Regulation of metabolic health by dietary protein and isoleucine

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Abstract:

People are living longer than ever before, which means age-related diseases are also on the rise. Thus, solutions are needed to promote healthy longevity and close the healthspan-lifespan gap. Exercise is a potent intervention that reduces the risk of a variety of obesity-related illness that increase in prevalence with age. Specifically, aerobic exercise is important for improving and maintaining cardiovascular fitness, reducing body weight, and enhancing other metabolic parameters, while resistance exercise is known to improve skeletal muscle health and function, increase bone strength, and promote healthy glucose homeostasis. To support the strengthening outcomes by resistance exercise, a high protein (HP) diet is typically recommended.

Not surprisingly, diet is also a significant determinant of metabolic health and lifespan. Recent work has clearly shown that a calorie is not just a calorie, and a variety of diets have been examined for their role in molecular control of health and longevity. Two such diets, protein restriction and specific isoleucine restriction, are examined in this work. Low protein (LP) diets have been shown to improve health and prolong lifespan in a variety of model organisms and in humans.

In **Chapter 2**, we explore the “protein paradox.” If a HP diet is recommended in people that perform resistance exercise, but those consuming an LP diet live longer and in better health, does resistance exercise protect against the negative side effects of a HP diet? We find that despite having lower food consumption than the LP group, HP-fed mice accumulate significantly more fat mass than LP-fed mice when not exercising, while weight pulling protects HP-fed mice from this excess fat gain. Weight pulling-induced strength increased more rapidly in the exercised HP-fed mice, but by the end of the study the LP-fed mice were just as strong in absolute terms, and were actually stronger relative to their body weight. The HP-fed weight pulling mice did, however, have significantly bigger muscles. Surprisingly, exercise did not

protect from HP-induced changes in glycemic control. Our results confirm that HP diets enhance muscle hypertrophy and accelerate absolute strength gain by resistance exercise while protecting against fat gain. However, trained mice fed the LP diet had better glycemic control and were relatively stronger. In the sedentary mice, LP diets were superior in terms of metabolic health.

As the essential branched-chain amino acids – leucine, isoleucine, and valine – are required for the benefits of LP diets, limitation of the BCAAs has been recently evaluated as a diet therapeutic in rodent models and also in two human clinical trials. Repletion of isoleucine in a BCAA-limited diet ablates the metabolic benefits observed, and both isoleucine intake and circulating levels have been linked to increased BMI and mortality in humans, respectively. Reducing dietary levels of isoleucine has also been shown to rapidly improve the metabolic health of male C57BL/6J mice with diet-induced obesity. We further explored amino acid restriction in context of an unhealthy Western diet in **Chapter 3**. Here, we tested the phenotypes of young male and female C57BL/6J and DBA/2J mice, and found a variety of global metabolic improvements despite some unique molecular sex- and strain-dependent signatures. Through multi-omics analyses, we discovered hepatic molecular mechanisms shared by all groups to be tested in greater detail in future experiments. We also determined that healthiest human diets are also naturally lowest in isoleucine.

Mechanistically, a low isoleucine diet stimulates secretion of liver-derived hormone FGF21 to stimulate protein intake. FGF21 also increases adipose tissue browning and energy expenditure, improves insulin sensitivity, as well as other systemic metabolic effects. As some of the benefits of LP diets have been shown to be dependent on FGF21, we suspected that low isoleucine diets would also require FGF21. So, we utilized whole-body FGF21 knockouts and compared them to wild type animals in an analysis of lifelong isoleucine restriction (**Chapter 4**). Here, we see that the benefits of isoleucine restriction are largely independent of FGF21.

In total, the works here suggest that a sedentary lifestyle can be optimized by diets lower in dietary protein and specifically, dietary isoleucine. Conversely, being active is protective against consequences associated with HP diets, but the influence that exercise and HP diets yield on lifespan remain unclear. Further, a low isoleucine diet can be protective from an otherwise unhealthy Western-style eating pattern that appears to be largely independent of FGF21. Isoleucine restricted diets result in a variety of up- and downregulated hepatic mechanisms that will undoubtedly be explored in the future. Additional future directions of this work may include human clinical studies of a low isoleucine diet as well as deeper molecular exploration of age-related mechanisms in response to this diet.

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Chapter 1: Introduction: Protein restriction and branched-chain amino acid restriction promote geroprotective shifts in metabolism

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Abstract

The proportion of humans suffering from age related diseases is increasing around the world, and creative solutions are needed to promote healthy longevity. Recent work has clearly shown that a calorie is not just a calorie – and that low protein diets are associated with reduced mortality in humans and promote metabolic health and extended lifespan in rodents. Many of the benefits of protein restriction on metabolism and aging are the result of decreased consumption of the three branched-chain amino acids (BCAAs), leucine, isoleucine, and valine. Here, we discuss the emerging evidence that BCAAs are critical modulators of healthy metabolism and longevity in rodents and humans, as well as the physiological and molecular mechanisms that may drive the benefits of BCAA restriction. Our results illustrate that protein quality – the specific composition of dietary protein – may be a previously unappreciated driver of metabolic dysfunction, and that reducing dietary BCAAs - specifically isoleucine - may be a promising new approach to delay and prevent diseases of aging.

Introduction

Around the globe, human life expectancy increased by almost 20 years between 1950 and 2017 (Collaborators, 2018). Despite the effect of the global COVID-19 pandemic, which has caused life expectancy in the US to slightly decline (Arias, 2021), advances in medicine have shifted population demographics, and humans over the age of 65 now represent the fastest growing age group worldwide (United Nations, 2019). As a result, the portion of deaths attributed to non-communicable diseases, such as age-related diseases, has risen and will continue to rise (Foreman et al., 2018).

Though human life expectancy has largely increased, the prevalence of obesity and related disorders has grown rapidly, threatening the quality and duration of healthy years for an ever-expanding aged population. Obesity more than tripled in men and doubled in women from 1975 to 2014, and 43% of American adults aged 40-59 are now obese (Collaboration, 2016). Obesity is increasingly impacting younger individuals, with about 40% of children now overweight or obese, predisposing them to chronic diseases at younger ages as well as lifelong challenges maintaining a healthy body weight (2020). This increase comes despite the fact that the Healthy Eating Index, a score of diet quality, has not shown significant declines since the early 2000s and in fact may be higher in recent years (2020). This suggests that perhaps another factor besides worsening food habits is at play. Creative solutions are needed to combat these conditions and preserve quality of life, as obesity is a risk factor for many age-related diseases, including metabolic, cardiovascular, neurodegenerative, and musculoskeletal conditions, depression, and some cancers (Blüher, 2019). One of these solutions may be targeting aging itself to promote healthspan rather than attempt to treat the myriad of age-related diseases present in the elderly (Partridge, 2014).

