Fibroblast Growth Factor 21 is a Novel Protein Sensor in Pregnancy

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FIBROBLAST GROWTH FACTOR 21 IS A
NOVEL PROTEIN SENSOR IN PREGNANCY

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
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by
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LIST OF ABBREVIATIONS

ACOG: American College of Obstetricians & Gynecologists

BAT: Brown adipose tissue

BLAST: Basic Local Alignment Search Tool

BMI: Body mass index

CDC: Centers for Disease Control and Prevention

CNS: Central nervous system

DIO: Diet induced obesity

DOHaD: Developmental origins of health and disease

EAR: Estimated average requirement

ELISA: Enzyme linked immunosorbent assay

FFA: free fatty acid

FGF21: Fibroblast growth factor 21

FGFR: Fibroblast growth factor receptor

g: Gram

GLUT1: Glucose transporter 1

HDL: high-density lipoprotein

HIV: Human immunodeficiency virus

HOMA-IR: Homeostatic model assessment- insulin resistance

ICV: Intracerebroventricular

IP: Intraperitoneal

LDL: low-density lipoprotein

MAPK: Mitogen-activated protein kinase
mTORC1: mammalian target of rapamycin complex 1

NEFA: Non-esterified fatty acid

OGTT: oral glucose tolerance test

PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PLH: Protein leverage hypothesis

PPARα: Peroxisome proliferator-activated receptor alpha

PPARγ: Peroxisome proliferator-activated receptor gamma

qPCR: Quantitative polymerase chain reaction

RDA: Recommended dietary allowance

RFPM: Remote food photography methods

RNA: Ribonucleic acid

SDHA: Succinate dehydrogenase complex, subunit A

T2DM: type 2 diabetes mellitus

TBP: TATA-box binding protein

UCP1: Uncoupling protein 1

WAT: White adipose tissue

ZDF: Zucker diabetic fatty
ABSTRACT

The twenty-first century has experienced a shift in cause of death worldwide from communicable diseases to noncommunicable diseases. Interestingly, many of these implicated chronic diseases, such as cancer, diabetes, and cardiovascular disease, have been shown to be programmed in the womb. As first posited by the Barker Hypothesis, adverse exposures in utero can increase an individual’s risk for chronic disease later in life. Therefore, pregnancy is an opportune time for intervention to improve the health of future generations. Studies of exposures known to negatively impact infant health, e.g. states of overnutrition (obesity, diabetes, excess gestational weight gain) and undernutrition (starvation, protein restriction), are critical to reveal the mechanisms of and identify markers for developmental programming. Numerous endocrine signals including insulin, leptin, and adiponectin have been extensively investigated during pregnancy with aberrant effects on offspring growth and metabolic function. A novel endocrine hormone, fibroblast growth factor 21 (FGF21), which has been recently implicated as a signal for protein restriction, has not yet been studied for a potential role in developmental programming of future disease. Therefore, we aimed to investigate the role of FGF21 in pregnancy. We hypothesized FGF21 may be a nutrient sensor and a signal for fetal nutrient insufficiency during pregnancy. In studies of healthy, pregnant women, we found FGF21 was acutely regulated by maternal macronutrient balance. We then found in both mice and human studies that FGF21 is elevated in response to low maternal protein intake in pregnancy. We also showed elevated maternal FGF21 correlated with decreased infant size in the first year of life, an outcome commonly associated with reduced maternal protein intake in pregnancy. Finally, we used the Protein Leverage
Hypothesis to directly test whether FGF21 is indeed a protein sensor in pregnancy and found that FGF21 is required for the hyperphagic response to low protein intake in pregnancy. In summary, these studies support the hypothesis that FGF21 is a protein sensor in pregnancy. Further studies in large clinical populations including fetal growth restriction are needed to discern whether FGF21 could be used as a marker for fetal nutrient insufficiency in the public health setting.
CHAPTER 1: GENERAL INTRODUCTION

1.1. DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

1.1.1. Global Health

The rise in obesity and obesity-related comorbidities is a global epidemic. In 2013, an estimated 37% of men and 38% of women worldwide were overweight or obese (body mass index, BMI ≥ 25kg/m²) (1). In the United States, the Centers for Disease Control and Prevention (CDC) reported an alarming 35% of American men and 40% of American women were obese (BMI ≥ 30kg/m²) in 2013-2014 (2). Consequently, the global population faces higher risks for chronic disease than ever before.

With substantial medical advancements in the treatment of infectious diseases and longer life expectancy, causes for disease and mortality are transitioning. In 2012, the Global Burden of Disease study revealed a shift in causes of disease and mortality from communicable diseases to non-communicable diseases between 1990 to 2010 (3, 4). Across these two decades, deaths by communicable diseases decreased by 17% (15,859,000 in 1990 to 13,156,000 in 2010) while deaths by non-communicable diseases increased by 30% (26,560,000 in 1990 to 34,540,000 in 2010) (5). This rise of nearly 8 million deaths indicates two out of every three deaths in 2010 were due to a non-communicable disease. Three major contributors to this 20-year rise are: a 38% increase in death by cancer, a 31% increase in death by cardiovascular and circulatory diseases, and most alarming a 93% increase in death by diabetes mellitus (5). Moreover, this shift is also attributed to a simultaneous decrease in death by diarrheal disease (-42%), lower respiratory infections (-18%), tetanus (-78%), measles (-80%), and neonatal disorders (-27%) (5).
As individuals live longer with less communicable disease, non-communicable diseases are increasing dramatically; particularly those caused by obesity originating from an obesogenic environment of calorie-rich, nutrient-poor diets and reduced physical activity. In 2010, overweight and obesity caused an estimated 3.4 million deaths and was responsible of 3.9% of years of life lost worldwide (3). Moreover, the incidence of obesity and obesity-related comorbidities has increased globally as demonstrated by the aforementioned drastic increases in death by cancer, cardiovascular disease, and diabetes mellitus (three co-morbidities of obesity) in only the last 20 years (5). Intriguingly, one theory may partially explain the documented rise in non-communicable diseases and introduce opportunities for invention and/or prevention to combat this rapidly growing trend: the Developmental Origins of Health and Disease (DOHaD) Hypothesis, also known as the Barker Hypothesis.

1.1.2. The Barker Hypothesis

The Barker Hypothesis, also referred to as the Developmental Origins of Health and Disease (DOHaD) Hypothesis, posits that exposures during prenatal (\textit{in utero}) or early postnatal development cause permanent changes to the physiology, metabolism, and epigenome of an individual which subsequently will affect their health and increase risk of disease in later life (6, 7). This hypothesis is primarily accredited to the late David Barker from his initial epidemiological studies of infant mortality/size at birth and death by cardiovascular disease. In 1986, Barker and Osmond published a pivotal epidemiological study of the mortality rates by geographical regions of England and Wales which revealed a positive, geographical relationship between death rates by ischemic heart disease during 1968-1978 and infant mortality rates approximately 50 years earlier (1921-1925) (8). As inadequate growth \textit{in utero} and in early life was a primary cause for infant mortality...
in the 1920’s, Barker and Osmond hypothesized inadequate growth early in life left an individual more susceptible to death by ischemic heart disease in adulthood. In 1989, Barker and colleagues published two additional observations in support of this hypothesis: 1) results of a study of 5,654 men born in Hertfordshire, England with recorded birth weights and causes of death found the highest rates of death by ischemic heart disease were in men who were smallest at birth and at one year of age (9) and, 2) an inverse relationship between birth weight and systolic blood pressure, independent of gestational age at birth (9) from a study of 9,921 ten-year-olds and 3,259 adults born in Britain with recorded birth weights and blood pressure. While Barker is credited by most for founding the DOHaD field, ten years prior to Barker’s initial observations, Ravelli et al. showed increased obesity in 19-year-old men who were exposed to the Dutch Famine early \textit{in utero} (first half of their mother’s pregnancy) (10). Moreover, this study showed a timing-specific exposure effect as significantly lower obesity rates were observed if famine exposure occurred later \textit{in utero} (second half of their mother’s pregnancy). Barker and Ravelli’s findings began what has grown into a vast field for researching and understanding the impact of early life exposures on disease risk in adulthood. Such early life exposures include, but are not limited to, under-nutrition, over-nutrition, malnutrition; teratogens including pollutants, drugs, alcohol, etc.; altered hormonal milieu resulting from maternal overweight, obesity, excess gestational weight gain, diabetes mellitus; maternal stress; oxidative stress from hypertension or placenta insufficiencies.

Thus far, evidence in support of the DOHaD hypothesis has been demonstrated through epidemiological, prospective, and interventional studies in animal models and humans. Early epidemiological work identified the relationship between intrauterine
exposures such as undernutrition or famine and development of disease including obesity (11, 12) and coronary heart disease (9, 13) in adulthood. Follow up studies have further characterized low birth weight (considered as a result of early life adverse exposure) to be a risk factor for adult chronic diseases, including impaired glucose tolerance, hypertension, cardiovascular disease and obesity (14-17). For example, a prospective study of 468 men born in Hertfordshire, England with known birth weights and glucose tolerance measured in adulthood (between 59 and 70 years old) found adult blood pressure and glucose tolerance measurements rose as birth weights fell (15). Other epidemiological and prospective studies of cohorts such as those in Uppsala, Sweden, Helsinki, Finland, and the Nurse’s Health Study, USA have also revealed correlations between small size at birth and the development of diabetes (18-23), cardiovascular disease (24-32), unfavorable alterations in body composition (33, 34), and hypertension (35, 36) later in life. Yet, while the observed association between low birth weight and adult chronic disease has been reproduced in many cohorts around the world, this supportive evidence for the DOHaD hypothesis relies heavily on the assumption that infant growth/size at birth is an indicator of adverse exposures in utero. Addressing this weakness, the DOHaD has also been supported by epidemiological studies in populations with known rather than assumed exposures, as documented in Ravelli’s study (10). For instance, studies of individuals exposed to the Dutch Famine in utero between December 1944 and April 1945, when the daily ration for adults was 400-800 calories, have observed direct relationships between famine exposure in utero (resulting in low birth weights) and the development of diabetes (37), high blood pressure (38), and unfavorable body composition (39) in the offspring later in life.
More recently, the ability of researchers to obtain longitudinal data from individuals within large cohort studies with known *in utero* exposures has proven to substantially advance the field. Data collected in addition to the important timing of conception include biospecimens allowing for the study of epigenetics (40, 41) and prospective assessments of body composition, blood pressure, cardiovascular risk factors, food intake, medical history and glucose tolerance. The Quebec Ice Storm, that occurred in 1998 and led to a loss of electrical power for up to 6 weeks in 1,400,000 households, provided a unique opportunity for longitudinal data collection from offspring of a known *in utero* exposure, i.e. objective and/or subjective maternal stress (42). Offspring of mothers who were pregnant during the ice storm have been studied extensively throughout life and have been shown to have adverse changes in growth and metabolism. These changes included smaller birth size (43), increased obesity in adolescence (44), increased insulin secretion (45), increased DNA methylation (46), and altered mental capacities with reduced IQs (47), lower motor scores (48), and higher Autism Spectrum scores (49). Observational and interventional clinical trials of populations experiencing adverse intrauterine exposures also contribute to the understanding of developmental programming. Gambian women have been part of such research because The Gambia experiences two agricultural and thus nutritional seasons annually; the rainy (hungry) season and the dry (harvest) season, leading to fluctuation in nutritional status. Research groups have established relationships with communities in The Gambia, and clinical trials to study the effects of nutritional status in this population (50) particularly in childbearing women. These studies have been ongoing since the 1970's. Finally, use of animal models permit induction of exposures during gestation (which would be unethical in humans)
allowing human intrauterine exposures and subsequent investigations of causative mechanisms to be tested. Various species, including mice, rats, guinea pigs, sheep, and non-human primates, have been used and their advantages and disadvantages are eloquently reviewed by Rabadan-Diehl and Nathanielsz (51). The totality of all the above mentioned models, epidemiological, longitudinal, observational, interventional, and animal studies, has allowed for investigation and the forward progress of the DOHaD field over the past 30 years.

1.1.3. Mechanistic Application of the Barker Hypothesis

The epidemiological and prospective studies supporting the DOHaD hypothesis have spurred investigations into causative mechanisms to explain how exposures in utero translate into permanent physiological changes in offspring. From these investigations, the genesis of the maternal-placental-fetal communication field of study has demonstrated cause-and-effect relationships between maternal circulatory factors and fetal outcomes. For example, maternal nutrient availability is a classic example of maternal-placental-fetal communication, as extensively studied and reviewed by Powell and Jansson (52). Conditions of nutrient scarcity or excess in the maternal circulation, such as hyperglycemia or amino acid deficiency, have been shown to correlate with alterations in respective placental nutrient transporter expression and activity, e.g. placenta of mothers with hyperglycemia express higher levels of glucose transporters compared to normoglycemic placentas. Consequently, these alterations in placenta nutrient transport coincide with the subsequently observed fetal over- and undergrowth respectively (52). Another example of this maternal-placental-fetal communication is the role of the maternal circulatory factor adiponectin in the fetal overgrowth phenotype observed with obese pregnancies. Adiponectin, a beneficial adipokine that acts as an
insulin sensitizer to improve glucose clearance, is elevated in healthy individuals and found to be reduced with overweight or obesity (53-55), including in the pregnant population (56-59). An elegant study by Aye et al. demonstrated a direct cause-and-effect role for maternal adiponectin in the overgrowth phenotype typical of offspring of obese pregnancies (60). Typically, in diet-induced obesity (DIO) C57BL/6 pregnancies maternal adiponectin concentrations are decreased and placental signaling (insulin and mTORC1) is increased leading to up regulation of placenta nutrient transport and subsequently fetal overgrowth. Investigators rescued this fetal overgrowth phenotype by replacing the reduced circulating adiponectin by adiponectin infusion. This in turn reversed the increase in placental signaling and nutrient transport and corrected the overgrowth phenotype of the offspring (60). This study exemplifies the capacity of the maternally cultivated intrauterine environment to directly impact fetal development. However, innumerable maternal factors are differentially regulated in and by pregnancy, e.g. hormones, blood flow, energy balance, stress, etc., and only with in-pregnancy studies can their role as mediators of the DOHaD hypothesis be elucidated. Undoubtedly, these examples should provide rationale for continued investigations into the role of maternal nutritional and endocrine factors as mechanisms of the “developmental programming” posited by the DOHaD hypothesis.

In summary, with the current rates of obesity and obesity related co-morbidities, our population is being conceived and developing within increasingly adverse intrauterine environments. Considering the extensive supporting evidence for the DOHaD hypothesis, this progressively hostile in utero development is contributing to the increased incidence of non-communicable diseases currently observed in adults worldwide. Indeed, three of
the largest contributors to the increase in non-communicable disease mortality are cancer, cardiovascular disease, and diabetes mellitus; and each of these chronic diseases have been repeatedly shown in human and animal studies to be “programmed” in adulthood by adverse exposures in utero. As chronic disease continues to increase and people continue to reproduce, future generations will not only develop within adverse intrauterine environments but grow in postnatal environments that provide continued exposures for metabolic disease. In turn, offspring will likely show evidence of chronic diseases earlier in life and as adults, will then conceive and program the subsequent generation with potentially increasingly hostile intrauterine environments. The cyclical pattern of programmed chronic disease across generations epitomizes the importance for studying the DOHaD hypothesis. Increasing our understanding of the mechanisms that transmit these increased chronic disease risks could allow for proactive intervention in adulthood of exposed offspring, as well as preventive intervention during pregnancy.

1.2. FIBROBLAST GROWTH FACTOR 21

Fibroblast growth factor 21 (FGF21) is a 19kDa, 209 amino acid long peptide produced by the liver, white and brown adipose tissue, muscle (smooth and skeletal), thymus, and endocrine and exocrine pancreas in humans. FGF21 is a member of the “endocrine” subgroup of the fibroblast growth factor family, along with FGF19 and FGF23, grouped together based on their similar structural homology and ability for endocrine action. FGF21 was discovered in 2000 and reported as a potential novel therapeutic for obesity and improved glucose and lipid regulation in 2005. Since that time, great effort has been expended to elucidate the metabolic effects of FGF21 at the molecular and whole-body level, to identify its mechanism(s) of action, and explore its therapeutic
potential. As a result, publications relating to FGF21 have increased nearly exponentially each year since 2005 (61) (Figure 1.1).

Figure 1.1 FGF21 publications from 2000 to 2015. “Results by Year” exported from PubMed.gov searched with title keywords: “FGF21”, “FGF-21”, and “Fibroblast growth factor 21” (accessed December 8, 2016).

However, the research in the past decade has produced a complex and conflicting body of literature, and thus, herein is a comprehensive summary of the current understandings of FGF21, its capabilities, and suggested reasoning for conflicting results. The overview will first describe the discovery of FGF21 which has shaped the subsequent decade of research. Next, to better elucidate the physiological role of FGF21, cross-sectional observations of FGF21 concentrations among various populations, phenotypes of mouse models overexpressing or lacking FGF21, as well as effects of pharmacological administration in animal models and humans will be presented. Finally, to better understand the actions of FGF21, a description of the induction and regulation of this novel hormone and tissue specific effects and mechanisms of action observed thus far will be presented.
1.2.1. Discovery

FGF21 was first identified in 2000 by Nishimura and colleagues from isolated cDNA of mouse embryo (62). Murine FGF21 has 75% homology with human FGF21 and 35% homology with human FGF19. At the time of its identification, FGF21 was reported to be preferentially expressed by the liver (62). Five years later, FGF21 was revealed to have an adipocyte-specific glucose sensitizing capability (63). In this initial study, FGF21 induced glucose uptake in vitro in 3T3-L1 adipocytes and primary human adipocytes, an action that was both insulin independent and additive (63). When administered to obese rodents (ob/ob mice, db/db mice, and obese Zucker diabetic fatty (ZDF) rats), FGF21 reduced plasma triglycerides, decreased fasting insulin, and improved glucose clearance in vivo. Investigators also developed a transgenic mouse model that overexpressed human FGF21 using the apoE promoter (63). At maturity (9 months), the FGF21 transgenic mice showed decreased body weight, decreased hepatic lipid, and increased brown adipose tissue, as well as decreased fastenot sud glucose and improved glucose clearance and insulin sensitivity compared to age matched, wild type mice. On high-fat, high-carbohydrate diets, FGF21 transgenic mice were resistant to diet-induced obesity. Notably, the plasma concentration of FGF21 in a healthy wild type mouse is approximately 1 ng/mL while in this transgenic strain the plasma concentration is 70-150 ng/mL. Although these results are very interesting, the physiological relevance of these observations is not clear.

