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Interplay between genetic predisposition and diet in advancing obesity and type 2 Diabetes in the Tallyho mouse

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INTERPLAY BETWEEN GENETIC PREDISPOSITION AND DIET IN ADVANCING OBESITY AND TYPE 2 DIABETES IN THE TALLYHO MOUSE

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APPROVAL OF THESIS

We, the faculty supervising the work of Jacaline Parkman, affirm that the dissertation [*Interplay between Genetic Predisposition and Diet in Advancing Obesity and Type 2 Diabetes in the TALLYHO Mouse*], meets the high academic standards for original scholarship and creative work established by the department of Biomedical Research and Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I would like to dedicate this dissertation to my parents Rob and Hye Kyong and my brothers Chris and Clay. Without you guys I would not have made it this far. I love you all.

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ABSTRACT

Obesity is a global epidemic, affecting all ages. It is one of the leading causes of preventable death, as it increases the risk of type 2 diabetes (T2D), hypertension, cardiovascular disease, non-alcoholic fatty liver disease, and some cancers. Obesity is a complex disease that is caused by a combination of genetic and environmental factors such as diets high in fat and sedentary life style. Despite our increased knowledge of obesity development and progression, current obesity treatments have not stopped the rise in obesity rates. There are still many unknowns related to the underlying mechanisms of obesity that need to be investigated and understood, so that treatment of obesity can be more effective. To deal with the numerous variables involved with such studies, animal models are recommended. My dissertation centers around characterizing the TALLYHO/Jng (TH) mouse, a polygenic model for T2D and obesity, and identifying obesity gene(s) in this model. The first study focused on investigating the effect of diets high in fat and sucrose for the development of obesity and T2D in TH mice. Compared to normal C57BL/6J (B6) mice, TH mice responded more sensitively to the obesogenic diets in the development of obesity and type 2 diabetes, demonstrating that diets are important modulators of genetic susceptibility to the diseases in this model. The second study was conducted in an effort to identify obesity gene(s) in TH mice. We generated congenic mouse strains carrying obesity quantitative trait loci on chromosome 1 derived from TH mice on B6 background. Using these mouse strains, we determined that the distal segment of chromosome 1 from TH mice is necessary to cause diet induced obesity. In the last study, we demonstrated that increased pro-inflammatory cytokine interleukin-6 levels and decreased mitochondrial respiration may be in part a mechanism underlying the gene-diet interaction in advancing obesity and type 2 diabetes in TH mice.

CHAPTER 1

INTRODUCTION

Abstract

Obesity is a worldwide epidemic, affecting 650 million adults. It is defined as excess adiposity, where one's body mass index is greater than or equal to 30 kg/m^2. Obesity is considered one of the leading causes of preventable death, as it is associated with an increased risk of comorbidities such as type 2 diabetes (T2D), hypertension, hyperlipidemia, cardiovascular disease, non-alcoholic fatty liver disease, and various cancers. Obesity is a complex disease caused by interactions between obesity promoting environmental factors, such as a high fat diet, and genetic factors. Excessive adiposity can lead to adipose tissue dysfunction, chronic, low-grade inflammation, and mitochondrial dysfunction, all of which contribute to the eventual development of insulin resistance and T2D. Obesity is polygenic and many of the genes involved and their underlying mechanisms, including gene-diet interactions, remain unknown. To identify genes and regions of interest, there are several different mapping techniques that can be used, such as genome-wide association studies or quantitative trait loci mapping. Once candidate genes are found, the mechanisms in which they act can be elucidated through cell and animal model work to identify potential therapeutic targets.

The Scope of the Problem

Obesity is an international health crisis. Worldwide, obesity rates have tripled since 1975, with 650 million adults and 1.9 billion people considered overweight in 2016. At the same time, 340 million children and adolescents were also reported to be obese or overweight (WHO, 2018). To classify individuals as overweight or obese in a population, body mass index (BMI), weight in kilograms divided by the square of height in meters, is commonly used. A BMI $\geq$ 25 signifies

"overweight" and a BMI ≥ 30 signifies "obese" (Nuttall, 2015). Obesity is considered one of the leading causes of preventable death, as it is associated with an increased risk of type 2 diabetes (T2D), hypertension, hyperlipidemia, cardiovascular disease, non-alcoholic fatty liver disease, and various cancers (Gallagher & LeRoith, 2015; Rhee, 2018). Obesity is a huge economic burden as the cost of obesity is estimated to be around $100 billion annually in the US (Panuganti & Lenehan, 2018).

Obesity is attributed to interactions between obesity promoting environmental factors, such as a high fat (HF) diet, and genetic factors (Yazdi, Clee, & Meyre, 2015). However, all the genes involved, their underlying mechanisms, and gene/environment interactions remain largely unknown (Albuquerque, Nobrega, Manco, & Padez, 2017; Xia & Grant, 2013). Obesity is characterized by adipose dysfunction, chronic, low-grade inflammation, and mitochondrial dysfunction. Ultimately, insulin resistance can develop, leading to the development of T2D (Trim, Turner, & Thompson, 2018).

Treating the obesity epidemic has proven difficult. The first line treatments are dietary and lifestyle modifications, but these long-term changes can be difficult to maintain (Brandt, Clemensen, Nielsen, & Søndergaard, 2018; Montesi et al., 2016). Other treatments include behavioral therapy, surgery, and medications (Panuganti & Lenehan, 2018). To find more effective therapeutic targets to treat an ever-increasing obese population, more research needs to be conducted.

Adipose Tissue

The two main forms of adipose tissue are brown adipose tissue and white adipose tissue.

Brown Adipose Tissue (BAT)

Brown fat, also termed "good fat," is involved in non-shivering thermogenesis to maintain core body temperature (Fenzl & Kiefer, 2014; Jastroch, Oelkrug, & Keipert, 2018). This process begins when lipids are broken down into free fatty acids (FFA), also known as lipolysis. The FFA are transported to the mitochondria through the carnitine shuttle, where they undergo β-oxidation to generate the electron carriers NADH and $FADH_2$, as well as acetyl coenzyme A (Houten & Wanders, 2010). Acetyl coenzyme A generates more electron carriers by entering the tricarboxylic acid (TCA) cycle (Fenzl & Kiefer, 2014). The electron carriers donate electrons to the electron transport chain (ETC) in the inner membrane of the mitochondria for oxidative phosphorylation. The electrons travel through the cytochrome complexes, pumping out protons and creating a proton gradient, until reaching the final electron acceptor oxygen (Fenzl & Kiefer, 2014). Normally, energy would be produced in the form of adenosine triphosphate (ATP) by ATP synthase due to the proton gradient; however, in brown adipocytes mitochondrial uncoupling protein 1 (UCP-1) disrupts the proton gradient by transferring protons back into the inner membrane of the mitochondria. Oxidative phosphorylation is uncoupled from ATP production, so the energy produced is released as heat, also known as thermogenesis, rather than ATP (Fenzl & Kiefer, 2014; Kajimura & Saito, 2014; Klingenberg & Huang, 1999).

Brown adipocytes get their color from the large number of mitochondria within the cell. Brown adipocytes also have multiple small lipid droplets in the cytoplasm (Hull, 1966; Park, Kim, & Bae, 2014). The biology of brown adipose has been extensively studied in the recent years and the browning of white adipose is proposed to be a potential therapeutic target in obesity as brown fat can increase energy expenditure and is inversely correlated with BMI (Townsend & Wright, 2018). Adult human BAT deposits exist, but the ability to harness the

function of BAT as a possible target for obesity remains elusive. More research needs to be conducted in order to pharmacologically induce browning in white adipose tissue (Devlin, 2015; Kajimura & Saito, 2014).

White Adipose Tissue (WAT)

WAT is an important endocrine organ that both stores energy for the body and regulates whole body metabolism. When energy intake is greater than energy expenditure, adipocytes store the excess energy as triglycerides. These triglycerides, along with cholesteryl esters, form one large lipid droplet within the adipocyte. When energy expenditure is greater than energy intake, energy can be supplied through lipolysis (Choe, Huh, Hwang, Kim, & Kim, 2016). Adipocytes from WAT also contain mitochondria; however, the numbers of mitochondria are much fewer than those in BAT and they lack UCP-1 expression (Fujimoto & Parton, 2011; Jastroch et al., 2018).

More recently, it has been discovered that WAT adipocytes have the potential to express UCP-1 under certain circumstances, for example from exposure to cold (Young, Arch, & Ashwell, 1984). These UCP-1 expressing WAT adipocytes are termed brite, "brown in white," or beige adipocytes and are being studied as a potential obesity treatment (Kajimura & Saito, 2014). Beige adipocytes have a white fat-like phenotype until stimulated to a brown fat-like phenotype and they reside in subcutaneous tissue (Bargut, Souza-Mello, Aguila, & Mandarim-de-Lacerda, 2017; Dehghani, Kargarfard, Rabiee, Nasr-Esfahani, & Ghaedi, 2018). They also have a molecular signature that differs from both mature white and brown adipocytes (Stanford, Middelbeek, & Goodyear, 2015). Aside from cold, stimulants of beige adipocytes include nutritional stimulants such as anthocyanins, flavonoids found in many fruits and vegetables, or curcumin, a chemical in turmeric, and certain medications such as Liraglutide, a glucagon-like

peptide 1 (GLP-1) receptor agonist used to treat T2D (Han et al., 2018; S. Wang et al., 2015; E. Zhu et al., 2016). Exercise has also been shown to stimulate browning. In the 1990s, it was discovered that rats that swam for 10 weeks had increased browning of WAT and increased mitochondrial enzyme activity (Stallknecht, Vinten, Ploug, & Galbo, 1991). This increase in thermogenic and oxidative capacities was repeatedly seen in multiple rodent exercise studies (Brenmoehl et al., 2017; Dehghani et al., 2018; Gollisch et al., 2009; Townsend & Wright, 2018). Regulation of the browning of WAT involves multiple transcription factors, signaling pathways, and hormones, including fibroblast growth factor 21 (FGF21), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), and bone morphogenetic proteins (BMPs), (Fisher et al., 2012; Schulz et al., 2013; Schulz et al., 2011). While research is promising, there have been issues translating work found in rodents to humans. For example, although browning have been seen in mice on a calorie-restricted diet (Fabbiano et al., 2016), this has not been found in humans. Rather, it has been found that effects on body fat from calorie-restricted diets are independent of browning/brown fat. (Barquissau et al., 2018). More research needs to be done to harness the energy expenditure of brown fat or brown-like fat.

In addition to adipocytes, adipose tissue is comprised of preadipocytes, adipocyte mesenchymal stem cells, red blood cells, endothelial cells, smooth muscle cells, pericytes, and immune cells, which make up the stromal vascular fraction (SVF) (Tanaka, Itoh, Ogawa, & Suganami, 2018). Adipose tissue deposition and distribution can vary based on gender and health. Visceral adipose is adipose located around the organs and subcutaneous adipose is adipose tissue beneath the skin. Increased visceral adipose tissue is linked to obesity, as visceral tissue can release cytokines and free fatty acids directly into portal circulation (Ibrahim, 2010; Ritchie & Connell, 2007).

Adipose tissue regulates whole body metabolism by secreting adipokines and chemokines like leptin, adiponectin, pro-inflammatory monocyte chemoattractant protein (MCP)-1, interleukin-6 (IL-6), tumor necrosis factor (TNF)-α, and anti-inflammatory IL-10, and by responding to hormonal signals, such as glucocorticoid, which promotes adipose tissue expansion, and the central nervous system (CNS) (G. H. Goossens, 2008; Kershaw & Flier, 2004). The CNS includes the parasympathetic nervous system (PNS), which promotes fat storage, and the sympathetic nervous system (SNS) which promotes lipolysis, increasing the release of FFA (Balistreri, Caruso, & Candore, 2010).

Adipocyte Development

Multipotent mesenchymal stem cells (MSCs) can differentiate into osteoblasts, chondrocytes, and adipocytes (Pittenger et al., 1999). Lineage commitment of MSCs into preadipocytes is dependent on extracellular factors such as the expression of bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and the silencing of Wnt and hedgehog signaling. Wnt and hedgehog signaling promote osteoblast differentiation (Chen et al., 2016; L. Hu et al., 2018). Based on lineage studies, brown adipocytes and skeletal muscle are derived from myogenic factor 5 (Myf5)-expressing precursor cells (Shan et al., 2013). Cell fate between brown adipocytes and muscle depends on transcriptional regulator PR domain containing 16 (PRDM16). PRDM16 binds to peroxisome proliferator-activated receptor gamma (PPARγ), activating brown adipogenesis (Seale et al., 2008). Studies have shown that white adipocytes may come from predominately Myf5 negative cells, but there is evidence of Myf5 positive precursor cells as well, indicating that white adipocytes can come from either (Peirce, Carobbio, & Vidal-Puig, 2014; Sanchez-Gurmaches & Guertin, 2014).

Once preadipocytes differentiate into adipocytes, a process known as adipogenesis, the cells become sensitive to insulin and begin to uptake glucose through glucose transporter 4 (GLUT4) (Kahn & Flier, 2000). The master regulators of adipogenesis are transcription factor PPARγ, which is activated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and transcription factor CCAAT-enhancer-binding protein alpha (C/EBPα) (L. Hu et al., 2018). These transcription factors induce the transcriptional cascade necessary for adipogenesis (Siersbaek, Nielsen, & Mandrup, 2010). Adipose tissue can adapt to over-nutrition by expanding through hypertrophy, increased adipocyte size, and/or hyperplasia, increased adipocyte number through adipogenesis (Siriwardhana et al., 2013). Healthy individuals have white adipose tissue made up of smaller, insulin sensitive adipocytes that secrete anti-inflammatory mediators such as adiponectin and IL-10 (Makki, Froguel, & Wolowczuk, 2013).

Causes of Obesity

Genetics

Obesity is a complex disease that is believed, in part, to be caused by many genetic factors. Genetics research involves twin studies, adoption studies, and more recently, candidate gene and genome-wide association studies (GWAS). Candidate gene studies and GWAS attempt to identify potential genes/gene variants that are linked to obesity. Once candidate genes are identified, researchers try to elucidate the mechanisms that underlie obesity (Choquet & Meyre, 2011). Monozygotic twin studies have shown fat mass concordance around 70% and dizygotic twins have shown fat mass concordance around 30% (Stunkard, Foch, & Hrubec, 1986; Turula, Kaprio, Rissanen, & Koskenvuo, 1990). In adoption studies, there is a strong correlation between the BMI of biological parents and those who were adopted instead of with the BMI of adoptive parents (Moll, Burns, & Lauer, 1991; Stunkard, Sorensen, et al., 1986). Candidate gene studies

for obesity were instrumental in the mid-90s for identifying genes responsible for monogenic obesity disorders, especially with the identification of the hormone leptin and its receptor, which were mutated in *ob/ob* and *db/db* mice respectively (Friedman, Leibel, Siegel, Walsh, & Bahary, 1991; G. H. Lee et al., 1996; Zhang et al., 1994). At least 20 forms of human monogenic obesity disorders have now been identified, and the studies have greatly contributed to the understanding of energy balance in obesity (O'Rahilly, 2009). The majority of obesity cases, however, are polygenic. GWAS scan genetic markers of thousands of individuals for gene variants that may play a role in obesity. The first obesity gene identified through GWAS was "fat mass and obesity-associated protein" (FTO) on human chromosome 16 (Frayling et al., 2007). FTO is a single-stranded DNA demethylase and a co-activator of C/EBPs. C/EBPs are involved with fat tissue expansion (Wu, Saunders, Szkudlarek-Mikho, Serna Ide, & Chin, 2010). FTO variants have also been shown to affect food intake (Melhorn et al., 2018). Studies have shown that the FTO risk allele rs9939609, also known as A allele, is associated with increased food intake from selection of more energy-dense foods, increased hunger, and decreased satiety after meals (Zhou, Hambly, & McLachlan, 2017).

Currently, 300 loci have been identified in polygenic obesity (Heymsfield & Wadden, 2017). Research is being conducted to identify obesity genes within the loci and investigate their role in obesity development.

There is also evidence that epigenetics increases obesity risk. In multiple studies using animal models, both in-utero undernutrition and maternal overnutrition increased the obesity risk in offspring (Jimenez-Chillaron et al., 2009; Khan et al., 2005; Vickers, 2014; Zhu et al., 2008). In addition, obesogenic endocrine disruptors (EDCs), or chemicals that increase adiposity (EDCs

further explained in the environment section) have been shown to have effects that are transgenerational (Chamorro-Garcia & Blumberg, 2014; Chamorro-García et al., 2013).

Environment

Diet-

While there are many factors that contribute to the development of obesity, diet is one of the most studied. To maintain body weight, there must be a balance between energy intake and energy expenditure. Even a small increase in energy intake can add up over time. For example, if energy intake is greater than 1% of energy expenditure over a year, or about 20 calories a day, a person can gain 2.6 kg or 5.7 lbs. during the year (Leng, 2014). Continuous over consumption will lead to continuous increased energy intake, increased adiposity, and the development of obesity.

Changes in food availability and manufacturing in industrialized countries have changed the past diet of low-calorie, high-fiber foods to a current diet of highly processed, energy-dense foods. Constant food cues from advertisements and the very addicting nature of certain foods, for example sugar which causes a dopamine stimulation similar to narcotics, have been cited as a challenge for maintaining or losing body weight (Berthoud, 2011; DiNicolantonio, O'Keefe, & Wilson, 2018). Furthermore, the sedentary, but stressful life style of the modern world may decrease energy expenditure and increase the chances of stress eating or mindless eating, both of which lead to overeating (Dallman, Pecoraro, & la Fleur, 2005; Horstmann et al., 2015).

There is currently a major debate about the role of increased dietary lipids versus increased carbohydrates in the obesity epidemic. If energy balance is in check, weight should not be gained, but the macro-composition of food could affect other areas of metabolism or insulin signaling. High-glycemic-index carbohydrates have been shown to be calorie dense, low in fiber,

and low in satiation, and may contribute to weight gain by promoting fat storage through continuous insulin release (Brand-Miller, Holt, Pawlak, & McMillan, 2002; Ludwig & Ebbeling, 2018; Radulian, Rusu, Dragomir, & Posea, 2009). Fructose, a common carbohydrate in the western diet, has been shown to induce leptin resistance, prevent fatty acid oxidation, and reduce energy expenditure (Shapiro et al., 2008). Fructose has been linked to obesity, T2D, and fatty liver disease (Hu & Malik, 2010; Lanaspa et al., 2012). While high-glycemic foods are associated with weight gain, low-glycemic index carbohydrates are seen as satiating and beneficial, as they contain more fiber and delay gastric emptying as well as decrease postprandial glycemia and insulin release (Schwingshackl, Hobl, & Hoffmann, 2015; Schwingshackl & Hoffmann, 2013). Multiple studies have found that a diet high in fat will increase adiposity as well as alter metabolic profiles by increasing blood glucose levels, lipid concentration, and insulin levels. Thus, diets high in fat have been associated with obesity (Boi et al., 2016; Glastras et al., 2016). Dietary fat; however, has been found to be important in satiety, so moderate fat intake is encouraged for weight loss (L. Wang, Bordi, Fleming, Hill, & Kris-Etherton, 2015). Diet studies that compared high carbohydrate and high fat diets showed differential effects on metabolism and insulin resistance, signifying the importance of the macronutrients and their different roles in metabolism alteration (Ble-Castillo et al., 2012; Chun et al., 2010).

A healthy diet is recommended to treat obesity, but there is conflicting information about what the ideal macronutrient composition of a healthy diet would be. Dietary guidelines are released by the United States Department of Agriculture and United States Department of Health and Human Services every five years, supporting low fat diet as the optimal diet for health. Based on studies that showed high variability in individuals' metabolic response to the same macronutrients, the reliance on one type of healthy diet would not be an effective treatment

(Korem et al., 2017; Zeevi et al., 2015). There are many who consume the high fat, western diet for example that do not become obese. Further research needs to be conducted on the effect of different macronutrient compositions in diets with genetics. With the incorporation of interpersonal variability, personalized diets may be successful in treating obesity.

Energy expenditure-

The development of obesity occurs when energy expenditure is less than energy intake. Energy expenditure includes basal metabolic rate, diet-induced thermogenesis, and overall physical activity (Westerterp, 2004). The shift from manual labor in industrialized countries to more sedentary jobs has decreased energy expenditure by more than 100 calories a day since 1960 (Church et al., 2011). In addition, the increase in modern technology with televisions and phones decreased energy expenditure even further and has been correlated with the owners' increased risk of obesity and type 2 diabetes (Lear et al., 2014). The combination of a more sedentary lifestyle with highly processed, energy dense food contributes to the development of obesity. For obesity treatment, a healthy diet with exercise is recommended. Studies and literature reviews showed physical activity improved metabolic parameters, reduced adiposity, reduced insulin resistance, and increased diversity of gut microbiota (Denou, Marcinko, Surette, Steinberg, & Schertzer, 2016; Paes, Marins, & Andreazzi, 2015; Son, Sung, Bharath, Choi, & Park, 2017). Physical activity includes not only vigorous workouts, but nonexercise activity thermogenesis (NEAT). NEAT includes everyday movements such as standing and walking. Increased NEAT is associated with decreased risk of metabolic syndrome, while decreased NEAT is associated with obesity (Malaeb, Perez-Leighton, Noble, & Billington, 2018; Villablanca et al., 2015).

Pollutants/chemicals-

While genetics, diet, and exercise are the major contributors to obesity, they do not fully explain the large increase in obesity worldwide. Other environmental factors and chemicals are believed to play a role as well.

Obesogens, or obesogenic endocrine disruptors, are classified as chemicals that can contribute to weight gain through altered metabolic processes such as increased adipogenesis and lipid accumulation (Darbre, 2017). An endocrine disruptor is a substance that disrupts the endocrine system and causes adverse effects (Legeay & Faure, 2017). Obesogens include environmental chemicals that people come into contact with such as pesticides and herbicides, detergents, plastics, and personal care products for hygiene and beauty (Darbre, 2017; Muscogiuri, Barrea, Laudisio, Savastano, & Colao, 2017). Exposure to the obesogens can happen through oral absorption, inhalation, or dermal absorption (Darbre, 2017). In addition, obesogens can be very detrimental to fetus development during pregnancy, increasing the risk of disease and developmental defects of the fetus (Braun, 2017).

Bisphenol A (BPA) is a well-known obesogen and was used to manufacture multiple polycarbonate plastic products such as water bottles and food can linings. Studies have found evidence of measurable BPA concentrations in adults, children, adolescents and fetuses (Chamorro-Garcia et al., 2012; Le Corre, Besnard, & Chagnon, 2015; D. K. Li et al., 2013; R. Y. Wang, Abbott, Zieba, Borowsky, & Kaplan, 2017). In 2010, studies showed that 90% of people in the US had detectable amounts of BPA in their system despite the ban of BPA in 2008 by the FDA (Vandenberg et al., 2010). Low levels of BPA were shown to increase adipogenesis by upregulating 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which is associated with increased visceral adipose tissue, and by upregulating PPAR-γ, a master regulator in adipogenesis (Siersbæk et al., 2010; J. Wang, Sun, Hou, Pan, & Li, 2013). To test the effect of

BPA in-utero, 2D and 3D human embryonic adipose tissue models were used. BPA was found to upregulate adipogenic genes in-utero as well (R. Y. Wang et al., 2017).

Air pollution has also been considered a potential obesogen. It has been associated with increased body weight through the increase of oxidative stress and inflammation in adipose tissue and through increased sedentary lifestyle. Air pollution is also associated with an increased risk of comorbidities such as cardiovascular disease due to pulmonary inflammation, oxidative stress, and elevated blood pressure (An, Zhang, Ji, & Guan, 2018; Meo & Suraya, 2015; Q. Sun et al., 2009; Y. Wang, Hollis-Hansen, Ren, Qiu, & Qu, 2016). Studies on fossil fuels and air pollution are mixed; with some showing positive correlation, some negative correlation, and some no correlation at all (An, Ji, Yan, & Guan, 2018).