Dietary interventions can promote healthy aging

Calorie restriction (CR), defined as a decrease in caloric intake without malnutrition, is often referred to as the 'gold standard' of nutritional interventions, for its potent ability to preserve healthspan and extend lifespan in diverse model organisms (Colman et al., 2014; Gribble and Welch, 2013; Lin et al., 2002; McCay et al., 1935; Weindruch et al., 1986). The hallmarks of CR in mammals include decreased mortality and also decreases in all major diseases of aging, including cancer, cardiovascular disease, kidney disease, diabetes, and neurodegenerative diseases (Green et al., 2022a). There has been significant interest in understanding the physiological and molecular mechanisms engaged by CR that are responsible for its beneficial effects. We have recently reviewed these in depth (Green et al., 2022a), but will briefly discuss a few of the key potential mechanisms that have been examined here.

CR has been proposed to work in part due to reduced production of reactive oxygen species; however, extensive studies in genetically engineered rodents suggest that reactive oxygen species likely do not play a major role in normal aging (Salmon et al., 2010). CR substantially reduces the risk of cancer in mice; however, while the loss of transcription factor NRF2 (Nuclear factor erythroid 2-related factor) blocks the effects of CR on cancer protection, it does not block the ability of CR to extend lifespan (Pearson et al., 2008). While CR improves insulin sensitivity in all mammals, this improvement is dispensable for the benefits of a CR diet on frailty and longevity in mice (Yu et al., 2019). CR may promote health through reduced activation of specific signal transduction pathways such as PI3K/AKT (Mercken et al., 2013) and possibly, mechanistic Target Of Rapamycin (mTOR) (Bjedov et al., 2010; Unnikrishnan et al., 2020), but this remains to be tested rigorously in mammals. We recently showed that many of the benefits of CR require prolonged fasting between meals, which is necessary for CR-induced improvements in insulin sensitivity, frailty, cognition, and longevity in mice (Pak et al., 2021).

In short, there are still many questions about the mechanisms by which CR functions to promote healthy aging. Further, the translatability of CR to humans is generally thought to be low, as most people are unlikely to be able to maintain lifelong adherence to such an abstemious diet. Thus, there is substantial interest in identifying alternative dietary regimens that will mimic the beneficial effects of CR without restricting calories.

Dietary protein in healthspan and aging

CR proportionally decreases the consumption of all three macronutrients (fat, carbohydrate, and protein), and for many years the contributions of restricting these individual macronutrients to the effects of CR have been explored. It is now generally believed that the restriction of protein in a CR diet is not great enough to fully explain the benefits of CR (Speakman et al., 2016). However, restriction of protein reproducibly extends the lifespan of flies (Bruce et al., 2013; Grandison et al., 2009; Lee et al., 2008; Mair et al., 2005) and rodents (Solon-Biet et al., 2014; Weindruch et al., 1986). Many studies have shown PR also improves metabolic parameters in rodents, such as glucose tolerance, insulin sensitivity, circulating triglycerides and other blood lipids (Fontana et al., 2016; Maida et al., 2016; Solon-Biet et al., 2014; Solon-Biet et al., 2015a).

This data in model organisms goes against trending dietary advice for humans, which has generally recommended that humans should be eating more protein to improve satiety and promote weight loss (Cuenca-Sánchez et al., 2015; Yu et al., 2020). High protein diets are indeed indicated for certain clinical conditions or life stages, such as pregnancy and old age, but epidemiological evidence suggests that overconsumption of protein outside of these conditions could be deleterious (Delimaris, 2013). A randomized controlled trial (RCT) of overfeeding in metabolically healthy individuals with low, normal, or high protein content found that low protein

feeding resulted in significantly less weight gain, though this was a result of lack of lean mass gain rather than reduced fat gain (Bray et al., 2012). In middle-aged overweight males, a RCT of protein restriction (PR) (feeding of a 7-9% protein diet without calorie restriction) for 6 weeks resulted in significant body weight and fat mass loss, as well as improvements in body mass index (BMI) (Fontana et al., 2016). As evidence regarding appropriate distribution of dietary protein, carbohydrate, and fat continues to develop, the US and Canadian Dietary Reference Intake Steering Committee are planning to re-investigate the Dietary Reference Intake (DRI) recommendations for energy and the macronutrients for humans (2020). In **Chapter 2**, the impact of both a low (7%) and high (36%) protein diet in a 12-week resistance-exercise regimen in mice is tested.

In summary, PR is an attractive regimen as it replicates many of the beneficial phenotypes of CR without requiring reduced calorie intake. Determining the mechanisms by which PR improves health is of great interest, as these can inform more specific dietary recommendations and potential drug targets to promote health and longevity.

The branched-chain amino acids

Decades ago, it was found that protein source can influence longevity; rats fed a soy protein-based diet lived 15% longer than rats fed a comparable casein-based diet (Iwasaki et al., 1988). One potential explanation for these different outcomes is that different protein sources have distinct amino acid profiles. For example, vegans are believed to naturally consume less methionine than meat eaters with a different balance of amino acids overall, though this idea was challenged in a recent study (MacArthur et al., 2021; McCarty et al., 2009; Schmidt et al., 2016). Methionine is a sulfur-containing essential amino acid with roles in methylation that was first observed to extend lifespan in rats in the 1990s (Orentreich et al., 1993; Richie et al., 1994) and

its lifespan extending effects have since been replicated in other models (Johnson and Johnson, 2014; Lees et al., 2017; Miller et al., 2005). A fuller discussion of the role of dietary methionine in healthy aging can be found elsewhere (e.g. (Brown-Borg and Buffenstein, 2017)), but the work on methionine has highlighted the possibility that the specific amino acid composition of the diet may play a critical role in metabolic health as well as longevity.

The branched-chain amino acids leucine, isoleucine, and valine are three of the nine amino acids that are known to be essential for non-ruminant mammals, including mice and humans. The BCAAs are abundant in high protein foods, making up approximately 20% of the amino acids found in meat, fish, eggs, and nuts. BCAAs are hydrophobic, and serve important roles at the molecular level in protein folding, substrate binding, lipid solubility, and interaction with nonpolar substrates; these amino acids are also common in coiled-coiled α helices which occur often in the proteins myosin, keratin, and some transcription factors (Brosnan and Brosnan, 2006).

