**: p. 36123.
128. Reynolds, R.M., et al., *Maternal BMI, Parity, and Pregnancy Weight Gain: Influences on Offspring Adiposity in Young Adulthood.* The Journal of Clinical Endocrinology & Metabolism, 2010. **95**(12): p. 5365-5369.
129. Gaillard, R., et al., *Risk factors and outcomes of maternal obesity and excessive weight gain during pregnancy.* Obesity (Silver Spring), 2013. **21**(5): p. 1046-55.
130. Myrskylä, M. and A. Fenelon, *Maternal Age and Offspring Adult Health: Evidence From the Health and Retirement Study.* Demography, 2012. **49**(4): p. 1231-1257.
131. Schulz, L.C., *The Dutch Hunger Winter and the developmental origins of health and disease.* Proceedings of the National Academy of Sciences, 2010. **107**(39): p. 16757-16758.
132. Drake, A.J. and R.M. Reynolds, *Impact of maternal obesity on offspring obesity and cardiometabolic disease risk.* Reproduction, 2010. **140**(3): p. 387-398.
133. Elsakr, J.M. and M. Gannon, *Developmental programming of the pancreatic islet by in utero overnutrition.* Trends Dev Biol, 2017. **10**: p. 79-95.
134. Brissova, M., et al., *Pancreatic islet production of vascular endothelial growth factor--a is essential for islet vascularization, revascularization, and function.* Diabetes, 2006. **55**(11): p. 2974-85.
135. Dai, C., et al., *Pancreatic islet vasculature adapts to insulin resistance through dilation and not angiogenesis.* Diabetes, 2013. **62**(12): p. 4144-53.
136. Kobayashi, M., et al., *Searching for genomic region of high-fat diet-induced type 2 diabetes in mouse chromosome 2 by analysis of congenic strains.* PLoS One, 2014. **9**(5): p. e96271.
137. Xu, W., et al., *Regenerating islet-derived protein 1 inhibits the activation of islet stellate cells isolated from diabetic mice.* Oncotarget, 2015. **6**(35): p. 37054-65.
138. Henquin, J.C., *Influence of organ donor attributes and preparation characteristics on the dynamics of insulin secretion in isolated human islets.* Physiol Rep, 2018. **6**(5).
139. McCurdy, C.E., et al., *Maternal obesity reduces oxidative capacity in fetal skeletal muscle of Japanese macaques.* JCI Insight, 2016. **1**(16): p. e86612.
140. Aimukhametova, G., et al., *The impact of maternal obesity on mother and neonatal health: study in a tertiary hospital of Astana, Kazakhstan.* Nagoya J Med Sci, 2012. **74**(1-2): p. 83-92.
141. Sewell, M.F., et al., *Increased neonatal fat mass, not lean body mass, is associated with maternal obesity.* Am J Obstet Gynecol, 2006. **195**(4): p. 1100-3.
142. Vickers, M.H., et al., *Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition.* Am J Physiol Endocrinol Metab, 2000. **279**(1): p. E83-7.
143. Hill, D.A., *Seasonal variation in the feeding behavior and diet of Japanese macaques (Macaca fuscata yakui) in lowland forest of Yakushima.* American Journal of Primatology, 1997. **43**(4): p. 305-320.
144. Elsakr, J.M., et al., *Maternal Western-style diet affects offspring islet composition and function in a non-human primate model of maternal over-nutrition.* Mol Metab, 2019.
145. Thorel, F., et al., *Normal Glucagon Signaling and β-Cell Function After Near-Total α-Cell Ablation in Adult Mice.* Diabetes, 2011. **60**(11): p. 2872-82.
146. Curry, D.L., *Glucagon Potentiation of Insulin Secretion by the Perfused Rat Pancreas.* Diabetes, 1970. **19**(6): p. 420.
147. Date, Y., et al., *Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion.* Diabetes, 2002. **51**(1): p. 124-9.

148. Hansen, A.M., et al., *Upregulation of alpha cell glucagon-like peptide 1 (GLP-1) in Psammomys obesus--an adaptive response to hyperglycaemia?* Diabetologia, 2011. **54**(6): p. 1379-87.
149. Taylor, P.D., et al., *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, 2005. **288**(1): p. R134-R139.
150. Theys, N., et al., *Maternal malnutrition programs pancreatic islet mitochondrial dysfunction in the adult offspring.* The Journal of Nutritional Biochemistry, 2011. **22**(10): p. 985-994.
151. Simmons, R.A., I. Suponitsky-Kroyter, and M.A. Selak, *Progressive accumulation of mitochondrial DNA mutations and decline in mitochondrial function lead to beta-cell failure.* J Biol Chem, 2005. **280**(31): p. 28785-91.
152. Park, J.H., et al., *Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1.* J Clin Invest, 2008. **118**(6): p. 2316-24.
153. Simmons, R., *Epigenetics and maternal nutrition: nature v. nurture.* Proceedings of the Nutrition Society, 2011. **70**(1): p. 73-81.
154. Ng, S.-F., et al., *Paternal high-fat diet consumption induces common changes in the transcriptomes of retroperitoneal adipose and pancreatic islet tissues in female rat offspring.* The FASEB Journal, 2014. **28**(4): p. 1830-1841.
155. Buenrostro, J.D., et al., *ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide.* Curr Protoc Mol Biol, 2015. **109**: p. 21.29.1-21.29.9.