Gene-Diet Interaction

Obesity is attributed to genetics, lifestyle factors such as diet, and gene-environment interactions. Studies have shown that a diet high in fat and/or carbohydrate may modify gene expression and increase the risk for obesity through changes in metabolic response and adiposity (Heianza & Qi, 2017; Qi, 2012). One study put Wistar Bonn Kobori (WBN/Kob) diabetic fatty (WBKDF) rats, which have a recessive mutation that causes leptin receptor deficiency, on a high-fat diet (HFD) and a fructose-rich diet (FRD). Both diets promoted obesity and dyslipidemia; however, only the HFD exacerbated T2D (Namekawa et al., 2017). High fat diets have been linked to weight gain and obesity in epidemiological, clinical, and basic science studies, especially diets where fat is at least 30 % of total energy of the diet, such as in the western diet (Bray & Popkin, 1998; Hammad & Jones, 2017; J. O. Hill, Melanson, & Wyatt, 2000). HFD may induce obesity due to increased energy intake and increased efficiency of energy storage compared to carbohydrates and protein. Increased dietary fat intake and storage

cause alterations in metabolism and energy homeostasis (Hammad & Jones, 2017). On the contrary, some studies have shown that an increase in dietary fat may prevent/treat obesity when accompanied by an increase in protein (Hariri & Thibault, 2010). The type of dietary fat, saturated fatty acids versus unsaturated fatty acids, has been shown to modify expression levels of genes related to adiposity and lipid metabolism. Saturated fatty acids upregulate lipogenic genes for example, while unsaturated fatty acids downregulate lipogenic genes (Reynes, Palou, & Palou, 2017; Sampath, Miyazaki, Dobrzyn, & Ntambi, 2007).

With a high carbohydrate diet, especially those involving high levels of fructose, lipid synthesis is promoted in the liver. This can lead to non-adipose lipid deposition and decreased insulin sensitivity (Stanhope, 2016). However, some conflicting studies have shown that the consumption of fructose does not cause excessive weight gain and that glucose has a similar effect on metabolism as fructose (van Buul, Tappy, & Brouns, 2014). Weight gain and insulin resistance may instead be due to an overconsumption of sugary beverages or foods (Musselman et al., 2011). Limiting the consumption of sugary foods/beverages seems to be an effective strategy for weight loss (Macdonald, 2016; Stanhope, 2016).

Dietary treatment may be complicated in obesity as many gene variants linked to obesity have been found to play a role in regulating appetite and food intake, thus genetic variants may modify dietary intervention. Meta-analysis studies have reported success in weight loss in individuals that decreased fat intake, decreased carbohydrate intake, and increased protein intake (Qi, 2014). It should be noted, there are interindividual responses to diet. For example, a study showed that individuals with predominantly smaller low-density lipoprotein (LDL) particles responded better to a low-fat diet than those with larger LDL particles, who developed more atherogenic LDL (Pérusse & Bouchard, 2000). Currently, gene-diet interaction studies are

relatively new and face several difficulties. More replicated studies and large-scale analysis need to be done. But, results thus far support the important role of gene-diet interactions in obesity development and treatment (Heianza & Qi, 2017).

Pathogenesis of obesity

Energy balance occurs when energy intake is equal to energy expenditure. Humans take in energy in the form of carbohydrates, protein, fat, and alcohol and expel energy through resting metabolic rate, diet-induced thermogenesis, and physical activity (Hill, Wyatt, & Peters, 2012). If there is positive energy expenditure there will be weigh loss. If there is positive energy intake, there will be weight gain. Circulating systemic signals and signals in the gut, inform the brain about energy levels and fat storage to regulate energy homeostasis in order to promote weight stability. Obesity is the result of continuous positive energy gain and a dysfunctional energy homeostasis system (Schwartz et al., 2017). There are short- and long-term homeostatic mechanisms to regulate weight.

The short-term homeostatic mechanism of regulation involves hunger and satiety hormones from the gastrointestinal tract (GI) and the CNS. Mechanoreceptor neurons in the stomach sense gastric distension. The distension is signaled through the vagal afferent and spinal sensory nerves to the hindbrain, which is involved with meal size control (Berthoud, Münzberg, & Morrison, 2017; Ritter, 2004). In addition, some satiation-inducing gut peptides such as cholecystokinin (CCK), which is secreted due to fat/protein metabolism, or glucagon-like peptide-1 (GLP-1), or peptides released by the pancreas such as amylin, likewise work through the vagal afferent nerve fibers. (Ahima & Antwi, 2008; Cummings & Overduin, 2007). If CCK, GLP-1, or amylin signaling is disrupted, satiety is affected and more food is consumed (Cummings & Overduin, 2007; Geary & Smith, 1982; Williams, Baskin, & Schwartz, 2009). On

the other hand, the peptide ghrelin, which is secreted from the gastric mucosa, promotes feeding. Ghrelin is expressed before the meal and declines during the meal (Cummings et al., 2001). If ghrelin is removed, mice continue to eat, indicating ghrelin may have a redundant role with appetite regulation (Wortley et al., 2004).

Long-term homeostatic regulation signaling is done by leptin and insulin. Leptin is secreted by adipocytes and signals changes in energy balance and stored fat via receptors in hypothalamus region, particularly the arcuate nucleus (ARC). The ARC is a major site for different energy signals like ghrelin or fatty acids. Leptin is involved in a negative feedback loop, limiting fat mass through decreased energy intake and promoting energy expenditure. If leptin is decreased, there is increased food intake and fat accumulation (Morton, Cummings, Baskin, Barsh, & Schwartz, 2006). The ARC and leptin-sensitivity are regulated by neurons expressing proopiomelanocortin (POMC), which release anorexigenic peptides and are stimulated by leptin or insulin, as well as neurons expressing neuropeptide Y (NPY)/ agouti-related peptide (AgRP), which are stimulated by ghrelin and inhibit POMC neurons, promoting feeding (Aponte, Atasoy, & Sternson, 2011; Luquet, Perez, Hnasko, & Palmiter, 2005; Morton et al., 2006).

Short periods of weight gain can be reversible rather quickly, but long periods of slow weight gain may be adapted into an upward reset energy homeostasis. Slow progression of obesity is the most common development. If weight is lost due to a change in lifestyle, it is not uncommon to gain the weight back to the newer reset energy homeostasis (Schwartz et al., 2017). One key player could be the hypothalamus. Obesity is associated with systemic low-grade inflammation as well as inflammation in adipose and the surrounding tissues due to infiltrating cytokines. Diet-induced obesity causes inflammatory mediator activation at the hypothalamus,

including in areas involved with energy homeostasis (Schenk, Saberi, & Olefsky, 2008). Intracellular serine kinases are activated, as they are intermediaries for inflammatory cytokines, which phosphorylate some elements involved with the insulin transduction pathway (Hotamisligil, 2003). Increased inflammation in the hypothalamus leads to insulin resistance and loss of effect of insulin through POMC neurons.

Adipose Tissue Dysfunction and Obesity

Obesity is a condition defined by excess white adipose tissue. This excess adiposity is due to increased energy consumption and/or decreased energy expenditure. The excess adipose tissue, especially excess visceral adipose tissue, is considered detrimental to health. Although adipose tissue expands to adapt to excess calorie consumption, there is a limit for its expansion capabilities.

When the storing capacity of adipocytes is exceeded, fatty acids spillover from visceral adipose tissue resulting in ectopic deposits and lipotoxicity in non-adipose organs, such as the heart or liver. Excess lipid deposition in the heart may promote plaque buildup and excess lipid deposition in the liver may lead to non-alcoholic fatty liver disease (NAFLD) (G. H. Goossens, 2008; Senoo et al., 2018; Serra, Mera, Malandrino, Mir, & Herrero, 2013).

Obesity is characterized by adipose tissue dysfunction. Adipose tissue dysfunction occurs through decreased expansion through adipogenesis and increased expansion through adipocyte hypertrophy, leading to increased inflammation (Kloting & Bluher, 2014). In adipose tissue, hypertrophied adipocytes may undergo apoptosis and hypoxia develops due to limited angiogenesis, increasing intracellular stress (Goossens & Blaak, 2015). Apoptotic adipocytes and hypoxia in adipose tissue lead to increased infiltration of macrophages, and increased secretion of pro-inflammatory cytokines, such as TNF-α and IL-6, by both the adipose tissue and

infiltrating macrophages. Low-grade chronic inflammation, impaired lipid metabolism, and mitochondrial dysfunction occur (Balistreri et al., 2010; Kahn & Flier, 2000; Yang, Eliasson, Smith, Cushman, & Sherman, 2012). Over time, chronic inflammation and dysfunction can result in insulin resistance as well as secondary organ damage (Kloting & Bluher, 2014).

Inflammation

Inflammation in Healthy Adipose

Lean adipose tissue tends to be anti-inflammatory. There are several mechanisms that contribute to inflammation in adipose tissue (Serbulea et al., 2018), for example, structural and functional changes, including extracellular matrix remodeling, that are necessary for the development of hypertrophic adipocytes. When expansion is healthy and gradual, there is acute inflammation, which is necessary for adipose remodeling. After expansion, the inflammation decreases as the tissue returns to metabolic homeostasis (Wernstedt Asterholm et al., 2014). In lean adipose tissue, "alternatively activated" class or the M2-type macrophages are the major population of immune cells (Wynn, 2015). M2 macrophages are immunosuppressive cells that help induce an anti-inflammatory state in lean adipose tissue. These macrophages remove dying/dead adipocytes and secrete angiogenic factors and anti-inflammatory cytokines such as IL-10 (A. A. Hill, Reid Bolus, & Hasty, 2014; Mantovani, Biswas, Galdiero, Sica, & Locati, 2013; Thomas & Apovian, 2017).

Inflammation in Obesity

Chronic low-grade inflammation is a hallmark of obesity (Trim et al., 2018). Obesity-associated inflammation is induced by several factors, including adipocyte hypertrophy. Adipocyte hypertrophy increases inflammation by activating inflammatory signaling pathways, increasing the release of pro-inflammatory cytokines and promoting recruitment of pro-

inflammatory immune cells (Pirola & Ferraz, 2017). Both adipocytes and immune cells release pro-inflammatory cytokines (Ferrante, 2013). Constant inflammatory stimulation leads to systemic cytokine elevation, and eventually insulin resistance and metabolic complications (Alligier et al., 2012; Boden, 2008; McQuaid et al., 2011). Hypoxia also contributes to inflammation as increased adipocyte size can lead to poor oxygenation of adipose tissue (Serra et al., 2013). Hypoxia can activate toll-like receptors and the nuclear factor-κB (NF-κB) pathway to induce downstream expression of pro-inflammatory cytokines (Boden, 2008; J. Ye, Gao, Yin, & He, 2007).

"Classically activated," or M1 macrophages increase with increased adiposity. In addition, there is a correlation between increased macrophage number and whole-body insulin resistance in humans (Travers, Motta, Betts, Bouloumie, & Thompson, 2015). Adipose tissue macrophages are derived from monocytes, which differentiate in response to growth factors and cell-stress markers such as IL-6, interferon-γ (IFN-γ), and TNF-α (A. A. Hill et al., 2015). These signals polarize macrophages into the inflammatory, M1 macrophages (Castoldi, Naffah de Souza, Camara, & Moraes-Vieira, 2015; Trim et al., 2018).

Fatty acids also act on the M1/M2 macrophage switch. Saturated fatty acids promote M1 cell development, while unsaturated fatty acids promote M2 cell development. The addition of dietary fish oils has been shown to decrease pro-inflammatory markers and increase anti-inflammatory markers (Pirola & Ferraz, 2017). Adiponectin, an adipokine secreted by adipocytes, enhances insulin sensitivity by decreasing liver gluconeogenesis and increasing fatty acid oxidation and glucose uptake. In addition, adiponectin is inversely correlated with inflammation, driving M2 polarization by increasing the expression of certain factors such as the

anti-inflammatory cytokine IL-10. Adiponectin has also been shown to decrease the expression of NF-κB, TNF-α, and reduce monocyte infiltration (Pirola & Ferraz, 2017; Tian et al., 2012).

Continuous energy overload causes inflammation to organs peripheral to adipose tissue as well. Liver is a major contributor to inflammation, disrupting hepatic insulin signaling and reducing insulin sensitivity (Cai et al., 2005; Shoelson, Lee, & Goldfine, 2006).

Mitochondria

Mitochondria in Healthy Adipose Tissue

Mitochondria are essential for cell and tissue function. In white adipose tissue, mitochondria are involved with metabolic homeostasis, producing ATP, branched-chain amino acid catabolism, fatty acid synthesis, and lipolysis (Boudina & Graham, 2014). Mitochondria are double membraned organelles that are directly involved in pyruvate oxidation, the tricarboxylic acid (TCA) cycle, fatty acid β-oxidation, and oxidative phosphorylation (OXPHOS) (Serra et al., 2013). The electron transport chain (ETC) for OXPHOS is in the inner membrane of the mitochondria. During cellular respiration, electron carriers transfer electrons to the ETC to produce ATP for energy. The ETC is made up of complexes I-IV: Complex I is NADH dehydrogenase, complex II is succinate dehydrogenase, complex III is cytochrome *bc1* complex (coenzyme Q), and complex IV is cytochrome oxidase, and then there is ATP-synthase, also known as complex V. The electrons move through the complexes, pumping out protons across the inner membrane space to create a proton gradient. Ubiquinone and cytochrome c transfer electrons between the different complexes (Sharma, Lu, & Bai, 2009). Oxygen is the final acceptor of the electrons and ATP synthase produces ATP by pumping protons down their concentration gradient. In addition to nuclear-encoded proteins, mitochondria have their own

maternally inherited circular genome that includes transcripts for the protein subunits of the ETC complexes (Ladoukakis & Zouros, 2017; Ryu et al., 2013).

There is evidence of a link between mitochondria and adipogenesis. During adipocyte differentiation, there is increased oxygen consumption and increased mitochondrial protein content, indicating increased mitochondrial biogenesis. There are also changes in mitochondrial morphology (Goldman, Zhang, & Jin, 2011; Ryu et al., 2013; Wilson-Fritch et al., 2003). In addition, studies have shown that adipogenesis and mitochondrial biogenesis are coordinated as PPARγ, C/EBPα, cAMP response element binding (CREB) protein, and PGC-1α are major regulators of both processes (Ryu et al., 2013). Normal mitochondrial function has been found to be necessary to drive adipogenesis in adipose tissue. Mitochondrial defects have been shown to limit adipocyte differentiation (Boudina & Graham, 2014; Kusminski & Scherer, 2012).

Mitochondrial Dysfunction in Obesity

Excess adipose tissue in obesity is known to be associated with mitochondrial dysfunction. In obese mice, a decrease in mitochondrial mass, mitochondrial function, and mitochondrial DNA is observed. In both mice and humans, lower oxygen consumption rates in preadipocytes and adipocytes are seen in obese subjects compared to lean ones (Boudina & Graham, 2014).

Mitochondrial dysfunction occurs when mitochondria are unable to manage the constant metabolic demands. This can lead to increased production of reactive oxygen species (ROS) and oxidative stress as oxygen plays a major role in the ETC. Oxidative stress from the mitochondria and from hypoxia in the adipose tissue further increase inflammation of inflammatory adipose tissue. ROS can also result in the fibrosis of adipose tissue. As adipose tissues become fibrotic, storage capacity and endocrine function are reduced, and ectopic lipid deposition occurs in non-

adipose tissue. The inflammation and reduced adipogenesis lead to reduced mitochondrial number and mass (Bournat & Brown, 2010; de Mello, Costa, Engel, & Rezin, 2018; Kusminski & Scherer, 2012).

Insulin Resistance

Insulin, a hormone released by the beta cells of the pancreas, regulates metabolism. Insulin is released after the ingestion of a meal and signals for glucose uptake by the liver, skeletal muscle, and adipose tissue through glucose transporters, while inhibiting lipolysis. Excess glucose can then undergo glycogenesis or lipogenesis (Goossens, 2008; Rutkowski, Stern, & Scherer, 2015; Wilcox, 2005). Insulin resistance can develop from continuous over-nutrition, where the insulin-sensitive tissues (liver, skeletal muscle, adipose) stop responding to insulin, linking obesity to type 2 diabetes development (Ye, 2013). Insulin resistance is in part attributed to chronic systemic inflammation, mitochondrial dysfunction, and adipose dysfunction. Beta cells in the pancreas try to adapt to insulin resistance by increasing insulin secretion and by increasing beta cell mass. This increase in insulin production and secretion eventually causes beta cell failure due to constant stress (Goossens, 2008).

Continuous over-nutrition can trigger multiple inflammatory pathways that lead to the activation of IκB kinase (IKK) and related kinases. IKKβ phosphorylates the insulin receptor substrate 1 (IRS-1) at several serine residues to inhibit cellular insulin signaling (Stafeev, Vorotnikov, Ratner, Menshikov, & Parfyonova, 2017). The inhibitor of IκB normally binds to NF-κB dimers, so NF-κB remains inactive in the cytoplasm. Phosphorylation promotes ubiquitination and degradation of IκB, freeing NF-κB. NF-κB translocates to the nucleus due to a nuclear localization signal and is able to target specific genes (Baker, Hayden, & Ghosh, 2011). NF-κB contributes to inflammation by driving the differentiation of monocytes to M1 or M2

macrophages (Baker, Hayden, & Ghosh, 2011). In addition to IKK, activation of c-Jun N-terminal kinase and mitogen-activated protein kinase can act as a negative regulator of insulin signaling by direct Ser/Thr phosphorylation of IRS-1 (Solinas & Karin, 2010). Dysregulation of insulin signaling as well as downregulation of glucose transporters and unregulated lipolysis results in insulin resistance (Kahn & Flier, 2000; Rutkowski et al., 2015).

Treatment of obesity

Prevention of obesity through education about healthy eating and exercise is the best way to treat obesity (Matwiejczyk, Mehta, Scott, Tonkin, & Coveney, 2018). With a rise in childhood obesity, treating obesity as early as possible results in the best outcome (Gortmaker & Taveras, 2014). Obese children have more adipocytes than lean children, but losing weight will only reduce adipocyte size and not number. (Knittle, Timmers, Ginsberg-Fellner, Brown, & Katz, 1979).

Whether in childhood or adulthood, it is recommended for the patient to visit a physician to check for any rare or endocrinological reasons for weight gain (Wickham & DeBoer, 2015). If there are no abnormalities for obesity development, then treatment of obesity involves lifestyle interventions in diet and exercise. Therapy may be recommended as well to address emotional eating (van Strien, 2018). Pharmacological medications are considered a secondary treatment option and are available in conjunction with lifestyle change; however, these are prescribed only if necessary for the patient to lose weight and maintain weight loss (Patel & Stanford, 2018). The medications involve suppressing appetite, decreasing dietary absorption, and increasing energy expenditure. Contrave (Orexigen Therapeutics, United States) is one such approved obesity drug that reduces appetite, where clinical trials showed weight loss ≥8% over 56 weeks. (Panuganti & Lenehan, 2018). The most extreme and third line of treatment, bariatric surgery, may be the most

successful in weight loss (Patel & Stanford, 2018). Weight loss surgery is associated with significantly decreased BMI for a patient overtime as gastric bypass reduces hunger through increased anorexigenic hormones, decreased gastric distension, and enhanced gut peptide signaling (Shin, Zheng, Pistell, & Berthoud, 2011). To qualify for surgery, certain criteria must be met and there is the risk of surgical complications (Wickham & DeBoer, 2015).

Despite our understanding of obesity, the lifestyle modifications and pharmacological modifications recommended are not as effective as expected, especially since the rates of obesity continue to rise. With dietary interventions, there is a lot of contradictory information on the best diet and people often have trouble with adherence to the change in lifestyle and the maintenance of weight loss long-term (Thom & Lean, 2017). In 2015, a study was published in *Cell* where investigators monitored glycemic responses after meals for a week for 800 people. They found high interpersonal variability among the group, despite everyone eating the same meals. They then created a machine-learning algorithm that incorporated various measured clinical parameters including gut microbiota profiles and self-reported lifestyle factors to predict glycemic responses. They tested their algorithm on 100 new people where they found the coefficient correlation equated to R=0.68. Next, they used the algorithm to test personal dietary interventions for 26 new participants. They found that those on the "good" diet had significantly reduced glycemic response compared to those on the "bad" diet, as well as improved glucose metabolism and changes in gut microbiota (Zeevi et al., 2015). The interpersonal variability may partly explain why diet studies can contradict one another. This study points to personally-tailored diets as the most effective diet for obesity and T2D. As for drug treatment, mechanisms involved with appetite and body weight regulation are rather complex and many discontinued anti-obesity drugs caused severe medical complications (Kang & Park, 2012). More research

needs to be done on the complex mechanisms underlying obesity in order to increase drug effectiveness and decrease side effects.

Type 2 Diabetes

Diabetes mellitus type 2 (T2D) is a complex metabolic disorder. The number of people who have diabetes has quadrupled from 108 million in 1980 to 422 million in 2014, and there are likely many more who have developed T2D but are unaware of their condition (Bell, Kivimaki, & Hamer, 2014; WHO, 2017). The classic symptoms of T2D are increased thirst and hunger and increased urination. A fasting blood glucose level of 126 mg/dL or higher indicates diabetes and a fasting blood glucose level of 100 to 125 mg/dL indicates prediabetes (Pour & Dagogo-Jack, 2011). Diabetes is associated with insulin resistance, hyperglycemia, dyslipidemia, oxidative stress, chronic inflammation, and renal dysfunction. Obesity is a risk factor for T2D and T2D can lead to heart disease, stroke, blindness, kidney failure, and amputations due to poor blood flow (Bell et al., 2014).

Causes of Type 2 Diabetes

Genetics

A genetic basis for T2D has been well documented. A child with one parent with T2D has a diabetes risk odds ratio (OR) of 3.4-3.5. The OR becomes 6.1 if both parents have T2D (Nair & Baier, 2015). Twin studies have shown if one monozygotic twin has T2D then the other has a 90% chance of developing T2D and a dizygotic twin has a 25 to 50% chance of developing T2D (Pour & Dagogo-Jack, 2011). Through GWAS and meta-analysis, 69 established genetic loci were linked with T2D in 2014 (Hivert, Vassy, & Meigs, 2014). More recently this has increased to >300 novel and established loci in total (Ingelsson & McCarthy, 2018; Scott et al., 2017), demonstrating the high polygenicity of T2D. The studies have also revealed that T2D is

characterized by many causal genetic variants with small additive effects on risk (Ingelsson & McCarthy, 2018). Many of the loci also show no known function in the pathophysiology of T2D, so further research is required (Nair & Baier, 2015). *TCF7L2*, which encodes transcription factor 4 (TCF4), is the most studied locus for T2D. *TCF7L2* is involved in the Wnt signaling pathway and Wnt is involved with pancreatic cell proliferation and differentiation. The risk alleles of *TCF7L2* seem to affect beta cell function and insulin production (Florez et al., 2006; Y. Zhou et al., 2014).

Medical drug treatments for T2D are very variable from patient to patient. Genetic variability found by GWAS may explain the variability experienced in patients on similar drug treatments (Pollastro, Ziviello, Costa, & Ciccodicola, 2015). Continued research will help increase the effectiveness of pharmacogenomics to treat T2D. Increased research may also help predict the risk of developing T2D from genetic information. A number of recent studies have tried to develop accurate genetic risk score models (X. Sun, Yu, & Hu, 2014).

Environment

T2D is associated with a sedentary lifestyle, as well as a poor diet. As with obesity, diet and exercise are key treatment options in treating T2D (Sami, Ansari, Butt, & Hamid, 2017). A systematic review of 60 cross-sectional and cohort studies, found decreased risk of T2D where there was space conducive to walking, greener neighborhoods, and quieter neighborhoods (Dendup, Feng, Clingan, & Astell-Burt, 2018). In addition, areas with higher air pollution, measured as particulate matter, had increased risk for T2D (Dendup et al., 2018). Epidemiological data indicate that chronic exposure to organic land pollutants (pesticides, herbicides) disturbs glucose metabolism and induces insulin resistance (Murea, Ma, & Freedman, 2012).