The BCAAs are strong agonists of the amino acid sensitive kinase mTOR Complex 1 (mTORC1). mTORC1 regulates a wide variety of downstream biological processes – most notably those related to growth and proliferation such as ribosomal, protein, nucleotide, and lipid synthesis through integration of nutrient and hormonal cues; this has been reviewed in great detail elsewhere (Babygirija and Lamming, 2021; Kennedy and Lamming, 2016). Put simply, amino acids promote the lysosomal localization of mTORC1. Binding of the BCAAs, especially leucine, to Sestrin2 relieves the inhibitory action of Sestin2 on the GATOR2 complex, allowing the Rag GTPases to bind to mTORC1 and recruit it to the lysosome (Chantranupong et al., 2014; Wolfson et al., 2016). Several other molecular mechanisms by which BCAAs, especially leucine, regulate mTORC1 recruitment by the Rag GTPases have been discovered (Han et al., 2012; He et al., 2018; Zhu et al., 2021a). At the lysosome, mTORC1's kinase activity is allosterically activated by the binding of Rheb-GTP (Yang et al., 2017).

The other amino acid sensor that is involved in BCAA metabolism is GCN2 (General control nonderepressible 2). Unlike mTORC1, GCN2 senses amino acid deprivation by binding to uncharged transfer RNA molecules (tRNA) and stalled ribosomes (Dong et al., 2000; Harding et al., 2019; Wek et al., 1995), and works to repress general translation and prioritize preferential production of ATF4, a transcription factor that upregulates genes necessary to adapt to PR, including the energy balance hormone fibroblast growth factor 21 (FGF21) (De Sousa-Coelho et al., 2012). Mice lacking GCN2 have a delayed metabolic response to PR, including a two week delay in the induction of FGF21 (Laeger et al., 2016).

Downstream of GCN2 and ATF4 is FGF21, which is secreted from the liver and other tissues in response to nutrient stress (Nishimura et al., 2000). FGF21 is induced by PR in rodents and humans, and has been described as a key regulator of the response to PR, increasing insulin sensitivity and energy expenditure (Fontana et al., 2016; Laeger et al., 2014). Recent studies show that FGF21 signaling in the brain is required to alter food intake and increase energy expenditure during protein restriction (Hill et al., 2017; Hill et al., 2019), and transgenic overexpression of FGF21 has been found to extend lifespan (Zhang et al., 2012). Discussion of the role of FGF21 in the response to total and specific restriction of BCAAs will be discussed in greater detail below.

BCAA catabolism

After consumption, BCAAs are absorbed in the intestine by classic Na^+ dependent co-transporters, and transported across other membranes by the essential amino acid antiporter L-type large neutral amino acid transporter 1 (LAT1) (Scalise et al., 2018). While many BCAAs are utilized as building blocks for protein synthesis, BCAAs in excess of those needed for protein translation are catabolized. The first catabolic step is reversible: deamination by branched-chain aminotransferase (BCAT), which catalyzes any BCAA and α-ketoglutarate to a branched-chain

keto acid (BCKA) and glutamate, respectively (Harper et al., 1984). BCAT is expressed in mitochondrial and cytoplasmic isoforms, BCATm and BCATc (Hutson et al., 1992), though most BCAA catabolism takes place in the mitochondria so intermediates can quickly enter the TCA cycle. Nearly all subsequent intermediates downstream of the BCKAs remain trapped in the mitochondria due to their conjugation to CoA, a process that is described in greater detail later (Neinast et al., 2019). BCATc is highly expressed in the brain and various CNS cells; this provides leucine-derived nitrogen needed for production of the neurotransmitter glutamate (Castellano et al., 2007; Hutson et al., 1998; Yudkoff, 1997).

As shown in **Figure 1**, BCAT catabolizes each BCAA to its respective ketoacid; leucine to α-ketoisocaproate (KIC), isoleucine to α-keto-methylvalerate (KMV), and valine to α-ketoisovalerate (KIV) (Harper et al., 1984). The next step in BCAA catabolism, which is irreversible and rate-limiting, is performed by the branched-chain keto acid dehydrogenase (BCKDH) complex, a member of the mitochondrial α-ketoacid dehydrogenase complex family (Matthews et al., 1981; Shimomura et al., 2001). BCKDH is comprised of multiple copies of three subunits: BCKA decarboxylase (E1), dihydrolipoamide acyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). BCKDH converts KIC to isovaleryl-CoA (IV-CoA), KMV to α-methylbutyryl-CoA (MB-CoA), and KIV to isobutyryl-CoA (IB-CoA). BCKDH is expressed in all tissues, including hepatocytes.

BCKDH is regulated by a kinase and phosphatase, and is inhibited by serine phosphorylation of the E1 subunit by BCKD Kinase (BCKDK) (Shimomura et al., 1990). Conversely, dephosphorylation of BCKDH is performed by the mitochondrial protein phosphatase 1K (PPM1K) (Lu et al., 2009). BCKDK and PPM1K link BCKDH activity to feeding; PPM1K is highly expressed in the fed state but lowered by fasting, while the reverse is true for BCKDK. Overexpression of the transcription factor ChREBPβ upregulates hepatic BCKDK and downregulates PPM1K expression (White et al., 2018). BCKDK is also allosterically inhibited by

BCKA excess (Shimomura et al., 2001), and PPM1K is inhibited by interaction with E2 of BCKDH (Dong et al., 2013).

Following processing by BCKDH, BCAA catabolism undergoes multiple additional steps, some of which are specific to the catabolism of a specific BCAA and others of which are shared between catabolic pathways. A key point is that with the exception of 3-Hydroxyisobutyrate (3-HIB) – a valine specific catabolic intermediate – the intermediates of BCAA catabolism are conjugated to CoA, and thus confined to the mitochondria (Jang et al., 2016). The final products are specific to each individual BCAA: ketogenic leucine is catabolized to acetoacetate and acetyl-CoA, glucogenic valine to propionyl-CoA, and isoleucine to both acetyl-CoA and propionyl-CoA. Propionyl-coA can enter the TCA cycle via conversion to succinate.