Important considerations when contemplating the therapeutic potential of FGF21 have also been assessed. Investigators evaluated the mitogenic effects of FGF21 by treating cells in culture, including murine, nonhuman primate, and primary human cell lines, with FGF21 and showed treatment did not induce cell proliferation. Moreover, a
concern of any glucose sensitizing therapeutic is its ability to induce hypoglycemia, though no evidence of hypoglycemia in the fed or fasted state following FGF21 administration was observed in obese and lean ZDF rats. To investigate mechanism(s) of action of FGF21, Kharitonenkov et al. observed glucose sensitizing effects of FGF21 were greatly diminished with cycloheximide treatment, leading to the postulation that the mode(s) of action requires transcriptional/translational activation (63). Consequently, treatment with FGF21 was found to increase GLUT1 expression (mRNA and protein) in white adipose tissue in vitro and in vivo and induce phosphorylation of MAPK through FRS-2 in 3T3-L1 adipocytes (63). In summary, this initial publication showed FGF21 plays a role as a metabolic regulator affecting glucose and lipid metabolism in vitro and when overexpressed or administered in vivo has both genomic and non-genomic actions on adipose tissue.

1.2.2. Physiological Role of FGF21

*FGF21 in the Population:* The first observation of FGF21 in humans was described in patients with type 2 diabetes mellitus (T2DM) (64). Paradoxical to its glucose and lipid regulatory effects, plasma FGF21 was increased in patients with diabetes compared to non-diabetic controls. Following that report, elevated serum FGF21 was observed in overweight and obese patients compared to lean controls and positively correlated with risk of metabolic syndrome, adiposity, fasting insulin, and triglycerides (n=232) (65). Investigators also found FGF21 expression in subcutaneous adipose tissue correlated with serum FGF21 in a subset of this population (n=29), giving rise to FGF21 as an adipokine. Introducing more complexity, Galman et al. reported that circulating FGF21 varied nearly 250-fold (454 ± 799 pg/mL) within a broad human population of 76 healthy males and females and failed to find relationships between FGF21 and BMI, lipids, or
plasma glucose (66). Since these early publications, FGF21 has been consistently observed to be elevated in patients with type 2 diabetes mellitus, dysregulated glucose homeostasis, adiposity, and an adverse metabolic phenotype (Table 1.1). Indeed, prospective, population-based studies across 5-years of observation have shown that FGF21 is a predictor of the development of metabolic syndrome [odds ratio 2.6 (67)], type 2 diabetes mellitus [odds ratio 1.8 (68)] and obesity [odds ratio 2.4 (67)].

Table 1.1 FGF21 trends in clinical populations

<table>
<thead>
<tr>
<th>FGF21 Trend (compared to healthy population)</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced</td>
<td>Type 1 diabetes mellitus (69) Latent autoimmune diabetes (69)</td>
</tr>
<tr>
<td>Elevated</td>
<td>Type 2 diabetes mellitus (64, 68-74) Overweight/Obesity (65, 70, 72, 75-78) Gestational Diabetes Mellitus(79, 80) NAFLD/NASH (75, 81) Coronary heart disease (74) Polycystic ovary syndrome (82) Chronic kidney disease (83)</td>
</tr>
<tr>
<td>No change</td>
<td>Overweight/Obesity (84) Gestational Diabetes Mellitus (85, 86) Preeclampsia (78)</td>
</tr>
</tbody>
</table>

Apart from clinical conditions summarized in Table 1.1, FGF21 is correlated with independent adverse markers of metabolic health, e.g. hyperinsulinemia, hyperglycemia, and hypertriglyceridemia (Table 1.2). These observations suggest obesity and its co-morbidities may induce a state of FGF21 resistance, similar to hyperinsulinemia or hyperleptinemia. Indeed, elevated FGF21 concentrations in ob/ob, db/db, and diet-induced obese (DIO) mice have been repeatedly reported (65, 87, 88).
Table 1.2 Correlation of FGF21 with markers of metabolic health

<table>
<thead>
<tr>
<th>FGF21 Correlation</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose Homeostasis</strong></td>
<td></td>
</tr>
<tr>
<td>Positive correlation</td>
<td>Fasting insulin (65, 71, 73, 74, 77)</td>
</tr>
<tr>
<td></td>
<td>Fasting glucose (70, 71, 74)</td>
</tr>
<tr>
<td></td>
<td>HbA1c (71, 73)</td>
</tr>
<tr>
<td></td>
<td>HOMA-IR (71, 73, 74, 77, 78, 85)</td>
</tr>
<tr>
<td>Negative correlation</td>
<td>Glucose tolerance (70, 89)</td>
</tr>
<tr>
<td></td>
<td>Insulin sensitivity (70)</td>
</tr>
<tr>
<td>No correlation</td>
<td>Fasting insulin (84)</td>
</tr>
<tr>
<td></td>
<td>Fasting glucose (84)</td>
</tr>
<tr>
<td></td>
<td>Insulin sensitivity (89)</td>
</tr>
<tr>
<td></td>
<td>Insulin secretion (89)</td>
</tr>
<tr>
<td><strong>Lipid homeostasis</strong></td>
<td></td>
</tr>
<tr>
<td>Positive correlation</td>
<td>Adiposity (65, 77, 89)</td>
</tr>
<tr>
<td></td>
<td>Triglycerides (65, 74, 80, 85, 89)</td>
</tr>
<tr>
<td></td>
<td>Total cholesterol (84, 89)</td>
</tr>
<tr>
<td>Negative correlation</td>
<td>HDL (65, 74, 85)</td>
</tr>
<tr>
<td></td>
<td>Adiponectin (77, 80, 85)</td>
</tr>
<tr>
<td>No correlation</td>
<td>HDL (84)</td>
</tr>
</tbody>
</table>

*Determined by hyperinsulinemic euglycemic clamp

Considered together, it could be suggested that the patterns of FGF21 observed in the metabolically unhealthy clinical populations cited in Table 1.1 are likely due to the imbalance of the metabolic markers that commonly accompany the clinical conditions cited in Table 1.2. In other words, FGF21 is not likely a product of obesity or type 2 diabetes but rather a product of the elevated triglycerides or glucose created by these pathological conditions.

**Phenotypes of Transgenic FGF21 Mouse Models:** A powerful method to better understand the function of a hormone is to create transgenic mouse models with the goal to either overexpress or reduce the hormone of interest in vivo. Multiple FGF21 transgenic mouse models have been developed over the past decade and their phenotypes are described below. As discussed earlier, transgenic FGF21-overexpressing mice (FGF21-TG^A^) exhibit a >50-fold increase in circulating FGF21 compared to control mice. FGF21
Transgenics are leaner than wild type controls, have increased brown adipose tissue, reduced subcutaneous adipocyte cell size, and are resistant to diet induced obesity (63). Furthermore, these mice have reduced fasting glucose levels, increased insulin sensitivity, and improved glucose clearance (63). A separate transgenic line (FGF21-TG\textsuperscript{B}) generated by Steve Kliwer's laboratory exhibit a more modest, 5-fold elevation of plasma FGF21 and has been phenotyped extensively (90-93). Similar to the FGF21-TG\textsuperscript{A} strain, the FGF21-TG\textsuperscript{B} mice are smaller than wild-type controls, though they exhibit increased adiposity and food intake normalized to body weight, suggesting FGF21 inhibits growth without inducing hypophagia. FGF21-TG\textsuperscript{B} mice have an extended lifespan (69) and show a metabolic profile similar to the FGF21-TG\textsuperscript{A} strain, including decreased serum glucose, insulin, triglycerides, and cholesterol, and decreased hepatic lipid. Considered together, FGF21-TG mice appear to exhibit a phenotype mimicking long term fasting: elevated free fatty acids (accompanied by elevated white adipose tissue lipase protein expression) and \(\beta\)-hydroxybutyrate in fed conditions, increased ketogenesis, impaired glycogenolysis and increased hepatic glycogen content. Moreover, during extended fasting, the FGF21-TG mice show reductions in physical activity and body temperature, and enhanced torpor compared to wild-type controls (90, 92). The phenotype(s) resulting from transgenic FGF21 overexpression illuminate a role for FGF21 in metabolic regulation, however these findings do not appear to be in agreement with the trends of FGF21 observed in the human population. It is intriguing to consider that in a transgenic animal model of elevated FGF21 that FGF21 appears to be responsible for lowering many of the adverse metabolic markers it is positively associated with in humans, e.g. glucose, insulin, triglycerides. Moreover, when considering evidence that FGF21 is highly variable in studies of healthy
participants (66), perhaps the role and regulation of FGF21 is reliant and complicated by energy and macronutrient status.

Notably, these transgenic strains have also revealed various untoward effects of FGF21. First, consistent with their reduced size, adverse effects from FGF21 overexpression is bone loss (93) and growth hormone resistance (94). FGF21-TG^B mice show decreased bone lengths and significant decreases in bone mass (bone mineral density, bone volume/tissue volume, bone surface, trabecular number and thickness) (93). Induction of growth hormone (GH) resistance is also a proposed adverse effect of FGF21 overexpression. FGF21-TG^B mice show elevated levels of GH but decreased circulating insulin-like growth factor-1 (IGF-1), a finding consistent with their small size. Finally, female FGF21-TG mice were shown to be infertile through hypothalamic-pituitary-ovarian axis responses similar to those induced by starvation (94). However, recent findings have shown this infertility may be driven by energy balance because it was shown to be restored with a high fat diet (95). These adverse effects should be taken into consideration when contemplating future applications for FGF21 as a pharmaceutical agent.

While the gain-of-function strains demonstrate a fairly consistent role of FGF21 as a metabolic regulator, loss-of-function models are less cohesive. The first loss-of-function model, using an adenoviral-mediated FGF21 knockdown (KD), found mice fed a ketogenic diet with a 72% knock-down of FGF21 displayed a dysfunctional lipid phenotype: increased serum cholesterol, triglycerides and non-esterified fatty acids (NEFAs), elevated hepatic lipid, and reduced hepatic ketogenic-related gene expression (96). A global FGF21 knock out (FGF21-KO^A) mouse confirmed these findings (97).
FGF21-KO\textsuperscript{A} mice have normal fasting and fed glucose and insulin levels but display impaired glucose homeostasis in response to a glucose tolerance test compared to wild-type mice. When challenged by a ketogenic diet, FGF21-KO\textsuperscript{A} mice exhibit increased weight gain and decreased physical activity; increased leptin, triglycerides, insulin, and glucose; as well as decreased ketosis and elevated hepatic glycogen content and hepatosteatosis (2-fold increase hepatic lipid) compared to wild-type mice also fed a ketogenic diet. In contrast to the lean, DIO-resistant phenotype of the FGF21-TG, these FGF21-null mice have increased body weight, lean mass, fat mass, and food intake with age, as well as an altered adipose phenotype with increased adipocyte cell size, decreased WAT expression of lipolytic genes, and increased BAT lipid stores. FGF21-KO\textsuperscript{A} mice also show attenuated weight loss following a 24hr fast, indicating mice without FGF21 have an inefficient ability to mobilize energy.

The FGF21-KO\textsuperscript{B} developed by Potthoff and colleagues replicated some of the abovementioned findings (92). Compared to wild-type controls, the FGF21-KO\textsuperscript{B} transgenic model exhibited reduced fasting glucose, elevations in triglycerides and non-esterified fatty acids (NEFAs) in the fasted state, elevated ketones in the fed state, as well as impaired ketogenesis. These mice did not differ in weight or fed glucose, insulin, triglycerides, or NEFA levels, from wild-type controls. Further studies showed a reduction in expression of key hepatic genes responsible for regulation of hepatic glucose and lipid metabolism in response to fasting in FGF21-KO\textsuperscript{B} versus wild-type mice. Complementing the loss of bone mass observed in FGF21-TG\textsuperscript{B} mice, FGF21-KO\textsuperscript{B} mice have high bone mass (93).
Table 1.3 Summary of FGF21 transgenic mouse models

<table>
<thead>
<tr>
<th>FGF21 Model</th>
<th>FGF21 Levels</th>
<th>Phenotype</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF21-TG&lt;sup&gt;A&lt;/sup&gt;</td>
<td>70-150 ng/mL</td>
<td>↓ body weight/size, ↓ fasting glucose, ↑ insulin sensitivity</td>
<td>-C57BL/6 background</td>
</tr>
<tr>
<td>Kharitonenko (63)</td>
<td></td>
<td></td>
<td>-Gene: human FGF21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Promoter: ApoE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Primary source: liver</td>
</tr>
<tr>
<td>FGF21-TG&lt;sup&gt;B&lt;/sup&gt;</td>
<td>20 ng/mL</td>
<td>↓ body weight/size, ↑ food intake, ↑ fat mass, ↑ lifespan, ↓ glucose (fasting), ↓ triglycerides (fasting), ↓ cholesterol, ↑ FFA (fed), ↑ β-hydroxybutyrate (fed), ↑ gluconeogenesis (fed), ↑ ketogenesis</td>
<td>-C57BL/6 background</td>
</tr>
<tr>
<td>Inagaki (90)</td>
<td></td>
<td></td>
<td>-Gene: murine FGF21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Promoter: ApoE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Primary source: liver</td>
</tr>
<tr>
<td>FGF21-KD</td>
<td>Undetectable</td>
<td>↑ body weight, ↑ fat mass, ↑ lean mass, ↓ glucose tolerance, X fasting response</td>
<td>C57BL/6 injected with shFGF21 or control adenovirus</td>
</tr>
<tr>
<td>Badman (96)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF21-KO&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Undetectable</td>
<td>- body weight, ↓ fasting glucose, ↑ ketones (fed state), X ketogenesis</td>
<td>-Targeted deletion of 3′ part of exon 1, all of exon 2, and the 5′ region of exon 3.</td>
</tr>
<tr>
<td>Badman (97)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF21-KO&lt;sup&gt;B&lt;/sup&gt;</td>
<td>Undetectable</td>
<td>↑ body weight, ↑↓ fat mass, ↑ bone mass, ↑ lean mass</td>
<td>-FGF21loxP x Meox-cre deletion</td>
</tr>
<tr>
<td>Potthoff (92)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF21-KO&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Undetectable</td>
<td>↑ adipocyte hypertrophy, ↓ adipocyte lipolysis (fed), ↓ NEFAs (fed), ↑ adipocyte lipolysis (fasted), ↑ NEFAs (fasted)</td>
<td>-Targeted deletion: majority of exon 1 and all of exons 2 and 3 of Fgf21 were replaced with the IRES-LacZ-polyA/PGK-neo cassette (606 of 630 bases)</td>
</tr>
<tr>
<td>Hotta (98)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X = dysregulated or dysfunctional, <sup>C</sup> = conflicting reports

A third global FGF21-KO<sup>C</sup> strain generated by Hotta et al. does not replicate the impaired hepatic ketogenesis or lipid metabolism that had been previously observed (98).
However, the FGF21-KO<sup>C</sup> mice show impairments in adipose tissue lipolysis. FGF21-KO<sup>C</sup> mice show decreased adipocyte lipolysis and decreased circulating NEFAs in the fed state and vice versa in the fasted state, indicating FGF21 plays a role in regulating lipolysis (stimulating lipolysis during feeding and inhibiting lipolysis during fasting).

Studies of these loss-of-function models contribute to our understanding of the role and regulation of FGF21, particularly when considered alongside the previously described overexpressing models and human population trends. Taken together, these gain- and loss-of-function models show FGF21 plays a role in regulation of metabolic processes, particularly in response to fasting, such as ketogenesis, lipolysis (hepatic fat oxidation), and physical activity. It also appears FGF21 is required for maximal hepatic lipid oxidation and ketogenesis induced by a ketogenic diet. Notably, the phenotypes of the three knockout models are much less cohesive compared to the reproducible findings across the transgenic strains. Moreover, the findings from transgenic overexpression of FGF21 are not consistently inversely reproduced as may be expected. However, the variability in reports of whole-body effects from a lack of FGF21 lead to important considerations for FGF21 biology. First, cause-and-effect conclusions in loss-of-function experiments can be more difficult to come to because whole-body physiology demands a rigorous redundancy built into life sustaining processes. If the role of FGF21 is considered be a metabolic regulator, it is biologically unreasonable to expect for it to be the sole hormone responsible for effects observed in the transgenic mice, e.g. glucose metabolism, lipid metabolism, energy expenditure regulation, etc. Second, many of the phenotypic effects observed in FGF21-KO mice only resulted from studies of “challenged” mice, i.e. mice on ketogenic diets, extended fasts, or advanced age. This is an intriguing observation when
considering the disparities between effects of elevated FGF21 in healthy, transgenic mouse stains and in metabolically unhealthy human populations. Perhaps, the differential roles and regulation of FGF21 described in “challenged”, i.e. unhealthy, versus healthy populations indicate various responsibilities and responses of FGF21 which are reliant on health status.

Effects of FGF21 Administration: Another useful model for elucidating the effects of an endocrine hormone is exogenous administration in vivo. The response to recombinant FGF21 administered in acute and chronic protocols has been observed in healthy, obese, and diabetic rodents, as well as in obese/diabetic non-human primates, and most recently in humans with obesity and diabetes. These findings are collated and summarized below (Table 1.4). As soon as two days following FGF21 administration (measured up to 8 weeks of continuous FGF21 infusion) to rodent models of diabetes and obesity (db/db mice, ob/ob mice, DIO mice, and ZDF rats), animals displayed decreased fasting and fed glucose, fasting insulin, and triglycerides, improvements in glucose tolerance and hepatosteatosis, and increases in energy expenditure (Table 1.4). Similarly, in non-human primates, FGF21 administration decreased fasting glucose, triglycerides, LDL-cholesterol, and body weight while increasing HDL-cholesterol. Interestingly, neonatal pups administered FGF21 show increased expression of thermogenic genes in BAT and increased body temperature (99). These observations were supported by elevated thermogenic gene expression (UCP1, DIO2) in BAT and even more so in iWAT of C57BL/6 adult mice (100). Proposed mechanisms accounting for these metabolic improvements observed with exogenous FGF21 administration include: upregulation of GLUT1 expression in WAT shown in in vitro and in vivo studies
(63), induction of adiponectin (101), increased insulin production, and altered expression of key genes involved in ketogenesis, lipid metabolism, and gluconeogenesis (See Action of FGF21 section below) (decreases in ACC1, ACC2, SCD1, GPAT, FAS, nuclear SREBP-1, PPARγ, ap2, CD36; increases in G6Pase, PEPCK, PGC1α, AMPKα1, CPT1α, CPT1β, FoxA2, BDH1, UPC2; no change in DGAT1, CPT1, LPAAT, PGC1α) (102-106).