To decrease the risk of developing T2D, obesity should be treated through increased physical activity by exercise and NEAT and by healthy eating. High physical activity is associated with decreased diabetes risk, increased insulin sensitivity, and increased glycemic control. While there are many conflicting short-term diet studies, generally, a more plant based diet with reduced intake of refined grains and sugar is beneficial (Kolb & Martin, 2017). Other factors include quality and duration of sleep (7-8 hours), moderate tea and coffee consumption, and moderate alcohol consumption to decrease the risk of T2D. (Kolb & Martin, 2017). In a large cohort study of overweight adults with impaired glucose tolerance, there was a 58% reduction in diabetes through lifestyle modification and weight loss. Despite the lifestyle modification, 11% of the test group developed diabetes in 4 years (Knowler et al., 2002). The development of diabetes may be due to other environmental or genetic factors, but the best interventions would be before the development of diabetes or prediabetes.

Pathophysiology of T2D

T2D is characterized by hyperglycemia, beta cell dysfunction, and insulin resistance.

Beta-cell dysfunction

Decreased beta cell mass and beta cell dysfunction, where the beta cell can no longer increase insulin secretion to compensate peripheral insulin resistance, are important to T2D development (Brereton, Rohm, & Ashcroft, 2016). Beta cell dysfunction occurs as a result of inflammation and insulin resistance from obesity. Chronic hyperglycemia leads to increased oxidative stress, damaging mitochondria, and increased inflammation, increasing cytokines released from infiltrating immune cells in the pancreas. Compensation for insulin resistance results in hyperinsulinemia as well as increased hyperplasia and hypertrophy of beta cells, increasing beta cell mass. However, overtime, oxidative stress and inflammation cause beta cell

destruction and increased apoptosis, decreasing insulin secretion and beta cell mass (Cerf, 2013). Eventually, the beta cell population declines through apoptosis and amyloid fibrils develop (Pandey, Chawla, & Guchhait, 2015).

Insulin resistance

Obesity-related insulin resistance is a precursor to T2D as described in previous sections. Insulin resistance can occur from dysregulation of insulin signaling in peripheral tissues and downregulation of glucose transporters, causing hyperglycemia from digestion and unregulated release of glucose into the blood. Decreased insulin secretion further exacerbates insulin resistance. It should be noted that studies have shown variability of insulin resistance and beta cell dysfunction in those who are obese (Pour & Dagogo-Jack, 2011).

People with a "normal" BMI can develop T2D as well. They tend to have greater visceral adiposity than those who have "normal BMI" and do not have T2D. Their T2D is believed to have developed from genetics, adipose dysfunction, insulin resistance, hyperglycemia from impaired insulin secretion, and low-grade inflammation in the intestinal tract (Hammarstedt, Graham, & Kahn, 2012; Vaag & Lund, 2007; J. Zhou et al., 2018).

There are several possible complications that could arise from T2D including coronary artery disease, diabetic nephropathy, and diabetic retinopathy (Murea et al., 2012).

Treatment of Type 2 Diabetes

As with obesity, prevention of T2D through education, diet, and exercise is the best route. Once a person is diagnosed as pre-diabetic or with T2D, treatment through lifestyle modification is recommended. Restricted calories and increased physical activity have been shown to reduce BMI and increase insulin sensitivity (Hoelscher, Kirk, Ritchie, & Cunningham-Sabo, 2013). In adults, these lifestyle modifications decrease glycemic levels, but there is little experimental

study in adolescence. The few cases available found adolescents may still lack control of glycemic levels (Zeitler et al., 2012). Uncontrolled glycemic levels may be due to the differences with the progression of T2D, as children develop hyperglycemia much more quickly and have more trouble adhering to lifestyle change (McGavock, Dart, & Wicklow, 2015).

The goal of diabetes treatment involves glycemic control in the long term. Along with exercise and diet, treatment can often include medication such as oral antidiabetic drugs (OAD) (Pulgaron & Delamater, 2014; Semiz, Dujic, & Causevic, 2013). OADs include sulphonylureas, which stimulate insulin release and increase glucose uptake in skeletal muscles, biguanides like metformin, which decrease hepatic gluconeogenesis and increase glucose uptake, and thiazolidinediones, which bind to PPARγ and increase fatty acid uptake, increasing insulin sensitivity (Semiz et al., 2013). Bariatric surgery, previously mentioned as an option for obesity, is now being studied for its effectiveness in treating T2D. Both insulin resistance and β-cell dysfunction improve after surgery; however, the mechanisms behind diabetes remission remains incomplete as improvements are seen before dramatic weight loss (Pok & Lee, 2014). Future research needs to examine the interindividual differences of treatment outcomes by further studying genetic variants related to diabetes and drug uptake. Studies also need to be done on elucidating mechanisms caused by bariatric surgery and how to achieve juvenile glycemic control.

Animal Models for Obesity Research

Animal models are extremely useful in obesity and T2D research for understanding the regulation of energy balance. The advantages of using animal models include whole genome sequence availability, the variety of strains, ease of genetic manipulation, ability to localize disease genes, and ease of variable control (Yazdi et al., 2015). There are many genetic models

available that can be monogenic and polygenic. The first animal models that were involved in obesity and T2D research include single gene mutations of leptin and the leptin receptor in *ob/ob* and *db/db* mice, respectively, which were developed in The Jackson Laboratories in the 1950s (Hummel, Dickie, & Coleman, 1966; Ingalls, Dickie, & Snell, 1950; Lee et al., 1996; Zhang et al., 1994). Most human obesity and T2D cases, however, are polygenic. Monogenic models are useful for studying severe phenotypes, but they are not an accurate representation of most human obesity and T2D phenotypes (Mittwede, Bergin, Clemmer, & Xiang, 2015; Nilsson, Raun, Yan, Larsen, & Tang-Christensen, 2012). There are several different approaches to learn more about energy regulation and the underlying mechanisms of obesity and T2D in polygenic models. One method is to study different mutations in rodents by exposing them to different mutagens. By exposing rodents for example to radiation, major loss-of-function mutations can be discovered more quickly. This method, however, involves a large number of random mutations and thus phenotyping all the various offspring can be costly (Speakman, Hambly, Mitchell, & Król, 2008). Another method is genetically engineering mutations for over-expression or knock-out of certain genes; these mutations can be global or cell or tissue specific. This method has led to the formation of several transgenic and knock-out and knock-in models. A potential issue with these models may be the formation of compensatory mechanisms due to the targeted mutations (Speakman et al., 2008). The next method involves selective breeding for obesity and T2D phenotype to generate polygenic models. Polygenic models for obesity/T2D have many of the same symptoms as humans including hyperinsulinemia, hyperlipidemia, hyperglycemia, hypertension, and insulin resistance. Several polygenic mouse strains are used for research, including the New Zealand Obese mouse (NZO), C57B6 late-onset obesity mouse (LOO), and the TALLYHO/JngJ (TH) mouse. (Joost & Schurmann, 2014; Lutz & Woods, 2012). There are

also studies on obesity-resistant models such as the rat strains S5B/PI and Lou/C or A/J mice (Nilsson et al., 2012). Rodents are the most commonly used animal model for obesity and T2D research, but other animals such as chickens, pigs, dogs, Göttingen minipigs, and non-human primates have been studied as well (Nilsson et al., 2012; Speakman et al., 2008). Non-human primates have the closest phylogenetic relationship to humans and thus may be good model for research, but the model has a long-life span and gestation period and would most likely provide a small sample size (Vaughan & Mattison, 2016).

When using animal models, quantitative trait loci (QTL) mapping can be conducted to locate regions of genome that are responsible for obesity or T2D phenotypes and then congenic lines and bioinformatics can be used to identify the possible candidate genes. Beyond genetics, environmental effects that contribute to obesity and T2D can be studied. For example, effects of diet can be studied using diet-induced obesity models (DIO) (Nilsson et al., 2012).

This thesis is focused on the TALLYHO/Jng mouse model. The TH mouse is a polygenic model for human obesity and T2D that develops hyperleptinemia, hyperinsulinemia, insulin resistance, glucose intolerance, hyperlipidemia, and hyperglycemia (Denvir et al., 2016). TH mice originated from male Theiler's original mice that were glycosuric and polyuric. They were imported to The Jackson Laboratory in 1992, where the male mice were selectively bred for hyperglycemia (Leiter, 2001). Previous studies report a genetic basis for obesity and T2D through QTL mapping in TH mice (Kim & Saxton, 2012b).

Objectives of the study

TH mice are a polygenic model for obesity and T2D, whose genetic basis has been well documented. Diet studies in TH mice have not been reported. The second chapter of this thesis is dedicated to studying the effect of nutritionally modified diets in the development of obesity and

T2D in TH mice, testing our hypothesis of the presence of interplay between genetic susceptibility and obesogenic diets in this model. The third chapter builds off previous QTL mapping studies performed on the F2 generation of mice from a TH and B6 cross. We hypothesized that a TH allelic difference at the obesity QTL on chromosome 1 confers susceptibility to obesity. To test this hypothesis, we generated congenic mouse strains carrying the chromosome 1 QTL derived from TH on a B6 background and characterized them. Finally, the fourth chapter involves a mechanism study in respect to inflammation and mitochondrial dysfunction in relation to obesity and T2D. We hypothesized that TH mice had increased inflammation and decreased mitochondrial respiration in advancing obesity and T2D.

CHAPTER 2

GENOTYPE-DEPENDENT METABOLIC RESPONSES TO SEMI-PURIFIED HIGH-SUCROSE HIGH-FAT DIETS IN THE TALLYHO/JNG VS. C57BL/6 MOUSE DURING THE DEVELOPMENT OF OBESITY AND TYPE 2 DIABETES

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Abstract

The co-epidemic of obesity and type 2 diabetes is associated with increased morbidity and mortality. Genetic factors are highly involved in the development of these diseases, in the form of interactions of multiple genes within obesogenic and diabetogenic environments, such as a high fat diet. The TALLYHO/Jng (TH) mouse is an inbred polygenic model for human obesity and type 2 diabetes. In order to further develop the TH mouse as a clinically relevant model, we investigated diet dependence of obesity and type 2 diabetes in TH mice vs. C57BL/6 (B6) mice.

TH and B6 mice were weaned onto a standard rodent chow, semi-purified high-sucrose low-fat (HSLF), or semi-purified high-sucrose high-fat (HSHF) diet and maintained on these diets throughout the study. Despite similar fat contents in HSLF diets and chow, both B6 and TH mice responded to HSLF diets, with increases in adiposity. TH mice, but not B6 mice, exhibited significantly higher adiposity with severely aggravated glucose intolerance and hyperglycemia on HSHF diets compared to the other diets. HSLF diets also advanced diabetes in TH mice compared to chow, but it did not surpass the effects of HSHF diets. The severe glucose intolerance and hyperglycemia in TH mice on both HSLF and HSHF diets were accompanied by significantly reduced *Glut4* mRNA levels compared to B6 mice. The present data demonstrate that diets are important modulators of genetic susceptibility to type 2 diabetes and obesity in TH mice. The interplay between heredity and dietary environment in TH mice appears to amplify insulin resistance, contributing to severe glucose intolerance and diabetes.

Introduction

Globally, the prevalence of obesity and type 2 diabetes is increasing dramatically. The estimated total numbers of adults who were overweight (BMI $\geq$25kg/m^2) or had obesity (BMI $\geq$30kg/m^2) in 2005 were 937 million and 396 million, respectively, and the respective number is

projected to be 1.35 billion and 573 million by 2030 (Kelly, Yang, Chen, Reynolds, & He, 2008). In 2013, 382 million people had diabetes and this number is expected to be 595 million by 2035 (Guariguata et al., 2014). Type 2 diabetes is the most common form of human diabetes, accounting for 90% to 95% of diagnosed diabetes patients (Gavin, Freeman, Shubrook, & Lavernia, 2011).

The etiology of obesity and type 2 diabetes involves multiple factors, including multiple susceptibility genes and environmental factors (Bouret, Levin, & Ozanne, 2015). The global epidemics of obesity and type 2 diabetes are attributed to the interaction between westernized environmental factors and a genetic predisposition to these diseases (Walker et al., 2015). A major contributing environmental factor of obesity and type 2 diabetes is a diet high in fats (Hariri & Thibault, 2010). The vulnerability to high-fat diets in developing obesity and type 2 diabetes differs among individuals, which is most likely explained by genetic factors.

The TALLYHO/Jng (TH) mouse is a genetic model for human obesity and type 2 diabetes (Kim & Saxton, 2012b). The genetic basis of obesity and type 2 diabetes in TH mice follows a polygenic pattern of inheritance (Kim & Saxton, 2012b), closely resembling human cases (Grarup, Sandholt, Hansen, & Pedersen, 2014). Obesity in TH mice develops in both males and females, whereas the type 2 diabetes develops in males, preceded by glucose intolerance (Kim & Saxton, 2012b; Kim et al., 2001b). To further develop the TH mouse as a clinically relevant model, in the present study we analyzed diet dependence of obesity and type 2 diabetes in TH mice vs. C57BL/6 (B6) mice. The data demonstrate the high sensitivity of TH mice to dietary high fat during the development of obesity and type 2 diabetes, providing strong evidence that diets are important modifiers of genetic susceptibility to type 2 diabetes and obesity.

Materials and Methods

Animals and diets

TH mice used in this study were from our breeding colony that has been maintained since 2001 (Kim & Saxton, 2012b). B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and/or bred in our facility. All mice were allowed free access to food and water in a temperature and humidity-controlled room with a 12-hour light/dark cycle. At 3-4 weeks of age, mice were weaned onto standard rodent chow (Purina 5001, PMI Nutrition, Brentwood, MO, USA), semi-purified high-sucrose low-fat (HSLF) diets (D12489B) or semi-purified high-sucrose high-fat (HSHF) diets (12266B) (Research Diets, New Brunswick, NJ, USA) and maintained on these diets throughout the study. The HSLF diets contain a comparable sucrose content but a lower fat content than HSHF diets and the fat calories are replaced mainly with cornstarch. Chow contains a similar fat content but a lower carbohydrate content than HSLF diets and the carbohydrate calories are replaced with proteins. Detailed composition of chow and semi-purified HSLF and HSHF diets are presented in Table 1. All animal studies were carried out with the approval of Marshall University Animal Care and Use Committee.

Body composition

Quantitative magnetic resonance imaging was used to assess body composition, including fat mass and lean mass in mice using EchoMRI-100 whole body composition analyzer (Echo Medical Systems, Houston, TX, USA). A median of quintuple measurements for each animal was used as recommended by the manufacturer.

Intraperitoneal glucose tolerance test

Mice were fasted overnight and injected with glucose in saline intraperitoneally (1mg/g body weight). Blood was collected via submandibular bleeding at 0, 15, 30, 60 and 120 minutes

after the injection. Blood glucose levels were measured and calculated using a One Touch Ultra2 Blood Glucose Monitoring System (Diagnostics Direct, Cape May Court House, NJ, USA). In this system, the maximal level of blood glucose that can be determined is 600 mg/dl.

Non-fasting glucose, triglyceride, total cholesterol, and insulin levels in the circulation

Blood was drawn in the morning via submandibular bleeding and plasma was obtained by centrifugation (1,200*g*) at 4ºC. Blood glucose levels were measured as mentioned above, and plasma levels of total cholesterol (Thermo Electron, Louisville, CO, USA) and free and total glycerol (Sigma, St. Louis, MO, USA) were determined using commercial colorimetric assays. Plasma true triglyceride concentrations were estimated by subtraction of free glycerol from total glycerol. Plasma insulin levels were determined using an ELISA kit (Crystal Chem, Downers Grove, IL, USA).

Indirect calorimetry, locomotor activity, and food intake

Heat production, respiratory exchange ratio (RER), food intake, and locomotor activity were measured in mice using an eight-chamber Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA) as described previously (Mao, Dillon, McEntee, Saxton, & Kim, 2014). Locomotor activity was determined as ambulatory count, the number of times different infrared beams were broken in either the x- or y-axes during an interval.

Real-time quantitative RT-PCR

Total RNA was isolated from epididymal adipose tissue using RNeasy Lipid Tissue Midi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was then reverse-transcribed with SUPERSCRIPT® RT using oligo d(T)12-18 as primer to synthesize first-strand cDNA according to manufacturer’s instructions (Thermo Fisher Scientific,

Waltham, MA, USA). The primers used for the real-time PCR were proprietary for the insulin receptor substrate 1 (*Irs1)* (Qiagen) or synthesized (Sigma) using sequences obtained from the published literature for insulin receptor substrate 2 (*Irs2*) (Arai et al., 2013) and glucose transporter 4 (*Glut4*) and *36B4* (Dalen, Ulven, Bamberg, Gustafsson, & Nebb, 2003). The real-time PCR reaction was carried out in 1x SYBR Green PCR core reagents as described previously (Stewart, Kim, Saxton, & Kim, 2010) using the StepOne™ Real-Time PCR system (Thermo Fisher Scientific). For each sample, duplicate amplifications were performed and the average measurements used for data analysis.

Statistical analysis

Physiological data analysis: Variables were analyzed with analysis of variance, using a fixed effects factorial model of strain and diet and interaction. To satisfy normality and equal variance requirements a few variables were log transformed. Variables measured at 14+ weeks of age were tested for age effects, and an age covariate added to the model when significant. Least squares means were compared using Fisher's protected LSD at a 5% significance level.

Real-time qRT-PCR data analysis: Duplicate threshold cycle times were averaged for each mouse, and differences tested using a two-way ANOVA in SAS software (Cary, NC, USA), with an interaction contrast (housekeeping vs. gene interaction with strain or diet) used to estimate ΔΔCt. Data are presented as relative fold-change (Livak & Schmittgen, 2001).

Results

In order to examine the sensitivity of TH mice to dietary high fat in the development of obesity and type 2 diabetes, TH and B6 mice were weaned onto a regular chow diet, semi-

purified HSLF diet, or semi-purified HSHF diet and maintained on these diets throughout the study.

	Chow		HSLF		HSHF	
kcal/gm	3.36		3.9		4.41	
	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	23.9	28.5	16.4	16.8	18.5	16.8
Carbohydrate	48.7	58.0	70.8	72.6	56.7	51.4
Fat	5.0	13.5	4.6	10.6	15.6	31.8
Total		100		100		100
Notable Ingredient						
Sucrose	3.7		24.6		27.8	
Starch	31.9		42.3		20.6	
Crude fiber	5.1					
Cellulose			2.5		2.8	

HSLF, high-sucrose low-fat diets; HSHF, high-sucrose high-fat diets

Table 1. Diet composition

Body weight and body fat changes

At 5-6 weeks of age, when the animals were on the diets for only about 2 weeks, HSHF diets increased adiposity in TH mice at a greater extent than B6 mice (Fig.1A and B). At this young age, TH mice also exhibited larger body weight and body fat in HSLF diets compared to

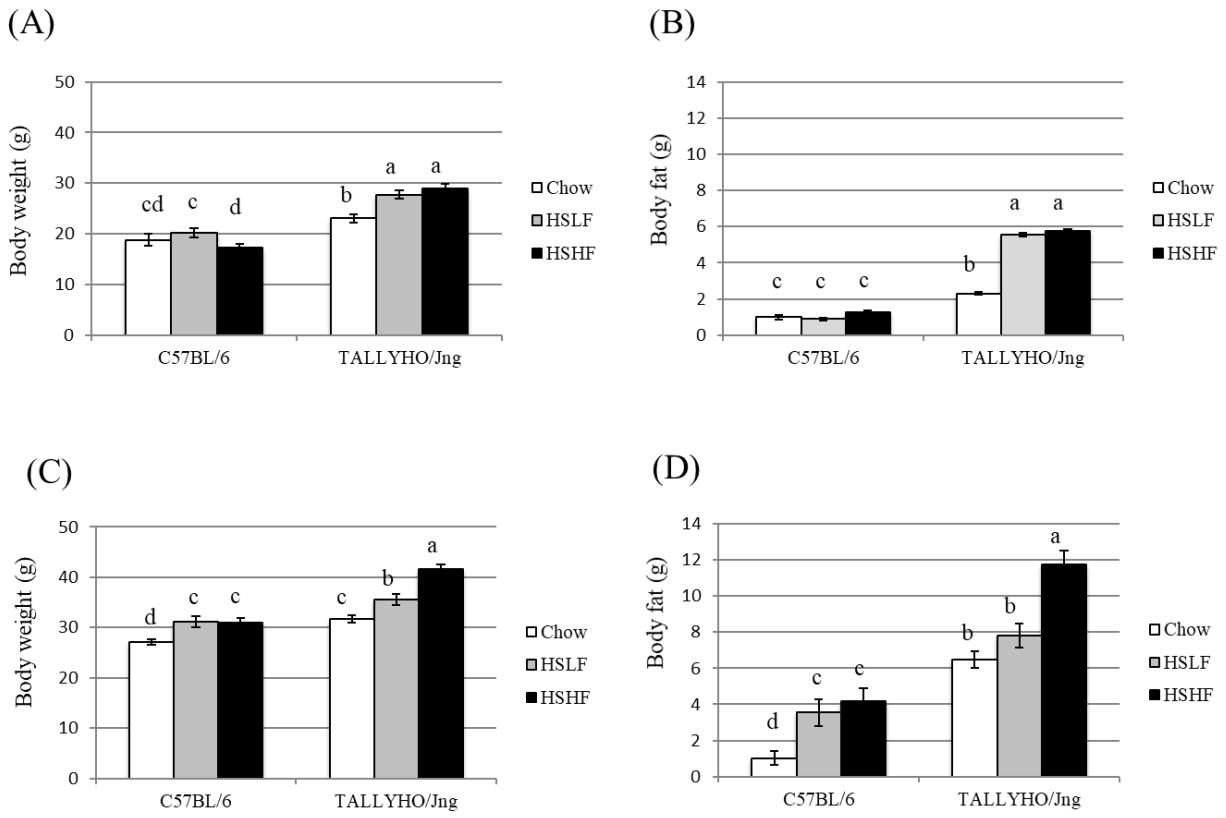

Figure 1. Body weight and body fat mass in C57BL/6 (B6) and TALLYHO/Jng (TH) mice fed chow, high-sucrose low-fat (HSLF) and high-sucrose high-fat (HSHF) diets. A and B present data at 5-6 weeks age and C and D present data at 16-17 weeks of age. Data were reported as means ± SEM. A and B: n=4 (chow), n=7 (HSLF), n=10 (HSHF) for B6 mice and n=9 (chow), n=8 (HSLF), n=7 (HSHF) for TH mice. C and D: n=45 (chow), n=12 (HSLF), n=12 (HSHF) for B6 mice and n=29 (chow), n=14 (HSLF), n=11 (HSHF) for TH mice. Group means labeled with different letters are significantly different ($P<0.05$).

the chow diet, while no diet effects were seen in B6 mice at this age (Fig. 1A and B). At 16-17 weeks of age, TH mice were highly responsive to HSHF diets both by increasing body weight and body fat (Fig. 1C and D). Interestingly, at this age HSLF and HSHF diets were equally obesogenic in B6 mice compared with chow (Fig. 1C and D).