Physiologically, BCAA catabolism is partitioned to tissues for different purposes. While the liver is a major metabolic hub for most amino acid metabolism, BCATm is not expressed in hepatocytes (Suryawan et al., 1998a; Sweatt et al., 2004). In contrast, skeletal muscle is both a consumer of BCAAs for protein synthesis and expresses both BCAT and BCKDH. As a result, the conventional model for amino acid catabolism has for many years suggested that although other amino acids are catabolized in the liver, BCAAs are primarily catabolized in skeletal muscle (Suryawan et al., 1998b). More recent work has shown that adipose tissue is also a key player in BCAA catabolism as transplanting wild-type adipose tissue into whole body *Bcatm*$^{-/-}$ mice is sufficient to decrease circulating BCAAs by 30-50% (Herman et al., 2010). BCAAs are also used to fuel thermogenesis in brown adipose tissue, and BCAA transport into BAT mitochondria is essential to raise body temperature after cold exposure (Yoneshiro et al., 2019).

A recent model of whole-body BCAA fates reconciled these recent findings with early models (Neinast et al., 2019). BCAA turnover is quite rapid once BCAAs enter the bloodstream, with transamination products visible after only three minutes, and end-catabolism into TCA products detectable within three to five minutes. While a majority of BCAA catabolism takes place

in skeletal muscle, 19% occurred in brown adipose tissue, with other tissues representing about 20% of flux. This new model verifies that even in more nuanced tracing experiments, muscle is the main tissue of BCAA catabolism, while also confirming new data on the catabolic capacity of adipose tissues. This understanding of the 'normal' catabolic process aids our understanding – and the potential for therapeutic modulation – of dysregulated BCAA catabolism in insulin resistance.

Modulating BCAA catabolism

Genetic and pharmacological manipulation of BCAA catabolism has been used to investigate how BCAAs regulate metabolic health. Whole-body deletion of BCKDK activates BCAA catabolism to promote new steady-state and tissue levels of BCAAs and BCKAs, but also impairs growth and neurological function (Joshi et al., 2006; Neinast et al., 2019). Conversely in lean mice, whole body deletion of *Ppm1k* decreases, but does not completely blunt BCAA catabolism, and actually improves insulin sensitivity, glucose tolerance, and weight (Lu et al., 2009). Additionally, deletion of *Bcatm* provides resistance to diet induced obesity, and promotes leanness and improved glucose tolerance (She et al., 2007a), though these mice need access to lower BCAA diets to avoid toxicity.

Resistance to diet-induced obesity was also replicated in a recent study of adipose- and iWAT-specific *Bcatm* knockout mice (Ma et al., 2022). Deletion of *Bcatm* in these tissues improved glucose tolerance, insulin resistance, and reduced circulating cholesterol, triglyceride, and free fatty acid levels. Mechanistically, this was found to be a result of increased thermogenesis and adipose tissue browning. Further study found that this was the result of PR/SET Domain 16 (PRDM16) acetylation by BCAA-derived acetyl-CoA, resulting in decreased binding of PRDM16 to Peroxisome proliferator-activated receptor γ (PPARγ). This leads to suppression of browning genes, and therefore contributes to diet induced obesity. Interestingly, this same study identified

that telmisartan, an FDA-approved anti-hypertensive medication, inhibits BCATm, increasing iWAT browning and energy expenditure, and reducing adiposity in mice.

Acute overexpression of *Ppm1k* in liver of Zucker fatty rats, which are obese, hyperphagic, and hyperinsulinemic, lowers hepatic triglycerides and improves glycemia, likely through action on ATP citrate lyase (ACL), rather than BCKDH, as described above (White et al., 2018). However, a greater focus has been placed on modifying BCKDK activity, as increasing BCAA disposal is logically potential therapy for treating insulin-resistant obesity. The compound 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid (BT2) is a recently identified allosteric inhibitor of BCKDK (Tso et al., 2014). In Zucker fatty rats, BT2 rapidly lowers hepatic triglycerides, improves glucose tolerance and insulin sensitivity (White et al., 2018), and treatment of *ob/ob* and diet-induced obese mice with BT2 restores BCAA catabolism and is sufficient to improve glucose tolerance and insulin resistance (Zhou et al., 2019). Quantitative tracing experiments show that BT2 treatment robustly increases BCAA oxidation in skeletal muscle, though it alters phosphorylation of BCKDH in liver and heart as well (Neinast et al., 2019).

Indeed, one of the most characterized applications of BT2 is to prevent BCAA accumulation in the heart and improve cardiac function. In the heart, high levels of glucose negatively regulate BCAA catabolic enzymes through inhibition of the transcription factor Krüppel-like factor 15 (KLF15), and BCAA accumulation along with high glucose levels produce insulin resistant cardiac tissue (Fillmore et al., 2018; Shao et al., 2018; Sun et al., 2016). This increases vulnerability to ischemic injury, accelerates oxidative stress and superoxide production, and has been linked to multiple types of heart failure in humans and mice (Li et al., 2017; Sun et al., 2016; Uddin et al., 2019; Wang et al., 2016). BT2 preserves cardiac function and prevents vulnerability to acute ischemia in mouse models of heart failure (Li et al., 2017; Sun et al., 2016), and can even restore cardiac function to hearts with preexisting dysfunction (Chen et al., 2019a). Additional

studies are warranted to find the specific mechanisms that allow improved cardiac function by BT2.

BCAA catabolism is also necessary for and altered by endurance exercise. Disruption of BCAA catabolism by *Bcatm* deletion in skeletal muscle impairs exercise performance and endurance (She et al., 2010). Conversely, mice genetically predisposed to high endurance catabolize BCAAs faster, and more efficiently; this is likely driven by increased PGC1α activation (Overmyer et al., 2015). When overexpressed in skeletal muscle, PGC1α drives BCAT and BCKD expression (Hatazawa et al., 2014), and dramatically increases the BCAA catabolic capabilities of muscle (Jang et al., 2016; Neinast et al., 2019). Clearly, perturbations in BCAA catabolism alter physiological metabolism, and may be a therapeutic target in insulin resistance and tissue-specific metabolic dysfunction.