Table 1.4: Effects of FGF21 administration in animal models and humans

<table>
<thead>
<tr>
<th>Effect</th>
<th>Healthy rodents</th>
<th>Obese/diabetic rodents</th>
<th>Diabetic nonhuman primates</th>
<th>Humans with diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased fasting glucose</td>
<td></td>
<td>(63, 102-104, 107-110)</td>
<td>(111)</td>
<td></td>
</tr>
<tr>
<td>Decreased fasting insulin</td>
<td>(105)</td>
<td>(103, 104, 107-109)</td>
<td>(108, 111)</td>
<td>(112)</td>
</tr>
<tr>
<td>Decreased fed glucose</td>
<td></td>
<td>(107, 110)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Improved glucose tolerance/Si</td>
<td>(108, 110)</td>
<td>(103, 104, 106-108, 110)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased lipids*</td>
<td>(105)</td>
<td>(63, 103, 104)</td>
<td>(108, 111)</td>
<td>(112)</td>
</tr>
<tr>
<td>Improved cholesterol***</td>
<td></td>
<td>(104, 108, 109)</td>
<td>(111)</td>
<td>(112)</td>
</tr>
<tr>
<td>Improved liver health****</td>
<td></td>
<td>(102, 104, 107)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased EE</td>
<td>(106)</td>
<td>(102, 104, 106, 109)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Si= insulin sensitivity, EE= energy expenditure
*triglycerides, FFAs, or NEFAs **Reduction in body weight or resistance to DIO weight gain; ***decrease in total cholesterol, LDL, or VLDL; or increase in HDL; ****reduction in hepatic lipid, hepatosteatosis

In summary, FGF21 administration has been demonstrated to improve body weight status, regulate lipid and glucose homeostasis, and increase energy expenditure in diabetic and obese rodent models (ob/ob mice, db/db mice, DIO mice, ZDR) as well as non-human primates and humans with obesity and/or diabetes. Furthermore, some of
these effects, e.g. decreased insulin, improved glucose tolerance, weight loss, and increased energy expenditure, have been demonstrated in healthy, lean mice as well. Notably, while effects of exogenous administration can illuminate the role(s) of a hormone, it is important to remain skeptical of the physiological relevance at pharmacological doses. It is encouraging however that these administration studies reproduced several effects of FGF21 observed in studies of transgenic, overexpressing mice. Finally, the differential effects of FGF21 administration in unhealthy, obese or diabetic models compared to healthy models further supports the observations in the transgenic, knockout, and population trend studies that the role and regulation of FGF21 may be reliant on energy or macronutrient status.

1.2.3. Regulation of FGF21

FGF21 is produced by the liver (62, 113-115), white adipose tissue (65, 113, 116), brown adipose tissue (113, 117, 118), muscle (119), and the endocrine and exocrine pancreas (113, 120, 121). An elegant study by Markan et al. employed a liver specific FGF21 knockout mouse model (FGF21^{fl/fl;Albumin-Cre}) to show the liver is the primary source of circulating FGF21 in vivo (115). Both fasting and refeeding studies in these FGF21 liver-specific knock out mice showed circulating FGF21 levels were abolished, despite FGF21 mRNA expression being present in other expected tissues. Commensurate with the numerous tissues which express FGF21, many mechanisms have been described with regard to regulation of its expression which contributes to the complexity of FGF21 biology. The diet is the most common known regulator of FGF21. In circulation, FGF21 has been shown to be elevated in conditions of fasting (66, 115, 122), ketogenic diets (122), amino acid deprivation or low protein diets (123-129), obesity and diabetes (88), and cold-exposure (117). As PPARα is expected to be elevated in many of the
abovementioned states (fasting, ketogenic diets, obesity), PPARα agonist administration has also been shown to increase circulating FGF21 (66, 72, 122, 130). Current tissue specific regulatory mechanisms are considered below.

Liver: In vitro studies of hepatocytes and in vivo studies of the liver have shown FGF21 to be regulated by fasting (90, 96, 116, 122, 127, 131-133), refeeding (76, 134), ketogenic diets (96, 122), amino acid deprivation/low protein diets (123-127), and obesity (65, 88, 131, 135). Pregnancy has also been shown to be a time of elevated hepatic FGF21 expression (114). Upstream hepatic molecular regulators that have been identified thus far include PPARα (90, 96, 116, 122, 131), CREB-H (132, 136), ATF4 (125, 126), eIF2 (125), and SIRT-1 (137).

Adipose: In vitro studies of white adipocytes and in vivo studies of WAT have shown FGF21 is regulated by fasting (122, 133), refeeding (116), ketogenic diets (116), obesity (65, 72) and cold-exposure (100). Key upstream WAT regulators of FGF21 expression include PPARγ (65, 72, 122) and SIRT-1 (65). In BAT, FGF21 is also regulated by cold-exposure (100, 117, 138), and downstream of PPARα (117) and β3-adrenergic stimulation (100, 117, 138). In muscle, FGF21 expression is induced by mitochondrial stress (139, 140).

Understanding the mechanisms of a hormone’s regulation can help illuminate its physiological role(s). It is important to consider that the two organs responsible for the majority of FGF21 peptide found in circulation, the liver and adipose tissue, are also two of the primary organs responsible for energy storage and mobilization, and thus energy sensing. Considering the suggested role of FGF21 as a metabolic regulator and its
potential sensitivity to energy status, it is logical that the production and secretion of FGF21 would rely on these organs and variables under different provocations.

1.2.4. Actions of FGF21

*FGF21 action is reliant on cell surface co-factor β-Klotho:* Traditionally, fibroblast growth factor proteins signal extracellularly through cell-surface fibroblast growth factor receptors (FGFRs). FGFRs are ubiquitously expressed in one of four common isoforms (FGFR1, 2, 3 and 4) (141). However, the structure of FGF21 (along with FGF19 and FGF23) has a unique C terminus missing a binding domain other FGFs rely on to bind to FGFRs. The absence of this binding domain disables FGF21 from binding efficiently to FGFRs which suggests the need of additional cell surface interaction(s) for effective FGF21 signaling. FGF21 requires a co-factor, β-Klotho, to securely bind and activate FGFR (142). Micanovic et al. confirmed the C terminus is responsible for the interaction between FGF21 and β-Klotho (143). This co-factor necessity confers signaling specificity of FGF21; only tissues that express β-Klotho respond to FGF21.

In early *in vitro* studies, fibroblasts (cells without β-Klotho) were shown to be unresponsive to FGF21 (144, 145), while treating Baf3 cells (cells typically without FGFRs and Klotho isoforms) with β-Klotho and FGFR1 knock-in with FGF21 can activate FGFR1 intracellular receptor signaling (146). siRNA knock-down of β-Klotho in 3T3-L1 adipocytes resulted in attenuation of FGF21 downstream signaling and effects (142, 147), while FGF21-mediated improvements in metabolism are elusive in global β-Klotho knock-out mice (109, 148). Moreover, a β-Klotho agonist (mimAb1) conferred the same beneficial FGF21-mediated effects when administered to non-human primates (149). These
investigators also found ablation of FGF21-mediated improvements in an FGFR1 knock-out mouse model, indicating FGF21 signals primarily through the FGFR1 isoform in conjunction with β-Klotho. Indeed, β-Klotho knock-in, knock-down, and global/tissue specific β-Klotho knock-out models have been essential in understanding the actions of FGF21.

*Tissue Specific Actions of FGF21:* As detailed above, β-Klotho expression confers the responsiveness of a given tissue to FGF21, and tissues known to express β-Klotho are the liver (113), white and brown adipose tissues (105, 142, 147) (113, 150), hypothalamus (151, 152), and the exocrine and endocrine pancreas (113). The tissue-specific effects of FGF21 are organized below by tissue of action.

*Adipose tissue:* The currently known primary actions of FGF21 on adipose tissue known thus far are: increased glucose uptake (63, 104), insulin sensitivity (104), energy expenditure (99), and browning (100). Indeed, the initial breakthrough for a role of FGF21 as a metabolic regulator was in murine and human white adipocytes, where it was shown to upregulate expression of the glucose uptake gene GLUT1 (63). Subsequently, in WAT, FGF21 has been shown to also effect expression of genes involved in lipid metabolism (lipogenesis and lipolysis) and browning (UCP1, DIO2, PGC1α, CIDEA Cox7a) (100, 124, 153). *In vivo* treatment with FGF21 in C57BL/6 mice resulted in increased UCP1-positive, multilocular adipocytes in inguinal and perirenal adipose tissue (100). Acute and chronic FGF21 administration also promotes sustained adiponectin secretion. In studies employing adiponectin knock-out mice, adiponectin has been demonstrated to be a key mediator of FGF21 benefits, such as decreased glucose levels, improved insulin sensitivity and hepatic steatosis, and elevated energy expenditure (101, 154). In BAT,
FGF21 increases oxygen consumption and glucose uptake and oxidation (99, 115, 148), while regulating thermogenic and glucose uptake gene expression (UCP1, DIO2, PGC1α, GLUT1, GLUT4, and CPT1α) (99, 100, 124, 153).

Mechanisms of FGF21 action in adipose tissue are thought to function by autocrine, paracrine, and endocrine means (100, 116). Dutchak et al. demonstrated, in the absence of elevated plasma FGF21, increased WAT FGF21 mRNA and protein expression alongside the expected increase in FGF21-mediated WAT activity with TZD administration (116). Investigators also showed in vitro TZD treatment increased FGF21 protein in culture media of white adipocytes. Initially, FGF21 was proposed to mediate its effects through transcriptional regulation (63); however, studies with bolus FGF21 injections have shown short-term effects of FGF21 on adipocytes (within 5-60 minutes of administration) as well (101, 104). While precise mechanisms of regulation and action continue to be elucidated, to date FGF21 has been shown to increase FRS2 and ERK1/2 phosphorylation in adipocytes (63, 147) and induce STAT3 phosphorylation and CREB phosphorylation (with subsequent PGC1α expression) in brown adipocytes (155). FGF21 has also been shown to be intricately interwoven with PPARγ signaling by suppressing sumolyation of PPARγ and thereby increasing its transcriptional activity (116), however this finding has yet to be repeated. Loss-of-function studies have revealed a critical significance of FGF21 action on adipose tissue for the whole body. Both lipodystrophic mice and mice without FGF21 receptors (β-Klotho or FGFR1c) in adipose tissue are refractory to the whole-body metabolic benefits of FGF21, including decreases in body weight, plasma glucose, insulin, and triglycerides, and improvements in glucose uptake and insulin sensitivity (148, 155-157).
Liver: The primary actions of FGF21 on the liver are in regulation of glucose and lipid homeostasis. FGF21 decreases hepatic glucose output, increases ketogenesis and fatty acid turnover while decreasing hepatic steatosis. Gain-of-function studies in transgenic overexpressing mice and elevated-FGF21 models (e.g. fasting, ketogenic diet, pharmacological administration) have shown FGF21 increases ketogenesis, lipid oxidation and circulating ketones, and decreases triglycerides and cholesterol in the liver (90, 92, 96, 104, 105). Loss-of function studies support this lipid regulation role: FGF21-KO mice display reduced ketogenesis (92), β-oxidation, and increased circulating FFAs and steatosis, while FGF21 knock-down mice exhibit fatty livers and decreased serum ketones on a ketogenic diet (96). In studies of transgenic overexpression and pharmacological administration in obesity, FGF21 reduces or reverses hepatic steatosis, potentially by decreased nuclear localization of SREBP-1 and downregulation of expression of genes involved in de novo lipogenesis (ACC1, ACC2, SCD1, GPAT, FAS, aP2, and CD36) (92, 102, 104).

The role of FGF21 in hepatic glucose regulation is controversial. The current consensus is that FGF21 plays a role in glucose flux, with additional roles, specifically gluconeogenesis, still to be elucidated. Studies of pharmacological administration of FGF21 to obese mice have consistently led to improved hepatic insulin sensitivity and improved glucose clearance (103, 104, 106, 158). Supporting a role for FGF21 in stimulating gluconeogenesis, Potthoff et al. reported increased gluconeogenesis (similar to fasted levels) in fed FGF21-transgenic mice and mild hypoglycemia in fasted FGF21-KO mice which investigators suggests indicate an impairment in gluconeogenesis (92). In support of these findings, Liang et al. also reported severe hypoglycemia and impaired
gluconeogenesis in FGF21-KO mice (152), and Fisher et al. found elevated hepatic

gluconeogenic gene expression (glucose-6-phosphatase (G6Pase) and phosphoenol
pyruvate carboxykinase (PEPCK)) two-hours following IP FGF21 injection of healthy wild-
type mice (105). However, in argument against FGF21 stimulating gluconeogenesis,
multiple studies have been unable to reproduce hypoglycemia in separately derived
FGF21-KO strains (97, 98, 159). In a recent study of the FGF21-KO strain used by
Potthoff and colleagues (92), mice were reported to show increased hepatic glucose
production and hepatic insulin resistance (160). Additionally, FGF21-KD mice on a high
fat diet exhibited a 75% increase in gluconeogenesis, accompanied by increases in
hepatic G6Pase and PEPCK expression (161). FGF21 administration studies in obese
rodents have shown a decrease in hepatic glucose production confirmed by in vitro
studies showing FGF21 suppresses glucose production in hepatocytes (104, 158, 162).
Likely stemming from studies using various pharmacological agents and different loss-of-
function mouse strains, along with inconsistent energy balance statuses, the literature
available on the role of FGF21 in hepatic gluconeogenesis is contradictory and
controversial; more work is needed to better elucidate its role.

The liver shares certain mechanisms of action of FGF21 with adipose tissue.
FGF21 has been shown to induce FRS2 and ERK1/2 phosphorylation in liver as well as
adipose tissue (87, 105, 163). Hepatic PGC1α has also been demonstrated to be induced
by FGF21 (92, 105), and many genes regulated by FGF21 are targets of PPARγ and
FoxO1 (105). Many of the mechanisms of action of FGF21 in the liver however appear to
be indirect. While the above downstream signaling effect demonstrates direct FGF21
actions on the liver, hepatic β-Klotho expression is very low compared to other primary
tissues of FGF21 action (113). Investigators have had difficulty reproducing in vivo observations in vitro (e.g. hepatocytes, liver perfusion studies) (92, 147), and tissue specific FGF21 receptor knockouts in the nervous system and adipose tissue have eliminated many of the hepatic effects of FGF21 (153, 157).

Central Nervous System: FGF21 is not synthesized by the brain but can cross the blood brain barrier in mice and humans (77, 151, 164). Considering that the suprachiasmatic nucleus and paraventricular nucleus of the hypothalamus and the area postrema and solitary nucleus in the hindbrain (dorsal-vagal complex) all express β-Klotho, the brain has become a tissue of great interest for FGF21 action (113). Indeed, studies employing brain specific β-Klotho-KO models (SVN-KO using Camk2a-Cre or DVC-KO using Phox2b-Cre) have revealed significant roles for FGF21 in and/or throughout the brain including suppression of physical activity, regulation of ketogenesis, and modulation of certain FGF21 metabolic outcomes previously described such as reduced body weight, bone growth, insulin, and cholesterol, and increased energy expenditure and BAT thermogenesis (151, 153). In support of these KO models, ICV injections in obese rats resulted in increased energy expenditure and improved insulin sensitivity, believed to result from decreased hepatic glucose output (162). Moreover, ablation of β-Klotho in these hypothalamic nuclei lead to differential expression of metabolic regulatory genes in both liver and adipose tissue (Lgfbp1, Pck1, Cyp2d6, Hsd3b5, Ucp1, Dio2, Pparγ, Atgl, Hsl, Scd1, Adipoq), demonstrating the ability of FGF21 to act centrally to exert effects on peripheral tissues.

Mechanisms of action of FGF21 in/throughout the brain are less defined as liver and adipose tissue. However, one well established conclusion is that FGF21 increases
circulating corticosterone by increasing hypothalamic Crf expression in mice (151, 152). Owen et al. also showed FGF21 directly increases sympathetic stimulation of BAT using multifiber sympathetic nerve recording following ICV or IP FGF21 administration (153). Finally, as also seen in liver and adipose tissue, FGF21 has also been shown to induce phosphorylation of ERK1/2 in the hypothalamus (165).

Pancreas: Many of the actions of FGF21 on the pancreas remain to be revealed, however, it is well established that this tissue produces FGF21 and expresses β-Klotho. Demonstrated early in FGF21 research, FGF21 was shown to increase the number of islets, number of β-cells, and insulin content within islets in the pancreas of db/db mice and healthy rats (110), as well as regulate glucose-sensitive insulin secretion (110). FGF21 also plays a protective role for the pancreas, as it has been shown to protect INS-1e cells from apoptosis, decrease caspase activity in β-cells in studies of isolated pancreatic islets, and reduce pancreatitis by protecting pancreatic acini from damage (110, 120). While likely an indirect effect of FGF21 mediated improvements in lipid and glucose regulation, it is worth noting FGF21 has been repeatedly shown to decrease glucagon secretion (63, 103, 111). Interestingly, treatment with a glucagon receptor agonist resulted in elevation of circulating FGF21 levels in mice and humans, suggesting a keen interplay between FGF21 and glucagon that deserve further investigation (166).

Reviewing the differential tissue-specific roles of FGF21 underscores the complexity of FGF21 biology. The actions of FGF21 common across tissues, particularly organs involved in energy and substrate regulation such as the liver, adipose tissue, and brain, emphasizes the significant role for FGF21 in metabolic regulation. In adipose tissue, liver, and the brain, FGF21 exerts either direct or indirect effects on regulation of
glucose metabolism, lipid handling, and the tissue’s responsiveness to insulin. FGF21 shares common effects on energy expenditure across tissues as well. Considering the inconsistent findings within the field of FGF21 regulation (likely complicated by the health and energy status of the employed model), mechanistic studies such as these provide clearer evidence for cause-and-effect relationships for FGF21 action. However, studies describing inter-organ effects (e.g. brain and BAT) serve as important reminders that many of these highly controlled models, such as cell culture experiments, are missing this potentially key, whole-body interplay.

1.2.5. Summary

FGF21 was initially revealed to have adipose-specific glucose sensitization properties. Since its discovery, FGF21 has been found to play diverse roles in metabolic regulation particularly in the liver, adipose tissues, and brain (β-Klotho expressing tissues). Similar to insulin or leptin, FGF21 is elevated in human populations with obesity, as well as type 2 diabetes mellitus, dysregulated glucose homeostasis, and an adverse metabolic phenotype. Regulation of FGF21 expression and secretion varies among tissues but overall appears to respond primarily to energy balance and nutrient intake, e.g. fasting, ketogenic diets, low protein diets, pregnancy, cold-exposure, obesity, etc. Gain- and loss-of-function models, as well as pharmacological administration studies, have revealed the role of FGF21 in regulation of energy expenditure, body weight, lipid homeostasis, glucose homeostasis, ketogenesis, lipolysis, and liver health (i.e. ectopic lips and hepatic steatosis).
1.3. PREGNANCY

Pregnancy is a series of physiological events which begins with the creation of an embryo, followed by the development of a fetus, and ending with delivery of a neonate. As reviewed in Chapter 1.1, in pregnancy the intrauterine environment fostered by a mother can impact the development, growth, and health of her offspring. Indeed, studies of the impact of pregnancy pathologies that can influence fetal growth, e.g. gestational diabetes, preeclampsia, or fetal growth restriction, have revealed adverse consequences for the long-term health of the offspring, such as cardiovascular disease, cancer, diabetes, and obesity (9, 11-17). Moreover, studies of less abrasive exposures, such as maternal nutrition imbalance or excess gestational weight gain, have also shown adverse effects on offspring health such as hypertension and obesity (38, 167-169). These findings reveal that the impact of the intrauterine environment is great, and that the intrauterine development of the fetus may be more delicate and long-lasting than previously thought. To better understand the contributions of the intrauterine environment to fetal development, a brief overview of the physiology of pregnancy is outlined below beginning with the development of the embryo/fetus, changes in the mother, and placental development and function.