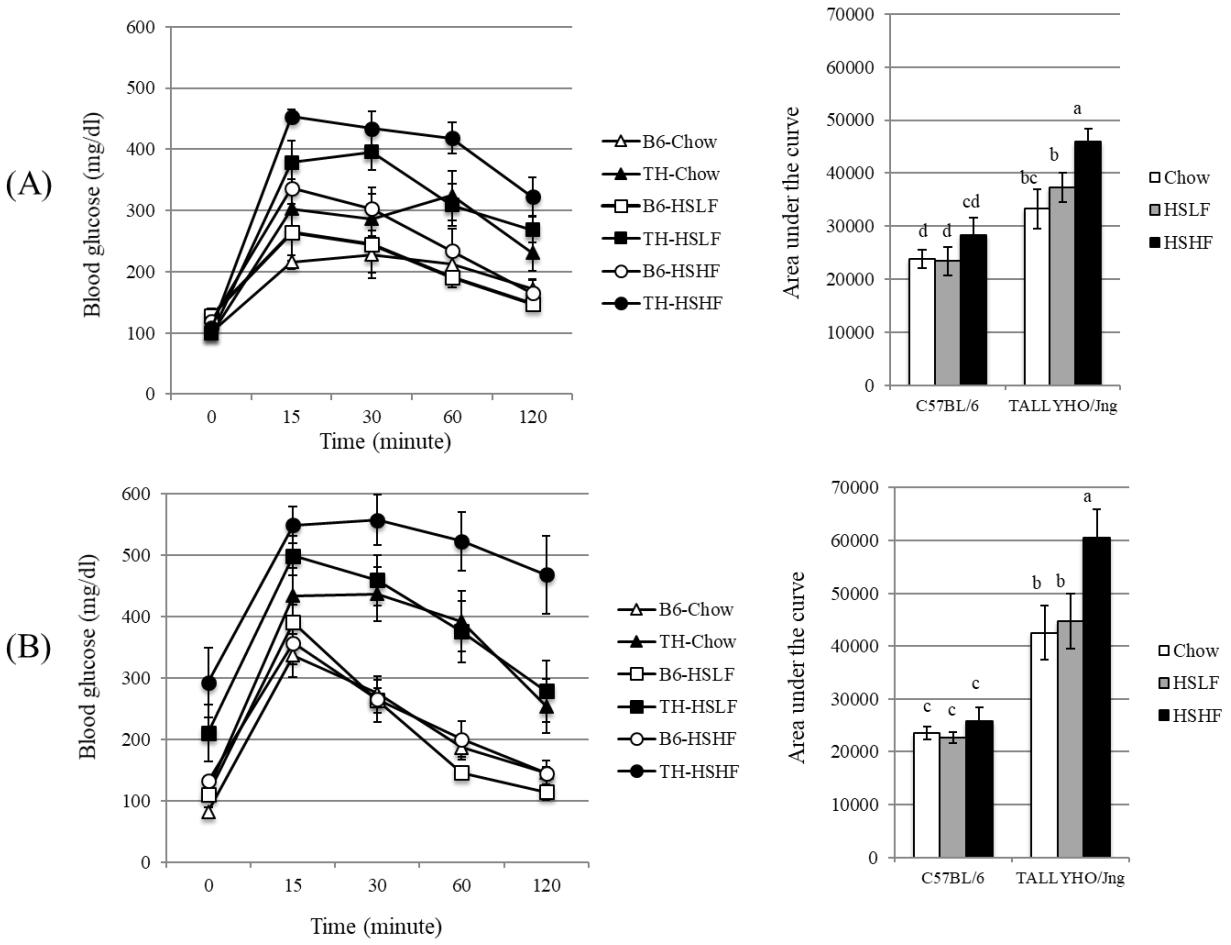

Figure 2. Intraperitoneal glucose tolerance test (IPGTT) in B6 and TH mice on chow, HSLF and HSHF diets at (A) 5-6 and (B) 16-24 weeks of age. Area under the curve of glucose levels during the IPGTT was also presented. Data were reported as means ± SEM. A: n= 4 (chow), n=6 (HSLF), n=8 (HSHF) for B6 mice and n=7 (chow), n=8 (HSLF), n=7 (HSHF) for TH mice. B: n=7 (chow), n=7 (HSLF), n=5 (HSHF) for B6 mice and n=6 (chow), n=9 (HSLF), n=8 (HSHF) for TH mice. Group means labeled with different letters are significantly different (P<0.05).

	B6			TH		
	Chow	HSLF	HSHF	Chow	HSLF	HSHF
6-10 week						
N	7	5	7	6	5	7
BW (g)	23.8 ± 0.50^{DE}	24.3 ± 0.60^{D}	22.8 ± 0.47^{E}	26.8 ± 0.54^{C}	34.3 ± 0.56^{B}	29.1 ± 0.47^{A}
Heat (kcal/hr)	0.40 ± 0.02^{D}	0.54 ± 0.02^{B}	0.44 ± 0.01^{C}	0.44 ± 0.02^{CD}	0.62 ± 0.02^{A}	0.58 ± 0.01^{AB}
RER	0.87 ± 0.02^{C}	0.99 ± 0.02^{A}	0.90 ± 0.02^{BC}	0.89 ± 0.02^{BC}	0.95 ± 0.02^{AB}	0.87 ± 0.02^{C}
Ambulatory count	12505 ± 1648^{B}	14247 ± 1958^{B}	26156 ± 1546^{A}	12647 ± 1764^{B}	13491 ± 1833^{B}	15130 ± 1555^{B}
Food (g)	4.36 ± 0.31^{A}	3.98 ± 0.37^{AB}	3.21 ± 0.29^{B}	4.04 ± 0.34^{AB}	3.95 ± 0.35^{AB}	3.64 ± 0.30^{AB}
17-19 week						
N	7	7	7	5	6	5
BW (g)	27.08 ± 0.89^{D}	32.97 ± 0.89^{C}	32.43 ± 0.89^{C}	37.38 ± 1.05^{B}	34.39 ± 0.96^{C}	43.97 ± 1.05^{A}
Heat (kcal/hr)	0.45 ± 0.02^{C}	0.56 ± 0.02^{B}	0.56 ± 0.02^{B}	0.55 ± 0.02^{B}	0.58 ± 0.02^{DB}	0.62 ± 0.02^{A}
RER	0.93 ± 0.01^{C}	0.98 ± 0.01^{A}	0.89 ± 0.01^{D}	0.91 ± 0.01^{CD}	0.95 ± 0.01^{AB}	0.84 ± 0.01^{C}
Ambulatory count	12535 ± 946^{B}	17284 ± 946^{A}	16163 ± 946^{A}	9439 ± 1119^{C}	10866 ± 1022^{BC}	10690 ± 1119^{B}
Food (g)	3.79 ± 0.31^{AB}	3.89 ± 0.31^{A}	3.33 ± 0.31^{AB}	4.19 ± 0.37^{A}	4.03 ± 0.34^{A}	2.89 ± 0.37^{B}

B6: C57BL/6, TH: TALLYHO/Jng, BW: body weight, RER: Respiratory exchange ratio. Data are means ± SEM. Group means labeled with different letters are significantly different ($P<0.05$). The values for B6 and TH mice on chow at 17-19 weeks were previously reported (Mao et al., 2014).

Table 2. Indirect calorimetry, locomotor activity, and food intake in B6 and TH mice on chow, HSLF, and HSHF diets over a 24-hour period

Intraperitoneal glucose tolerance test

When we evaluated mice at the young age of 5-6 weeks, HSHF diets induced earlier development of glucose intolerance in TH mice (Fig. 2A). At 19-24 weeks of age, TH mice exhibited a significantly impaired glucose tolerance compared with B6 mice on chow (Fig. 2B). While the severity of glucose intolerance was maintained on HSLF diets, it became markedly worse on HSHF diets in TH mice (Fig. 2B). Fasting blood glucose levels (values at 0 minute during IPGTT) were also significantly elevated in TH mice on HSHF diets compared to chow diets (293±34 vs. 117±40 mg/dl) (Fig. 2B).

Indirect calorimetry, locomotor activity, and food intake

In order to determine if the differences in body mass and composition resulted from differences in energy intake and/or energy expenditure, we assessed food intake, locomotor activity, and respiratory parameters using CLAMS. Overall, with increased body mass the heat production (kcal/hr) per animal increased at both young (6-10 week) and old (17-19 week) ages (Table 2). However, when the heat production was normalized by body weight (kcal/kg/hr) (Tschop et al., 2011), TH mice showed decreased heat production on HSLF diets at young age and on chow and HSHF diets at old age (Table 2).

RER reflects the whole-body oxidation rate of carbohydrate and fat; ~0.7 suggests complete reliance on fat oxidation and ~1.0 carbohydrate oxidation. Average RER over a 24-hour period reflected the dietary energy sources in our study, exhibiting the highest on HSLF diets without strain differences at both young and old ages (Table 2). Mice usually exhibit a stable fasting RER that is considerably lower than fed RER during the 12-hr light phase, as they primarily consume food during the 12-hr dark phase (Vaitheesvaran, LeRoith, & Kurland, 2010). Interestingly, RER during the dark phase was significantly lower in TH mice than B6 on HSLF and HSHF diets at both ages (Table 2).

There were no significant differences in food intake (gram consumed) among groups over a 24-hr period at both ages (Table 2). Notably, % of total food intake during the light phase was overall higher on HSLF and HSHF diets than on chow, with greater extend in TH mice (Table 2). The concomitant decrease in percentage of food consumed during the dark phase may be reflected as the decreased RER during the dark phase in TH mice shown above.

TH mice showed decreased locomotor activity compared to B6 mice especially at older age, for all diets (Table 2). The overall decrease in activity was attributed to specific reduction in activity during the dark phase, but not from the light phase (Table 2).

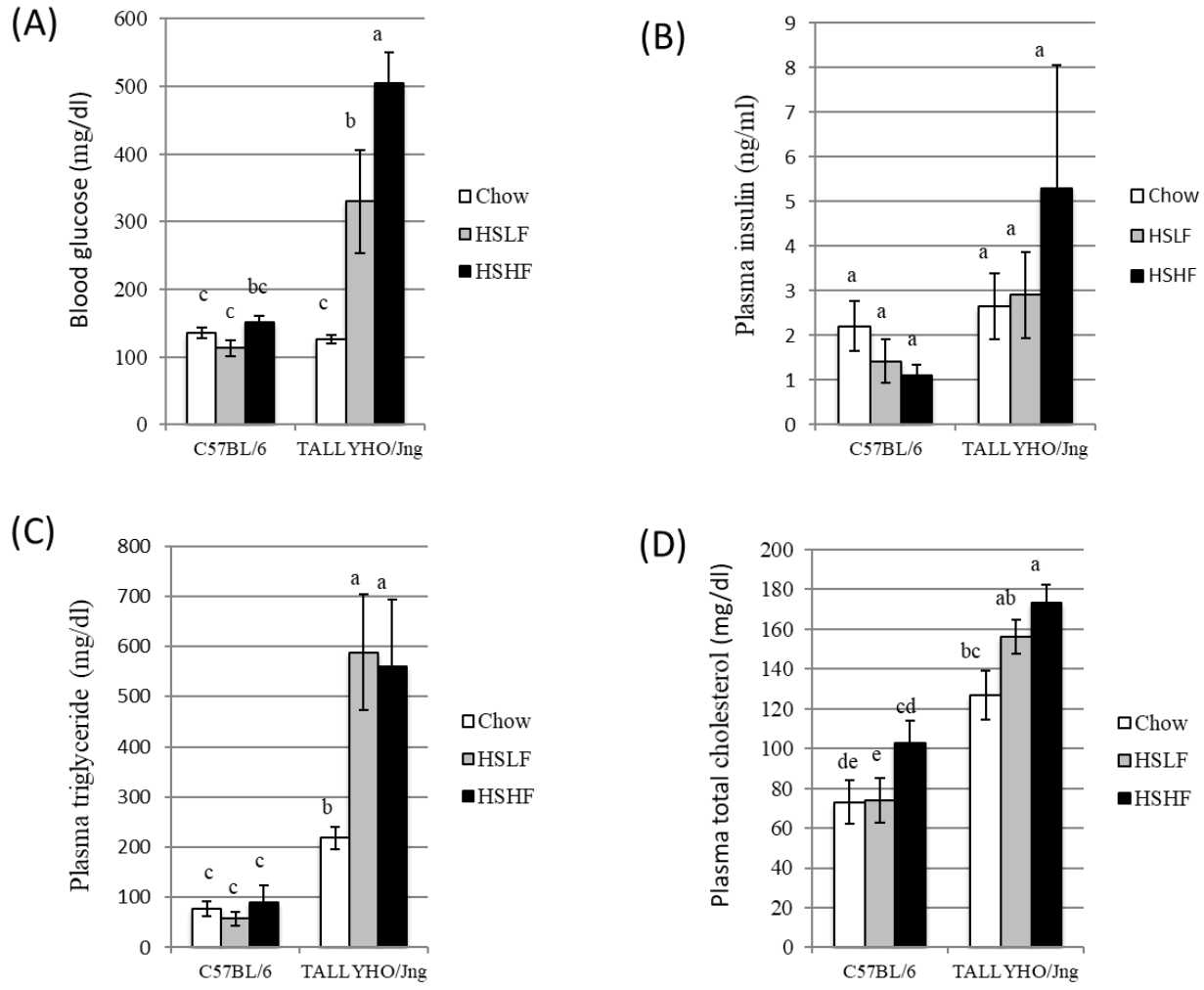

Figure 3. Circulating (A) glucose, (B) insulin, (C) triglyceride, and (D) total cholesterol levels in B6 and TH mice on chow, HSLF and HSHF diets at 14-28 weeks of age (non-fasting). Data were reported as means ± SEM. N=7 (chow), n=7 (HSLF), n=8 (HSHF) for B6 mice and n=8 (chow), n=10 (HSLF), n=9 (HSHF) for TH mice. Group means labeled with different letters are significantly different (P<0.05).

Circulating glucose, insulin, triglyceride, and cholesterol levels

Non-fasting glucose, insulin, triglyceride, and total cholesterol levels in circulation were measured at 14-28 weeks of age. Unlike reported in earlier publications (Kim et al., 2001b; Kim et al., 2006), TH mice maintained at the Marshall University vivarium did not develop severe hyperglycemia when fed chow (Fig. 3A). However, blood glucose levels were significantly elevated in TH mice fed HSLF or HSHF diets, with a greater value in HSHF, compared to chow (Fig. 3A). Along with the advanced hyperglycemia, plasma insulin levels were also higher in TH mice on HSLF and HSHF diets compared to chow but did not reach statistical significance possibly due to the large variability (Fig. 3B). The severe hyperglycemia was accompanied by an increase in plasma triglyceride in TH mice on HSLF and HSHF diets, while there were no changes in plasma triglyceride across the diets in B6 mice (Fig. 3C). Whereas total plasma cholesterol levels were overall significantly higher in TH mice than B6 mice, both strains trended towards higher cholesterol values when fed HSHF diets vs. chow (Fig. 3D).

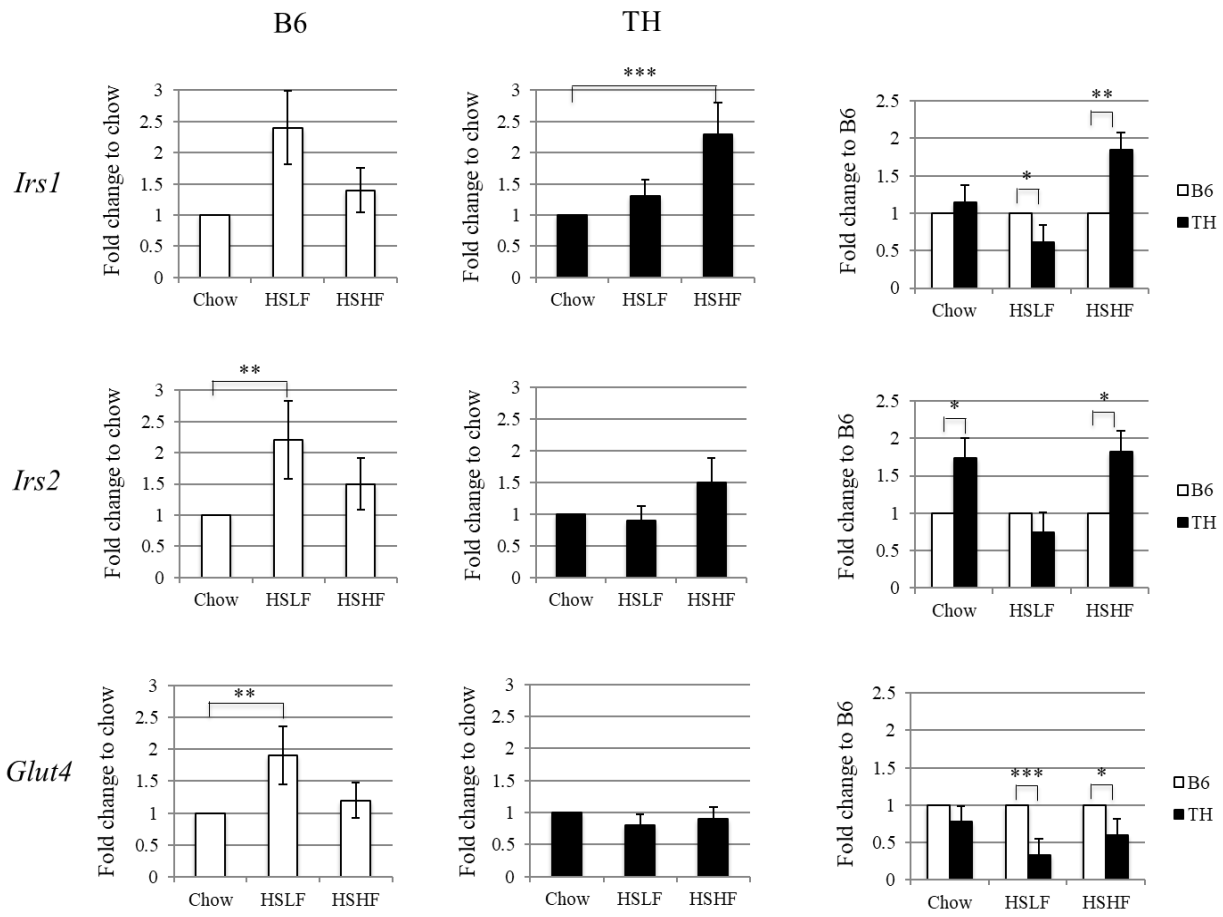

Figure 4. Gene expression in epididymal adipose tissue from B6 and TH mice on chow, HSLF and HSHF diets. Total RNA was extracted from epididymal fat pad and cDNA synthesized. The gene expression of Irs1, Irs2 and Glut4 was measured by real-time PCR. The values were normalized to *36B4* gene expression in each sample. Panels in the first and second columns represent diet effects in each strain; changes in gene expression were expressed as fold change relative to mean values for mice on chow in each strain. Panels in the third column represent strain effects; changes in gene expression were expressed as fold change relative to mean values for B6 mice in each diet. N=4 (chow), n=3 (HSLF) and n=3 (HSLF) for B6 mice and n=4 (chow), n=5 (HSLF) and n=5 (HSHF) for TH mice. **P*<0.05, ***P*<0.01, ****P*<0.001

Gene expression of insulin resistance-related genes in adipose tissue

We measured expression levels of genes related to insulin resistance including Irs1, Irs2 and Glut4 in adipose tissue of mice at 22-28 weeks of age. There were significant interactions of strain with diet. In B6 mice, mRNA levels of the Irs1, Irs2 and Glut4 genes were significantly

elevated on HSLF diets compared to chow (Fig. 4, first column). However, these increases were blunted in TH mice on HSLF diets (Fig. 4, second column). On the other hand, HSHF diets did not significantly affect mRNA levels of these genes in either strain compared to chow, except the elevation of Irs1 in TH mice. Focusing on strain differences within diets, TH mice had lower expression of the Irs1 gene on HSLF diets, but higher expression on HSHF diets compared to B6 mice (Fig. 4, third column). TH mice also had higher expression of the Irs2 gene compared to B6 mice on chow and HSHF diets. Despite these divergent changes in Irs1 and Irs2, TH mice exhibited significantly lower Glut4 gene expression compared to B6 mice on HSLF and HSHF diets.

Discussion

The present data demonstrate that diets are important modulators of genetic susceptibility to type 2 diabetes and obesity in TH mice. The HSHF diets used in this study were a purified form of the condensed milk rodent diet (Lauterio, Bond, & Ulman, 1994) that was known to be effective in identifying obesity-susceptible and obesity-resistant populations (Levin, Triscari, & Sullivan, 1983a, 1983b). The fat content of the HSHF diets is moderate compared with that of other high-fat diets (Lauterio et al., 1994) and more closely mimics the average American diet consisting of 35% of energy from fat (Last & Wilson, 2006). In our study, it was clear that chow diets have weight-reducing and diabetes-delaying effects compared to semi-purified diets, as previously reported (Mirhashemi et al., 2011). In B6 mice, despite the similar fat content with chow, semi-purified HSLF diets exerted a weight-increasing effect to the degree of HSHF diets at older ages studied. This similar degree of adiposity increasing effects of these two diets may be due to the moderate fat content in HSHF diets because other purified high-fat diets with higher fat content were effectively obesogenic compared with purified low-fat diets in B6 mice

(Black et al., 1998; C. M. Hill et al., 2015; Opara, Petro, Tevrizian, Feinglos, & Surwit, 1996). It is also possible that these effects are due to the short duration of HSHF diet feeding. Indeed, other studies reported that the same HSHF diets effectively increased adiposity in B6 mice with longer duration of feeding (Heber et al., 2014), but not with short duration (Cope et al., 2007).

HSLF diets also increased adiposity in TH mice at young age, but this effect did not surpass the effect of HSHF diets at older age. These data suggest that the obesogenic effect of semi-purified (and sucrose) diets precedes the obesogenic effect of dietary fat in TH mice. Similar phenomenon was also observed in obese NZO mice in which semi-purified diets (with or without sucrose) effectively increased adiposity compared to chow at early stage of feeding (Mirhashemi et al., 2011). In the NZO mouse, a weight gain in response to high fat diets began to show at later stage of the feeding; but the study was terminated before it became statistically significant (Mirhashemi et al., 2011). Chassaing et al. (2015) observed a rapid obesogenic effect of semi-purified low-fat diets compared to chow in mice and demonstrated that this effect was associated with lack of soluble fiber in semi-purified diets, which causes cecal and colonic atrophy and microbiota-dependent promotion of adiposity. As dietary fibers, only insoluble fiber, cellulose, is present in the HSLF and HSHF diets (Table 1), and therefore we speculate that the early obesogenic effect of HSLF diets in TH mice may be attributed to the lack of soluble fiber in this semi-purified diet. In future, it is a great interest to test whether this effect is microbiota dependent.

TH mice showed reduced locomotor activity levels, especially at older ages, compared with B6 mice without diet differences, suggesting that the reduced locomotor activity may not directly correlate with increased body mass in response to high-fat diets in TH mice. Currently, the significance of reduced locomotor activity in the pathogenesis of obesity and diabetes in TH

mice is unclear. It is known that there is wide variation among inbred mouse strains in the level of locomotor activity (Mhyre et al., 2005). Among multiple inbred strains tested, the B6 strain was near the top of locomotor activity measures (Mhyre et al., 2005). Therefore, it is possible that the cage activity differences between TH and B6 mice may reflect genetic background differences of these two strains. It is also possible that the presence of physical limitations secondary to the increased body mass may cause the hypo-activity in TH mice.

Although our food intake measurement was limited to the short time period while animals were housed in the CLAMS, daily food intake in TH mice was overall comparable with B6 mice. Therefore, we hypothesize that TH mice have a higher energy storage efficiency than B6 mice through unknown mechanisms. A potential mechanism may include an alteration in energy partitioning. For example, fat in the body may be in line for oxidative processes in skeletal muscle or for storage as triglyceride in adipose tissue, such that preferential partitioning of energy to storage over oxidative processes may be overwhelming in the development of obesity. This hypothesis, however, remains to be tested. A weight gain usually results in compensatory increases in energy expenditure (Leibel, Rosenbaum, & Hirsch, 1995). In our study, although the observed heat production (kcal/hr) was comparable between TH and B6 mice or even greater in TH mice, heat production normalized by body weight (kcal/kg/hr) was lower in TH mice especially at old ages, possibly reflecting insufficient compensatory increases in energy expenditure. Collectively, our working hypothesis is that the positive energy balance in TH mice may be attributed to the combined effect of increased energy retention efficiency and inadequate energy expenditure.

When the major energy source was carbohydrates, as found in HSLF diets, B6 mice significantly increased insulin receptor substrates *Irs1* and *Irs2* and glucose transporter *Glut4*

mRNA in the adipose tissue. These transcriptional elevations were totally absent in the adipose tissue from TH mice on HSLF diets, which led to lower levels of *Irs1* and *Glut4* mRNA in TH mice than B6 mice. TH mice also had lower *Glut4* mRNA expression than B6 mice on HSHF diets. Glucose uptake by adipose tissue is dependent on insulin-mediated glucose transport, mainly provided by insulin-regulated GLUT4 glucose transporter (Scheepers, Joost, & Schurmann, 2004). Therefore, we infer that down-regulation of *Glut4* mRNA expression is one of the mechanisms by which HSLF and HSHF diets induce the severe hyperglycemia in TH mice.