BCAAs are associated with insulin resistant obesity

Over fifty years ago, it was discovered that plasma levels of BCAAs are positively correlated with insulin resistance and obesity in humans (Felig et al., 1969). This has been expanded upon in more recent studies of obese and insulin resistant humans around the world (Chen et al., 2019b; Huffman et al., 2009; Newgard et al., 2009; Xu et al., 2013) as well as in laboratory models of diabetes and obesity (Lynch and Adams, 2014; She et al., 2007b). High BCAA levels can also be predictive of diabetes onset (Wang et al., 2011), especially in post-renal transplant recipients (Osté et al., 2020). This prognostic association has also been observed in adolescents (McCormack et al., 2013). More recently, elevated BCAAs have been associated with negative cardiovascular outcomes (Du et al., 2018; Le Couteur et al., 2020; Portero et al., 2021; Sun et al., 2017).

Normal BCAA levels can be restored through weight loss; a comparison of plasma amino acid levels in over 1,000 individuals from two randomized dietary weight loss trials found that pounds of weight lost correlated well with decreased BCAAs (Zheng et al., 2016). Other studies have found that plasma BCAA levels during weight loss interventions are correlated with improvements in glucose homeostasis and insulin sensitivity (Laferrere et al., 2011; Shah et al., 2012). This correlative effect between BCAAs and metabolic parameters has raised a question among researchers: are BCAAs pathogenic in insulin resistant obesity, or a by-product of accelerating metabolic syndrome?

To answer this question, several groups have combined data from genome-wide association studies (GWAS) with BCAA and insulin levels. One meta-analysis found several single nucleotide polymorphisms (SNPs) in genomic regions of BCAA catabolism and connected these SNPs to elevated BCAAs and insulin resistance (Lotta et al., 2016). Two similar studies presented opposing results, postulating that though BCAAs are elevated in type II diabetes, this is caused by genetic risk for insulin resistance (Mahendran et al., 2017; Wang et al., 2017). Recent research in this field has concentrated on what increases circulating BCAAs, how this interacts with dysregulated metabolic states such as insulin resistance, and what methods are most effective at restoring BCAA levels.

Another recently published GWAS study utilized the generation of a genetic risk score (GRS) of five common BCAA metabolic pathway SNPs in conjunction with dietary BCAA intake to parse out determinants of T2DM risk. This study was conducted in a Chinese population of almost 10,000 individuals and determined that a high GRS plus high intake of BCAAs conveyed the greatest risk for T2DM development. Interestingly, high BCAA intake positively correlated with HbA1c and circulating BCAA levels in participants with a high, but not a low GRS (Wang et al., 2021). In a cohort of over 1600 Mexican adults, rare SNPs in both BCATm and BCKDH genes were associated with elevated body weight and BMI, fasting blood glucose and blood pressure,

compared to their peers with more common alleles (Vargas-Morales et al., 2021). Individuals with the rare variants also had higher amounts of isoleucine, methionine, proline, and aspartate in circulation, though follow-up studies are needed to determine how this impacts overall health. Together, this data suggests that a personalized nutrition approach with specific attention to the BCAAs might be warranted, particularly in those with a high genetic risk for diabetes development or with certain SNPs in the BCAA catabolism pathway.

BCAA catabolism is altered by insulin resistance, and this likely perpetuates a cycle where elevated BCAAs promotes further insulin resistance and keeps BCAAs elevated. This is exemplified in adipose tissue, where BCAA catabolic enzymes decrease in activity and expression in insulin resistant WAT (Lackey et al., 2013; She et al., 2007b). Furthermore, disrupting BCAA catabolism or preventing BCAA transport in BAT is sufficient to lower body temperature and accelerate insulin resistant, obese phenotypes in mice (Yoneshiro et al., 2019). This was confirmed in tracing experiments of two insulin resistant mouse models, where catabolism was shifted away from adipose tissues, with increased reliance on skeletal muscle and heart (Neinast et al., 2019).

In both humans and rodents with insulin resistant obesity, elevated BCAAs are associated with decreased levels of glycine, particularly in muscle tissue (Newgard et al., 2009; White et al., 2016). It's postulated that glycine is depleted in an attempt to clear lipid metabolites that accumulate in muscle as a hallmark of insulin resistance. However, lipotoxicity is further exacerbated by the increased requirement for BCAA catabolism in muscle. Recently, a catabolite of valine, 3-hydroxy-isobutyrate (3-HIB), was discovered to promote fatty acid trans-endothelial uptake into skeletal muscle, providing a key and previously unknown link between BCAA catabolism and fatty acid transport in muscle (Jang et al., 2016); this catabolite is also associated with an increased future incidence of insulin-resistant obesity, even after adjustment for BCAAs

(Mardinoglu et al., 2018). By this mechanism, increased dependence on skeletal muscle for BCAA catabolism only furthers insulin resistance.

The liver is another site of dysregulated metabolism in insulin resistance; though liver represents less than 10% of BCAA oxidation, this tissue represents 27% of BCAA disposal into protein synthesis (Neinast et al., 2019). Elevated hepatic BCAAs prevent GCN2 mediated repression of SREBP1c and fatty acid synthase (FAS), which drive lipogenesis (Guo and Cavener, 2007). Additionally, the BCKDH regulators BCKDK and PPM1K also target ACL in the liver, another key enzyme in lipogenesis, and in insulin resistant conditions, high BCKDK and low PPM1K inhibit BCKDH while promoting ACL activity (White et al., 2018). By these mechanisms, elevated BCAAs can increase insulin resistance by promoting hepatic lipogenesis in inappropriate conditions. Indeed, diets with reduced levels of total BCAAs, isoleucine, or valine reversed hepatic lipid accumulation in Western diet-induced obese mice, even as the mice continued to consume an otherwise high-fat, high-sucrose Western diet (Cummings et al., 2018; Yu et al., 2021a). As the liver, muscle, and adipose tissues all alter BCAA catabolism in insulin resistant states, some researchers have explored altering these catabolic pathways in an attempt to treat these conditions and learn more about the pathogenicity of elevated BCAAs.

BCAA disposal in protein and BCAA restriction

The field of BCAA metabolism has been advanced with the use of large, multi-omic data analyses and metabolic tracing experiments that can determine tissue specific use of AAs. The new model proposed by Neinast *et al*. is certainly a more comprehensive assessment of whole-body BCAA catabolism (Neinast et al., 2019), however, there are still a few outstanding questions. Although this updated model seems to agree with previous literature, it only accounts for approximately 50% of whole-body BCAA disposal; for example, one major metabolic organ not evaluated was the gut, home to intestinal microbiota that can produce BCAAs and other essential

amino acids (Lynch and Pedersen, 2016; Pedersen et al., 2016; Ridaura et al., 2013). Furthermore, there are discrepancies in the literature regarding the expression and modification of BCAA catabolic enzymes, and how this relates to tissue specific BCAA oxidation. It will be interesting to see how models of BCAA catabolism evolve, especially as delineation of sexual dimorphisms is prioritized in research.