1.3.1. Fetus

*In utero* development can be studied in two stages: the embryonic stage and the fetal stage. The embryonic stage begins with the release of a secondary oocyte from the ovary (ovulation). The oocyte travels through the fallopian tube where it is entered into by a sperm cell, eventually causing the fusion of two haploid cells and creating a single diploid zygote. Rapid mitotic divisions occur that results in a blastocyst approximately 5 days following fertilization. The blastocyst attaches to the endometrium of the uterus
between days 6 and 7 to establish the pregnancy. In the next week, trophoblast cells (cells which will make up the placenta, amniotic sac, and umbilical cord) begin to differentiate, the yolk sac is established, and the inner cell mass differentiates into the bilaminar embryonic disc. In the third week after fertilization, the placenta and chorionic villi begin to develop and gastrulation and neurulation occur. During the following month, all major organs are established, albeit not yet functional, by the end of the eighth week (organogenesis). Week 8 marks the end of the embryonic stage and the beginning of the fetal stage. During the fetal stage, the fetus will develop for approximately 32 additional weeks growing from an estimated 30 grams to a full-term 3400 grams as the organs established during the embryonic stage differentiate and mature.

1.3.2. Mother

During pregnancy, many physiological changes occur in the mother to accommodate for and support the growing fetus. A woman with a normal body mass index (BMI) at conception is recommended to gain approximately 25-35 pounds (170). Thirty-eight percent of mass gained is due to the products of conception (fetus, placenta, amniotic fluid), 27% due to maternal energy stores (fat mass), and the remaining 35% can be attributed to increased blood volume and extracellular fluid, increased breast tissue, and the enlarged uterus (171, 172). Estimations of tissue deposition in a healthy pregnancy are 925g of protein gain and 3345g of fat gain [Table 1.5, adapted from (173)]. The energy cost of this tissue deposition is estimated to be a total of 41,518 kcal in pregnancy. Weight gain above or below the recommended amount can lead to adverse consequences of mother and her fetus (170).
Table 1.5 Estimated gestational tissue depositions and respective energy costs (173)

<table>
<thead>
<tr>
<th>Products of Conception</th>
<th>Protein Gain (grams)</th>
<th>Fat gain (grams)</th>
<th>Total (calories)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetus</td>
<td>440</td>
<td>440</td>
<td>6644</td>
</tr>
<tr>
<td>Placenta</td>
<td>100</td>
<td>4</td>
<td>598</td>
</tr>
<tr>
<td>Fluid (amniotic + blood)</td>
<td>138</td>
<td>20</td>
<td>963</td>
</tr>
<tr>
<td>Uterus and breasts</td>
<td>247</td>
<td>16</td>
<td>1536</td>
</tr>
<tr>
<td>Maternal Energy Stores</td>
<td>0</td>
<td>3345</td>
<td>31778</td>
</tr>
<tr>
<td>Totals</td>
<td>925</td>
<td>3825</td>
<td>41519</td>
</tr>
</tbody>
</table>

In 2009, the Institute of Medicine (170) released updated weight gain recommendations classified by preconception BMI. A woman entering pregnancy with a BMI between 18.5 and 24.9 kg/m² is recommended to gain between 25 and 35 pounds, entering pregnancy with a BMI of 25 to 29.9 kg/m² between 15 and 25 pounds, and entering pregnancy with a BMI greater than 30 kg/m² between 11 and 20 pounds. The Food Nutrition Board (173) has published energy and macronutrient recommendations during pregnancy with special considerations for increased conservation of nutrients during pregnancy and the increased nutrient demand of pregnancy. However, as the data available is insufficient for determining estimated average requirements (EAR) in pregnancy, many of the intake recommendations are based on requirements for a weight gain of 16 kg (the average gestational weight gain reported by Carmichael et al. (174). Therefore, the recommended energy intake for a woman between the ages of 19 and 50 years is 2403 kcal/day in the first trimester, 2743 kcal/day in the second trimester, and 2855 kcal/day in the third trimester. Others have created a mathematical model that can estimate individual energy requirements for each trimester of pregnancy that can foster healthy weight gain (175). The EAR for carbohydrate intake is 135 g/day, for fiber is 28 g/day, and for protein is 0.88 grams/kg/day. While there is not a total fat intake recommendation, 13 g/day of n-6
polyunsaturated fatty acids and 1.4 g/day of n-3 polyunsaturated fatty acids are recommended during pregnancy.

1.3.3. Placenta

The placenta is a transient organ present only in pregnancy that serves as a gateway between the mother and her fetus. The placenta transports nutrients and oxygen to the fetus and waste from the fetus, and equally as important, the placenta can prevent transport of pathogens from the mother. The placenta is responsible for serving as the respiratory, endocrine, immune, and gastrointestinal systems for the fetus. It is also an endocrine organ responsible for producing hormones to be secreted into the maternal and/or fetal circulation. Below is a detailed description of placenta invasion and development (Table 1.6) as well as placenta transport.

Table 1.6 Placenta morphological development

<table>
<thead>
<tr>
<th>Month</th>
<th>Placenta Weight</th>
<th>Placenta diameter</th>
<th>Maternal-fetal diffusion distance</th>
<th>Total villous volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>30 g</td>
<td>50 mm</td>
<td>35 μm</td>
<td>0.3 m²</td>
</tr>
<tr>
<td>4</td>
<td>70 g</td>
<td>75 mm</td>
<td>20 μm</td>
<td>0.6 m²</td>
</tr>
<tr>
<td>5</td>
<td>120 g</td>
<td>100 mm</td>
<td>12 μm</td>
<td>1.5 m²</td>
</tr>
<tr>
<td>6</td>
<td>190 g</td>
<td>125 mm</td>
<td>10 μm</td>
<td>2.8 m²</td>
</tr>
<tr>
<td>7</td>
<td>260 g</td>
<td>150 mm</td>
<td>-</td>
<td>4.0 m²</td>
</tr>
<tr>
<td>8</td>
<td>320 g</td>
<td>170 mm</td>
<td>-</td>
<td>7.0 m²</td>
</tr>
<tr>
<td>9</td>
<td>400 g</td>
<td>200 mm</td>
<td>-</td>
<td>10.0 m²</td>
</tr>
<tr>
<td>10</td>
<td>470 g</td>
<td>220 mm</td>
<td>-</td>
<td>12.5 m²</td>
</tr>
</tbody>
</table>

Invasion and Development: Beginning eight days after fertilization, the trophoblast cells develop into two layers: syncytiotrophoblast and cytotrophoblast. The function of the syncytiotrophoblast will be to transport nutrients, gases, and waste and synthesize hormones, while the cytotrophoblasts will serve as the progenitor cells for the placenta. The syncytiotrophoblasts secrete enzymes that digest the endometrial cells of the uterine lining which allows the blastocyst to become buried in the endometrium. As the
trophoblasts erode into the decidua, the trophoblastic blastocyst cover subdivides into 3 layers: primary chorionic plate which is composed mainly of cytotrophoblasts, the lacunar system, and the cytotrophoblast shell which is composed mainly of syncytiotrophoblasts covering cytotrophoblasts. Fingerlike projections called chorionic villi that will contain the fetal blood vessels begin to form. By week 3, fetal blood vessels develop in the chorionic villi which will fully connect to the embryonic heart through the umbilical arteries and vein by the end of 12 weeks. During weeks 4-7, fetal capillaries continue to develop within chorionic villi which contain nucleated fetal erythrocytes. The trophoblastic shell transforms into the basal plate hallmarked by mixing of trophoblastic and decidual cells. By the end of week 7, feto-placental circulation is established but resistance is high and most of the fetal erythrocytes are nucleated. There is no maternal arterial circulation to the placenta yet. During weeks 8-12, extravillous cytotrophoblasts begin to invade and remodel maternal uterine arteries (a process that completes about week 22) and all chorionic villi are vascularized but only occupy 4% of the villous space. The presence of nucleated fetal erythrocytes declines rapidly and complete feto-placental circulation is established as well as maternal arterial circulation to the placenta. In the second trimester, once these structures and the maternal-fetal exchange have been established, the remaining placenta development is generally a continuation of the following processes: development of fetal capillaries into the chorionic villi, maturation of immature villi into immediate and stem villi, growth of villi diameter to accompany increased blood volume/flow from fetus, cytotrophoblasts surrounding the villi are replaced by syncytiotrophoblasts, and fibrinoid deposition begins. In the third trimester, the same processes continue from the second trimester, however the villi mature further into
terminal villi as well. By the end of gestation, 40% of villous volume is terminal villi and the total villous volume is about 12.5 m². See the table below for month-by-month details in placenta morphological development.

**Transport:** The ability of the placenta to provide nutrients for the fetus relies primarily on nutrient availability in the maternal circulation, the expression of nutrient transporters by the placenta, and utero-placenta blood flow. Indeed, there is increasing support to show the placenta can sense nutrient availability in the maternal circulation and respond by up- or down-regulating nutrient transporters (52). While placental surface area is the primary determinate for transport of permeable molecules, e.g. oxygen, the placenta uses active and passive transport systems to transport less permeable substrates, e.g. glucose, amino acids, and ions. The primary barrier between mother and fetus in the placenta is a single layer of syncytiotrophoblasts. Intriguingly, these polarized cells have differential regulation of nutrient transporters in the maternal (microvillous membrane) versus the fetal side (basal membrane) which contributes to the regulation of transfer and fetal nutrient availability.

The three primary macronutrients, protein, carbohydrate and fat, each cross the placenta by independent transport systems. The most complex system is the transport of amino acids. There are over 20 proteins responsible for amino acid transport and they can be classified into two categories: accumulators and exchangers. The accumulators, also called System A, co-transport non-essential, neutral amino acids into the cell with sodium. The primary transport proteins in System A of the human placenta are SNAT1, SNAT2 and SNAT4. The exchangers, also called System L, then use these intracellular non-essential amino acids and exchange them to transport extracellular essential
(aromatic or branch chained) amino acids against their concentration gradients into the cell. The primary transport proteins in System L of the human placenta are LAT1 and LAT2. Fatty acids are hydrolyzed from triglycerides within maternal lipoproteins primarily by lipoprotein lipase and endothelial lipase. These fatty acids are then transported across the syncytiotrophoblast by five fatty acid transport proteins (FATP) in the human placenta: FATP1, FATP2, FATP3, FATP4, FATP6. Once inside the syncytiotrophoblast, fatty acids are trafficked by four fatty acid binding proteins (FABP1, FABP3, FABP4, and FABP5) for transport to the fetus, beta-oxidation, or esterification. Finally, the simplest nutrient transport system is the facilitated diffusion of glucose by glucose (GLUT) transporters. The higher concentration of maternal glucose facilitates the diffusion of glucose towards the fetus. GLUT1 is the primary glucose transporter, however GLUT3, GLUT4, GLUT8, GLUT9, GLUT10, and GLUT12 are also expressed in the human placenta. Interestingly, GLUT expression in the basal membrane (fetal side) is much lower compared to the microvillous membrane (maternal side of the syncytiotrophoblast), suggesting basal membrane transport is the rate limiting step for transfer of glucose to the fetus. These three systems collectively supply the primary macronutrients necessary for fetal development and growth, and changes to these systems in response to maternal malnutrition, maternal pathology, or placenta pathology can be highly informative for studies of developmental programming. In summary, fetal development from an embryo to neonate requires a highly complex and coordinated set of processes. At the heart of these events is the placenta whose intricate functions facilitates the transfer of the maternal environment to the growing fetus which can impact health at birth and beyond.
1.4. PROJECT SUMMARY

Due to the enduring obesity epidemic, the global population is now heavier and faces higher risks for chronic disease than ever before. Unfortunately, reproductive aged women are not spared in this epidemic, and as a result the DOHaD hypothesis would suggest our population is now participating in a deadly cycle: conceiving children within unhealthy early-life environments (maternal overweight, obesity, diabetes, hypertension, stress, etc.) which are “programming” the infant to herself grow up with increased risks of these co-morbidities; subsequently, when the adversely “programmed” child reaches reproductive age, she is more likely to present with these complications and expose her own offspring to the very environmental offenders which contributed to her own. Reflecting the impact of this cycle, a dramatic shift in the causes of global mortality has been observed over the past 20 years from communicable diseases to non-communicable diseases, such as those shown to be “programmable” through in utero exposures. As evidence grows to show that the propensity for chronic, non-communicable diseases can begin in the womb, so too grows the need to discover and understand the causative mechanisms linking the intrauterine environment of an infant and his or her risk for disease in adulthood.

One field aiming to understand the mechanisms of the DOHaD is the study of the maternal-placental-fetal unit. Indeed, many hormones and nutrients in the maternal circulation are altered under conditions associated with adverse developmental programming. For example, adiponectin is decreased in pregnant women with obesity and insulin and glucose can be increased in pregnant women with diabetes. As the maternal circulation comes into contact with the placenta, these factors communicate to
alter its development and activity which could subsequently affect the nutrients, hormones, oxygen, etc. the fetus receives during development.

FGF21 is a novel endocrine hormone differentially regulated in non-pregnant populations with conditions that are well-established offenders in adverse developmental programming. For example, FGF21 has been shown to be elevated in individuals with obesity, individuals with diabetes, and under conditions of malnutrition and protein deprivation. Moreover, murine and human placenta have been demonstrated to express FGF21 receptors (FGFR1 and β-Klotho). Taken together, the work included in this dissertation will explore the potential role of FGF21 to act as a mediator of the DOHaD hypothesis. For the first time, our results describe FGF21 in a healthy, pregnant population. We extended these observations to explore the role of FGF21 as a protein sensor in pregnancy and a potential mechanism operating through the maternal-placental-fetal unit to impact fetal development.
CHAPTER 2: FIBROBLAST GROWTH FACTOR 21 IN PREGNANT WOMEN

2.1. INTRODUCTION

Fibroblast growth factor 21 (FGF21) is secreted in response to energy imbalance for the regulation of energy and nutrient metabolism. First discovered as a glucose sensitizer of adipose tissue, FGF21 has since been shown in both animal models and human studies to regulate glucose and lipid metabolism under various states of energy balance (63). Metabolically challenged transgenic models overexpressing FGF21 and pharmacological administration of FGF21 to obese and diabetic animals and humans have revealed FGF21 to be capable of reducing body weight, protecting against diet induced obesity, improving glucose tolerance, and improving lipid profiles (63, 102-104, 106-112). Intriguingly, cross sectional studies of the human population show that FGF21 is elevated in clinical conditions of energy or nutrient excess, such as obesity and type 2 diabetes (64, 65, 68-78). FGF21 is also positively correlated with metabolically unfavorable characteristics, such as hyperinsulinemia, insulin resistance, hypertriglyceridemia, and total circulating cholesterol (65, 71, 73, 74, 77, 78, 80, 85, 89).

Pregnancy is a state of energy flux with energy and macronutrient demand increasing at variable rates and in response to various cues throughout gestation. Considering the impact of energy status and macronutrient balance on FGF21 expression and action, understanding how FGF21 is regulated and/or acting in pregnancy could be highly informative to FGF21 biology. However, descriptions of FGF21 in the pregnant population are surprisingly limited to cross sectional studies in pregnant women with gestational diabetes or preeclampsia, and the majority of these studies show FGF21 to be elevated in these conditions (79, 80, 176-178). These reports also confirm well
established associations between FGF21 and an untoward metabolic milieu described in the non-pregnant state. For example, FGF21 in late pregnancy positively correlates with triglycerides and insulin resistance and inversely with adiponectin and HDL-cholesterol (80, 176, 177). The current knowledge of FGF21 biology in pregnancy is therefore in need of more in depth studies in healthy populations throughout pregnancy. The aim of this study was to describe FGF21 in a healthy population of pregnant women and to understand the role of maternal energy stores and the placenta on FGF21 secretion. We hypothesized FGF21 concentrations would be higher in women with increased body mass index, adiposity, and glucose intolerance.

2.2. MATERIALS AND METHODS

2.2.1. Study population

One hundred and fourteen pregnant women enrolled in the Expecting Success Study (n=54, NCT01610752) or the MomEE study (n=60, NCT01954342) at Pennington Biomedical Research Center in Baton Rouge, Louisiana were potentially eligible for this ancillary study. As detailed elsewhere, participants were recruited primarily from obstetrical offices, augmented by print and social media advertisements (179). For entry into a parent study, participants were required to be healthy, overweight and obese women (BMI>25kg/m²) aged 18-40 years with a single, viable, first trimester pregnancy (<14 weeks gestation). Medical clearance for participation was also required by a primary care obstetrician or midwife and the Medical Investigator. Patients were excluded for pregnancy-related conditions (known fetal anomaly, planned termination of pregnancy or adoption of infant, history of ≥3 consecutive miscarriages), hypertension, diabetes (diagnosis prior to pregnancy, elevated HbA1c, or first trimester OGTT diagnosis of
diabetes), psychological criteria (history or current psychotic disorder, current major depressive episode, bipolar disorder, history of anorexia or bulimia, current eating disorder, or actively suicidal), medications (metformin, systemic steroids, antipsychotic agents, anti-seizure medications, or medications for ADHD), HIV, severe anemia, contraindications to exercise (180), prior or planned (within one year of expected delivery) bariatric surgery, or recent history of or current nicotine, alcohol, or drug use. Of the 114 participants in the parent studies, 43 satisfied inclusion criteria for this ancillary study, provided consent for future use of biospecimens (i.e. blood, placenta), and had the required clinical data available at one or more time point for analysis. Briefly, the Expecting Success Study (NCT01610752) was an interventional study testing the efficacy of a lifestyle intervention designed to help overweight and obese pregnant women gain the recommended amount of weight in pregnancy. The MomEE Study (NCT01954342) was an observational study determining energy requirements of overweight and obese women across pregnancy. Both parent studies and this ancillary study were approved and monitored by the Pennington Biomedical Research Center Institutional Review Board and all participants provided verbal and written consent prior to study initiation.

2.2.2. Clinic Assessments

The parent studies enrolled women in the first trimester of pregnancy (<14 weeks gestation) and study visits for data collection coincided with gestational age to allow for one assessment visit per trimester, i.e. 1st trimester (<16 weeks), second trimester (25-26 weeks), and third trimester (35-36 weeks). Study visits were conducted in the morning following an overnight fast and allowed for collection of body weight, body composition, and blood. All data were obtained in accordance with standard operating procedures of Pennington Biomedical Research Center to ensure scientific rigor and reproducibility.
2.2.3. **Body weight and body mass index**

Maternal body weight was recorded twice after a 10-hour fast with the participant wearing a hospital gown and undergarments only. The two recorded weights were averaged and the hospital gown weight subtracted. Body mass index was calculated as body weight (kg) divide by the square of study-measured height (m²). Pregravid weight was determined by self-report recorded by questionnaire and confirmed by interview.