We have maintained our inbred colony of TH mice since 2001, which was derived from The Jackson Laboratory (Kim & Saxton, 2012b). In the present study, it was noticed that while the obesity and glucose intolerance were sustained, the hyperglycemia was largely reduced in our colony of TH mice on chow maintained at Marshall University, compared with that reported in our previous publications on TH mice (Kim et al., 2001b; Kim et al., 2006). This reduced hyperglycemia may reflect either a genetic divergence that occurred in our sub-colony after many generations (Leiter, 2001) or an environmental factor reducing hyperglycemia. Phenotypic divergence in TH mice was also previously observed among sub-colonies at different institutions, including the earlier onset of hyperglycemia in the Calgary-based colony (Cheng, Jiang, Ding, Severson, & Triggle, 2007). Nonetheless, the present data demonstrate that the interplay between heredity and dietary factors in TH mice can maximize and/or synergize the susceptibility to hyperglycemia.

In summary, this study provides important information about diet dependence of obesity and type 2 diabetes in TH mice. TH mice are very sensitive to the dietary fat content in advancing obesity and diabetes. The TH mouse will be a clinically relevant model for obesity

and type 2 diabetes where genetic factors interplay with dietary factors, serving as a useful tool in developing and testing therapeutic targets.

CHAPTER 3

CONGENIC MICE DEMONSTRATE THE PRESENCE OF QTLS CONFERRING OBESITY AND HYPERCHOLESTEROLEMIA ON CHROMOSOME 1 IN THE TALLYHO MOUSE

A manuscript published in *Mammalian Genome*
Parkman, J. K., Denvir, J., Mao, X., Dillon, K. D., Romero, S., Saxton, A. M., & Kim, J. H. (2017). Congenic mice demonstrate the presence of QTLs conferring obesity and hypercholesterolemia on chromosome 1 in the TALLYHO mouse. *Mamm Genome, 28*(11-12), 487-497. doi:10.1007/s00335-017-9719-2

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Abstract

The TALLYHO (TH) mouse presents a metabolic syndrome of obesity, type 2 diabetes, and hyperlipidemia. Highly significant quantitative trait loci (QTLs) linked to adiposity (proximal) and hypercholesterolemia (distal) were previously identified on chromosome (Chr) 1 in a genome-wide scan of F2 mice from C57BL/6J (B6) x TH. In this study we generated congenic mouse strains that carry the Chr 1 QTLs derived from TH on a B6 background; B6.TH-Chr1-128Mb (128 Mb in size) and B6.TH-Chr1-92Mb (92 Mb in size, proximally overlapping). We characterized these congenic mice on chow and high fat (HF) diets. On chow, B6.TH-Chr1-128Mb congenic mice exhibited a slightly larger body fat mass compared with B6.TH-Chr1-92Mb congenic and B6 mice, while body fat mass between B6.TH-Chr1-92Mb congenic and B6 mice was comparable. Plasma total cholesterol levels were significantly higher in B6.TH-Chr1-128Mb congenics compared to B6.TH-Chr1-92Mb congenic and B6 mice. Again, there was no difference in plasma total cholesterol levels between B6.TH-Chr1-92Mb congenic and B6 mice. All animals gained more body fat and exhibited higher plasma total cholesterol levels when fed HF diets than fed chow, but these increases were greater in B6.TH-Chr1-128Mb congenics than in B6.TH-Chr1-92Mb congenic and B6 mice. These results confirmed the effect of the 128Mb TH segment from Chr 1 on body fat and plasma cholesterol values and showed that the distal segment of Chr 1 from TH is necessary to cause both phenotypes. Through bioinformatic approaches we generated a list of potential candidate genes within the distal region of Chr 1 and tested *Ifi202b* and *Apoa2*. We conclude that Chr 1 QTLs largely confer obesity and hypercholesterolemia in TH mice and can be promising targets for identifying susceptibility genes. Congenic mouse strains will be a valuable resource for gene identification.

Introduction

Obesity is associated with many comorbidities such as type 2 diabetes and coronary heart disease and affects 13% of the adult population worldwide, making it a major global health concern (Flynt & Daepp, 2015). Hyperlipidemia characterized by increased plasma lipids, namely cholesterol and triglyceride, also signifies an increased risk of cardiovascular disease and often coexists with obesity (Erion & Corkey, 2017). An important component in the development of obesity (Loos & Janssens, 2017; Yazdi et al., 2015) and hyperlipidemia (Dron & Hegele, 2016) is genetic factors. Although there are rare monogenic forms of obesity and hyperlipidemia, the genetic susceptibility to common forms is polygenic (Dron & Hegele, 2016; Loos & Janssens, 2017). Environmental factors are also a critical component in the development of obesity and hyperlipidemia, which is more likely the result of interplay between genes and environments with varying contributions in each individual (Doo & Kim, 2015; Marais, 2013). Among many environmental factors, nutrition is a strong connection between these interplays and especially, consuming food high in fat content is well known to promote obesity and hyperlipidemia (Doo & Kim, 2015; Marais, 2013).

The TALLYHO/Jng (TH) mouse is a polygenic model for human obesity and type 2 diabetes and characterized by increased adiposity, insulin resistance, hyperglycemia, and hyperlipidemia (Kim et al., 2001a). In previous mapping study using F2 progeny of C57BL/6 (B6) and TH mice, we have identified two major quantitative trait loci (QTLs) on chromosome (Chr) 1; one linked to increased fat pad weight near *D1Mit215* and the other linked to hypercholesterolemia near *D1Mit113* (Stewart et al., 2010). Later, we named these QTLs; *Tabw3* for the adiposity peak and *Tachol1* for the hypercholesterolemia peak (Kim & Saxton, 2012b). To confirm the QTLs and develop a strategy for positional cloning of the responsible

gene(s), in the present study, we generated and characterized congenic mice carrying these QTLs derived from the TH strain on a B6 genetic background.

Materials and Methods

Animals and diets

B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facility and TH mice used for construction of congenic strain were from our breeding colony. All mice had free access to food and water in a temperature and humidity-controlled room with a 12-h light/dark cycle. At 4 weeks of age, mice were weaned onto standard rodent chow (14% kcal from fat, Purina 5001, PMI Nutrition, Brentwood, MO) or high fat (HF) diets (32% kcal from fat, 12266B, Research Diets; New Brunswick, NJ) and stayed on these diets throughout the study. Mice were euthanized by CO2 asphyxiation, and blood was collected by cardiac puncture and plasma obtained by centrifugation (1,200g) at 4ºC and stored at −20°C. Tissues were collected, frozen in liquid nitrogen and stored at −80°C. All animal studies were carried out with the approval of Marshall University Animal Care and Use Committee.

Construction of congenic mice

Mice congenic for the Chr 1 QTL genomic region were generated by marker-assisted backcrossing (Kim et al., 2005). Briefly, B6 female and TH male mice were crossed to yield F1 and then F1 was backcrossed with B6. The progeny were then genotyped with single sequence length polymorphism (SSLP) markers to select heterozygotes for the QTL. Selected heterozygotes were backcrossed again with B6 mice. This procedure was repeated 10 times, at which point female and male heterozygotes were crossed to obtain offspring homozygous for TH

alleles. Homozygous mice were then interbred to maintain congenic lines. The congenic segment stretched from *D1Mit213* to *D1Mit113*.

Genotyping by PCR

Genomic DNA was isolated from tail tips of mice using Gentra Puregene Mouse Tail Kit (Qiagen, Valencia, CA) for genotyping. SSLP primers synthesized (Sigma, St. Louis, MO) based on sequences from Mouse Genome Informatics (http://informatics.jax.org/) were assayed after PCR of genomic DNA by 3% agarose (Amresco, Solon, OH) gel electrophoresis and visualized with ethidium bromide (Thermo Fisher Scientific, Marietta, OH) staining.

Body composition

Quantitative magnetic resonance imaging was used to assess body composition including fat mass and lean mass in mice using EchoMRI-100 whole body composition analyzer (Echo Medical Systems, Houston, TX). A median of quintuple measurements for each animal was used (Parkman et al. 2016).

Indirect calorimetry, locomotor activity, and intake of food and water

An eight-chamber Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH) was used to measure heat production, respiratory exchange ratio (RER), food intake, water intake, and locomotor activity (Stewart et al., 2012). All mice were acclimatized to monitoring cages for 24 hours prior to an additional 48 hours of recordings under the regular 12-hour light-dark cycle. In this system, heat production (kcal/hr) is calculated by multiplying the calorific value [CV = 3.815 + (1.232 x RER)] by the observed VO_2 (Heat = CV x VO_2). Heat production was then normalized by body weight to calculate energy expenditure (kcal/kg/hr) (Tschop et al., 2011). RER is the ratio between the VCO_2 and VO_2

(RER = VCO_2/VO_2). Locomotor activity was determined as ambulatory count, the number of times different infrared beams were broken in either the x- or y-axes during an interval.

Intraperitoneal glucose tolerance test

Mice were fasted overnight and injected with glucose in saline intraperitoneally (1mg/g body weight). Blood was collected via submandibular bleeding at 0, 15, 30, 60 and 120 minute after the injection. Blood glucose levels were measured and calculated using a One Touch Ultra2 Blood Glucose Monitoring System (Diagnostics Direct, Cape May Court House, NJ).

Non-fasting plasma insulin, triglyceride, and total cholesterol levels

Plasma insulin levels were determined using an ELISA kit (Crystal Chem, Downers Grove, IL). Plasma levels of total cholesterol (Thermo Electron, Louisville, CO) and free and total glycerol (Sigma) were determined using commercial colorimetric assays. Plasma true triglyceride concentrations were estimated by subtraction of free glycerol from total glycerol.

RNA isolation and real-time quantitative PCR (qRT-PCR)

Total RNA was isolated from epididymal adipose tissue and liver using RNeasy Plus Universal Midi Kit and RNeasy Midi Kit, respectively, according to the manufacturer's instructions (Qiagen). Total RNA (2 μg) was reverse-transcribed with SUPERSCRIPT RT using oligo dT as primer to synthesize first-strand cDNA in 20-μl volume according to manufacturer's instructions (Invitrogen, Waltham, MA). The primers used for the qRT-PCR for the interferon activated gene 202B (*Ifi202b*) gene were synthesized (Sigma) using sequences obtained from the published literature (forward: 5′-GGCAATGTCCAACCGTAACT-3′ and reverse: 5′-TAGGTCCAGGAGAGGCTTGA-3′) (Kimura et al., 2014) and the primers for apolipoprotein A-II (*Apoa2*) gene were proprietary (PPM05347B, Qiagen). The real-time PCR reaction was carried out in a 25-μl volume in 1x SYBR Green PCR core reagents (Qiagen) containing 1 μl

cDNA template diluate (1:5, v/v) and 6 pmol primers using the StepOne^{TM} Real-Time PCR system (Thermo Fisher Scientific). For each sample, duplicate amplifications were performed and the average measurements used for data analysis. The *36B4* gene was used as a control to calculate the difference in average threshold cycle (ΔCt) values (Stewart et al. 2010).

RT-PCR and sequencing of the *Ifi202b* gene

The diluate cDNA described above was PCR amplified for *Ifi202b*^{NZO} exon 1 and part of exon 2 using the Expand Long Template PCR system (Roche, Indianapolis, IN). The specific primers were forward (5′-CCCTCT TCCTTTACACCCAAC-3′) and reverse (5′-GCCTGGGACAGATGTCTC TT-3′) derived from Mus Musculus *Ifi202b* gene mRNA sequences (GenBank: JX945582.1) (H. Vogel et al., 2013). The PCR products were directly sequenced with primers originally used to amplify the PCR products. Sequencing was carried out automatically with fluorescent tags (Marshall University Genomics Core Facility).

Statistical analysis

Physiological data analysis: Variables were analyzed with analysis of variance, using a fixed effects factorial model of strain and diet and interaction. Least squares means were compared using Fisher's protected LSD at a 5% significance level.

Real-time qRT-PCR data analysis: Duplicate threshold cycle times were averaged for each mouse, and differences tested using a two-way ANOVA in SAS software (Cary, NC, USA), with an interaction contrast (housekeeping vs. gene interaction with strain or diet) used to estimate ΔΔCt. Data are presented as relative fold-change (Livak & Schmittgen, 2001).

Bioinformatic analysis

We used a previously published catalog of sequence variants in the TH mouse (relative to the B6 strain) (Denvir et al., 2016) to list all variants lying between the markers *D1Mit498*

(position 135,687,493 bp on Chr 1 in the GRCm38 genome build) and *D1Mit361* (186,261,999 bp on Chr 1). We restricted these to variants with the potential to alter the protein sequence: namely those with sequence ontology terms "frameshift_variant," "inframe_deletion," "inframe_insertion," "missense_variant," "stop_gained," "stop_lost," "initiator_codon_variant," "splice_region_variant," "splice_acceptor_variant," or "splice_donor_variant." We extracted molecular function and biological process terms for the genes containing each of these variants from the Gene Ontology (GO) database (Ashburner et al., 2000; The Gene Ontology, 2015). We further restricted this collection of genes to contain only those that were associated to GO terms containing any of "cholesterol," "fat," "lipid," "lipoprotein," "lipase," "glucose," or "metabolic process," to obtain a list of potential susceptibility genes.

Results

Generation of congenic mice for the obesity and hypercholesterolemia QTLs

In previous mapping study, we have identified two major QTLs on Chr 1; one linked to fat pad weight (*Tabw3*) near *D1Mit215* and the other linked to plasma cholesterol levels (*Tachol1*) near *D1Mit113* (Kim & Saxton, 2012b; Stewart et al., 2010). We established a congenic line by introgressing a TH-derived genomic fragment containing both *Tabw3* and *Tachol1*, defined by the flanking markers *D1Mit213* and *D1Mit113* (minimum of 128 Mb in size), onto B6 background for 10 generations. After the 10^{th} backcross generation, the offspring were intercrossed to generate B6.TH-Chr1-128Mb congenic mice (Fig. 5). Using this congenic line we generated a second congenic line, B6.TH-Chr1-92Mb, carrying a smaller fragment of Chr 1 with only *Tabw3*, defined by the flanking markers *D1Mit213* and *D1Mit498* (minimum of 92 Mb in size) (Fig. 5).

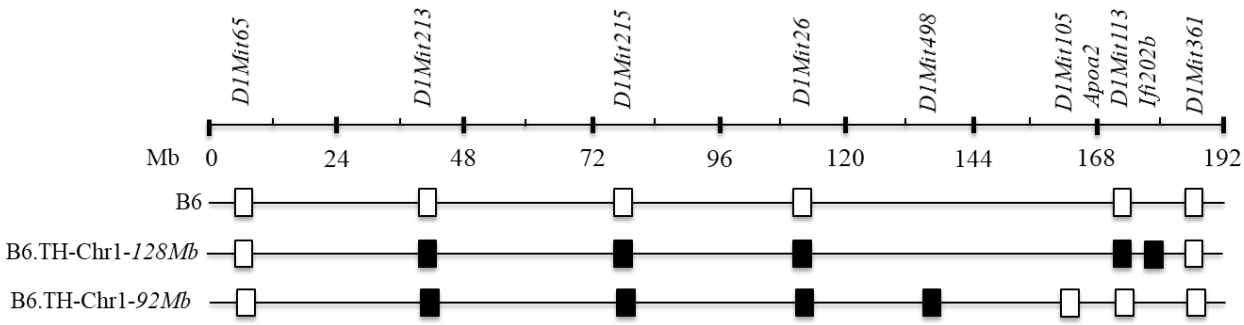

Figure 5. B6.TH-Chr1 congenic intervals on chromosome 1. Genetic markers shown at the top were used to allelotype the congenic interval. The open and filled boxes represent a C57BL/6 (B6) and TALLYHO (TH) allele, respectively. Mb: Megabase

Increased adiposity and plasma cholesterol levels in B6.TH-Chr1-128Mb, but not in B6.TH-Chr1-92Mb, congenic mice

When animals were fed a standard rodent chow, B6.TH-Chr1-128Mb congenic mice exhibited a small increase in body fat mass compared with B6.TH-Chr1-92Mb congenic and B6 mice, while body fat mass between B6.TH-Chr1-92Mb congenic mice and B6 mice was comparable (Fig. 6A). When fed HF diets, all animals had more body fat than when fed chow, but the increase was greater in B6.TH-Chr1-128Mb congenic mice than either B6.TH-Chr1-92Mb congenic or B6 mice (Fig. 6A). Again, body fat mass between B6.TH-Chr1-92Mb congenic and B6 mice was comparable on HF diets (Fig. 6A). The increased adiposity in B6.TH-Chr1-128Mb congenic mice on HF diets was accompanied by moderate increases in plasma insulin levels (Fig. 6B) and slightly impaired glucose tolerance during IPGTT (Fig. 6C), indicating some degree of insulin resistance.

Plasma total cholesterol levels were significantly higher in B6.TH-Chr1-128Mb congenic mice compared to B6.TH-Chr1-92Mb congenic and B6 mice on chow (Fig. 6D), confirming the presence of the *Tachol1* hypercholesterolemia QTL in the distal region of Chr 1.

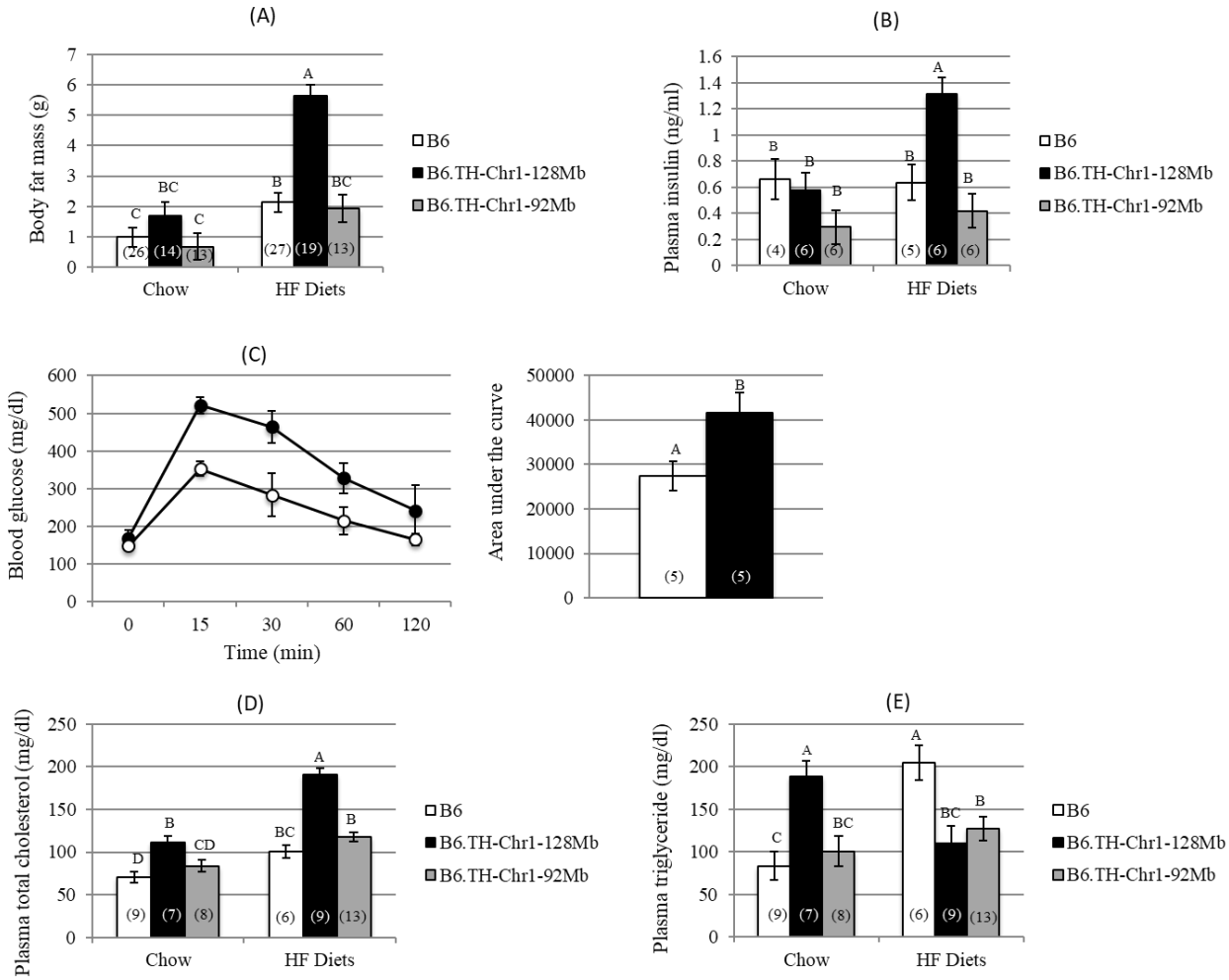

Figure 6. (A) Body fat mass (14 wk), (B) plasma insulin levels (14-25 wk), (C) intraperitoneal glucose tolerance test (14-18 wk), and plasma (D) total cholesterol and (E) triglyceride levels (20-27 wk) in B6, B6.TH-Chr1-128Mb congenic, and B6.TH-Chr1-92Mb congenic mice fed chow and high fat (HF) diets (males). Data were reported as means ± SEM (N is indicated in each bar). Group means labeled with different letters are significantly different (P<0.05). wk: weeks of age

HF diets induced an increase in total plasma cholesterol in all three groups compared to chow, with the greatest degree in B6.TH-Chr1-128Mb congenic mice (Fig. 6D). Plasma triglyceride levels were also significantly higher in B6.TH-Chr1-128Mb congenic mice compared to B6.TH-Chr1-92Mb congenic and B6 mice on chow (Fig. 6E). While HF diets

induced an increase in plasma triglyceride levels in B6 mice, no increases were shown in both congenic mice.

These results confirmed the effect of the 128Mb TH segment from Chr 1 on body fat and plasma cholesterol values and showed that the distal segment of Chr 1 from TH is necessary to cause both phenotypes.

Energy expenditure, locomotor activity, and food and water intakes

We measured energy expenditure and intake and locomotor activity of B6, B6.TH-Chr1-128Mb and B6.TH-Chr1-92Mb mice on chow and HF diets by CLAMS and summarized in Table 3. Overall, with increased body mass the heat production (kcal/hr) per animal increased with HF diet feeding compared to chow. However, when the heat production was normalized to body weight (kcal/kg/hr), B6.TH-Chr1-128Mb congenic mice exhibited lower values than B6.TH-92Mb congenic and B6 mice on both chow and HF diets.

RER values were generally lower when animals were placed on HF diets compared to chow, demonstrating that whole body substrate metabolism was shifted towards fat oxidation. Additionally, compared to chow, in general animals consumed less food in grams on HF diets, balancing the total kcal intake. Water intake was also overall lower on HF diet. However, there was no genotype effect on RER, food intake, or water intake in either diet.

B6.TH-Chr1-128Mb congenic mice showed somewhat decreased locomotor activity compared to B6 and B6.TH-Chr1-92Mb mice on both diets, but it did not reach statistical significance.