APPENDIX

Table A-1. **Comparison of Rodent Models of Maternal Overnutrition**

Legend: CTR = control diet; HFD = high fat diet; HFDG = HFD during gestation only; HFDG# = HFD during week X of gestation; HFDL = HFD during lactation only; HFDL# = HFD during week X of lactation; HFDGL = HFD during gestation and lactation; HFDGL# = HFD during gestation and week X of lactation; HFDP = HFD postnatally; HFDG-P = HFD during gestation and postnatally; HSu = high Sucrose diet; GTT = glucose tolerance test; AUC = area under the curve; wk = weeks of age; P = postnatal day. Adapted from Elsakr and Gannon, *Trends in Developmental Biology*, 2017

Animal model	**Diet/Experimental groups**	**Glucose tolerance/ Fasting glucose**	**Insulin tolerance/ Fasting insulin**	**Endocrine cell mass/ratios**	**Notes**	**Reference**
C57/BL6J mouse	CTR (11.5% kcal from fat) or HFD (62% kcal from fat) from conception to weaning. Offspring placed on CTR from 4-6wks then CTR or HFD	Impaired at 6wk in HFD offspring. In males and females at 14wk and 20wk, HFD/HFD was worse than CTR/HFD. In males, HFD/CTR worse than CTR/CTR	At 14 and 20 wks, HFD/HFD had worse insulin tolerance in both males and females	Maternal HFD decreased islet area in males but increased islet area in HFD/HFD females (with higher proportion of large islets).	Greater body weight at birth and from 4-6wks. From 6-20wks HFD/HFD females (only) weighed more. Islet insulin content and *Pdx1* mRNA was decreased in HFD/HFD males, but elevated in females.	[60]
C57/BL6 mouse	CTR (17% from fat-soybean oil); HFD (49%-soybean oil as in CTR plus additional fat in lard). CTR or HFD starting at 4 wks for 8 weeks before mating. Diet maintained through lactation; then	Glucose tolerance Impaired (with higher AUC) in F1 and impaired at two time points in F2. F1 HFD had fasting hyperglycemia.	F1 and F2 had fasting hyperinsulinemia.	F1 and F2 HFD had higher islet mass, higher α-cell mass and much higher β-cell mass. Both F1 and F2 had reduced Pdx1 immunolabeling.	F0 and F1 HFD were overweight before and during pregnancy.	[55]

	offspring on CTR for 2 generations (F1 and F2). Male offspring evaluated at 3 months.			HFD F1 and F2 had α cells in the core of islet.		
Animal model	**Diet/Experimental groups**	**Glucose tolerance/Fasting glucose**	**Insulin tolerance/Fasting insulin**	**Endocrine cell mass/ratios**	**Notes**	**Reference**
C57BL/6 mouse with or without the agouti yellow mutation (obese but euglycemic)	Mothers (Obese = agouti yellow mutation; CON = wild type) were fed normal chow during pregnancy. Offspring fed normal chow until 15 wks, then CTR (22% from fat) or HFD (60% cal from fat) starting from 15wks to make four groups: CON/CTR, Obese/CTR, CON/HFD, Obese/HFD	50-wk obese/HFD females had impaired glucose tolerance relative to CON/HFD.	Obese-exposed females (Obese/HFD and Obese/CTR) had less insulin secretion during GTT at 50 wk. At 30 and 50 wk, female obese/HFD offspring had reduced insulin secretion during GTT relative to CON/HFD. Isolated islets from obese/HFD females had similar basal insulin secretion but impaired response to stimuli compared to CON/CTR	Not analyzed.	Offspring of obese mothers had higher birth weight, but this normalized in adulthood. HFD increased islet insulin content in CON/HFD but not obese/HFD. Protein levels of select metabolic enzymes (Transketolase, GAPDH, PFK) were reduced in obese/CTR and obese/HFD islets.	[57]
Wistar rats	CTR (10/15/75% kcal from fat/protein/carbs) HFD (40/14/46) with fat from saturated animal fat. Groups: HFDG1, HFDG2, HFDG3 or HFDG. Examined at birth.	HFDG1 and HFDG2 were hypoglycemic. HFDG were hyperglycemic	No differences in plasma insulin levels.	β-cell volume and numbers were reduced in HFDG neonates. α-cell volume, size, and number were increased in HFDG.	HFDG1 had low birth weight	[52]
Wistar rats	CTR and HFD as above. Groups: HFDG, HFDGL1, HFDGL2,	Only CTR, HFDG and HFDGL were normoglycemic.	HFDG, HFDGL1, and HFDGL were hypoinsulinemic.		HFDG weighed less than controls, HFDL weighed more.	[51]