2.2.4. **Body composition**

Maternal body composition was assessed by air displacement plethysmography using a BOD POD® (COSMED). Fat mass and fat free mass were calculated from body volume as determined by the BOD POD® with equations by van Raaij et al. (181, 182).

2.2.5. **Blood chemistry**

Serum glucose and insulin were assayed with the Beckman Coulter DXC 600 Pro (Beckman Coulter Inc., Brea, CA). Fibroblast growth factor 21 was measured in duplicate by sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (RD191108200R, Fibroblast Growth Factor Human ELISA, Biovendor, Brno, Czech Republic). Serum was diluted 1:2 (125μL of serum in 125μL of dilution buffer) before analysis. The detectable concentration by the assay is 0.03 ng/mL. The intra-assay coefficient of variation was 3.9%.

2.2.6. **Placenta collection**

Placenta samples were collected, dissected and frozen within two hours of delivery. Samples were dissected at four separate sites of the placental disc and stored by section (basal plate, villous tissue, and chorionic plate) at -80°C until being thawed for study. Experiments in this study used villous tissue snap frozen within two hours of delivery and pooled from the four collection sites.
2.2.7. Quantitative real time PCR

Placenta tissue mRNA was isolated from flash-frozen tissue with the RNeasy Mini Kit (QIAGEN). Samples were quantified by Nanodrop and all samples had 260:280 and 260:230 ratios greater than 1.75. Two thousand nanograms of RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) into cDNA. Quantitative real-time PCR was performed with 20 ng of cDNA, 300 nM of the forward and reverse primers, and iTaq universal SYBR green mastermix. The PCR protocol was performed on a 7900HT PCR Machine (ThermoFisher Scientific, Waltham, MA) beginning with one cycle at 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 59°C for 1 minute, and ended with a dissociation curve analysis. Primers sequences unique for each target gene were designed with primer BLAST to span exon-exon junctions. The geometric mean of PPARα and FATP1 was used as an endogenous control.

Table 2.1 Primer sequences (human)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF21</td>
<td>GCTTCGGACTGGTAAACATTG</td>
<td>GGAGTCAAGACATCCAGGTT</td>
</tr>
<tr>
<td>FGF21</td>
<td>TGGATCGCTCCACTTTGACC</td>
<td>GGGCTTCGGACTGGTAAACA</td>
</tr>
<tr>
<td>PPARα</td>
<td>GGCAAGACAAGCTCAGAAC</td>
<td>TTATCTATGAAGCAGGCAC</td>
</tr>
<tr>
<td>FATP1</td>
<td>AGGTGGTCAGTACATCGGG</td>
<td>AGAACTCCCGATTTGCG</td>
</tr>
</tbody>
</table>

2.2.8. Statistical analysis

Data are presented as mean ± standard error. Fasting insulin and HOMA-IR were logarithmically transformed. Statistical significance was determined by linear regression analyses or paired student’s t-tests when appropriate. Tests were performed with significance level α=0.05, and findings considered significant when P<α.
2.3. RESULTS

2.3.1. Participants

The study cohort was 29±5 years old at the time of enrollment and was comprised of participants who self-identified as Caucasian (n=37), black (n=5) and other (n=1). Using first measured study height and weight (<14 weeks gestation), 14 participants were overweight (BMI 25-29.9kg/m²) and 29 participants were obese (BMI ≥ 30 kg/m²) (16 within Class I, 7 within Class II, and 6 within Class III). Enrollment weight and BMI were in close agreement with self-reported pregravid weight documented in the physician office at the confirmation of pregnancy. Population characteristics are detailed in Table 2.2.

Table 2.2 Participant characteristics

<table>
<thead>
<tr>
<th></th>
<th>Pre-pregnancy</th>
<th>1st Trimester</th>
<th>3rd Trimester</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at enrollment</td>
<td>29.1 (5.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity (0/1/2/3/4)</td>
<td>20/20/0/2/1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>5 (11.6%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>37 (86.1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1 (2.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI Class</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overweight</td>
<td>13</td>
<td>14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Obese- Class I</td>
<td>17</td>
<td>16</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Obese- Class II</td>
<td>8</td>
<td>7</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Obese- Class III</td>
<td>5</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.8 (5.4)</td>
<td>33.5 (5.3)</td>
<td>36.6 (4.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.8 (14.5)</td>
<td>88.6 (14.5)</td>
<td>96.9 (13.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>40.4 (10.5)</td>
<td>41.8 (9.7)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Percent fat (%)</td>
<td>45.0 (5.4)</td>
<td>42.6 (5.0)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>48.3 (6.1)</td>
<td>55.3 (6.1)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>84.6 (8.0)</td>
<td>81.5 (7.6)</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Fasting insulin (uIU/mL)</td>
<td>12.4 (7.9)</td>
<td>16.8 (9.3)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.7 (2.0)</td>
<td>3.4 (2.1)</td>
<td></td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values reported as mean (± standard deviation); *differences between first and third trimesters
2.3.2. Pattern of FGF21 in human pregnancy

Circulating FGF21 concentrations are highly variable among humans (66). In fasting serum samples collected from our cohort of healthy women in the first and third trimesters, FGF21 was highly variable as well. In the first trimester FGF21 concentrations ranged from 0.035 to 0.256 ng/mL (Figure 2.1A) and there was an even larger degree of variability observed in the third trimester (range 0.056-0.850 ng/mL, Figure 2.1B). First trimester FGF21 concentrations showed ten-fold less variance compared to third trimester concentrations, 0.003 versus 0.033, respectively. FGF21 concentrations were more than 2-fold higher in the third trimester of pregnancy compared to concentrations measured in the first trimester, 0.105 vs. 0.248 ng/mL, respectively (Figure 2.1C).

Figure 2.1 Serum FGF21 concentrations in the first and third trimesters of our study population. (A) Distribution of FGF21 in the 1st trimester (<16 weeks) (n= 29), (B) Distribution of FGF21 in the 3rd trimester (35-36 weeks) (n=43), (C) Individual and mean ± SEM serum FGF21 measured across pregnancy, 1st trimester: 0.105 ± 0.01 ng/mL, 3rd trimester: 0.248 ± 0.03 ng/mL, *p<0.0001.

2.3.3. FGF21 is correlated with maternal body size and adiposity throughout pregnancy

The literature in non-pregnant individuals shows FGF21 is elevated with higher BMI and, hence, increased adiposity. In this population, FGF21 concentrations were significantly and positively correlated with maternal BMI in both the first (r=0.48, p=0.008) and third trimesters (r=0.38, p=0.01) (Figure 2.2A and B). However, FGF21
was not correlated to body weight at either time point during gestation \( p=0.07 \) and 0.23, Figure 2.2C and D respectively),

![Figure 2.2](image)

Figure 2.2 Serum FGF21 is significantly correlated with maternal body mass index but not body weight throughout pregnancy. (A) 1\textsuperscript{st} trimester BMI, \( n=29 \), \( r=0.48 \), \( p=0.008 \), (B) 3\textsuperscript{rd} trimester BMI, \( n=43 \), \( r=0.38 \), \( p=0.01 \), (C) 1\textsuperscript{st} trimester body weight, \( n=29 \), \( r=0.34 \), \( p=0.07 \), and (D) 3\textsuperscript{rd} trimester body weight, \( n=43 \), \( r=0.19 \), \( p=0.23 \).

FGF21 concentrations were strongly and significantly correlated with maternal adiposity reported as total fat mass (kg) in both the first and third trimesters (1\textsuperscript{st} trimester: \( r=0.51 \), \( p=0.005 \); 3\textsuperscript{rd} trimester: \( r=0.33 \), \( p=0.03 \)) (Figures 2.3A and B). However, FGF21 concentrations were not significantly correlated with maternal fat free mass in either trimester (1\textsuperscript{st} trimester: \( r=-0.02 \), \( p=0.92 \), 3\textsuperscript{rd} trimester: \( r=-0.14 \), \( p=0.38 \)) (Figures 2.3C and D). Considering FGF21 is strongly correlated with adiposity
and not body weight or fat free mass, these results suggest that FGF21 regulation may be more related to energy balance rather than body size or products of conception (i.e. placenta and fetus).

Figure 2.3 Serum FGF21 is significantly correlated with maternal adiposity throughout pregnancy. (A) 1st trimester fat mass, n=29, r=0.51, p=0.005, (B) 3rd trimester fat mass, n=43, r=0.33, p=0.03, (C) 1st trimester fat free mass, n=29, r= -0.02, p=0.92, and (D) 3rd trimester fat free mass, n=43, r= -0.14, p=0.38.

Maternal BMI prior to pregnancy was significantly correlated with FGF21 concentrations measured during pregnancy, regardless of body weight or composition changes during gestation. Maternal pregravid BMI was significantly correlated with FGF21 concentrations in the first trimester (r=0.51, p=0.005) (Figure 2.4A) and third trimester (r=0.38, p=0.01) (Figures 2.4C). Maternal pregravid body weight also correlated with first trimester, but not third trimester, FGF21 concentrations (1st: r=0.39, p=0.04; 3rd: r=0.23, p=0.14) (Figures 2.4B and D).
Figure 2.4 Serum FGF21 measured during pregnancy is significantly and positively correlated with pre-pregnancy BMI and body weight. (A) Pregravid BMI and 1st trimester FGF21 (n=29, r=0.51, p=0.005), (B) pregravid body weight and 1st trimester FGF21 (n=29, r=0.39, p=0.04), (C) pregravid BMI and 3rd trimester FGF21 (n=43, r=0.38, p=0.01), and (D) pregravid body weight and 3rd trimester FGF21 (n=43, r=0.23, p=0.14).

2.3.4. FGF21 is not correlated with glucose homeostasis in normal pregnancy

FGF21 has been shown to positively correlate with fasting glucose, fasting insulin, and insulin resistance in animal models and non-pregnant humans (70, 71, 73, 74, 77). In our healthy population of pregnant women with normal glucose tolerance and maternal overweight or obesity, there were no significant relationships between FGF21 and fasting glucose (p=0.55 and 0.10, Figures 2.5A and D respectively), fasting insulin (p=0.18 and 0.48, Figures 2.5B and E respectively), or HOMA-IR (p=0.20 and 0.38, Figures 2.5C and F respectively) were observed in either the first or third trimesters.
Figure 2.5 First and third trimester serum FGF21 is not correlated with maternal glucose homeostasis in early or late pregnancy. (A) 1st trimester glucose and FGF21, n=29, r=0.12, p=0.55, (B) 1st trimester insulin and FGF21, n=29, r=0.26, p=0.18, (C) 1st trimester HOMA-IR and FGF21, n=29, r=0.25, p=0.20, (D) 3rd trimester glucose and FGF21, n=42, r=0.26, p=0.10, (E) 3rd trimester insulin and FGF21, n=42, r=0.11, p=0.48, and (F) 3rd trimester HOMA-IR and FGF21, n=42, r=0.14, p=0.38.
2.3.5. Change in glucose, not adiposity, correlates with change in FGF21 across pregnancy

In an effort to understand if changes in maternal characteristics throughout gestation may explain the increase in FGF21, we tested for relationships between the change in FGF21 (absolute and percent) and change in known contributors of FGF21 in the non-pregnant state, namely adiposity and metabolic status. As expected, body weight, fat mass, fat free mass, and fasting insulin each increased from the first to the third trimester while surprisingly, fasting glucose decreased slightly (Table 2.2). Although we observed significant relationships between body composition, namely adiposity at single time points, the changes in these parameters across pregnancy were not associated with the changes in FGF21 concentrations measured across the same time interval (Figures 2.6A-C). However, the change in FGF21 concentrations was significantly and negatively correlated with the change in fasting glucose (n=29, r= -0.40, p=0.03) (Figure 2.6D). There was no relationship observed between change in FGF21 and change in insulin or HOMA-IR (n=29, r= -0.23, p=0.22 and n=29, r= -0.28, p=0.14 respectively) (Figures 2.6E and F).
Figure 2.6 Change in FGF21 across pregnancy is correlated with change in maternal fasting glucose but not change in maternal body size or adiposity. Percent change in FGF21 from first to third trimester compared to (A) percent change in body weight (n=29, r=0.12, p=0.52), (B) percent change in fat mass (n=29, r=0.02, p=0.93), (C) percent change in fat free mass (n=29, r=0.18, p=0.35), (D) percent change in glucose (n=29, r= -0.40, p=0.03), (E) percent change in insulin (n=29, r= -0.23, p=0.22), and (F) percent change in HOMA-IR (n=29, r= -0.28, p=0.14).
2.3.6. The placenta is not a primary source for FGF21 production in pregnancy

FGF21 has been reported to be produced by the human placenta by two separate laboratories, in one study by ELISA of placenta explant media (79) and two other studies by qPCR of flash frozen human placenta samples (86, 183). We therefore hypothesized that the two-fold increase in FGF21 concentrations in the third trimester compared to the first trimester may originate from the placenta. Despite the three earlier reports of FGF21 production by human placental tissue, we were not able to detect meaningful and replicable quantities of FGF21 transcript in human placenta by qPCR utilizing two different sets of primers.

2.4. DISCUSSION

In this study, we describe FGF21 in a healthy pregnant population with pregravid overweight or obesity and normal glucose tolerance. The three novel observations from this body of work are that circulating FGF21 measured in fasting conditions was correlated with maternal body mass index and adiposity before and throughout pregnancy, FGF21 concentrations increased across pregnancy, and this increase in FGF21 was not related to change in maternal energy stores but with changes in maternal glucose concentrations.

The relationship of FGF21, BMI, and adiposity has been well reported in non-pregnant animal models and human populations (65, 70, 72, 75-78, 89). In the current study, FGF21 concentrations positively correlated with maternal body mass index and adiposity in pregnant women, not only at the onset of pregnancy but also in both the first and third trimesters. Considering pregnancy is a time for increased energy deposition in preparation for postpartum energy demands, e.g. lactation, these findings show the relationship between FGF21 and adiposity seen in non-pregnant states holds in and
across pregnancy. However, it is important to note the individual change in adiposity across pregnancy did not predict FGF21 concentrations; this finding suggests the physiological drivers of maternal energy deposition in fat mass are independent of changes in FGF21.

Notably, we were unable to confirm that high concentrations of FGF21 are present in individuals with increasing levels of glucose intolerance. Lack of support for this relationship in our population could be due to insufficient statistical power because we excluded participants with pregravid maternal diabetes and impaired glucose tolerance in their first trimester (HbA1c< 6.5%). This exclusion limited the variability in glucose dysfunction within our cohort despite the large range of maternal BMI. While fasting insulin and HOMA-IR showed moderate distribution among participants across pregnancy (see Table 2.2), glucose regulation appeared highly controlled. Indeed, the percent change in fasting glucose, from the first to third trimester, ranged from -20% to +9%, with fasting glucose decreasing in two-thirds of the participants. Therefore, our population had normal glucose tolerance, and there was limited variability in measures of glucose homeostasis for comparison with FGF21. This explanation is further corroborated by reports of increased FGF21 in women with gestational diabetes (79, 80), a condition we did not include in our study. Future studies need to be conducted in pregnant women with varying degrees of glucose tolerance to further elucidate the changing role of FGF21 in response to maternal glucose homeostasis throughout pregnancy.

We observed FGF21 concentrations were less variable and significantly lower in the first trimester compared to the third trimester of pregnancy. Given the previous reports of FGF21 expression in the human placenta, it was reasonable to hypothesize that the
increase in circulating FGF21 could be attributed to placenta tissue. Following in extensive qPCR experiments in a subset of term human placentas from our cohort, we were unable to quantify expression of FGF21. We therefore conclude that the human placenta is not contributing meaningful amounts of FGF21 into the maternal circulation. However, because FGF21 correlates with maternal BMI and adiposity, it is logical to hypothesize that the rise in FGF21 throughout pregnancy can be explained on the bases of concurrent and variable increase in maternal energy stores. The increase in FGF21 was not found to be correlated with change in body weight or adiposity across pregnancy.

FGF21 has been regularly shown to correlate with glucose homeostasis in non-pregnant and pregnant populations with glucose dysfunction which could be another possible explanation for the change in FGF21 across pregnancy. Despite the limited variability of glucose homeostasis within our cohort, we observed a significant negative correlation between change in FGF21 and change in fasting glucose, implying that the more FGF21 increased, the more fasting glucose decreased during pregnancy. As seen at single time points during pregnancy, there was not a significant correlation between change in insulin or HOMA-IR and change in FGF21 either. The relationship between FGF21 and glucose regulation in pregnancy merits further investigation as we made these observations within a healthy population with little variability and the original role of FGF21 was indeed as a glucose regulator.

Therefore, since the changes in FGF21 were associated with changes in fasting glucose and not with the changes in body energy stores (i.e. weight or fat mass), we hypothesize changes in energy status, that is, the maternal diet, which influences both tissue deposition in tissues and glucose regulation, might be involved. Specifically,
changes in dietary protein intake can result in changes in glucose regulation, such that consumption of high protein diets can significantly lower glucose concentrations, while conversely, diets low in protein raise blood glucose levels (184-187). Indeed, FGF21 has been shown to be regulated by various dietary states (e.g. fasting, ketogenic diets, amino acid deprivation, etc.). Most recently, the nutrient-driven responses of FGF21 have been proposed to be due to protein intake (125, 129). Though our study did not administer a dietary intervention, naturally occurring variations in protein accretion, protein demand, and self-selected protein intake within our cohort produced the opportunity to study the effects of low and high protein availability in pregnancy. In our study, participants are enrolled and FGF21 measured in the first trimester when classic studies of leucine kinetics and $^{15}$N lysine tracers have demonstrated little to no protein accretion (188, 189). However, by the end of pregnancy, an approximated 925 grams of protein is deposited which requires increased estimated daily depositions of 3.6 g/day and 7.2 g/day in the second and third trimesters respectively (173, 190). Therefore, by the third trimester, we hypothesize the increased need for protein is achieved by those few individuals who adequately increase protein intake. This thereby would result in majority of women consuming a reduced protein diet, having poor regulation of glucose homeostasis (increased fasting concentrations), and responding by induction of FGF21.