	Chow			HF diets		
	B6	B6.TH-128Mb	B6.TH-92Mb	B6	B6.TH-128Mb	B6.TH-92Mb
N	17	15	9	7	4	13
BW (g)	27.92± 0.57^{C}	30.73± 0.61^{B}	30.16± 0.79^{B}	32.43± 0.89^{B}	37.35± 1.18^{A}	30.95± 0.66^{B}
Heat						
kcal/hr	0.45± 0.01^{C}	0.44± 0.01^{C}	0.46± 0.01^{C}	0.56± 0.01^{A}	0.55± 0.01^{A}	0.51± 0.01^{B}
kcal/kg/hr	15.97±0.26^{BC}	14.44±0.28^{D}	15.37±0.36^{C}	17.28±0.41^{A}	14.82±0.54^{CD}	16.68±0.30^{AB}
RER	0.92± 0.01^{AB}	0.91± 0.01^{BC}	0.93± 0.01^{A}	0.89± 0.01^{CD}	0.87± 0.01^{D}	0.89± 0.01^{CD}
Food g	3.93±0.15^{AB}	3.90±0.16^{BC}	4.45±0.21^{A}	3.33±0.24^{CD}	3.17±0.32^{D}	3.31±0.18^{D}
kcal	13.20±0.57^{A}	13.11±0.60^{A}	14.95±0.78^{A}	14.68±0.89^{A}	13.98±1.17^{A}	14.61±0.65^{A}
Drink (ml)	3.54±0.17^{A}	3.22±0.18^{AB}	3.78±0.23^{A}	2.72±0.26^{BC}	2.40±0.34^{C}	2.87±0.19^{BC}
Ambulatory count	16210±1106^{AB}	13623±1178^{AB}	17395±1520^{A}	16163±1724^{AB}	11648±2280^{B}	16284±1265^{AB}

RER: respiratory exchange rate
ABCD means in a row with no common letter differ (P<0.05)

Table 3. Energy expenditure (heat production), respiratory exchange rate (RER), locomotor activity (ambulatory count), and food and water intakes in B6, B6.TH-Chr-128Mb congenic and B6.TH-Chr1-92Mb congenic mice on chow and HF diets over a 24-hour period (males, 19-23 weeks of age)

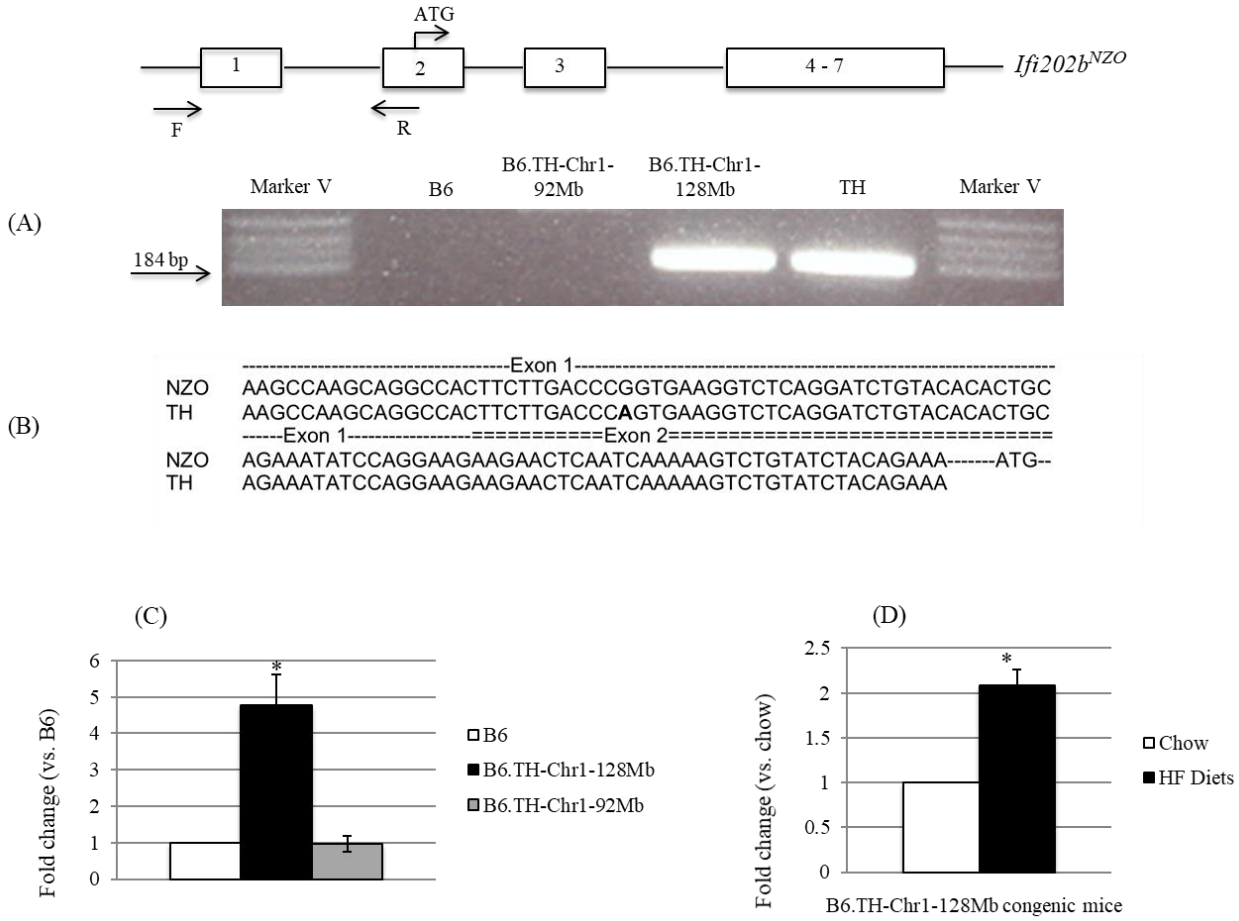

Figure 7. Expression of Ifi202b in B6 and congenic mice. (A) PCR amplification of exon 1 and part of exon 2 from cDNA (epididymal adipose tissue) of B6, B6.TH-Chr1-92Mb, B6.TH-Chr1-128Mb, and TH mice using a primer set of F and R as indicated in the scheme, (B) sequence comparison of the PCR product of TH to the sequence of NZO derived from GenBank accession number JX945582, and (C) the mRNA levels of *Ifi202b* in epididymal adipose tissue. Data were reported as means ± SEM (N=3-4 each group, 17-27 weeks of age). **P*<0.05.

The *Ifi202b* gene and *Apoa2* gene in B6.TH-Chr-128Mb congenic mice

The *Ifi202b* gene maps to the distal region of mouse Chr 1, near the *D1Mit113*, *Tachol1* QTL. It has been known that the gene expression levels of *Ifi202b* are closely associated with the obesity phenotype mediated by *Nob3* obesity QTL identified from F2 population of NZO and B6 strains; mRNA of *Ifi202b* was generated by the NZO allele but not by the B6 allele (Vogel et

al., 2012). The B6 allele of *Ifi202b* contains a deletion that comprised exon 1 and the 5′-flanking region of *Ifi202b*, causing the lack of mRNA (Vogel et al., 2017; Vogel et al., 2012).

We performed PCR with primers corresponding to exon 1 and exon 2 of *Ifif202b* on the cDNA from adipose tissue in B6, congenic, and TH mice. B6.TH-Chr1-128Mb congenic and parental TH mice produced a PCR product, while B6 and B6.TH-Chr1-92Mb congenic mice did not as expected (Fig. 7A). We sequenced the PCR product of TH mice and found that they are the same as NZO except for one SNP in exon 1 (Fig. 7B). We then measured gene expression levels of *Ifi202b* in adipose tissue of B6 and congenic mice. Once the data were normalized to B6 mice having an expression value of one, B6.TH-Chr1-128Mb congenic mice had a relative expression value over 4 folds that of B6 mice, while B6.TH-Chr1-92Mb congenic mice had a comparable value to that of B6 mice on chow (Fig. 7C). The gene expression of *Ifi202b* was significantly up-regulated in adipose tissue of B6.TH-Chr1-128Mb congenic mice when fed HF diets compared to chow (Fig. 7D).

The *Apoa2* gene is located near the *Tachol1*. An *Apoa2*^{b} allele, characterized by Ala-to-Val substitution at amino acid residue 61, has been proposed to be hypermorphic in increasing plasma cholesterol levels in mice (J. Suto, Takahashi, & Sekikawa, 2004; J. I. Suto & Kojima, 2017). The TH mouse carries the *Apoa2*^{b} allele, table 4 (Stewart et al., 2010). We examined the *Apoa2* gene expression in liver from B6 and B6.TH-Chr1-128Mb congenic mice. While the *Apoa2* gene expression levels were comparable between B6 and B6.TH-Chr1-128Mb congenic mice on chow, they were about 2-fold higher in B6.TH-Chr1-128Mb congenic than B6 mice on HF diets (Fig. 8).

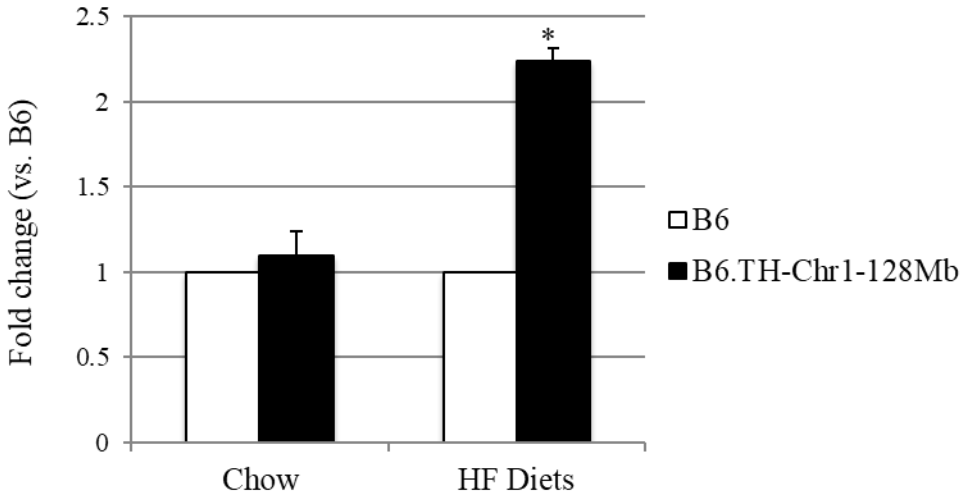

Figure 8. The mRNA levels of Apoa2 in liver from B6 and B6.TH-Chr1-128Mb congenic mice on chow and HF diets (males, 15-24 weeks of age). Changes in gene expression were expressed as fold change relative to mean values for B6 mice. Data were reported as means ± SEM (N=5-7 each group). **P*<0.05.

Bioinformatic analysis of the distal region of Chr 1

We previously conducted a whole genome sequencing analysis for the TH mouse and generated a complete catalog of variants (relative to the B6 strain) (Denvir et al., 2016). We used this catalog to search potential candidates in the distal region of Chr 1. There were 167,833 SNPs and 36,284 indels in the region flanked by *D1Mit498* and *D1Mit361*; of these, 934 SNPs and 89 indels had the potential to directly alter the protein product. These variants occurred in 252 unique genes, of which 223 had molecular function or biological process terms associated with them in the GO database. This list of genes included *Apoa2* and *Ifi202b*. There were three missense variants in *Apoa2* and 11 missense variants in *Ifi202b* (Table 4).

Nucleotide change (B6-TH)	Amino acid change (B6-TH)	Amino acid position
Apoa2		
T-A	D-E	43
A-G	M-V	49
C-T	A-V	61
Ifi202b		
A-C	S-A	432
T-A	T-S	379
A-G	L-P	350
A-T	F-I	292
T-C	K-E	204
G-A	T-I	187
T-A	I-F	142
A-C	I-M	141
T-A	I-F	127
T-C	E-G	109

Table 4. Missense variants occurring in *Apoa2* and *Ifi202b*.

We further filtered the list of 223 genes to include only those whose associated GO terms included "cholesterol," "fat," "lipase," "lipoprotein," "metabolic process," or "glucose." The filter resulted in a list of 36 genes. These genes, the type of variant(s) that occur in them, and their relevant associated GO terms are shown in Appendix C.

Discussion

In this study, we generated congenic mice for previously mapped Chr 1 QTLs linked to adiposity (*Tabw3*) and hypercholesterolemia (*Tachol1*) derived from a TH fragment on a B6 background. On the B6 background, the TH derived Chr1 128Mb segment, containing the *Tabw3* and *Tachol1* QTLs, produced obesity and hypercholesterolemia in B6.TH-Chr1-128Mb congenic mice. However, contrary to our expectations, B6.TH-Chr1-92Mb congenic mice capturing the proximal segment corresponding with the *Tabw3* QTL failed to develop obesity. The lack of obesity may suggest that the effect of *Tabw3* alone may not be large enough or it

may require interacting with other loci in the distal segment, potentially *Tachol1* or other obesity QTLs undetected from our original genome-wide study (Stewart et al., 2010). In this context, the obesity in B6.TH-Chr1-128Mb congenic mice may reflect this complexity. Generating congenic mice containing only the distal segment of Chr 1 may allow us to test this complexity.

Daily food intake of B6.TH-Chr1-128Mb congenic mice was comparable with that of B6 mice on both chow and HF diets, suggesting greater energy storage efficiency in the congenic mice than B6. Energy expenditure determined by heat production normalized with body weight (kcal/kg/hr) was lower in B6.TH-Chr1-128Mb congenic mice than B6 mice. These findings are consistent with the characterization of parental TH strain (Parkman et al., 2016).

Based on the published knowledge, we tested *Ifi202b* and *Apoa2* genes as potential candidate genes within the distal region of Chr 1. It has been reported that the gene expression levels of *Ifi202b* are positively associated with the development of obesity in mice (Vogel et al., 2017; Vogel et al., 2012). The *Ifi202b* (also called *p202*) is known to play a role in cell proliferation and differentiation including adipocyte differentiation (Li, Liu, Guo, Zhu, & Jiao, 2014). The mRNA levels of *Ifi202b* were significantly higher in adipose tissue from B6.TH-Chr1-128Mb than B6 mice and they were significantly upregulated in response to HF diets, suggesting a potential gene-diet interaction.

Several studies have identified highly significant cholesterol QTLs in the *Tachol1* interval and proposed the *Apoa2* gene as a likely candidate (Machleder et al., 1997; J. Suto et al., 2004; J. I. Suto & Kojima, 2017). APOA2, synthesized in liver, has an antagonist effect on cellular cholesterol efflux (Bandarian, Daneshpour, Hedayati, Naseri, & Azizi, 2016). Transgenic mice overexpressing *Apoa2* develop hypercholesterolemia (Warden, Hedrick, Qiao, Castellani, & Lusis, 1993) while knockout of the gene in mice reduces plasma cholesterol levels

(Weng & Breslow, 1996). Further, transgenic mice overexpressing *Apoa2* have larger fat mass than controls (Castellani, Goto, & Lusis, 2001). The TH mouse carries the APOA2-Val61 allele that is putatively hypermorphic in increasing cholesterol levels. This polymorphism, however, does not seem to directly alter the mRNA levels of *Apoa2* as they were comparable between B6 and B6.TH-Chr1-128Mb congenic mice on chow. It was interesting to observe that the mRNA levels of *Apoa2* were significantly higher in B6.TH-Chr1-128Mb congenic than B6 mice on HF diets.

Through bioinformatic approaches using a catalog of sequence variants between B6 and TH mice we generated a list of potential candidate genes (Table 5). Among these, genes that cause obesity in knockout mice include the ATPase, Na^+/K^+ transporting, alpha 2 polypeptide gene (Kawakami et al. 2005) and poly (ADP-ribose) polymerase family, member 1 gene (Devalaraja-Narashimha & Padanilam, 2010). On the other hand, transgenic mice overexpressing the upstream transcription factor 1 gene exhibit lower adiposity and lower plasma cholesterol levels than wild type mice (S. Wu et al., 2010).

In addition to the genes for which obesity or cholesterol phenotype was found in knockout or transgenic mice, other genes are also noticeable in the list based on known function. The hydroxysteroid (17-beta) dehydrogenase 7 (*Hsd17b7*) gene encodes an enzyme required for the cholesterol biosynthesis in liver and is known to be downregulated in a hypocholesterolemic rodent model (Nemoto et al., 2013). The phospholipase A2, group IVA (cytosolic, calcium-dependent) (*Pla2g4a*) gene encodes a calcium activated enzyme that catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid, mediating eicosanoid-driven inflammation; polymorphism in *Pla2g4a* was associated with obesity resistant phenotype in a primate model (Harris et al., 2016). The C-reactive protein (*Crp*) gene and serum amyloid P-component (*Apcs,*

also called *Sap*) gene are in close proximity on Chr 1. CRP and SAP are a superfamily of proteins that are characterized by pentraxin domain at the carboxyl-terminus and play key roles in innate immunity and inflammation (Garlanda, Bottazzi, Bastone, & Mantovani, 2005). Human circulating CRP (Yudkin, Stehouwer, Emeis, & Coppack, 1999) and SAP (Jenny, Arnold, Kuller, Tracy, & Psaty, 2007) concentrations were positively associated with BMI. Bochud et al. (2009) also suggested CRP as a causal factor for the obesity in human.

In conclusion, by analysis using congenic strains, we confirmed the effect of the 128Mb TH segment from Chr 1 in increasing body fat and plasma cholesterol values and demonstrated that the distal segment of Chr 1 from TH is necessary to cause both phenotypes. Our studies provided logical information regarding potential candidate genes for the Chr 1 QTL. The congenic strains will be a valuable resource for the identification of genes underlying these complex traits and their interactions with diets.

CHAPTER 4

IL-6 LEVELS AND MITOCHONDRIAL RESPIRATION IN TALLYHO AND B6.TH-CHR1-128MB CONGENIC MICE

This chapter contains unpublished data

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Abstract

Obesity is a global epidemic and one of the leading causes of preventable death. The development of obesity is most likely due to interactions of multiple genes with obesogenic environments such as a high fat diet. Obesity is defined as excess adiposity that causes chronic secretion of pro-inflammatory cytokines, contributing to systemic low-grade inflammation. At the cellular level, expanded adipose tissue may cause decreased angiogenesis and hypoxia, increasing reactive oxygen species formation and dysfunctional mitochondria. Mitochondrial dysfunction and inflammation are known contributors of adipose dysfunction in obesity. The TALLYHO/Jng (TH) mouse is a polygenic model for obesity and type 2 diabetes. Previously, we demonstrated that diets are important modulators of genetic susceptibility to obesity and type 2 diabetes in TH mice and congenic mice carrying obesity QTL on chromosome 1 derived from TH mice (B6.TH-Chr1-128 Mb). In this study, we measured pro-inflammatory cytokine IL-6 levels and mitochondrial respiration in TH and congenic mice to elucidate mechanisms mediating the gene-diet interaction in the development of obesity and type 2 diabetes. TH mice had significantly higher IL-6 levels in plasma than B6 mice on chow, which was exaggerated on high-sucrose high-fat diets (HSHF). The congenic mouse also had significantly higher levels of IL-6 in the plasma than B6 mice. We then examined mitochondrial respiration of white adipose tissue of TH, B6, and the congenic mice on chow by using the XFp Seahorse analyzer, which measured the oxygen consumption rate (OCR) of cells. Stromal vascular cells (SVC) from TH mice had significantly lower spare and maximal respirations compared to B6. SVC from B6.TH-Chr1-128Mb congenic mice also had significantly lower spare respiration. In summary, this study showed that the obesity in TH and congenic mice is associated with increased systemic inflammation and decreased mitochondrial respiration.

Introduction

Obesity is a worldwide epidemic, associated with an increased risk of type 2 diabetes (T2D), hypertension, hyperlipidemia, cardiovascular disease, and various cancers (Gallagher & LeRoith, 2015; Rhee, 2018). In the United States, nearly 60 % of the population is considered overweight, BMI is ≥25kg/m2 or obese, BMI is ≥30kg/m2 (Berrios, 2016) . Obesity is a complex disease that is attributed to interactions between obesity promoting environmental factors, such as a high fat (HF) diet, and genetic factors (Yazdi et al., 2015).

Obesity is characterized by excess adipose tissue accumulation. This expansion is due to increased adipocyte size (hypertrophy) and/or number (hyperplasia) through adipogenesis (Shao et al., 2018). Adipose tissue, a storage site for excess energy in the form of triglycerides, is an endocrine organ involved in energy homeostasis. Adipose tissue releases multiple adipocytokines including leptin, adiponectin, interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-alpha) (Castro, Macedo-de la Concha, & Pantoja-Meléndez, 2017). Increased adipose tissue mass results in increasing cytokine release (Monteiro & Azevedo, 2010). Increased adipose mass also leads to hypoxia, followed by the apoptosis of adipocytes and the formation of reactive oxygen species (ROS) in the tissue (Trayhurn & Alomar, 2015). The structural stress due to hypertrophy, increased ROS, and increased adipocyte apoptosis leads to increased macrophage infiltration of adipose tissue and an increased number of cytokines, resulting in a pro-inflammatory environment (Alcala et al., 2017). Ultimately, chronic low-grade systemic inflammation develops (Majdoubi, Kishta, & Thibodeau, 2016).

There is emerging evidence linking mitochondrial dysfunction to adipose dysfunction and inflammation in obesity and T2D. Mitochondria in white adipose tissue regulate several metabolic processes including lipogenesis, lipolysis, and fatty acid (FA) re-esterification.

Mitochondria are also suggested to be important in adipogenesis as studies have shown that adipogenesis and mitochondrial biogenesis initiation are synchronized and many of the same regulatory proteins are used for both processes (Boudina & Graham, 2014). When there is excess adipose mass and inflammation, there is decreased mitochondrial content and oxidative capacity. Mitochondrial dysfunction also causes increased ROS formation in the electron transport chain and impaired substrate oxidation, both of which contribute to adipose dysfunction (Vernochet et al., 2014).

The TALLYHO/Jng (TH) mouse is a polygenic model for human obesity and T2D. It is characterized by increased adiposity, insulin resistance, hyperlipidemia, and hyperglycemia (Kim & Saxton, 2012a). In addition, the penetrance of genetic susceptibility to obesity and T2D is modulated by dietary fat in TH mice (Parkman et al., 2016). In this study, we determined whether the gene-diet interaction in the development of obesity and T2D in TH mice could be attributed to inflammation and mitochondrial dysfunction. We also included congenic mice carrying obesity QTL on chromosome 1 derived from TH mice on C57BL/6J (B6) background (B6.TH-Chr1-128Mb) (Parkman et al., 2017; Stewart et al., 2010).

Materials and Methods

Animals and diets

All mice including TH, B6.TH-Chr1-128Mb congenic, and B6 mice, were bred in our animal facility. All mice had access to food and water ad libitum in a temperature and humidity-controlled room with a 12-h light/dark cycle. At 4 weeks of age, mice were weaned onto standard rodent chow (14% kcal from fat, Purina 5001, PMI Nutrition, Brentwood, MO), semi-purified high-sucrose low-fat (HSLF) diet (D12489B) (except the congenic mice), or semi-purified high-sucrose high-fat (HSHF) diet (D12266B) (Research Diets, New Brunswick, NJ,

USA). The diets were maintained throughout the study. Mice were euthanized by CO_2 asphyxiation. Blood was collected by cardiac puncture and underwent centrifugation (1,200*g*) at 4ºC to collect plasma, which was stored at −20°C. Tissues were collected, frozen in liquid nitrogen, and stored at −80°C. All animal studies were carried out with the approval of Marshall University Animal Care and Use Committee.

RNA isolation and real-time quantitative PCR (qRT-PCR)

Total RNA was isolated from epididymal adipose tissue using RNeasy Plus Universal Midi Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Total RNA (2μg) was reverse-transcribed with SUPERSCRIPT RT using oligo dT as primer to synthesize first-strand cDNA according to the manufacturer's instructions (Invitrogen, Waltham, MA, USA). The real-time PCR reaction was carried out with 1x SYBR Green PCR core reagents (Qiagen) using the StepOne™ Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA for interleukin 6 (*Il-6)* (forward: 5'- GAGAAAAGAGTTGTGCAATGGC-3' and reverse: 5'- CCAGTTTGGTAGCATCCATCAT-3') and *36B4* (forward: 5'-GAGGAATCAGATGAGGATATGGGA -3' and reverse: 5'-AAGCAGGCTGACTTGGTTGC -3'). Duplicate amplifications were performed, and the average measurements used for data analysis.

Western blot analysis

Protein extracts were prepared from the epididymal adipose tissue of B6, B6.TH-Chr1-128Mb, and TH mice as well as liver tissue from B6 and B6.TH-Chr1-128Mb mice with radioimmunoprecipitation assay (RIPA) lysis buffer (Millipore, Temecula, CA, USA). Total protein (50μg) from each sample was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to hydrophilic polyvinylidene difluoride

(PVDF) membranes (Millipore Sigma, Burlington, MA, USA) using a mini trans-blot apparatus (Bio-Rad, Hercules, CA, USA). After blocking with 5% dry milk in Tris Buffered Saline with Tween 20® (TBST), the membranes were probed with anti-IL-6 (sc-1265, Santa Cruz Biotechnology, 1:200), followed by incubation with horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG (sc-2020, Santa Cruz Biotechnology, 1:5000). Membranes were also probed with HRP-conjugated anti-GAPDH (sc-25778, Santa Cruz Biotechnology, 1:1000). Bound antibody was detected by enhanced chemiluminescence (Mruk & Cheng, 2011) and then imaged using the Chemidoc^{TM} Imaging System. The bands were quantified by Image Lab software (Bio-Rad, USA).