	HFDGL3, HFDGL, and HFDL. Offspring examined at weaning.				Glucokinase mRNA and immunoreactivity reduced in HFDGL, HFDGL2 and HFDGL3	
Animal model	**Diet/Experimental groups**	**Glucose tolerance/Fasting glucose**	**Insulin tolerance/Fasting insulin**	**Endocrine cell mass/ratios**	**Notes**	**Reference**
Wistar rats	Same diet as above. Groups: HFDG neonates and adolescents, HFDL weanlings, HFDGL weanlings, HFDP adolescents, HFDG-P adolescents	HFDP were hyperglycemic in adolescence but not HFDG.	HFDP and HFDG-P were hyperinsulinemic in adolescence.	No differences in β-cell mass among adolescents. No change in ratios. Increased islet cell proliferation in HFDP. Otherwise, no differences in proliferation.	HFDGL were the heaviest group in adolescence, then HFDL. HFDG were similar to CTR. HFDG neonates had reduced pancreas:body weight ratio. HFDG-P pancreas:body was increased at weaning but decreased at P90.	[50]
Sprague-Dawley rats	CTR (10.9/70% kcal from fat/carbs) and HFD (59.5/24.4% from fat/carbs). Diet from postnatal day 24 through gestation and lactation (mated at P120). Progeny weaned onto CTR or HSu diet	At P90, HFD/CTR and HFD/HSu were glucose intolerant. At P120, HFD/CTR, CTR/HSu, and HFD/HSu had elevated plasma glucose.	Higher plasma insulin in HFD fetuses at term. Plasma insulin was higher in HFD/CTR compared to CTR/CTR from P40 onward. At P120, plasma insulin was high in HFD/CTR and HFD/HSu relative to respective controls.	Not analyzed	Islets from HFD fetuses secreted more insulin at high glucose or in the presence of Arginine or Leucine (insulin secretagogues). HFD/CTR and HFD/HSu had increased body weight from P30 to P120. P120 islets exposed to HFD *in utero* OR HSu diet	[58]

					after weaning had higher basal insulin and lower fold increase at high glucose.	
Animal model	**Diet/Experimental groups**	**Glucose tolerance/Fasting glucose**	**Insulin tolerance/Fasting insulin**	**Endocrine cell mass/ratios**	**Notes**	**Reference**
Albino Wistar rats	CTR (22% protein, 5% fat, 31% polysaccharide, 31% simple sugars). Maternal obesity (MO) (23.5% protein, 20% animal lard, 5% corn oil, 21% polysaccharide, 21% simple sugars). Diets from P21 to P120, then through pregnancy and lactation. Offspring weaned to CTR.	No differences in plasma glucose levels	In MO male offspring, plasma insulin was higher at P110. Otherwise, no differences. P110 islets secreted less insulin at basal (5 mM) glucose.	MO offspring had higher proportion of β cells (% of total islet cells) at P36, but fewer at P110. MO had higher proportion of α cells at both P36 and P110.	No effect on pup weight, but MO offspring had higher % body fat.	[54]
Wistar rats	CTR (45 g/kg fat); HFD (200 g/kg fat from vegetable oil) for one week before mating, then during pregnancy and lactation. Offspring examined at weaning.	HFD pups had lower plasma glucose at birth, but higher glucose at weaning	No differences in plasma insulin	Not analyzed	Mothers had no difference in weight gain during pregnancy and CTR group ate more kcal/week. HFD offspring weighed more and had more body fat.	[56]
Sprague Dawley rats	CTR (5% fat by weight) or HFD (CTR supplemented with 20% lard by weight).	Trend towards elevated fasting glucose at 6 months in HFD group.	6-month-old HFD offspring had elevated fasting insulin, and 1-year-old offspring were	Not analyzed	On Elecron Microscopy, HFD group β cells had a greater proportion of	[59]

	Fed diet 10 days before pregnancy and during pregnancy and lactation.		insulin resistant (clamp studies). HFD islets secreted less insulin in response to 20 mM glucose and had lower islet insulin content.		immature insulin granules. α-cell granules did not differ between groups. No obvious differences in islet morphology on H&E. HFD offspring had increased abdominal adiposity.	
Animal model	**Diet/Experimental groups**	**Glucose tolerance/Fasting glucose**	**Insulin tolerance/Fasting insulin**	**Endocrine cell mass/ratios**	**Notes**	**Reference**
Wistar rats	Mothers fed high saturated fat (SAFA) or high carb (CHO) diet from 9 to 12 wks, then through gestation and lactation. Offspring were fed either diet to make four groups, plus a fifth that was exposed to SAFA *in utero*, then nursed by CHO moms and maintained on CHO post-weaning. SAFA (60% energy from fat, 25% carbs, 15% protein). CHO (10/75/15).	No differences in plasma glucose levels at 14 wks	No differences in plasma insulin levels at 14 wks. SAFA during gestation was negatively correlated with offspring islet insulin secretion in response to stimuli. SAFA during lactation was positively correlated. No correlation with post weaning diet. No differences in perifusion	Not analyzed	No difference in litter size or average weight at birth. No differences in weight and food intake throughout the study. Another study by the same group showed no differences in insulin sensitivity between groups.	[53]