In summary, we observed maternal FGF21 is positively correlated with maternal body mass index and adiposity before and throughout pregnancy, and FGF21 is likely responsive to short-term changes in macronutrient balance induced by maternal diet rather than long-term changes in energy balance reflected in the maternal energy stores. We propose FGF21 may be a signal of nutrient insufficiency; particularly an insufficiency
arising from reduced maternal protein intake in pregnancy in the presence of elevated total intake and subsequent gain in adiposity.
3.1. INTRODUCTION

Diet is the most commonly described regulator of fibroblast growth factor 21 (FGF21). FGF21 concentrations are elevated in response to fasting (66, 115, 122), ketogenic diets (122), amino acid deprivation or low protein diets (123-129), and overfeeding (88). However, the novel discovery that FGF21 concentrations in circulation are robustly increased in states of protein restriction, even while controlling for energy intake (129), suggest that the elevations in FGF21 induced by ketogenic diets and fasting are likely due to the coincident reduction of protein in these conditions rather than ketogenesis or energy restriction itself. Moreover, Solon-Biet et al. completed a study of >800 mice on 25 various diets and, in support of Laeger’s finding (125), corroborated that the conflicting reports of FGF21 regulation in the literature are likely due to differences in nutrient intake. That finding points to dietary protein as the primary regulator of FGF21 (129).

As reported in Chapter 2, serum FGF21 concentrations were significantly lower and less variable in the first trimester of pregnancy compared to the third trimester in healthy pregnant women with overweight and obesity. We posit that the increase in FGF21 across trimesters is due to the increased protein demand of late pregnancy, and thereby, related to the failure of many pregnant women to increase dietary protein and consume a reduced protein diet in late pregnancy. In support of this idea, classic studies employing leucine kinetics and $^{15}$N lysine tracers in pregnant women have demonstrated little to no protein accretion during the first trimester compared to the second and third trimesters (188, 189). Assuming a typical gestational weight gain of 12.5 kg, including a
fetus weight of 3.3 kg, a total of 925 grams of protein is expected to be deposited across pregnancy (173). Whole body potassium studies further estimate for adequate protein accretion, daily protein depositions double from 8.4 g/day in the second trimester to 16.7 g/day in the third trimester (190). Based on these data, the estimated average requirement of protein for pregnant women increases from 0.80 grams of protein per kilogram of body weight per day (g/kg/d) in the first trimester (the same recommendation for non-pregnant women) to 0.88 g/kg/d in both the second and third trimesters (173). The recommended dietary allowance is more strict and recommends 1.1 g/kg/d of protein during the last two trimesters of pregnancy (173). Epidemiological studies of global and US populations revealed an overall decrease in protein intake over past decades (191); a finding that likely includes pregnant women. Therefore, considering that women are likely entering pregnancy eating diets with reduced protein content and that many women fail to adequately increase protein intake to match protein demands during pregnancy, we hypothesize that pregnancy is a state of unintentional, self-inflicted protein insufficiency for many women.

If FGF21 is indeed a sensor of protein intake, we hypothesize FGF21 concentrations will be increased when consuming a low protein diet in pregnancy when compared to intakes of normal or high protein diets in pregnancy. This hypothesis was tested in two studies: in 35 pregnant women with self-selected low, normal, or high third trimester protein intake and in C57BL/6 dams fed a low or normal protein diet throughout gestation. Finally, inadequate intake of protein by mothers during pregnancy has been empirically shown to cause fetal growth restriction (192). Because many maternal hormones have known effects on fetal growth, it is important to understand the effects of
maternal FGF21 on the fetus, especially if FGF21 is functioning as a nutrient sensor of low maternal protein intake. We explored this possible role by investigating the impact of maternal FGF21 on infant size and infant growth throughout the first year of life. We hypothesized maternal FGF21 measured late in pregnancy (i.e. close to delivery) would correlate with fetal size at birth and growth in the first year of life.

3.2. MATERIALS AND METHODS

Study 1: Preclinical study of maternal protein intake during pregnancy and FGF21

3.2.1. Animal care and diets

Animal experiments were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee. Virgin, 8-week old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and fed either low or normal protein diets (by Research Diets, D11092305 and D11092307 respectively). Diets were designed to be isocaloric, maintaining fat content and altering protein and carbohydrate content (Table 3.1). By energy, the “normal protein” diet contained 21% protein (as 24% casein) and the “low protein” diet contained 7% protein (as 8% casein). Animals were single-housed in a climate-controlled facility with a 12-hour light/dark cycle and ad libitum access to food and water unless otherwise indicated. Animals were maintained on standard chow until set up for breeding. Male and female mice were multi-housed (2 females: 1 male) for two dark cycles (approximately 40 hours) to breed and were fed the female’s respective randomized low or normal protein diet during this time. Females were then single-housed and the randomized diet (low or normal protein diet) was continued and measured daily until animals were sacrificed on gestational day 18.5 (first night of breeding considered 0.5). Mice were sacrificed in the fed state by acute CO₂ exposure
during the mid-light cycle. Blood was collected by cardiac puncture and the uterus was immediately excised. Each pup and placenta unit were carefully dissected, weighed, and flash frozen for future analyses, while liver, WAT depots, BAT, and muscle from the dams were simultaneously collected and also snap frozen. Blood was allowed to clot at room temperature and serum collected after centrifugation at 3000g.

Table 3.1 Detailed compositions of mouse diets

<table>
<thead>
<tr>
<th>Ingredient (g)</th>
<th>Low Protein Diet (D11092305)</th>
<th>Normal Protein Diet (D11092307)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>80</td>
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</tr>
<tr>
<td>L-Cystine</td>
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<td>3.6</td>
</tr>
<tr>
<td>Corn Starch</td>
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<td>315.2</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
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<td>150</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>107.1</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>50</td>
</tr>
<tr>
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</tr>
<tr>
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<td>75</td>
</tr>
<tr>
<td>Mineral Mix S10022G</td>
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</tr>
<tr>
<td>Mineral Mix S10022C</td>
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<td>3.5</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
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<td>12.5</td>
</tr>
<tr>
<td>Calcium Phosphate, Dibasic</td>
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<td>0</td>
</tr>
<tr>
<td>Potassium Citrate, 1 H2O</td>
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<td>3.6</td>
</tr>
<tr>
<td>Potassium Phosphate, Monobasic</td>
<td>6.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Sodium Chloride</td>
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<td>2.6</td>
</tr>
<tr>
<td>Vitamin Mix V10037</td>
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<td>10</td>
</tr>
<tr>
<td>Choline Bitrartrate</td>
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<td>2.5</td>
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<td>Protein (gram%)</td>
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<tr>
<td>Carbohydrate (gram%)</td>
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<td>58</td>
</tr>
<tr>
<td>Fat (gram%)</td>
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<td>10</td>
</tr>
<tr>
<td>Protein (kcal%)</td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>Carbohydrate (kcal%)</td>
<td>71</td>
<td>57</td>
</tr>
<tr>
<td>Fat (kcal%)</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Kcal/gram</td>
<td>4.1</td>
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</tr>
</tbody>
</table>

3.2.2. **FGF21**

Fibroblast growth factor 21 was measured in duplicate by sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions for humans
(RD191108200R, Fibroblast Growth Factor 21 Human ELISA, Biovendor, Brno, Czech Republic) and mice (RD291108200R, Fibroblast Growth Factor 21 Mouse/Rat ELISA, Biovendor, Brno, Czech Republic). Human serum was diluted 1:2 (125μL of serum in 125μL of dilution buffer) and murine serum was diluted 1:5 (50μL of serum in 200μL of dilution buffer) before analysis. The detectable concentration by the human assay is 0.03 ng/mL and mouse assay is 0.04 ng/mL. The intra-assay coefficient of variation was 3.9 for the human assay and 3.0 for the mouse assay.

3.2.3. Quantitative real time PCR

mRNA was isolated from flash-frozen tissue with the RNeasy Mini Kit (QIAGEN) and was quantified by Nanodrop. All samples had 260:280 and 260:230 ratios greater than 1.75. Two thousand nanograms of RNA was reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR was performed on 20ng of cDNA for human tissues and 4ng of cDNA for murine tissues, 300nM of each of forward and reverse primer, and iTaq universal SYBR green mastermix on a 7900HT PCR Machine (ThermoFisher Scientific, Waltham, MA). The PCR protocol began with one cycle at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 59°C for 1 minute, and ended with a dissociation curve analysis. Primer sequences unique for each target gene were designed with primer BLAST to span exon-exon junctions. Cyclophilin A was used as the endogenous control for murine samples and the geometric mean of PPARα and FATP1 was used as an endogenous control for human samples.
Table 3.2 Murine primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
<tbody>
<tr>
<td>FGF21</td>
<td>CAAATCCTGGGTGTCAAAGC</td>
<td>CATGGGCTTCAGACTGGTAC</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>CTTCGAGCTGTTTGCAGACAAAAGT</td>
<td>AGATGCCAGGACCTGTATGCT</td>
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</table>

Table 3.3 Human primer sequences

<table>
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<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
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<td>KLB</td>
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<td>CCACAGACTCGGGCTTAAGAA</td>
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<tr>
<td>FGFR1</td>
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</tr>
<tr>
<td>PPARα</td>
<td>GGCAAGACAAGCTCAGAAC</td>
<td>TTATCTATGAAGCAGGAAGC</td>
</tr>
<tr>
<td>FATP1</td>
<td>AGGTGGTCAGTACATCGGG</td>
<td>AGAACTCCCCGATTGGC</td>
</tr>
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</table>

Study 2: Cross-sectional study of maternal protein intake, FGF21 concentrations, and fetal growth

3.2.4. Study population

Thirty-five pregnant women were included in this ancillary study based on provision of consent for future use of biospecimens (i.e. blood and/or placenta) as well as data availability collected from two main studies: the Expecting Success Study (n=54, NCT01610752) or the MomEE study (n=60, NCT01954342), completed at Pennington Biomedical Research Center in Baton Rouge, Louisiana. Participants were healthy, overweight or obese women (BMI ≥ 25kg/m²), aged 18-40 years with a single, viable, first trimester pregnancy (<14 weeks gestation). For enrollment into this ancillary study, participants must have been enrolled in a parent study (Expecting Success or MomEE), provided consent for use of biospecimens in future research, and had archived serum and data necessary to address study aims collected in the third trimester (35-36 weeks). Both parent studies and the ancillary study were approved and monitored by the Pennington Biomedical Research Center Institutional Review Board and all participants provided verbal and written consent prior to study initiation.
3.2.5. Clinic assessments

The parent studies enrolled women in the first trimester of pregnancy (<14 weeks gestation) and study visits for data collection coincided with gestational age to allow for one assessment visit per trimester, i.e. 1\textsuperscript{st} trimester (<15 weeks gestation) and third trimester (35-36 weeks gestation). Study visits were conducted in the morning following an overnight fast and allowed for collection of body weight, body composition, and blood. All data were obtained in accordance with standard operating procedures of Pennington Biomedical Research Center to ensure scientific rigor and reproducibility.

3.2.6. Protein intake determination

Protein intake during pregnancy was determined by the Remote Food Photography Method (RFPM), an objective assessment of dietary intake collected over 5-7 days between 35 and 36 weeks gestation. Briefly, RFPM requires participants to capture images of their food selection and plate waste within a Smartphone application. The images are sent to a server in real-time and later analyzed using semi-automated procedures that rely on both computer automation and human operators (registered dieticians) from validated methodology (193). The validity of the RFPM has been tested and procedures are published (194, 195). Utilizing these data, average protein intake was determined as percent of energy (kilocalories per day), grams per day, and grams per kilogram of body weight per day.

3.2.7. Protein intake classifications

The Dietary Reference Intake recommendations for protein intake in pregnancy from the Institute of Medicine are published as total daily intakes, i.e. as grams per day (g/d) and grams per kilogram of body weight per day (g/kg/d): EAR= +21g/d or 0.88g/kg/d, RDA= 1.1 g/kg/d (173). We chose to use g/kg/d for classification of protein intake and
based our upper and lower classification bounds on the recommended EAR and RDA: participants who reported consuming less than 0.88 g/kg/d of protein were classified as having low protein intake, 0.88-1.1 g/kg/d were classified as normal protein intake, and greater than 1.1 g/kg/d as high protein intake.

3.2.8. Infant phenotyping

Infant length was measured by placing the infant on an infant length board with a built-in centimeter scale, stationary head-board and moveable foot board. Infant body weight was measured with the infant undressed by electrical infant scale. Head circumference was measured by a standard measuring tape around the infant's head. All measures were repeated in duplicate and averaged. Percentiles were calculated for the infant measurements considering the gender, date of birth, and date of measurement according to the World Health Organization growth charts (http://www.cdc.gov/growthcharts/whocharts.htm).

3.2.9. Placenta collection

Placenta samples were collected, dissected and frozen within two hours of delivery. Prior to dissection, tissue was kept refrigerated or on ice. Samples were dissected from the placental disc at four separate sites and stored by section (basal plate, villous tissue, and chorionic plate) at -80°C until being thawed for study (Figure 3.1). Additionally, gross morphology (photography, placenta shape and cord insertion site), weight, membrane sample, cord tissue sample and cord blood were collected. Experiments in this study used snap frozen villous tissue pooled from four sites on the placenta collected within two hours of delivery.
3.2.10. **Statistical analysis**

Data are presented as mean ± standard error. To determine statistical significance, for Aim 1 (investigating protein diet effects on FGF21 concentrations in pregnancy) students t-test’s, analysis of variance, or linear regression analyses were employed where appropriate. The PROOF study, which showed for the first time that FGF21 is induced by low protein feeding in humans, was used for power calculations and sample size estimates in the current study (196). In PROOF, FGF21 increased by 171.5% in participants consuming a low protein diet and by 14.8% in participants eating normal protein diets, giving an observed difference of over 150%. Based on the SD from the PROOF data (106%), to detect a difference of 150% in FGF21 between diet groups and ensuring 80% power, nine participants per group were required (Table 3.4). For Aim 2 (investigating the impact of maternal FGF21 on infant size and infant growth throughout the first year of life), partial correlations were estimated adjusting for gestational age at delivery, infant gender, and randomization (intervention or control). Tests were performed with significance level $\alpha=0.05$, and findings considered significant when $P<\alpha$. 
Table 3.4 Power Calculations

<table>
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<th>Power (%)</th>
<th>% Difference FGF21</th>
<th>SD</th>
<th>N Per Group</th>
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<tr>
<td>80</td>
<td>50</td>
<td>106</td>
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<td>80</td>
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<td>80</td>
<td>150</td>
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</table>

3.3. RESULTS

3.3.1. In mice, FGF21 is elevated with low protein intake during pregnancy

In serum from C57BL/6 female mice on day 18.5 gestation (Figure 3.2), FGF21 concentrations were 20-fold higher in mice fed low (7%) versus normal (21%) protein diets throughout gestation (22.760 ± 7.416 ng/mL vs. 1.673 ± 0.176 ng/mL, *p=0.006, n=5-7).

Figure 3.2 Serum FGF21 is elevated in states of low protein intake during pregnancy in mice. Serum FGF21 concentrations from C57BL/6 mice on day 18.5 gestation fed low (7%) or normal (21%) protein diets, (22.760 ± 7.416 ng/mL vs. 1.673 ± 0.176 ng/mL, *p=0.006, n=5-7)
3.3.2. In mice, the liver, not placenta, is the primary source of protein-inducible FGF21 production in pregnancy

To understand the tissue(s) responsible for the increase in FGF21 during pregnancy, we measured the expression of FGF21 in placenta and liver of the mice fed low and normal protein diets. We were unable to identify meaningful and reproducible levels of FGF21 transcript by qPCR in the placenta in mice which replicates our finding in pregnant women (Chapter 2). Hepatic FGF21 expression at day 18.5 of gestation was 7.6-fold higher in mice fed a low versus normal protein diet throughout gestation (p=0.002) (Figure 3.3).

![Figure 3.3](image)

Figure 3.3 Hepatic FGF21 mRNA expression in pregnant C57BL/6 mice fed a low versus normal protein diet, relative expression: 9.565 ± 1.86 vs. 1.26 ± 0.39, n=5, *p<0.01.

3.3.3. Classification of maternal protein intake

To determine whether maternal FGF21 is induced by low protein diets in pregnant humans, self-reported dietary intake data collected in the third trimester (35-36 weeks gestation) was used to classify participants as eating low, normal, or high protein diets
(classification bounds described in Section 3.2.7). The distribution of third trimester protein intake is shown as percent of total energy and grams per kilogram of body weight per day (Figure 3.4A and B). The majority of participants (60%, or n=21) reported consuming a low protein diet which had a mean group protein intake of 0.70 ± 0.12 g/kg/d. Eight participants (23%) reported consuming a normal protein diet which had a group mean intake of 0.98 ± 0.09 g/kg/d, and six participants (17%) reported consuming a high protein diet which had a mean group intake of 1.39 ± 0.20 g/kg/d (Figure 3.4C).

Figure 3.4 Distribution and classification of third trimester protein intake in study population. (A) Distribution of protein intake reported as percent of total energy, (B) Distribution of protein intake reported as grams of protein per kilogram of body weight per day, (C) Classifications of low (n=21), normal (n=8), and high (n=6) protein intake calculated as g/kg/d from self-reported third trimester food intake, *p<0.0001.
3.3.4. **FGF21 is elevated with low protein intake during pregnancy in humans**

A cross-sectional analysis of FGF21 concentrations in pregnant women according to their self-reported protein intake relative to dietary recommendations revealed that FGF21 was significantly higher in those individuals who self-selected a low versus normal versus high protein diet (Low: 0.302 ± 0.163 ng/mL, Normal: 0.206 ± 0.155 ng/mL, High: 0.133 ± 0.061 ng/mL, p=0.04) (Figure 3.5).

![Figure 3.5](image)

Figure 3.5 Serum FGF21 is elevated in states of low protein intake during pregnancy in humans. Serum FGF21 concentrations from humans during 35-36 weeks gestation self-selecting low (0.302 ± 0.163 ng/mL), normal (0.206 ± 0.155 ng/mL) or high (0.133 ± 0.061 ng/mL) protein diets, *p=0.04.

3.3.5. **Placental expression of β-Klotho is regulated under variable protein intake**

Although the placenta does not appear to synthesize amounts of FGF21 consequential to circulating concentrations, it is possible that the placenta is capable of responding to the FGF21 of the mother. For example, β-Klotho, the cell surface co-receptor to FGFR1 required for FGF21 signaling (142, 145, 147), has been reported to be differentially regulated in various states of energy and nutrient balance. In populations with proposed FGF21-resistance (obesity and diabetes) where FGF21 is found to be
elevated, β-Klotho expression is decreased in adipose tissue and liver (88). Moreover, rescuing this reduced expression has been shown to proportionally restore FGF21 action (197). In a subset of our human study cohort with food intake data as well as archived placenta samples (n=19), we found no difference in FGFR1 expression and elevated β-Klotho expression with reduced maternal protein intake in the third trimester (Figure 3.6), a state of elevated circulating FGF21 as we previously reported. However, this trend failed to reach significance, likely due to high variability and small sample size.