Plasma IL-6 levels

Plasma IL-6 levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Mouse IL-6 DuoSet ELISA R&D Systems, MN, USA) according to the manufacturer's instructions.

Mitochondrial respiration assay

Stromal vascular fraction (SVF) cells were isolated from the epididymal adipose tissue of B6, TH, and B6TH-Chr1-128Mb mice using procedures described in Lee et al (M. J. Lee & Fried, 2014). Briefly, adipose tissue was minced and washed with PBS. The minced tissue was digested at 37 °C for 2 hours in *Collagenase* I solution (*Gibco*® *Collagenase* I, Invitrogen, Grand Island, NY, USA, 1mg/ml) in Corning™ cellgro™ Hanks Balanced Salt Solution (Media Tech Inc, Manassas, VA, USA). Digested tissue was strained and centrifuged at 500g for 10 minutes. The supernatant was aspirated and the pellet was resuspended in culture media (Dulbecco's modified Eagle's medium with L-glutamine, 4.5 g/L glucose and sodium pyruvate (Mediatech Inc, Manassas, VA) with the addition of 10 % (v/v) fetal bovine serum (Sigma, St.

Louis, MO) and 100 units/ml penicillin and 100 mg/ml streptomycin (Mediatech Inc, Manassas, VA)). Cells were centrifuged a second time and the aspiration and resuspension were repeated. XFp cell culture plate wells (Seahorse Bioscience, Agilent, Billerica, MA, USA) were coated with 0.1% gelatin (Eastman Kodak Company, Rochester, NY, USA). 50,000 cells were seeded in the XFp cell culture plates in 200 µL culture media and placed in a 37°C incubator with 5% CO_2 for 24 hours. After incubation, culture media was removed and the cells were washed twice with assay medium (XF base medium,10 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate; pH 7.4, Seahorse Bioscience). Assay medium (180ul) was added to each well and the plate was incubated in a non- CO_2 incubator for 1 hour. The prepped seahorse cartridge was loaded to a final concentration of 1 µM oligomycin, 1 µM carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP), and 1 µM rotenone & antimycin A in the A, B, and C injector ports respectively (Cell Mito stress kit, Seahorse Bioscience). The cell mito stress test assay was run on the XFp analyzer (Seahorse Bioscience). Total protein concentration was analyzed by BCA assay. Data was normalized by total protein concentration. Individual parameters for basal respiration, proton leak, ATP production, maximal respiration, and spare respiratory capacity were calculated from % oxygen consumption rate (OCR). % of OCR was calculated using basal measurements as 100%. Individual parameters were then calculated (Tan et al., 2015).

Statistical analysis

Data analysis was conducted by ANOVA with GraphPad Prism (version 7.03). Student's unpaired t-tests were used when only two groups were compared. All data were presented as means ± SEM. Significance was considered at $P<0.05$. (Livak & Schmittgen, 2001).

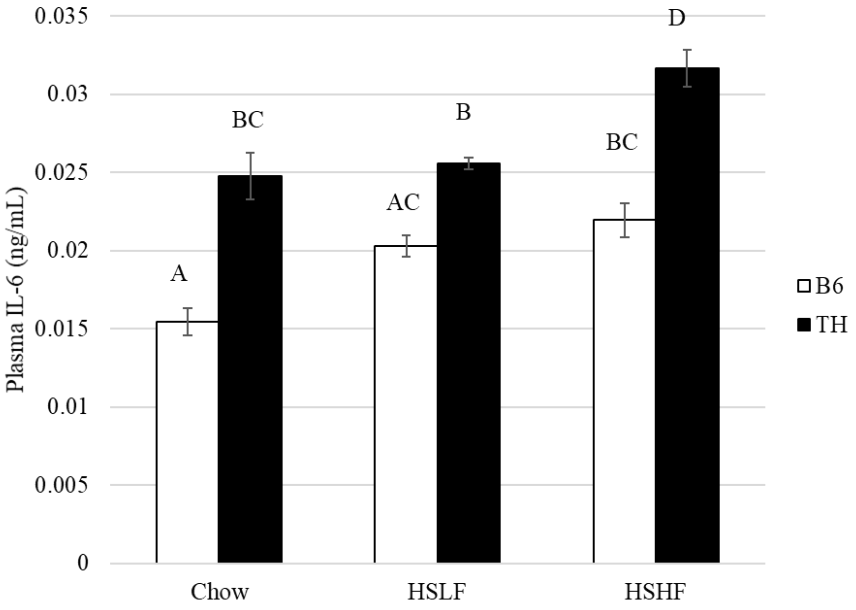
Results

IL-6 levels in TH and Congenic mice

Plasma IL-6 levels were significantly higher in TH mice than B6 mice on chow and HSHF (Figure 9A). In addition to strain effect, there was diet effect; B6 mice on HSHF had significantly higher levels of IL-6 compared to B6 mice on chow and the same was true for TH mice. In epididymal adipose tissue, there was a trend of increased IL-6 protein expression levels in TH mice on all diets, compared to B6 mice, however it did not reach statistical significance (Figure 9B).

Plasma IL-6 levels were also significantly higher in B6.TH-Chr1-128Mb congenic mice compared to B6 mice on chow (Figure 10A).

A



B

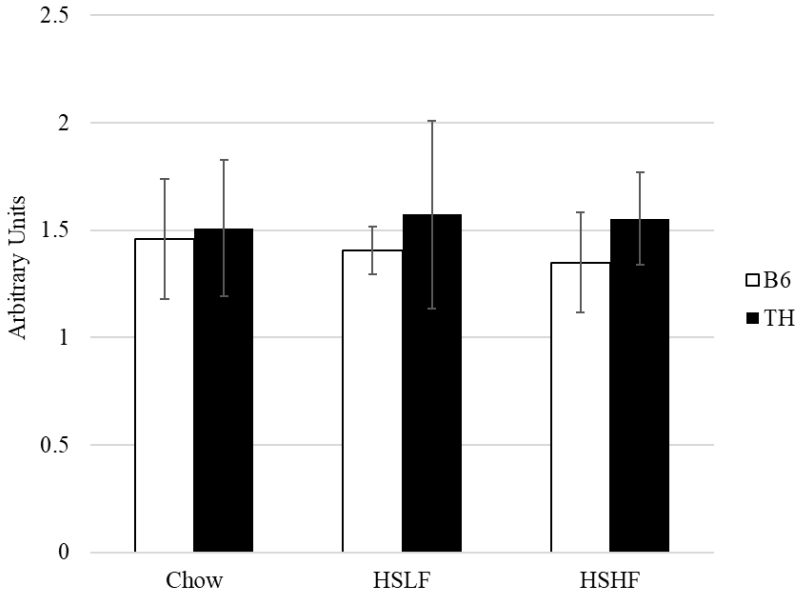


Figure 9. IL-6 expression (A) Plasma IL-6 levels (17 to 27 weeks n= 5,7 respectively) measured by ELISA and (B) Western blot analysis for IL-6 in epididymal adipose tissue (20-25 weeks of age, *n*=4 for each group) in male B6 and TH mice fed chow, HSLF, and HSHF diets. Mean IL-6 content of adipose tissue is displayed as arbitrary units after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content levels in the same blot. Data were reported as means ± SEM. Group means labeled with different letters are significantly different ($P<0.05$).

In epididymal adipose tissue, there was a trend of increased *Il-6* gene expression levels in B6.TH-Chr1-128Mb congenic mice compared to B6 mice, however, it did not reach statistical significance (Figure 10B). The same trend was seen in the IL-6 protein expression levels in epididymal adipose tissue (Figure 10C), as well as in liver (Figure 10D).

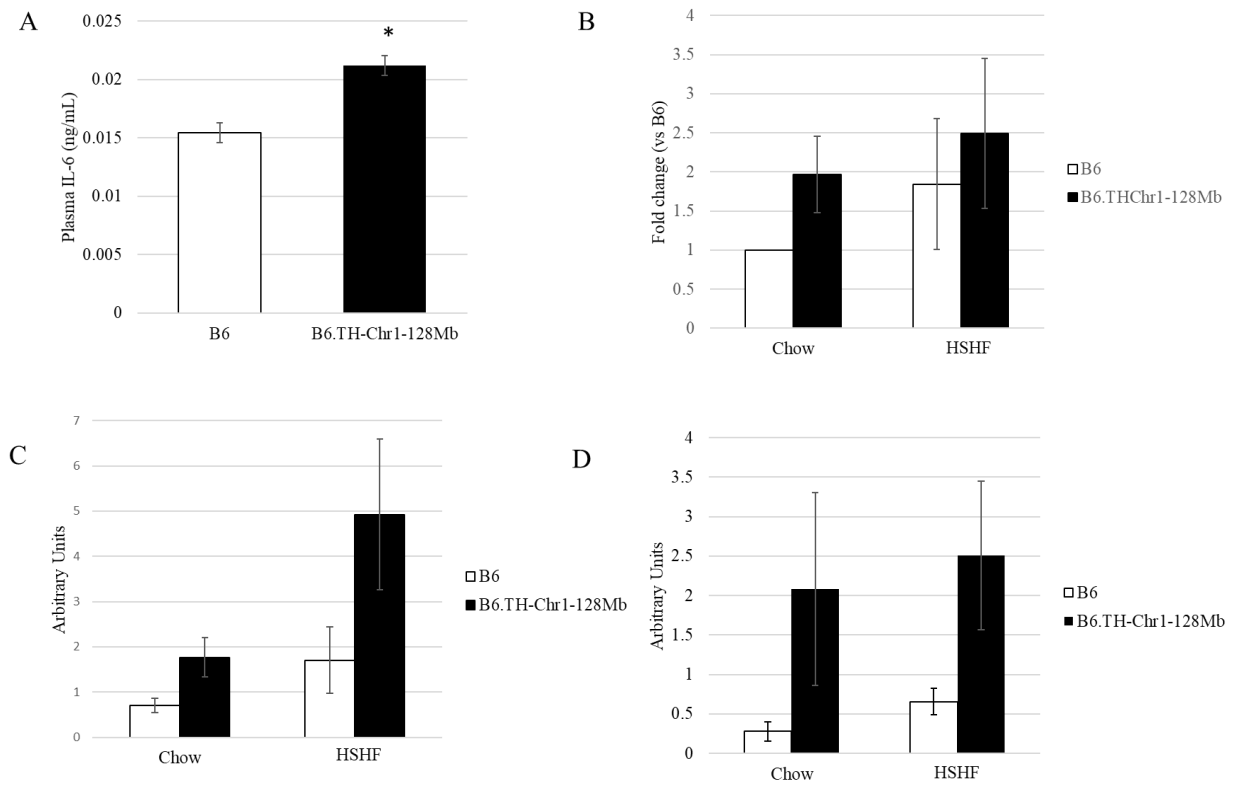


Figure 10. IL-6 expression (A) Plasma IL-6 levels in male B6 and B6.TH-Chr1-128Mb congenic mice fed chow (16 to 19wks n= 5,6 respectively). (B) The mRNA levels of Il-6 in epididymal adipose tissue (18-24 weeks of age, n=5 to 6 mice) where changes in gene expression were expressed as fold change relative to mean value of B6 mice on chow, (C) western blot analysis for IL-6 in epididymal adipose tissue (20-24 weeks of age, *n*=4 for each group), and (D) western blot analysis for IL-6 in liver tissue (20-24 weeks of age, *n*=4 for each group), in male B6 and B6.TH-Chr1-128Mb congenic mice fed chow and HSHF. Mean IL-6 content of adipose tissue is displayed as arbitrary units after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content levels in the same blot. Data were reported as means ± SEM. **P*<0.05

Mitochondrial respiration in adipose tissue from the TH and congenic mice

To measure the mitochondrial function in adipose tissue we used the Seahorse Extracellular flux analyzer (XFp). We isolated the SVF from epididymal adipose tissue of TH and B6 mice and plated the cells in a gelatin coated culture plate. The first injection was oligomycin (1 μM), an inhibitor of ATP synthase, followed by FCCP (1 μM), a mobile ion carrier that disrupts the membrane potential, and finally rotenone/ antimycin A (1 μM), complex I and III inhibitors respectively (Figure 11A). Individual parameters were then calculated from % OCR (Figure 11B). However, TH mice had significantly lower maximal and spare respirations compared to B6 mice.

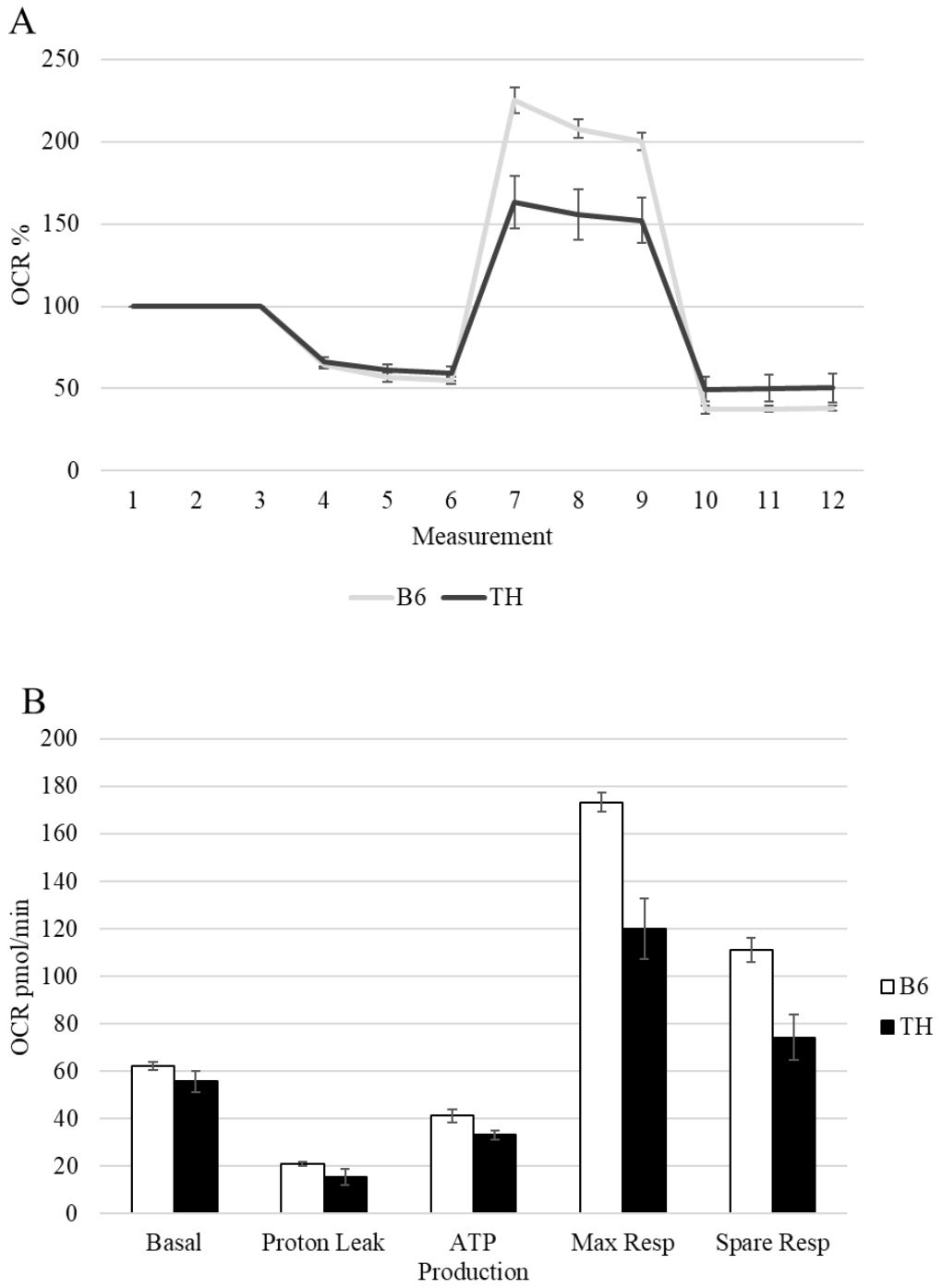


Figure 11. Measurements of mitochondrial respiration (A) % oxygen consumption rate (OCR) by extracellular flux analysis and (B) individual parameters for basal respiration, proton leak, ATP production, maximal respiration, and spare respiratory calculated from %OCR. Adipose tissue-derived SVF from B6 and TH mice on chow were seeded to 50,000 cells/well. OCR was measured under basal conditions. Oligomycin (1 μM), FCCP (1 μM), rotenone/ antimycin A (1 μM) were sequentially added. Data are expressed as means ± SEM, n =6,5 respectively (males, 19 to 23wks old). *P<0.05

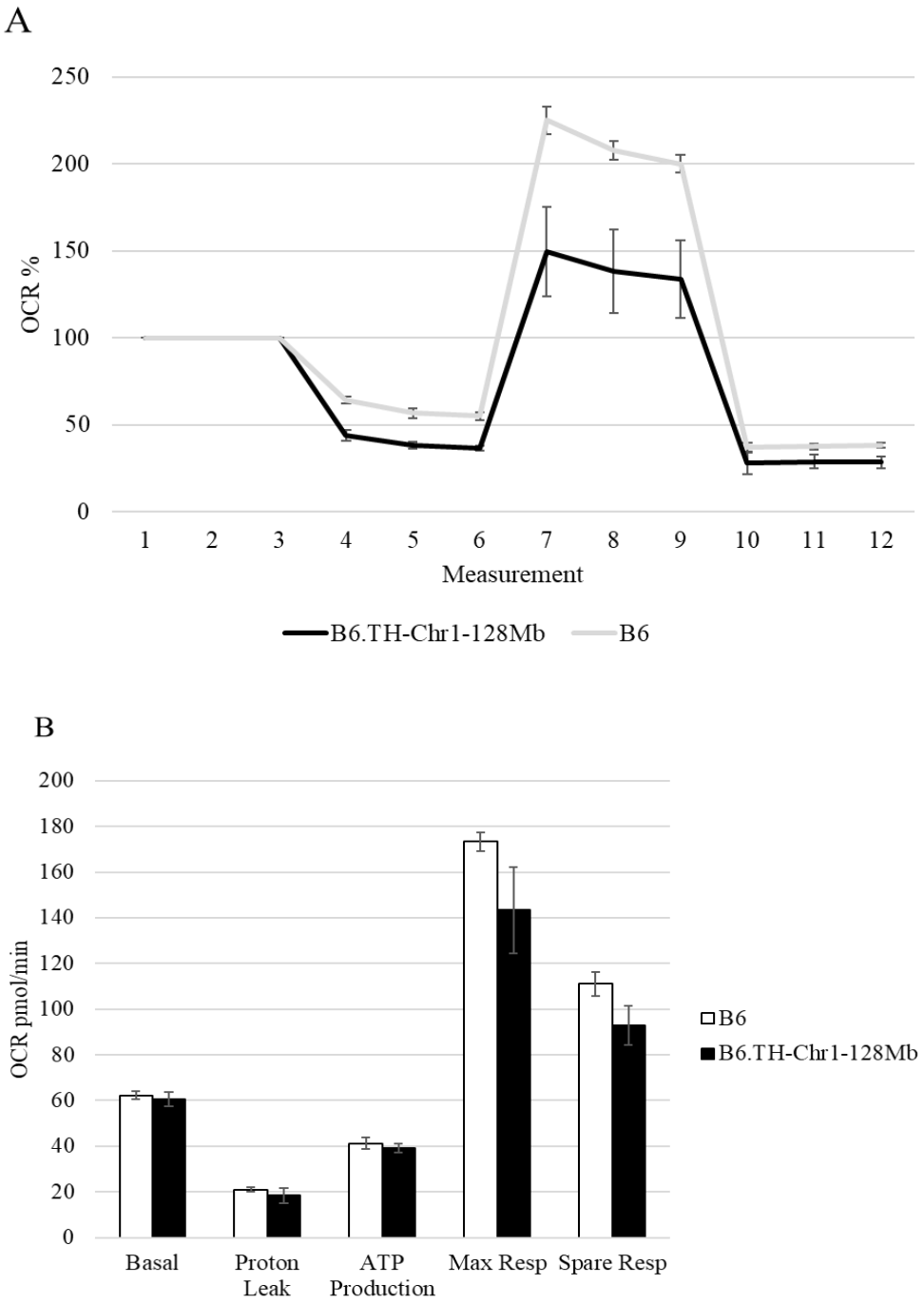


Figure 12. Measurements of mitochondrial respiration (A) % oxygen consumption rate (OCR) by extracellular flux analysis and (B) individual parameters for basal respiration, proton leak, ATP production, maximal respiration, and spare respiration that were calculated from % OCR. Adipose tissue-derived SVF from B6 and B6.TH-Chr1-128Mb congenic mice on chow were seeded to 50,000 cells/well. OCR was measured under basal conditions and oligomycin (1 μM), FCCP (1 μM), rotenone/ antimycin A (1 μM) were sequentially added. Data are expressed as means ± SEM, n = 6, 4 respectively, (males, 16 to 23wks old). *P<0.05

We saw a similar result when we compared SVF cells from B6 and B6.TH-Chr1-128Mb congenic mice (Figure 12B). B6.TH-Chr1-128Mb congenic mice had similar basal respiration, proton leak, and ATP production with B6 mice, but had a trend of lower maximal respiration and had significantly lower spare respiratory compared to B6 mice.

Discussion

This study examined the inflammatory marker IL-6 as well as the oxygen consumption rate of adipose tissue in TH and B6.TH-Chr1-128Mb congenic mice compared to B6.

Plasma IL-6 levels were significantly increased in the TH mouse model. IL-6 levels were further exacerbated when the mice were on HF diets. The finding of increased IL-6 expression on HF diets is in agreement with current studies of obesity (Ghosh & Ashcraft, 2013; Minihane et al., 2015; Shi et al., 2014). Under healthy circumstances, inflammation is seen as a protective mechanism. In healthy adipose tissue, inflammation is necessary for extracellular matrix (ECM)-remodeling and angiogenesis to expand adipose tissue (Wernstedt Asterholm et al., 2014). The inflammation is acute and involves mainly M2 macrophage infiltration. However, in obesity the inflammation becomes chronic with M1 macrophage infiltration (Braune et al., 2017). IL-6 has been shown to be increased in both obese mice and humans in adipose tissue and plasma. IL-6 increases lipolysis and fat oxidation, and increased concentrations of IL-6 have been linked to insulin resistance, T2D, and cardiovascular disease (Castro et al., 2017; Greenberg & Obin, 2006). In tissues, IL-6 regulates T-cells by promoting differentiation, activation, and tissue infiltration. Additionally, IL-6 stimulates M1 macrophage polarization. Both actions contribute to the pro-inflammatory environment of adipose tissue (Braune et al., 2017; Xu et al., 2017). IL-6, along with TNF-α, and IL-1β, promotes insulin resistance in peripheral tissues as well. These cytokines act in a paracrine and autocrine manner to activate the c-Jun N-terminal kinase (JNK)

and nuclear transcription factor kappa B (NF-kB) pathways which phosphorylate the insulin receptor substrate-1 (IRS-1), reducing expression and decreasing insulin signaling (Castro et al., 2017; Eder, Baffy, Falus, & Fulop, 2009). For example, TNF-α stimulates increased adipocyte lipolysis, increasing fatty acids in the blood stream. The fatty acids interact with muscle and liver and activate the NF-kB pathway, promoting insulin resistance (de Luca & Olefsky, 2008; Greenberg & Obin, 2006). Overall, increased cytokine release by adipose and macrophages, including IL-6, contribute to a pro-inflammatory microenvironment which contributes to adipose dysfunction and obesity.