In insulin resistant conditions, BCAA catabolism is decreased in adipose tissue, and shifted towards muscle. However, it is unknown how BCAA disposal and oxidation change in protein and BCAA restriction. When fewer BCAAs are fed, BCAA incorporation into protein would presumably proportionally increase, oxidation would decrease, and the distribution of BCAA uptake would also likely change. BCKA oxidation is elevated in liver of Zucker lean rats and elevated in muscle of Zucker fatty rats (White et al., 2016), which agrees with a model that BCAA oxidation is shifted towards muscle in insulin resistant conditions.

Dietary intake of BCAAs affects health and longevity

As early as the 1980s, scientists reported that modifying the BCAA content of diet can regulate activity of catabolic enzymes (Block et al., 1985). Dietary BCAA intake is associated with increased weight and adiposity in both aged male mice and humans (Ribeiro et al., 2019), and decreasing BCAA consumption through PR reduced blood levels of BCAAs in two human trials (Fontana et al., 2016; Maida et al., 2017). In a large nutritional geometry diet composition study, circulating BCAAs were the only amino acids (AAs) that correlated with dietary protein intake in mice (Solon-Biet et al., 2014). The use of AA defined diets has allowed specific modification of BCAA intake in several rodent and human trials during the last decade.

BCAA supplementation in rodents

Though it may seem paradoxical to the literature presented so far, there have been several instances where BCAA supplementation has been reported to improve health. Specific supplementation of leucine in drinking water prevented hyperglycemia and decreased weight and fat gain from high fat diet feeding in mice, not by decreasing energy intake, but by increasing resting energy expenditure and UCP3 (Uncoupling Protein 3) expression in adipose and muscle (Zhang et al., 2007). Additionally, supplementation of BCAAs (plus 8 other amino acids) in drinking water to mice from 9 months of age onwards slightly increased lifespan in a single study; this extension was attributed to increased mitochondrial biogenesis, decreased reactive oxygen species, and improved exercise capacity (D'Antona et al., 2010).

However, more recent and carefully controlled studies have reported that BCAA supplementation, particularly in the context of a Western diet, increases glucose intolerance and insulin resistance in rodents (Cummings et al., 2018; Newgard et al., 2009). In rats, this supplementation also increased muscle mTORC1 activity, and BCAA-induced insulin resistance was acutely reversed by rapamycin treatment (Newgard et al., 2009). Another recent study found that doubling dietary BCAAs promoted hyperphagia, obesity, and early mortality (Solon-Biet et al., 2019). A time-of-day feeding study also identified that feeding BCAA-enriched meals at the end of wake periods in mice resulted in enhanced cardiovascular growth and detrimental remodeling in a circadian-clock dependent manner (Latimer et al., 2021). These studies differ in means and degree of supplementation, which may explain some of these seemingly contradictory results.

BCAA restriction promotes metabolic health in rodents

Both BCAA restriction and deprivation have been studied in rodents; as BCAAs are essential, deprivation experiments are only sustainable short term. Initial deprivation experiments examined the effects of feeding mice leucine-free, isoleucine-free, or valine-free diets for up to a week. These deprivation regimens rapidly improved glycemic control and liver insulin sensitivity

(Xiao et al., 2011; Xiao et al., 2014). These diets appeared to influence canonical pathways of PR, as all three diets were associated with decreased mTORC1 and increased AMPK activity in the liver. Activation of mTORC1 signaling via S6K1 or whole body deletion of *Gcn2* was sufficient to reduce benefits of BCAA deprivation on insulin sensitivity. In valine deprived diet conditions, at least some of these effects appear to be mediated by GCN2, as whole-body *Gcn2*$^{-/-}$ mice were slightly less insulin sensitive than wild-type mice when fed a valine-free diet.

More recent studies have focused on the more physiologically relevant reduction of dietary BCAAs. These studies typically examined a 50-80% restriction of BCAAs, which in contrast to complete removal of one of these essential amino acids, is sustainable over the entire lifespan. A 67% restriction of all three BCAAs initiated at 9 weeks of age in male mice – approximately equivalent to a human teenager (Flurkey et al., 2007) – improves metabolic health and recapitulates many effects of decreasing consumption of PR (Fontana et al., 2016; Yu et al., 2021a). These mice weighed less despite increased food intake, primarily as a result of increased energy expenditure and reduced fat mass accretion. BCAA restriction also improved glucose and pyruvate tolerance equivalently to a PR diet.

As BCAA restriction was extremely successful in promoting metabolic health without negative side effects and could be fed for sustained periods of time in mice, we and others have tested the effects of restricting dietary BCAAs on diet-induced obese mice and other diabetic rodent models. In agreement with the hypothesis that elevated BCAAs contribute to insulin resistance by increasing muscle lipotoxicity, a study using a 45% restricted BCAA diet completely normalized accumulation of fatty acyl CoAs and restored skeletal muscle insulin sensitivity in 6-week old Zucker fatty rats to levels found in lean rats (White et al., 2016). BCAA restriction also improves fatty acid oxidation and triglyceride levels in hearts of Zucker fatty rats and shifts fuel selection from glucose to fatty acid catabolism (McGarrah et al., 2020).

Mice eating a Western diet were transitioned to Western diets in which the three BCAAs or all amino acids were restricted by 67% at 18 weeks of age, approximately equivalent to a human in their middle twentys (Flurkey et al., 2007). These mice rapidly returned to a normal body composition, losing the adipose mass and weight gained during the previous 12 weeks of Western diet feeding in about 4 weeks. They also demonstrated substantial improvements in glucose tolerance and insulin sensitivity. Thus, decreasing BCAA consumption is potent enough to counteract an otherwise unhealthy Western diet and rescue a metabolically unhealthy mouse (Cummings et al., 2018).

BCAA restriction promotes fitness and longevity in mice

As a BCAA restricted diet is quite effective and pervasive in improving metabolic health, recapitulates many of the effects of a PR diet, and BCAAs are agonists of mTOR signaling, researchers have investigated the effects of a BCAA restricted diet on longevity. Consistent with the negative effect of BCAAs on longevity, dietary supplementation with extra BCAAs leads not only to impaired metabolic health, but to decreased lifespan (Cummings et al., 2018; Mu et al., 2018; Newgard et al., 2009; Solon-Biet et al., 2019). Mice fed a 50% or 80% restricted BCAA diet from 12 weeks of age did not live longer (Solon-Biet et al., 2019); similarly, a 67% BCAA restricted diet improved the metabolic health and reduced the frailty of male and female mice when started at 16 months of age, but did not increase lifespan (Richardson et al., 2021).