Figure 3.6 Correlation of human placental (A) FGFR1 and (B) β-Klotho mRNA expression and maternal protein intake reported between 35-36 weeks gestation (n=19, p=0.186 and p=0.139 respectively)

3.3.6. Maternal FGF21 influences infant size and growth

In order to further explore the potential role of FGF21 as communicator of nutrient status between mother, placenta, and fetus, we explored the relationship of maternal FGF21 and fetal size and growth. For a subset of participants (n=27), infants of enrolled mothers were also enrolled in our study for collection of anthropometric assessments through the first year of life. Maternal third trimester FGF21 (an assumed value for the end of gestation) was found to be negatively correlated with measures
of infant length at birth \((p=0.03, r=-0.45)\) and length percentile at birth \((p=0.02, r=-0.46)\) (Figures 3.7A and B) such that mothers with high concentrations of FGF21 (and low protein intake) delivered smaller infants.

Figure 3.7 Maternal third trimester FGF21 concentration is negatively correlated with infant length at birth. (A) Maternal 3\(^{rd}\) trimester FGF21 and infant birth length \((n=27, p=0.03, r=-0.45)\) and (B) maternal 3\(^{rd}\) trimester FGF21 and infant birth length percentile \((n=27, p=0.02, r=-0.46)\).

Maternal FGF21 measured in late pregnancy was also found to negatively correlate with infant growth, i.e. infant anthropometrics collected throughout the first year of life. Serum FGF21 measured in the third trimester of pregnancy negatively correlated with infant weight \((r=-0.51, p=0.02)\) (Figure 3.8A), infant length \((r=-0.44, p=0.04)\) (Figure 3.8B), and infant head circumference \((r=-0.66, p<0.01)\) (Figure 3.9A) measured between 4 and 8 weeks of age.
Figure 3.8 Maternal third trimester FGF21 concentration is negatively correlated with infant size at 4-8 weeks of age. (A) Maternal 3rd trimester FGF21 and infant weight (n=25, r= -0.51, p=0.02) and (B) maternal 3rd trimester FGF21 and infant birth length percentile (n=25, r= -0.44, p=0.04).

Considering head circumference is a robust marker for intrauterine nutritional sufficiency that can be measured at birth and persists throughout adulthood (198), it was interesting to find the negative correlation of maternal FGF21 and infant head circumference and head circumference percentile at 1-2 months of age (cm: p<0.01, r= -0.66, percentile: p=0.03, r= -0.45; Figures 3.9A and B) extended through 6 months of age (cm: p=0.01, r= -0.53, percentile: p=0.01, r= -0.58, Figures 3.9C and D) and 1 year of life (cm: p<0.01, r= -0.66, percentile: p<0.01, r= -0.66) (Figures 3.9E and F).
Figure 3.9 Maternal third trimester FGF21 concentration is negatively correlated with infant head circumferences through the first year of life. (A) Maternal 3\textsuperscript{rd} trimester FGF21 and infant head circumference (n=25, $r=-0.66$, $p<0.01$) and (B) percentile (n=25, $r=-0.45$, $p=0.03$) at 4-8 weeks of age, (C) maternal 3\textsuperscript{rd} trimester FGF21 and infant head circumference (n=24, $r=-0.53$, $p=0.01$) and (D) percentile (n=24, $r=-0.58$, $p=0.01$) at 6 months of age, and (E) maternal 3\textsuperscript{rd} trimester FGF21 and infant head circumference (n=24, $r=-0.66$, $p<0.01$) and percentile (n=24, $r=-0.66$, $p<0.01$) at 1 year of age.
3.4. DISCUSSION

In this study, we investigated the role of FGF21 as a novel endocrine signal of nutrient status in pregnancy. Using cross-sectional analyses in pre-clinical and clinical studies, the key finding is that maternal FGF21 is induced by low protein intake in pregnancy. We also observed reduced protein intake correlated with increased β-Klotho expression in the placenta. Based on the elevated circulating FGF21 and FGF21 coreceptor placenta expression, we hypothesized that FGF21 could exert effects on the placenta resulting in downstream effects on fetal development. In our investigations of this mechanism, we found maternal FGF21 measured late in pregnancy was negatively correlated with fetal size at birth and growth during the first year of life.

The relationship between FGF21 and diet (e.g. fasting (66, 115, 122), ketogenic diets (122), amino acid deprivation or low protein diets (123-129), and overfeeding (88)) has been well described. Recent studies have demonstrated that FGF21 is robustly and specifically responsive to protein intake (125, 129). Pregnancy is an intriguing model for the study of FGF21 biology because of the pregnancy-driven increase in protein demand (173). In our study, FGF21 concentrations were elevated in response to low protein intake in pregnancy in both mice and humans. An important and alternative explanation to consider is the altered carbohydrate content in the low and normal protein diets. While we attribute the observed induction of FGF21 to the alterations in protein intake, Solon-Biet et al. observed that low protein intake coupled with high carbohydrate intake results in an exacerbated elevation in FGF21 compared to when carbohydrate is held constant. Therefore, due to the formation of the diets, our study cannot exclude a potential role for carbohydrate, either independently or in addition to protein. Interestingly, neither mouse
or human placenta appear to be contributing to this increase in serum FGF21 in the mother. However, mice showed a robust increase in FGF21 transcript in the liver of pregnant dams consuming a low protein diet in pregnancy. These findings suggest that, like non-pregnant models, FGF21 is responding to the nutritional status of the mother with the liver and without additional sensing from the placenta. It was also intriguing to find β-Klotho expression in the human placenta was negatively correlated with maternal protein intake. Other reports have shown decreased β-Klotho expression in fat and liver in states of FGF21-resistance (88). However, our findings suggest low maternal protein intake may contribute to an elevated sensitivity to FGF21 by the placenta by increasing co-receptor expression. Nevertheless, due to high variability and small sample size, our results failed to reach significance and the role of the regulation of β-Klotho in communications between maternal FGF21, placental function, and the fetus requires further investigation.

There is a well established, causative relationship between low maternal protein intake during pregnancy and fetal growth restriction. However, to our knowledge, FGF21 has yet to be measured in these conditions. Considering our new evidence that FGF21 is elevated with low maternal protein intake in pregnancy, it would be advantageous to explore if FGF21 could be a clinical test to identify women and infants at risk for low levels of fetal growth. As detailed in Chapter 1, individuals born small have an increased risk for chronic disease in adulthood. Identifying a biological marker for detection of growth restriction during gestation, coupled with proactive intervention, could have long term health benefits for the infant. In our study, we hypothesized that elevated FGF21 may be a maternal signal of insufficient fetal growth. Using maternal FGF21 measured in the third trimester as an assumed indicator of FGF21 concentrations at the end of pregnancy, we
found an inverse association with infant size at birth (length) and growth in the first year of life (length, weight, and head circumference). Interestingly, head circumference at birth and throughout infancy is used as a clinical indicator of intrauterine nutritional sufficiency as 60-70% of adult head circumference is achieved by the time a fetus is born and 80-90% by age two (165). In our study, we observed that head circumference was decreased in infants of mothers with elevated FGF21 in late pregnancy. This finding further supports our hypothesis that FGF21 is up regulated in pregnancies that have inadequate maternal protein intake.

In sum, we have reported maternal FGF21 positively correlates with maternal BMI and adiposity in pregnancy and negatively correlates with infant size at birth and infant growth in the first year of life. While these findings can appear contradictory, we propose FGF21 may be acting as a mediator and/or signal of the Protein Leverage Hypothesis (PLH). The PLH posits that total energy intake is driven by protein requirement, i.e. when consuming a low protein diet, an individual will over-consume all macronutrients in order to satisfy daily protein balance and thereby increasing total energy intake. The PLH creates a model of elevated energy intake (hyperphagia) and inadequate protein intake. Indeed, in a randomized controlled trial, Gosby et al. observed an elevation in circulating FGF21 commensurate with an elevated *ad libitum* energy intake in humans consuming a low protein diet (128). Consequently, we propose FGF21 to be a signal of fetal nutrient insufficiency; particularly an insufficiency arising from reduced maternal protein intake in pregnancy in the presence of elevated total intake and subsequent gain in adiposity. Future studies to directly test this proposed role of FGF21 in pregnancy would include animal studies to, first, establish the PLH in pregnancy with low and adequate protein
diets during gestation. Furthermore, replicating this experiment in an FGF21 knock out mouse model would enable direct study of whether the increased energy intake observed with a low protein diet is FGF21 dependent.
CHAPTER 4: FIBROBLAST GROWTH FACTOR 21 IS A MEDIATOR OF PROTEIN LEVERAGE

4.1. INTRODUCTION

The Protein Leverage Hypothesis (PLH) theorizes that total energy intake is driven by protein requirement, i.e. when consuming a low protein diet, an individual or animal will over-consume all macronutrients in order to satisfy daily protein balance and thereby increase total energy intake. This hypothesis was originally proposed by Simpson and Raubenheimer as a contributor to the obesity epidemic (191). Simpson et al. described decreased ratios of protein intake to carbohydrate and fat in the common diet in epidemiological studies (191). Mechanistic studies in rodents and humans have since demonstrated protein-induced hyperphagia by provision of low protein diets and assessment of food intake (199, 200). However, the PHL however has not yet been tested in pregnancy.

Pregnancy is a normal physiological state that demands an increase in the protein requirement and, as such, without proper dietary modifications to increase protein intake, pregnancy can lead to an unintentional protein restriction. It is approximated that the 925 grams of protein is deposited during a human pregnancy that requires an increase in protein deposition of 3.6 g/day and 7.2 g/day in the second and third trimesters respectively (173, 190). Simpson and others have described changes in the composition of adult diets globally and found them to be lacking in adequate protein intake (191). Given changes in the available food sources, we hypothesize that many women unintentionally consume a reduced protein diet. Pregnancy adds an increased protein demand and thereby becomes an advantageous model to test the PLH. Our recent finding that circulating fibroblast growth factor 21 (FGF21) increases throughout pregnancy and
in concert with acute changes in glucose status could suggest that FGF21 is acting as a nutrient sensor in pregnancy. It is also plausible that FGF21 is responding specifically to maternal protein intake, leading us to ask if FGF21 is a mediator of the PLH in pregnancy.

The first goal of our study was to determine whether low protein-induced hyperphagia (as predicted by the PLH) is present during pregnancy. We hypothesized pregnant, C57BL/6 mice would have a higher energy intake when fed a low versus normal protein diet throughout gestation. The second goal of our study was to determine whether FGF21 serves as a mediator of the PLH. First, as proof of concept, we employed protein choice experiments with non-pregnant C57BL/6 and FGF21 null mice. We hypothesized C57BL/6 mice would consume an average protein intake within a set point, while mice without FGF21 would have erratic protein intake. Second, to investigate the causative role of FGF21 in protein leverage in pregnancy, we aimed to determine whether low protein-induced hyperphagia is present in FGF21KO pregnancies. We hypothesized FGF21KO mice would not differ in energy intake when fed a low protein versus normal protein diet throughout gestation.

4.2. MATERIALS AND METHODS

4.2.1. Animal care

Female wild type (C57BL/6) and FGF21 knock out (FGF21KO) mice were used in studies. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and FGF21KO mice on the C57BL/6 background were provided by Steven Kliewer (University of Texas Southwestern, Dallas, Texas, USA) (92). For all experiments, animals were single-housed in a climate-controlled facility with a 12-hour light/dark cycle and allowed ad libitum access to food and water unless otherwise indicated. All studies were approved.
by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

4.2.2. Effect of low protein diet on food intake in pregnancy

Overview: Eight week old, virgin female C57BL/6 mice (n=12) were bred with C57BL/6 males and provided a low or normal protein diet throughout gestation. Daily food intake was measured starting on gestation day (GD) 1.5 until animals were sacrificed on GD18.5.

Design: Male and female mice were multi-housed (2 females: 1 male) for two dark cycles to breed (approximately 40 hours). The first night of breeding is considered GD0.5. Females were randomized to receive either a low (7% protein) or normal (21% protein) protein diet throughout gestation (Table 4.1). In order to feed the respective randomized diet throughout pregnancy, diets commenced at the onset of breeding (GD0). Following breeding, females were single housed on GD1.5 and continued on their randomized diet (low or normal protein). Body weight was measured weekly and food intake was measured at the same time daily until animals were sacrificed on GD18.5. Mice were sacrificed in the fed state by acute CO₂ exposure during the mid-light cycle. Blood was collected by cardiac puncture, allowed to clot at room temperature, and serum collected after centrifugation at 3000g. Necropsy was performed for collection of liver, WAT depots, BAT, and muscle from the dam, while simultaneously each pup and placenta unit were carefully dissected, weighed, and flash frozen.

Diets: Diets were formulated by Research Diets (D11092305 and D11092307 respectively) and were designed to be isocaloric (4.1 kcal/g), maintaining fat content (22%) and altering protein and carbohydrate content (Table 4.1). By energy, the normal
protein diet contained 21% protein (as 24% casein) and the “low protein” diet contained 7% protein (as 8% casein).

Table 4.1 Mouse Diet Compositions

<table>
<thead>
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<th>Low Protein Diet (D11092305)</th>
<th>Normal Protein Diet (D11092307)</th>
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<tr>
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<tr>
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<td>3.5</td>
</tr>
<tr>
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<td>12.5</td>
</tr>
<tr>
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</tr>
<tr>
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<td>3.6</td>
</tr>
<tr>
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**Statistical Analysis:** Data are presented as mean ± standard error. At sacrifice, number of pups were counted for each dam and food intake data were normalized for (divided by) litter size and reported as grams per day per pup (g/d/pup). For measure of food intake during “early” and “late” gestation, three day averages of normalized food intake were calculated using GD3.5-5.5 and GD14.5-16.5 respectively. To measure elevations in pregnancy-driven food intake, “early” food intake was subtracted from “late”
food intake. Statistical significance was determined between low and normal protein groups with unpaired students t-test's. All tests were performed with significance level α=0.05, and findings considered significant when P<α.

4.2.3. Role of FGF21 in protein sensing in female mice

Diets: Diets were formulated by Research Diets (D11092301, D11092304, and D11092303) and were designed to be isocaloric, maintaining fat content (22%) and altering protein and carbohydrate content (Table 4.2).

Table 4.2 Mouse Diet Compositions

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<th>Ingredient (g)</th>
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<th>36% Protein (D11092304)</th>
<th>55% Protein (D11092303)</th>
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<td>Carbohydrate (gram %)</td>
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<td>Kcal/gram</td>
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</tr>
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</table>
Overview: Eight week old, virgin C57BL/6 and FGF21KO female mice were provided a choice of two diets variable in protein content and were allowed to eat *ad libitum*. Food was weighed daily and protein intake calculated.

Design: Female C57BL/6 (n=19) and FGF21KO (n=20) mice were single-housed for 14 days and provided two diets of variable protein content simultaneously, one with low protein content (4% protein) and another with high protein content (either 36 or 55% protein) (Table 4.2). Mice were allowed to eat *ad libitum*. Diet choices were separated by the water trough and swapped daily to control for side preference. For each diet, food intake was measured at the same time daily. Mice were sacrificed by acute CO₂ exposure and decapitation at the end of study.

Statistical Analysis: Data are presented as mean ± standard error. To determine protein intake, each diet was weighed daily. The weights were multiplied by the respective percent protein content, and these two values were added together to calculate total grams of protein intake per day. As a measure of protein sensing, two-day averages of protein intake were calculated for study days 4-10; study days 1-3 were excluded to allow for acclimation to single-housing and diet change. A logistic model for repeated measures was constructed with fixed effects for diet composition, mouse type, day, and the interaction between mouse type and day. The repeated binary response indicated whether the two-day running average percentage of protein intake was within 20±5%. The running average was used to help determine if there was day-to-day compensation for high or low intake. A random mouse effect was included to account for within-animal correlations over time. For each mouse genotype, probabilities of achieving desired protein intake (20±5%) across any two-day sequence were estimated from the logistic
model. Additionally, odds of hitting the target protein range were compared between the two mouse types using a t-test of the log odds ratio. All tests were performed with significance level $\alpha=0.05$, and findings considered significant when $P<\alpha$.

4.2.4. Role of FGF21 in protein leverage in pregnant mice

To investigate the causative role of FGF21 in protein leverage in pregnancy, the *Effect of low protein diet on food intake in pregnancy* experiment was repeated in FGF21KO mice (See Section 4.2.2 for design and statistical analysis). Briefly, eight week old, virgin female FGF21KO mice ($n=11$) were bred with FG21KO males and provided a low or normal protein diet throughout gestation (Table 4.1). Food intake was measured daily from gestation day (GD) 1.5 until animals were sacrificed on GD18.5.

4.3. RESULTS

4.3.1. Evidence of protein leverage in pregnancy in C57BL/6 mice

C57BL/6 female mice were fed low or normal protein diets throughout gestation to determine whether the hyperphagia previously documented with low protein feeding in male mice (201-204) is present in pregnancy. C57BL/6 mice fed a low protein diet consumed more throughout gestation compared to mice fed a normal protein diet (Figure 4.1A). However, this elevation in food intake was only significantly higher than those consuming normal protein diets in late pregnancy (Figures 4.1A and B).
Figure 4.1 C57BL/6 mice respond to the increased protein requirement of pregnancy with hyperphagia when fed a low protein diet. (A) Average daily food intake of pregnant C57BL/6 mice consuming a low (gray, n=5) and normal (black, n=7) protein diet, (B) 3-day average food intake of pregnant C57BL/6 mice consuming a low (gray, n=5) and normal (black, n=7) protein diet in early pregnancy (GD3.5-5.5) and late pregnancy (GD14.5-16.5), **p=0.009, *p=0.02

4.3.2. FGF21 is required for protein sensing in female mice

C57BL/6 and FGF21KO mice were allowed to eat ad libitum for 14 days from two diets provided simultaneously; one diet was low in protein content, 4% energy, and the other diet was high in protein content, 36% or 55% energy. As hypothesized, C57BL/6 mice regulated protein intake. Representative patterns of daily protein intake using three example C57BL/6 mice are shown in Figure 4.2A. Protein intake oscillated in response to the protein intake consumed on the previous day. When protein intake is averaged across the week, each mouse consumed approximately 20% protein. In contrast, daily oscillations in food intake were not observed in the FGF21KO mice suggesting that these mice were incapable of regulating daily intake on the basis of protein. As such, FGF21KO mice consumed the provided diets at random without responding to the protein intake from the previous day (three example mice, Figure 4.2B).
Figure 4.2 Female C57BL/6, and not FGF21KO, mice defend certain level of protein intake. Daily self-selected protein intake of three example (A) C57BL/6 and (B) FGF21KO mice on study days 4-10, (C) Two-day average self-selected protein intake of C57BL/6 and FGF21KO cohort, n=19-20, red line: estimated protein requirement.

To measure protein sensing, we calculated the two-day average of protein intake (Figure 4.2C) and employed a repeated measures logistic model to determine whether mice were compensating, or sensing, protein intake from day to day. C57BL/6 mice averaged 20.7% protein intake across two-day intervals and had a 74% probability of consuming within 20±5% protein across two-day intervals. FGF21KO mice averaged 30.4% protein intake across two-day intervals and were only 12% likely to consume 20±5% protein across two-day intervals. The difference in probability of the mice to
choose a diet that provided 20±5% over consecutive 2-day periods was significantly different (p<.0001).