Adipose mitochondria are known to play a role in multiple metabolic processes. Changes in mitochondrial oxidative capacity can lead to changes in metabolic homeostasis, which can contribute to the development of various diseases (Boudina & Graham, 2014; Brand & Nicholls, 2011). While mitochondrial basal respiration in non-stressful circumstance was unchanged, maximum and spare respirations were significantly reduced in TH mice compared to B6 mice. Both decreases in respiration indicate possible mitochondrial dysfunction (Brand & Nicholls, 2011). Decreased expression could also be indicative of a hypoxic white adipose tissue as oxidative phosphorylation (OXPHOS) is aerobic and an anaerobic environment would cause a shift to glycolysis (Schottl, Kappler, Fromme, & Klingenspor, 2015). Mitochondrial dysfunction is a hallmark of obesity and may be a response to energy overload (Schottl, Kappler, Fromme, et al., 2015). Continuous excess nutrition causes cellular stress from hypertrophy in the form of inflammation and increased ROS, as well as a metabolic switch to lipid storage and increased ATP production (De Pauw, Tejerina, Raes, Keijer, & Arnould, 2009). Adaptive changes are made to alter the mitochondrial network. Eventually, in both humans and mice, it has been shown that obese individuals have decreased mitochondrial number, decreased function,

decreased biogenesis, and decreased mitochondrial DNA including genes involved in OXPHOS. There is also an increase in ROS in the electron transport chain which can further increase lipid accumulation and hypertrophic adipocytes and decrease oxygen consumption, resulting in increased inflammation, adipose dysfunction, and mitochondrial dysfunction (Kusminski & Scherer, 2012).

For this study, all the mice tested with Seahorse XFp analyzer were on a chow diet. Although there was no change to ATP production between the groups, a future study involving mice on the HSHF diet may exhibit decreased ATP production as high fat increases adiposity and inflammation, exacerbating mitochondrial dysfunction. In the future, it would also be beneficial to study the protein expression levels of different complexes of the electron transport chain for TH on different diets.

In conclusion, the obesity and T2D in TH mice are associated with decreased oxygen consumption and increased inflammation, which may in part contribute to the gene-diet interaction for the disease susceptibility in this model.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

Obesity, defined as the accumulation of excess adipose tissue, is considered one of the leading causes of preventable death, as it is associated with an increased risk of type 2 diabetes, hypertension, hyperlipidemia, cardiovascular disease, and certain cancers (Kanasaki & Koya, 2011). Obesity is a complex, multifactorial disease that is attributed to a number of variables, including genetics and environment (Wright & Aronne, 2012). The genes that contribute to obesity, their mechanisms, and their interactions with the environment, however, remain largely unknown (Chu, Malinowska, Jura, & Kozak, 2017). In obesity research, animal models provide a platform to control environmental variables, such as diet, and the ability to use genetic manipulation more easily (Kanasaki & Koya, 2011). This dissertation first focused on studying gene-diet interactions in the development of obesity and type 2 diabetes in the TALLYHO/Jng (TH) mouse. Next, congenic mouse strains were developed in an effort to identify obesity gene(s) in TH mice. Finally, we looked into inflammation and mitochondrial respiration in TH and congenic mice.

Diet dependence of obesity and type 2 diabetes in TH mice

The TH mouse is a polygenic model for human obesity and type 2 diabetes (Kim & Saxton, 2012b). The genetic basis of obesity and type 2 diabetes in TH mice has been well studied previously by our research group. In this study, we examined diet dependence of obesity and type 2 diabetes in TH mice compared to C57BL/6J (B6) mice. The data demonstrate high sensitivity of TH mice to dietary high fat in advancing obesity and type 2 diabetes, which provides evidence that diets are important modifiers of genetic susceptibility to obesity and type

2 diabetes in this model. The interplay between heredity and dietary environment in TH mice appears to amplify insulin resistance, contributing to severe glucose intolerance and diabetes.

The effect of various macronutrients on body weight regulation has been studied and debated for years. If the effects of protein, fat, and carbohydrates on the body can be elucidated, dietary intervention for obesity and type 2 diabetes will be more effective. The difficulty of studying the effects of macronutrients lies with the various other variables/contributors such as genetics, epigenetics, or the gut microbiome that are involved. The multiple variables can lead to variability in the effects of diet on individuals, resulting in various phenotypes from increased adiposity to decreased adiposity (Zeevi et al., 2015). Hu et al. (2018) reported a study where B6 mice were given 29 different diets of various percentages of carbohydrates, protein, and fat. They found that only dietary fat content was responsible for increased adiposity and energy intake. They repeated the experiment with 4 other mouse strains, DBA/2, BALB/c, FVB, and C3H with the same results (S. Hu et al., 2018).

Many diet-induced obesity model studies use extreme macronutrient diets such as 60% calories from fat. While there is plenty to learn from extreme phenotypes due to extreme diets, the development of a majority of human obesity and type 2 diabetes cases progresses much more gradually on a western style diet (Della Vedova et al., 2016; Mitchell, Catenacci, Wyatt, & Hill, 2011). The high fat diet used in our study is a representation of the standard western diet, with high fat (31.8 kcal%) and high carbohydrate (51.4 kcal%) (Table 1). The TH mouse is a clinically relevant model for obesity and type 2 diabetes where genetic factors interplay with dietary factors, serving as a useful tool in developing and testing therapeutic targets.

Obesity gene(s) in TH mice

Our previous mapping studies using F2 mice from TH and B6 mice identified quantitative trait loci (QTL) linked to obesity and hypercholesterolemia on chromosome 1 (Kim & Saxton, 2012b; Stewart et al., 2010). To identify obesity gene(s) in the QTL, we generated a congenic mouse strain carrying the QTL derived from TH mice on B6 background; B6.TH-Chr1-128Mb. A nested second congenic line (proximally overlapping), B6.TH-Chr1-92Mb, was also generated from the first line (Figure 5). With a high fat diet (HFD) feeding, B6.TH-Chr1-128Mb congenic mice exhibited greater adiposity than B6.TH-Chr1-92Mb or B6 mice (Figure 6). B6.TH-Chr1-128Mb congenic mice also had increased plasma insulin levels, slightly impaired glucose tolerance, and increased total plasma cholesterol on HFD compared to B6.TH-Chr1-92Mb or B6 mice (Figure 6). B6.TH-Chr1-128Mb congenic mice had lower energy expenditure, than B6.TH-Chr1-92Mb or B6 mice on chow or HFD (Table 3). The findings suggest that B6.TH-Chr1-128Mb congenic mice may have increased energy efficiency and decreased energy expenditure compared to B6.TH-Chr1-92Mb and B6 mice, resulting in the increased fat mass observed. From this study we concluded that the distal segment of chromosome 1 from TH mice is necessary to cause obesity. For fine mapping, we have begun to construct nested recombinant subcongenic lines carrying various regions of the distal segment of the QTL and established 3 lines (B6.TH-Chr1(7)-TTT, -TT, and -T) (Fig. 13) (unpublished data). Each nested subcongenic line is genetically distinct from the others for the length and the boundaries of the TH introgressed region.

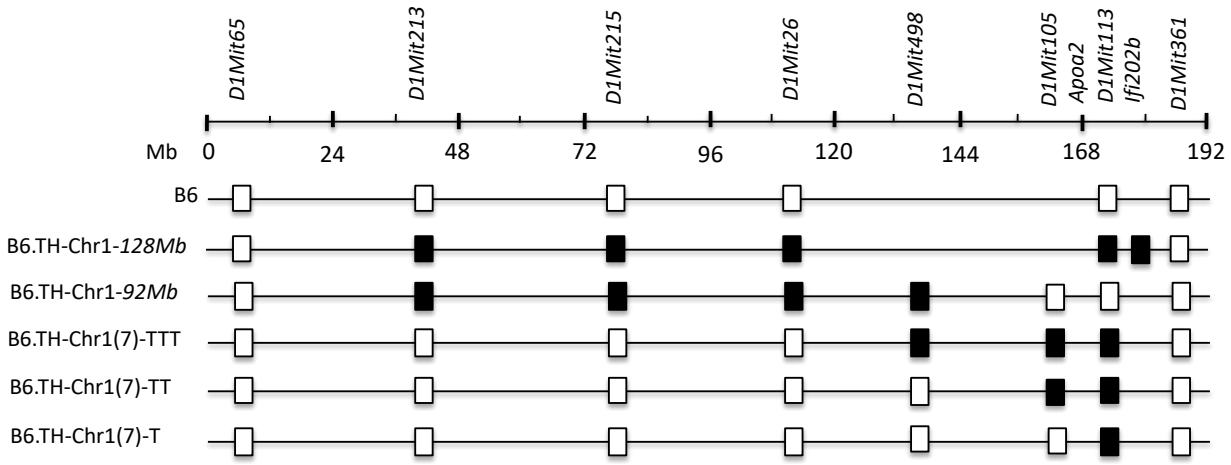


Figure 13. B6.TH-Chr1 congenic intervals on chromosome 1. Genetic markers shown at the top were used to allelotype the congenic interval. The open and filled boxes represent a C57BL/6J (B6) and TALLYHO (TH) allele, respectively. Mb: Megabase

B6.TH-Chr1(7)-TTT, B6.TH-Chr1(7)-TT, and B6.TH-Chr1(7)-T mice had significantly higher fat mass than B6 mice on HFD, defining the candidate region of the obesity QTL to marker *D1Mit113*. Based on the known biological function, *Ifi202b* and *Apoa2* genes are plausible positional candidate genes, and genetic variants of these genes are present between TH and B6 mice. We will also search candidate genes using the published catalog of sequence variants in the TH mouse (relative to the B6 strain). In this study, we created new congenic mice for obesity that can be a valuable resource for the identification of obesity gene(s) and their interactions with diets.

Higher inflammation and lower mitochondrial respiratory capacity in TH mice

The excess adipose tissue in obesity develops inflammation through hypoxia from increased mass but decreased vascularization, increased apoptosis of hypertrophic cells, and increased infiltration of macrophages (Gutierrez, Puglisi, & Hasty, 2009). Both adipose tissue and macrophages secrete pro-inflammatory cytokines such as IL-6 and TNF-α (Jung & Choi, 2014). Excess free fatty acids from digestion and spill-over from adipocytes form deposits on

peripheral organs, increasing lipotoxicity and contributing to the development of insulin resistance (Sears & Perry, 2015). Cytokines from adipose tissue and peripheral organs circulate and cause systemic, chronic low-grade inflammation (Jung & Choi, 2014). Increased inflammation and oxidative stress, due to increased nutritional intake, disrupt mitochondrial homeostasis and lead to mitochondrial dysfunction, as mitochondria are unable to generate and sustain necessary ATP production (de Mello et al., 2018). Increased inflammation, adipose tissue dysfunction, and mitochondrial dysfunction exacerbate metabolic dysfunction and insulin resistance (de Mello et al., 2018; Gutierrez et al., 2009; Jung & Choi, 2014; Sears & Perry, 2015).

We investigated inflammation and mitochondrial respiratory activity in TH and B6.TH-Chr1-128Mb congenic mice compared to B6 mice. It was hypothesized that TH mice would have increased inflammation compared to B6 mice, especially on HSHF. We found that plasma IL-6 levels were significantly higher in TH mice than B6 mice on chow and HSHF (Figure 9). Both B6 and TH mice had higher plasma IL-6 levels on HSHF compared to chow. Plasma IL-6 levels were also significantly higher in B6.TH-Chr1-128Mb congenic mice compared to B6 mice on chow (Figure 10).

Basal respiration, proton leak, and ATP production in SVF cells from adipose tissue were comparable between B6 vs TH mice or B6.TH-Chr1-128Mb congenic mice (Figure 11, 12). However, TH mice had significantly lower maximal and spare respiratory capacity compared to B6 mice. B6.TH-Chr1-128Mb congenic mice had a significantly lower spare respiratory capacity compared to B6 mice. Our results using SVF cells are in line with previous studies using adipocytes from diet-induced or genetic obese models, where adipocytes were shown to have decreased maximal respiration and spare respiratory capacity (Pardo et al., 2011; Schottl,

Kappler, Braun, Fromme, & Klingenspor, 2015). Spare respiratory capacity is the difference between basal and maximal respiratory activity and is necessary for sudden large energy requirements. If the spare respiratory capacity cannot meet demands, then the cells may die or become damaged (Desler et al., 2012).

Limitations

In chapter 4, oxygen consumption rate (OCR) of adipose tissue-derived SVF from TH, B6.TH.Chr1-128Mb, and B6 mice were measured with the Seahorse XFp analyzer; however, we were limited to chow-fed samples only. Differences in mitochondrial respiration were found between TH and B6.TH-Chr1-128Mb versus B6 mice on chow, and we anticipate that we would observe a marked difference if HFD fed samples had they been available. The plasma IL-6 levels of Chr1-128Mb congenic mice were also limited to chow only and should be further investigated on HFD compared to B6. In addition to the diet limitation, we only plated and examined SVF cells that contain preadipocytes for the assay. However, previous studies have been done comparing SVF cells before and after differentiation, resulting in a similar trend in OCR (LeMieux et al., 2016). Finally, this dissertation focused on male mice. There is sexual dimorphism with adipose deposition in humans, and more studies need to be done addressing these differences so effective treatments for both genders can be found (Giles, Jackman, & MacLean, 2016).

Conclusion

This dissertation sought to characterize the TH mouse as a relevant, clinical model for human polygenic obesity and T2D. This work was the first to examine the effect of diet on the TH mouse model and identify that TH mice are highly sensitive to dietary fat in advancing obesity and T2D. The interplay between heredity and dietary environment in TH mice appears to

amplify insulin resistance and inflammation. We were also able to identify obesity candidate loci in TH mice. TH mice will be a useful model for further candidate gene identification and mechanistic studies in order to advance obesity and T2D understanding and the development of more effective treatments.

Future Studies

In chapter 2, a diet study was conducted with TH and B6 mice on standard rodent chow, semi-purified high-sucrose low fat (HSLF) diet, and semi-purified high-sucrose high-fat (HSHF) diet. We found that semi-purified diets promoted weight gain before dietary fat promoted weight gain. The semi-purified diet effect may be due to lack of soluble fiber, which causes cecal and colonic atrophy and microbiota-dependent promotion of adiposity (Chassaing et al., 2015). In the future, it would be worthwhile to test whether this effect is microbiota dependent.

The results in chapter 3 confirmed previous mapping studies for obesity QTL on chromosome 1 in TH mice. By utilizing subcongenic mice analysis combined with bioinformatics, we will identify obesity gene(s) derived from TH genome. We currently have *Apoa2*, a candidate gene, knockout mice and bred them to our B6.TH-Chr1(7)-T sub-congenic mice. By knocking out the *Apoa2* gene in B6.TH-Chr1(7)-T sub-congenic mice, we can investigate to what extent *Apoa2* contributes to increased fat mass and plasma cholesterol seen in the congenic model.

Chapter 4 began looking into adipose tissue inflammation and mitochondrial health very briefly. Further studies need to be done to examine the cytokine profile of TH mice on chow and HFD. OCR should be examined once more using the Seahorse XFp to see the effect of HFD on the mitochondrial parameters for TH and B6. In addition, with the availability of the Seahorse XFe24 analyzer, adipose tissue can be added directly to the wells of the microplate. Various

white adipose depots could be studied as well as brown adipose tissue. If there is evidence of mitochondrial dysfunction, further experiments can be conducted to uncover the location(s) of the dysfunction in the mitochondria by testing the different complexes of the electron transport chain.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

March 12, 2019

Jacaline Parkman
Robert C Byrd Biotechnology Science Center, Rm 407
Marshall University

Dear Ms. Parkman:

This letter is in response to the submitted dissertation abstract entitled *"Interplay Between Genetic Predisposition and Diet in Advancing Obesity and Type 2 Diabetes in the Tallyho Mouse."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #508. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity

APPENDIX B: LIST OF ABBREVIATIONS

11β-HSD1…11β-hydroxysteroid dehydrogenase type 1

AgRP…Agouti-related peptide

Apoa2… Apolipoprotein A

ARC…Arcuate nucleus

ATP… adenosine triphosphate

B6…C57BL/6J or C57BL/6

BAT…Brown adipose tissue

BMI…Body mass index

BMPs… Bone morphogenetic proteins

BPA…Bisphenol A

C57BL/6J…The same as C57BL/6

C/EBPα …CCAAT-enhancer-binding protein alpha

CCK…Cholecystokinin

Chr1…Chromosome 1

CLAMS…Comprehensive Laboratory Animal Monitoring System

CNS…Central nervous system

CREB…cAMP response element binding protein

CRP…C-reactive protein

ΔCt…average threshold cycle

DIO…Diet induced obesity

ECM…Extracellular matrix

EDCs…Endocrine disrupting chemicals

ELISA… Enzyme-linked immunosorbent assay

ETC… Electron transport chain

FCCP…Carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone

FFA…Free fatty Acids

FGF21… Fibroblast growth factor 21

FRD…Fructose-rich diet

FTO… Fat mass and obesity-associated protein

GAPDH… Glyceraldehyde-3-phosphate dehydrogenase

GI…Gastrointestinal tract

GLP-1… Glucagon-like peptide 1

GLUT4…Glucose transporter 4

GO…Gene Ontology

GWAS… genome-wide association studies

HFD…High fat diet

HRP…Horseradish peroxidase (HRP)

Hsd17b7...Hydroxysteroid (17-beta) dehydrogenase 7

HSHF… Semi-purified high-sucrose high-fat

HSLF…Semi-purified high-sucrose low-fat

Ifi202b…Interferon activated gene 202b

IKK…IκB kinase

IFN-γ…Interferon-γ

IL-10…Interleukin-10

IL-6…Interleukin-6

IPGTT…Intraperitoneal glucose tolerance test

IRS-1…Insulin receptor substrate 1

IRS-2...Insulin receptor substrate 2

JNK… c-Jun N-terminal kinase

LDL…Low-density lipoprotein

LOO… C57B6 late-onset obesity mouse

MCP-1…Monocyte chemoattractant protein

MSCs… Multipotent mesenchymal stem cells

Myf5…Myogenic factor 5

NAFLD…Non-alcoholic fatty liver disease

NEAT… Nonexercise activity thermogenesis

NF-κB …Nuclear factor-κB

NPY…Neuropeptide Y

NZO…New Zealand Obese mouse

OAD…Oral antidiabetic drugs

OCR…Oxygen Consumption Rate

OR…Odds ratio

OXPHOS…Oxidative phosphorylation

PGC-1α …Peroxisome proliferator-activated receptor-γ coactivator-1α

Pla2g4a… The phospholipase A2, group IVA

PNS…Parasympathetic nervous system

POMC…Proopiomelanocortin

PPARγ…Peroxisome proliferator-activated receptor gamma

PRDM16…PR domain containing 16

PVDF…polyvinylidene difluoride

qRT-PCR…Real-time quantitative polymerase chain reaction

QTL…Quantitative trait loci

RER…Respiratory exchange ratio

RIPA… Radioimmunoprecipitation assay

ROS…Reactive oxygen species

SAP…Serum amyloid P-component

SDS-PAGE…sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNS…Sympathetic nervous system

SSLP…single sequence length polymorphism markers

SVC…Stromal vascular cells

SVF… Stromal vascular fraction

T2D…Type 2 diabetes

Tabw3…TALLYHO associated body weight 3

Tachol1…TALLYHO associated cholesterol 1

TBST…Tris Buffered Saline with Tween 20®

TCA… Tricarboxylic acid

TCF4…Transcription factor 4

TH…TALLYHO/Jng

TNF-α…Tumor necrosis factor alpha

UCP-1… Uncoupling protein 1

WAT…White adipose tissue

WBKDF… Wistar Bonn Kobori rats

XFp…Extracellular flux analyzer

APPENDIX C:

TABLE 5. GENES IN THE REGION FLANKED BY D1MIT498 AND D1MIT361

Gene	Variant type(s)	Go Terms
Abl2	missense_variant splice_donor_variant	positive regulation of phospholipase C activity
Acbd3	missense_variant	fatty-acyl-CoA binding; lipid metabolic process
Aldh9a1	splice_region_variant	metabolic process; carnitine metabolic process; cellular aldehyde metabolic process
Apcs	splice_region_variant	negative regulation by host of viral glycoprotein metabolic process; negative regulation of glycoprotein metabolic process
Apoa2	missense_variant	negative regulation of cholesterol import; high-density lipoprotein particle remodeling; cholesterol transporter activity;apolipoprotein receptor binding; high-density lipoprotein particle receptor binding; low-density lipoprotein particle remodeling; high-density lipoprotein particle clearance; negative regulation of cholesterol transport; cholesterol efflux; phospholipid efflux; phospholipid binding; fatty acid metabolic process; positive regulation of lipid catabolic process; phospholipid catabolic process; lipid transporter activity; triglyceride-rich lipoprotein particle remodeling; negative regulation of very-low-density lipoprotein particle remodeling; negative regulation of lipid catabolic process; lipoprotein metabolic process; cholesterol binding;lipid transport; cholesterol transport; positive regulation of cholesterol esterification; regulation of intestinal cholesterol absorption; reverse cholesterol transport; lipid binding; response to glucose; cholesterol metabolic process; negative regulation of lipase activity; high-density lipoprotein particle assembly; lipase inhibitor activity; high-density lipoprotein particle binding; negative regulation of cholesterol transporter activity; cholesterol homeostasis
Atp1a2	missense_variant splice_region_variant	ATP metabolic process
Bpnt1	missense_variant splice_region_variant	nucleobase-containing compound metabolic process
Crp	missense_variant	negative regulation of lipid storage; low-density lipoprotein particle receptor binding; regulation of low-density lipoprotein particle clearance; cholesterol binding; low-density lipoprotein particle binding
Ephx1	missense_variant splice_region_variant	cellular aromatic compound metabolic process
Eprs	missense_variant splice_region_variant	metabolic process
Exo1	splice_region_variant	nucleobase-containing compound metabolic process; DNA metabolic process
Fh1	missense_variant splice_region_variant	fumarate metabolic process; malate metabolic process
Hhipl2	missense_variant	carbohydrate metabolic process

	splice_acceptor_variant splice_region_variant	
Hsd17b7	missense_variant	cholesterol biosynthetic process; lipid metabolic process
Itpkb	missense_variant splice_region_variant	inositol trisphosphate metabolic process
Kmo	intron_variant missense_variant splice_region_variant	NAD metabolic process; kynurenine metabolic process
Lamc1	missense_variant splice_region_variant	glycosphingolipid binding
Marc1	missense_variant splice_region_variant	nitrate metabolic process
Marc2	splice_region_variant	nitrate metabolic process
Mark1	missense_variant splice_region_variant	lipid binding
Mia3	inframe_insertion missense_variant splice_region_variant	lipoprotein transporter activity; lipoprotein transport
Ncf2	splice_region_variant	superoxide metabolic process; response to glucose
Ncstn	missense_variant	beta-amyloid metabolic process
Nit1	missense_variant splice_region_variant	nitrogen compound metabolic process
Npl	splice_region_variant	metabolic process; carbohydrate metabolic process
Parp1	splice_region_variant	mitochondrial DNA metabolic process; DNA metabolic process
Pex19	splice_region_variant	negative regulation of lipid binding
Pla2g4a	splice_region_variant	metabolic process; calcium-dependent phospholipase A2 activity;calcium-dependent phospholipid binding; phospholipase A2 activity; phospholipid catabolic process; lipid metabolic process; lysophospholipase activity; phospholipase activity; lipid catabolic process; arachidonic acid metabolic process
Ppox	splice_region_variant	protoporphyrinogen IX metabolic process
Psen2	missense_variant splice_region_variant	cellular protein metabolic process; beta-amyloid metabolic process
Rab3gap2	missense_variant splice_region_variant	positive regulation of protein lipidation
Rnasel	missense_variant splice_region_variant	fat cell differentiation; positive regulation of glucose import in response to insulin stimulus
Soat1	missense_variant splice_region_variant	cholesterol O-acyltransferase activity;cholesterol efflux; cholesterol binding; cholesterol metabolic process; cholesterol storage; very-low-density lipoprotein particle assembly; lipid metabolic process; steroid metabolic process; cholesterol homeostasis; cholesterol esterification
Tnr	missense_variant	sphingolipid binding
Uap1	missense_variant splice_region_variant	metabolic process; UDP-N-acetylglucosamine metabolic process
Usf1	inframe_deletion	glucose metabolic process; positive regulation of transcription from RNA polymerase II promoter by glucose; lipid homeostasis

Table 5. Genes in the region flanked by D1Mit498 and D1Mit361 with protein-altering variants that are associated with obesity-related Gene Ontology terms.