However, we have found that lifelong restriction of BCAAs by 67% extends the longevity of two short-lived mouse models of Hutchinson-Gilford Progeria Syndrome (Richardson et al., 2021). In wild-type mice, dietary restriction of BCAAs by 67% initiated at weaning reduces frailty and extends the lifespan of male, but not female, mice by over 30%. These animals displayed reduced mTORC1 signaling in multiple tissues, specifically in males (Richardson et al., 2021). In combination, these studies suggest that the precise level of restriction, time of diet initiation, and sex may play a role in determining if BCAA restriction will extend lifespan. Further, while the sex-

specific effects of BCAA restriction on mTORC1 signaling may explain the male-specific benefits of BCAA restriction on lifespan, the effect of BCAAs on mTORC1 activity are likely dispensable for the effects of reduced BCAA diets on metabolic health.

Dietary BCAAs in human health and longevity

In humans, acute BCAA supplementation has been extensively studied as a way for athletes and the elderly to build or preserve muscle mass. BCAA supplementation before and after exercise promotes muscle protein synthesis and decreases exercise induced muscle damage in humans (Howatson et al., 2012; Shimomura et al., 2004). In mice, BCAA supplementation improves body composition when wheel access and exercise is allowed (Platt et al., 2016), indicating that BCAA supplementation may benefit those who are regularly exercising. In **Chapter 2**, we examine high vs low protein diets in response to a validated model resistance training; the high protein diet provides 36% of calories from amino acids, including the BCAAs.

As more studies focus on circadian biology and nutrient timing, it will be interesting to see if BCAA supplementation yields improvements or disadvantages based on the time of administration even in non-exercising models. Elevated BCAAs are specifically associated with poor health outcomes in humans overall, and higher blood levels of isoleucine are associated with increased mortality while in humans higher dietary levels of isoleucine are associated with body mass index (Deelen et al., 2019; Yu et al., 2021a). However in the elderly, especially the frail elderly, BCAAs are decreased (Adachi et al., 2018; Chaleckis et al., 2016; Ottestad et al., 2018; Ter Borg et al., 2019). Similarly, in the elderly increased protein or essential amino acid supplementation improves frailty outcomes (Dillon et al., 2009), blood glucose control (Solerte et al., 2008), and lean mass (Solerte et al., 2008).

In addition to frailty, the risk of dementia, related neurological complications, and neurodegenerative diseases increase with age. Blood levels of BCAAs are elevated in the blood of humans with Alzheimer's disease (AD) as well as mouse models of the disease, and brain BCAA catabolism is impaired in the brains of models of AD by downregulation of *Bcat1* expression (Li et al., 2018a). Dietary supplementation of BCAAs leads to increased cognitive deficits, increased phosphorylation of Tau, and, when combined with a high-fat diet, leads to the premature death in the 3xTg mouse model of AD (Tournissac et al., 2018). Dietary restriction of BCAAs instead improves the cognitive performance of 3xTg AD mice (Tournissac et al., 2018). It's likely that increasing BCAA consumption may improve health outcomes or quality of life in some specific conditions or life stages, and future research should try to determine what factors predispose humans to respond positively to BCAA supplementation.

BCAA restriction as a clinical intervention

There are many factors that need to be considered in the application of experimental diets to human patients, mainly safety and feasibility. BCAA restricted diets could conceptually be used as a weight loss and insulin sensitizing intervention or to promote healthy longevity. Restricting BCAAs in diet induced obese mice induced rapid weight loss (Cummings et al., 2018), and this dramatic effect would likely not be tolerated well or sustained in humans. Though mice of both sexes tolerate lifelong BCAA restriction well, some females who began the diet in midlife suffered early mortality, which is an obvious safety concern. However, young mice and diet-induced obese mice do not adversely react to a diet restricted by two-thirds BCAAs, and this appears to be a safe dietary intervention if weight is carefully monitored.

Clinical experiments utilizing dietary BCAA restriction in humans are sparse; to date, there are two trials using this dietary regimen. The first study was conducted in metabolically healthy individuals and utilized whole foods in addition to medical-grade foods and formulas that are engineered for individuals with Maple Syrup Urine Disease (MSUD). MSUD is an autosomal

recessive disease in which mutations in BCKDH genes require limitation of the BCAAs to prevent neurotoxic build-up of BCAAs and BCKAs. Over the course of a week, the intentional reduction of dietary BCAAs resulted in reduced circulating BCAAs by 50%. Additionally, the intervention slightly reduced insulin resistance as measured by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) (Ramzan et al., 2020). Presumably, a longer intervention would yield significant improvements in insulin resistance and overall glucose homeostasis, but more clinical trials are needed, especially in populations with underlying metabolic conditions.

In a second clinical trial of BCAA restriction, BCAAs were reduced by supplementing subjects eating a low protein diet with either a complete AA mixture or one lacking BCAAs for one month. BCAA-restricted humans demonstrated lowered circulating BCAAs in several feeding states, especially during a mixed-meal tolerance test, and improved some measures of metabolic health. Postprandial insulin secretion was lowered, oral glucose sensitivity was improved, and FGF21 increased by 21% (Karusheva et al., 2019). These exciting results prove efficacy and feasibility of decreasing BCAA consumption in humans, at least for a short period of time.

However, a recent study of mice feeding periodized PR found that many benefits were transient and reversed after PR ended, and it's unknown if this will apply to humans on a protein or BCAA restricted diet (Li et al., 2018b). Future studies should extend BCAA restricted periods, and track metabolic parameters after the diet ends, to see how interventions like these affect long term health. Other solutions could involve registered dietitian designed diets that naturally control BCAA intake. The outcomes of dietary BCAA restriction are summarized in **Figure 2**.