Table 4.3 Probability from repeated measures logistic model

<table>
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<td>22.0</td>
<td>&lt;0.0001</td>
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<tr>
<td>FGFR21 KO</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.3. **FGF21 is a mediator of protein leverage induced hyperphagia during pregnancy**

FGF21KO female mice were fed low or normal protein diets throughout gestation to determine whether FGF21 is responsible for signaling the hyperphagia observed with low protein feeding in pregnant C57BL/6 mice (Figure 4.1B). No difference in daily food intake was observed in pregnant FGF21KO mice fed a low versus normal protein diet (Figure 4.3A). Moreover, the elevated food intake coinciding with the increased protein demand in late pregnancy observed in C57BL/6 mice was absent in mice lacking FGF21 (Figures 4.3B and 4.4).

![Figure 4.3](image)

Figure 4.3 FGF21KO mice fail to respond a low protein diet with hyperphagia in pregnancy. (A) Average daily food intake of pregnant FGF21KO mice consuming a low (gray, n=6) and normal (black, n=5) protein diet, (B) 3-day average food intake of pregnant FGF21KO mice consuming a low (gray, n=6) and normal (black, n=5) protein diet in early pregnancy (GD3.5-5.5) and late pregnancy (GD14.5-16.5).
Figure 4.4 C57BL/6, not FGF21KO, pregnant mice exhibit hyperphagia in late pregnancy when fed a low protein diet. *p=0.006

4.4. DISCUSSION

In this study, we tested the protein leverage hypothesis in pregnancy and investigated the role of FGF21 as a potential mediator of the protein leverage hypothesis. Our key findings are that protein leverage can occur during pregnancy in mice and that FGF21 plays a role in protein sensing in mice. Considered together, we hypothesized FGF21 could serve as a mediator of the protein leverage hypothesis in pregnancy. We used FGF21 knock out mice to investigate this mechanism and observed that the low-protein induced hyperphagia associated with protein leverage in wild type mice was absent in mice lacking FGF21, suggesting FGF21 is required for protein leverage in pregnancy.

The Protein Leverage Hypothesis describes a link between protein and energy intake and specifically demonstrates that protein intake is prioritized over other macronutrients. This prioritization is consistent with the essential roles that proteins play
in all cellular processes, as well as the fact that the body does not maintain a protein reservoir that is rapidly mobilizable for energy analogous to the storage of fat. Protein deficiency therefore promotes counter regulatory mechanisms leading to adaptive increases in total energy intake in an effort to restore protein balance (191). For this reason, total food intake served as the primary outcome variable in our studies to detect effects of protein leverage. Although the leveraging effect of protein has been demonstrated in animal models and humans (199, 200), to our knowledge, the protein leverage hypothesis has not been directly studied in pregnancy. This work warrants investigation during gestation because diets with reduced protein over long periods would lead to increased energy intake and excess weight gain resulting in an increased risk for adverse maternal and fetal outcomes. For example, a study of the Helsinki Birth Cohort revealed children of overweight and obese mothers have an increased risk of developing cardiovascular disease, cancer, and type 2 diabetes in adulthood (205). Moreover, a study from the Dutch Famine Cohort has shown children of mothers consuming a low protein diet in pregnancy had elevated blood pressure in adulthood, one well known precursor to cardiovascular disease (38). As, epidemiological studies have shown protein intake as a percent of total energy consumed has declined across the general population (191), these considerations make this a question applicable to the pregnant population consuming a modern-day western diet.

In our study, we found evidence of protein leverage in pregnancy. We observed an increase in food intake of wild type mice fed low protein diets throughout gestation, which became more pronounced at the end of pregnancy. A plausible explanation for this could be the elevated protein requirement in late gestation (173, 190). Perhaps the
low protein diet that provided 7% of energy from protein was not sufficient to induce a protein restriction severe enough to elicit the protein leverage response until exacerbated by the increased protein demand of late gestation. For this reason, we explored the pattern of food intake across pregnancy, subtracting the three-day average food intake from early pregnancy from the three-day average food intake from late pregnancy. This approach revealed a significant increase in food intake with the presumptive increased protein demand and subsequent amplified protein restriction caused by late gestation.

The recently elucidated role of protein in FGF21 regulation in non-pregnant models alongside our new evidence of elevated FGF21 in response to low protein intake during pregnancy (Chapter 3), lead us to investigate the potential role of FGF21 in protein leverage in pregnancy. First, as proof of concept, we applied this question to a non-pregnant model. Pregnancy undoubtedly presents a unique physiological state which merits study. However, as protein balance was the primary variable for investigation, the products of conception, i.e. litter size, would introduce too much variability in altered pregnancy-driven protein requirement making a non-pregnant rodent model ideal. We observed FGF21 null mice could not regulate protein intake like wild type mice. These findings suggest that FGF21 is required for an animal to sense their protein balance and regulate food intake accordingly. Following this proof of concept, i.e. FGF21 acting as a protein sensor, we found that the increased food intake that accompanies a low protein diet in pregnancy was ablated in the absence of FGF21. Taken together, these findings demonstrate FGF21 to be a protein sensor and necessary for protein leverage during pregnancy.
The primary limitation of our study is utilization of a rodent model rather than conducting a human trial. Studies to test the PLH in humans are complex as the primary outcome could be influenced by behavior, i.e. energy intake. Research participants with knowledge of participating in studies aimed to assess food intake are likely to become more mindful eaters which could convolute results. Indeed, the Simpson Laboratory has gone to great lengths to alter recipes and disguise food to control for human bias (200). Also, studies in free-living conditions are problematic because measurement of self-reported food intake, particularly total energy intake, in clinical trials is notoriously unreliable (206). Finally, studies have repeatedly demonstrated the low protein-induced hyperphagia predicted by the PLH in male rats and mice (201-204). Taken together, rodents were a suitable model for initial studies to examine PLH in pregnancy. Moreover, the availability of FGF21 knock out (FGF21KO) mice allows for investigation of the direct and causative role of FGF21 in the PLH in pregnancy.

In summary, protein leverage appears to be present in states of protein restriction during pregnancy in mice. Moreover, FGF21 is required for protein sensing in non-pregnant female mice and is a mediator of the protein leverage hypothesis in pregnant dams. Future studies are needed to translate these findings into the human population and to understand the impact on fetal development and offspring growth after birth.
CHAPTER 5. SUMMARY AND CONCLUSIONS

The overarching goal of this work was to describe for the first time the role of fibroblast growth factor 21 in pregnancy. In our studies, we first described FGF21 in a healthy, pregnant population of overweight and obese women and observed that FGF21 was acutely regulated by maternal macronutrient balance. We next studied the role of protein specifically in FGF21 regulation during pregnancy with pre-clinical (rodent) and clinical (human) studies. We found that FGF21 was regulated by maternal protein intake in both mice and humans. Finally, we investigated the potential role that FGF21 might play under protein imbalance; asking whether FGF21 could be a mediator of the protein leverage hypothesis in pregnancy. Utilizing FGF21 null mice, we observed that FGF21 is needed for the low-protein induced hyperphagia associated with protein leverage in pregnancy. In total, within this body of work, our data reveal FGF21 to be a novel protein sensor in pregnancy.

In Chapter 2, we conducted a cross-sectional and longitudinal study to describe FGF21 in a population of healthy pregnant women. Maternal FGF21 concentration was measured in the first and third trimesters along with measures of body composition and other circulating factors, e.g. glucose and insulin. Our study showed that the well described relationships between FGF21 and adiposity are also evident in the pregnant state, i.e. FGF21 increases as adiposity and BMI increase. However, the longitudinal study of FGF21 across pregnancy showed that circulating levels of FGF21 are not responsive to the long-term changes in energy balance (reflected in the maternal fat mass gain) in pregnancy, but to short-term changes in gluco-regulatory function that reflect macronutrient balance induced by acute changes in the maternal diet. From these
studies, we proposed FGF21 to be a signal of nutrient insufficiency; particularly an insufficiency arising from maternal macronutrient imbalance in pregnancy.

To investigate FGF21 as a signal for fetal nutrient insufficiency in pregnancy, in Chapter 3 we first asked if the low-protein induced elevation of circulating FGF21 previously shown in male rodents was present during pregnancy. Indeed, we found a robust increase in serum FGF21 in both mice and human studies with low protein intake in late gestation. Considering both that maternal diets of low protein result in in utero growth restriction and maternal FGF21 was elevated with low protein intake in our studies, we next asked if maternal FGF21 could serve as a marker of fetal nutrient insufficiency. Indeed, we found maternal FGF21 inversely correlated with the size of offspring at birth and their growth through the first year of life. Together, these studies demonstrate FGF21 to be a signal of protein restriction and, subsequently, small fetal size and slow infant growth.

In Chapter 4, we built upon the data presented in Chapter 3 and conducted studies to implicate FGF21 in a direct and causative role as a protein sensor. Employing protein choice experiments, mice were allowed to self-regulate protein intake by ad libitum consumption of two provided diets, one diet low in protein and another high in protein. Wild type mice exhibited a great affinity for protein sensing and were highly capable of consuming a combination of both diets which would result in the overall consumption of their protein requirement, i.e. approximately 20% protein. Repeating this experiment in FGF21 knockout (FGF21KO) mice showed that mice were not capable of regulating protein intake without FGF21. These experiments demonstrated FGF21 plays a direct role in protein sensing in mice.
Finally, also in Chapter 4, we applied our findings to a model of protein restriction, the Protein Leverage Hypothesis, for practical testing of this newly elucidated role of FGF21 in protein sensing. The PHL posits that protein intake is prioritized over other macronutrients, insofar as an animal or individual will over-consume all macronutrients in order to attain or near their protein requirement. Evidence of this effect in an experiment or study is measured by elevated energy intake (hyperphagia) when consuming a low protein diet. However, to our surprise, the PHL had yet to be tested in pregnancy. Therefore, in order to test the role of FGF21 in protein leverage in pregnancy we first needed to replicate, as seen in non-pregnant models, the low-protein induce hyperphagia characteristic of protein leverage in pregnancy. When fed a low protein diet in late pregnancy, wild type mice did indeed increase food intake compared to pregnant mice fed normal protein diets. Next, we repeated this experiment in FGF21KO and as we hypothesized, the low-protein induce hyperphagia observed in late pregnancy in wild type mice was absent in mice without FGF21. These experiments serve was direct and causative evidence for the role of FGF21 as a protein sensor in pregnancy.

In summary, this body of work provides novel and compelling evidence for the role of FGF21 as a protein sensor in pregnancy. However, future epidemiological and molecular studies would allow for more translational and mechanistic insights respectively. As shown in a small sample in Chapter 3, FGF21 may be able to serve as a biomarker for protein and/or fetal growth restriction during pregnancy. While it is dangerous and unethical to randomize women to a diet with restricted protein content during pregnancy, observational studies could be conducted in populations faced with food scarcity. Indeed, an informative study for further confirmation of the role of FGF21
as a protein and/or fetal growth restriction biomarker would be measurement of serum FGF21 and protein balance in these individuals. To further mechanistic insights, molecular studies of the maternal-placental-fetal unit could better elucidate the effect of FGF21 when it is elevated in response to protein intake. Considering the placenta expresses receptors for FGF21, it could be highly informative to measure expression of placenta nutrient transporters under maternal protein restriction and/or elevated maternal FGF21. It would also be interesting to measure circulating FGF21 in offspring of phenotyped pregnancies to determine whether FGF21 overexpression is translated from mother to infant during protein restricted pregnancies, thereby programming the effect of protein restriction into offspring whether or not it persists past parturition. Given the increasing prevalence of obesity in children worldwide and the concomitant reduction in protein in maternal diets, further exploration of the protein leverage hypothesis in pregnancy and its role in maternal hyperphagia and gestational weight gain are warranted.
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APPENDIX: IRB APPROVAL

Pennington Biomedical Research Center
LOUISIANA STATE UNIVERSITY SYSTEM

PBRC IRB FWA 00006218  ACTION ON PROTOCOL APPROVAL REQUEST

TO:    Leanne Redman, Ph.D. and Corby Martin, Ph.D.
FROM:  Paula J. Geiselman, Ph.D., Chairman
PBRC Institutional Review Board for Research with Human Subjects
RE:    IRB #PBRC 11024  DATE:  October 24, 2011
Title:  Expecting Success: Personalized management of body weight during pregnancy

New Protocol/Modification:  Initial – Modification #1
Protocol (version 10/24/11); Informed Consent for Adult (version 10/24/11); Informed Consent for Minor (version 10/24/11); Child Guardianship Form; HIPAA (version 9/30/11); Protocol Appendix

Review Type:  Full Board - Conditional Approval  Review Date:  October 19, 2011
Review Type:  Expedited Approval of Board Requested Revisions Modification # 1
Expedited Approval Review Date:  October 24, 2011

Approved  X Disapproved

Date of Approval:  October 24, 2011  Approval Expiration Date:  October 18, 2012
Re-review frequency:  (annual unless otherwise stated) Continuing Review report due:  8/31/12
Number of subjects approved:  306 pregnant females and 306 infants born throughout the study

By:  Paula J. Geiselman, Ph.D.  Signature

Continuing Approval is CONDITIONAL on:

1.  Adherence to the approved protocol, familiarity with and adherence to the ethical standards of the Belmont Report and PBRC's Assurance of Compliance with DHHS regulations for the protection of human subjects.
2.  Prior approval of a change in protocol, including an increased number of volunteers over that approved.
3.  Obtaining renewed approval (or submittal of a termination report), prior to the approval expiration date, upon request by the IRB Office (regardless of when the project actually begins); notification of project termination.
4.  Retention of documentation of informed consent and study records for at least 3 years after the study ends.
5.  Continuing attention to the physical and psychological well-being and informed consent of the individual participants including notification of new information that might affect consent.
6.  A prompt report to the IRB of any adverse event affecting a participant and potentially arising from the study.

6400 Perkins Road, Baton Rouge, Louisiana 70808-4121  •  Phone: (225) 763-2500, Fax: (225) 763-2525
WOMAN'S HOSPITAL FOUNDATION
INSTITUTIONAL REVIEW BOARD
Support Services Building
9050 Airline Highway
Baton Rouge, Louisiana 70815

Peggy Dean, RPh, MBA, Chair

(225) 231-5359

December 2, 2011

Leanne Redman, PhD.
Pennington Biomedical Research Center
LSU System
6400 Perkins Road
Baton Rouge, LA 70808-4142

Dear Dr. Redman:

On behalf of the Woman’s Hospital Foundation Institutional Review Board, I have received and reviewed the revisions to the informed consent forms and authorization forms for RP-11-006, Expecting Success: Personalized Management of Body Weight During Pregnancy, requested by the full Board at its November 7, 2011, meeting.

The revisions were approved on December 1, 2011. You are granted permission to conduct your study as described effective immediately. The study is subject to continuing review on or before November 7, 2012, unless closed before that date; however, we recommend that it be presented two months prior to this date to avoid a delay in enrollment in the case of unforeseen circumstances.

Attached are the informed consents and authorization forms with the IRB stamp of approval in the lower right hand corners. Please note that these consents are the official copies and consents for future participants must be reproduced from these originals.

Please note that any further changes to the study as approved must be promptly reported and approved. Some changes may be approved by expedited review; others require full board review. Contact Ericka Seidemann, Human Protections Administrator, at (225) 924-8516 if you have any questions or require further information.

Sincerely,

Peggy Dean, RPh., MBA
IRB Chair
Cc: Elizabeth Frost, Pennington Biomedical Research Center
    Karen Elkind-Hirsch, PhD
IRB Certificate of Approval

FWA # 00006218

Date of Approval: September 23, 2013
Study Expiration Date: May 14, 2014
Submission Type: Initial
Review Frequency: annual
Number of Subjects Approved: 60
Review Type: Expedited Approval of Board Requested Revisions
Approval Status: Approved
Continuing Report Due 60 days prior to expiration date

Principal Investigator: Leanne Redman, Ph.D.
IRB # PBRC 13020 MomEE
Title: Determinants of gestational weight gain in obese pregnant women
Sponsor: NIH

Approval Includes: Protocol (9/23/13); Informed Consent (9/23/13); HIPAA

In Investigators and study staff must comply with the Human Research Protection Program policies and procedures that apply to IRB members and staff, which can be found at www.pbrc.edu/HRPP

Paula Geiselman, Ph.D., Chairman
IRB Certificate of Acknowledgment

FWA #00006218

Date of Acknowledgement: June 28, 2016
Submission Type: Exemption
Exemption valid until: June 27, 2019

Principal Investigator: Leanne Redman, Ph.D.
IRB #: 2016-062-PBRC
Title: Role of FGF21 in Protein Leverage and Placenta Function during Pregnancy
Sponsor: NIDDK

Acknowledgment Includes: FGF21 and Placenta Submission 0 Protocol (Protocol)

Federal Regulations as published in the Federal Register of January 26, 1981 Part 46 of 45CFR46.101(b) list exemptions to regulations of the Department of Health and Human Services governing research on human subjects. It is the opinion of the Chairman that your study is exempt since it falls into one of the categories listed, specifically number 4. Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects. (For research conducted, funded, or otherwise subject to regulation by any federal agency “existing” means “existing at the time the research is proposed.” Otherwise, it means “existing at the time the research is proposed or will exist in the future for non-research purposes.”)

THE INVESTIGATOR agrees to report to the Board any emergent problems or procedural changes that may affect the status of the investigation, and that no such change will be made without Board Approval, except where necessary to eliminate apparent immediate hazards.

If you plan to continue the research past the expiration date listed above, please submit an ‘Exempt Continuing Review Report’ through IRBManager before the expiration date.

Investigators and study staff must comply with the Human Research Protection Program policies and procedures that apply to IRB members and staff, which can be found at www.pbrc.edu/HRPP

Signed Tuesday, June 28, 2016 1:52:09 PM ET by Geiselman, Paula Ph.D.
VITA

Elizabeth Frost Sutton was born in October 1988 in Baton Rouge, Louisiana to Lisa and Gregory Frost. Elizabeth grew up in Baton Rouge and graduated with honors from Episcopal High School in 2006. That fall, Elizabeth began her undergraduate studies at Louisiana State University majoring in Biological Sciences. In the spring of 2009, Elizabeth joined the Energy Metabolism Core of Pennington Biomedical Research Center as an undergraduate student worker under the supervision of Dr. Eric Ravussin. Following graduation in 2010, she continued at Pennington Biomedical Research Center and began a career as a research associate with Drs. Ravussin and Leanne Redman. In the fall of 2012, she entered the graduate program at Louisiana State University in the Department of Biological Sciences as a doctoral student under the mentorship of Drs. Leanne Redman and Jacqueline Stephens. During her doctoral studies, Elizabeth was awarded a Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship (F31HD084199) by the Eunice Kennedy Shriver National Institute of Child Health and Human Development to support the conduction of this body of work. Elizabeth anticipates graduating with a Doctor of Philosophy degree in Biological Sciences in August 2017 and plans to pursue a career in academia.