Distinct metabolic effects of the individual BCAAs

While BCAAs are commonly grouped together and referred to collectively, there is evidence that these AAs can have individual effects, as alluded to previously. To test the effects

of individual BCAA restriction on metabolic health, mice were fed a new series of individually restricted BCAA diets. Restricting leucine instead of all BCAAs does not improve metabolic health—rather, it slightly increases adiposity (Fontana et al., 2016; Yu et al., 2021a). Interestingly, valine and isoleucine restriction both improved metabolic health. Specific valine deprivation has also been shown to reduce leukemic burden and increase survival in mice with acute lymphoblastic leukemia (ALL) by reducing expression of valine tRNAs that are typically upregulated in this condition (Thandapani et al., 2022). Furthermore, adding isoleucine or valine back to a diet with low levels of all other AAs markedly reduced the benefits to metabolic health. Overall, while reduction of isoleucine and valine proved to drive the benefits of a BCAA-restricted diet, and leucine often had no effect when manipulated in diet alone, isoleucine restriction elicited the most potent improvements to metabolic health in all conditions tested. Through use of genetically altered mice, it was determined that isoleucine restriction improves metabolic health independently of hepatic mTORC1 and GCN2, and that FGF21 may play a role in these benefits (Yu et al., 2021a).

Further, when male and female C57BL/6J mice were given either a normal fat control, high fat control, high fat low isoleucine, or high fat low BCAA diet (**Figure 3A**), a low isoleucine diet resulted in greater weight loss than a low BCAA diet (**Figure 3B-C, F-G**). Despite this, both the BCAA restricted and low isoleucine diet increased energy expenditure (**Figure 3 J, L**). Overall, these data show that while BCAAs are often referred to collectively and grouped together in analysis, they yield distinct physiological roles.

It’s curious that the individual BCAAs do not produce equal effects when restricted. The catabolism of these AAs is regulated by the same kinases, though their end products differ by glucogenic and ketogenic properties. In a standard chow diet, leucine is the most abundantly fed BCAA, and isoleucine is the least abundant, so it’s tempting to think that when restricted by 67%, isoleucine and valine have crossed some threshold that results in improved metabolic health.

Indeed, when leucine is restricted by 80-85%, there are benefits to metabolic health, and FGF21 was only induced after 85% leucine restriction (Lees et al., 2017; Wanders et al., 2015). However, even in experiments that eliminate individual BCAAs, differences among their effects were still observed. Xiao *et al*. showed that though eliminating any individual BCAA rapidly reduces weight and improves insulin sensitivity, only valine and isoleucine elimination improved glucose tolerance and lowered fed blood glucose levels, possibly through decreased expression of key gluconeogenic genes (Xiao et al., 2011; Xiao et al., 2014). Furthermore, one study of methylmalonyl-coA mutase heterozygosis in mice, which prevents complete valine and isoleucine oxidation to succinyl-CoA, resulted in susceptibility to insulin-resistant obesity (Lerin et al., 2016). However, the distinctions among the BCAAs that influence metabolic heath are still unclear, and future work should determine if and how diets limited in leucine, isoleucine, and valine are differently sensed.

Finally, as isoleucine restriction produces such potent effects compared to the other BCAAs, and as BCAA restriction extends longevity, feeding an isoleucine restricted diet and testing the effects on longevity is a logical next step. A recent study examined this in *Drosophila,* and found that intermittent cycles of Ile deprivation increases lifespan (Fulton et al., 2024). Further, an Ile-restricted diet was recently found to prolong lifespan in heterogenous mice (Green et al., 2023b), and the mechanism behind this extension is further examined in FGF21 WT and KO mice in **Chapter 4**. Though isoleucine is the BCAA most influential in improving metabolic health, metabolic health is not always linked to increased lifespan. Several groups have now shown that insulin sensitivity and longevity can be uncoupled in CR (Arriola Apelo et al., 2020; Lamming et al., 2012; Selman et al., 2008; Yu et al., 2019), however, BCAA restricted mice of both sexes are also less frail with age. It will be interesting to determine if isoleucine restriction recapitulates these benefits on frailty and longevity.

Sexual dimorphism in response to protein and BCAA restriction

Sex differences in longevity have been observed in almost every species studied, but there is a clear lack of research evenly into both sexes (Austad and Fischer, 2016; Zucker and Beery, 2010). Modern longevity studies have found substantial effects of sex on lifespan and disease burden (Le Couteur et al., 2018; Mitchell et al., 2016), so it is perhaps unsurprising that sexually dimorphic effects have also been found in mice subject to protein and BCAA restriction.

As previously discussed, consumption of a 67% BCAA restricted diet starting in midlife improved metabolic health and reduced frailty in both sexes, though this diet only extended lifespan in males when initiated at a young age (Richardson et al., 2021). Intriguingly, this was also the case with respect to PR; a PR diet started early in lifespan only extended the lifespan of males (Richardson et al., 2021). The only other study evaluating BCAA restriction and longevity to date did not find any difference in either sex with longevity, and found most effects were not dimorphic, though this study used a more strict level of BCAA restriction than ours (Solon-Biet et al., 2019). Some insights into these results can be drawn from research into dietary protein intake.

A recent publication tested how different levels of dietary protein impacted the metabolic health and molecular profile of multiple strains of male and female mice. While some phenotypes were conserved across strains and sexes, including increased glucose tolerance and energy expenditure, there was large variability in adiposity, insulin sensitivity, and circulating hormones with sex, strain and age of onset (Green et al., 2022b). This study also demonstrated that short-term PR was effective at improving metabolic health when started much later in life, and the pattern of sexual dimorphism was altered at old age.

Another experiment testing different protein:carbohydrate ratios in both sexes on reproduction and lifespan found that female mice maximize reproductive health and longevity at different ratios compared to male mice. Male longevity was optimized at lower ratios than females, indicating that the degree of PR increasing male lifespan is more severe than in females.

Additionally, reproductive function is maximized on higher ratios in both sexes, meaning that higher protein intake is needed for optimal reproductive health (Solon-Biet et al., 2015b). This has also been demonstrated in *Drosophila melanogaster*, as females suppress egg production on low protein, high carbohydrate diets (Lee, 2015).

These results are useful when thinking about BCAA restriction, as it's conceivable that to increase lifespan in females, a different levels of limitation may be needed. At the two-thirds level of restriction tested, females did not have diminished mTORC1 activity (Richardson et al., 2021). Future experiments could test different degrees of BCAA restriction and measure how mTORC1 signaling and lifespan respond in female mice. Furthermore, effects on reproductive health may differentially influence female vs. male longevity, and it would be interesting to determine how BCAA restriction influences reproductive health in both sexes.

Future directions in BCAA research metabolism and aging

The role of FGF21

Probably the largest remaining question in the work presented so far is the role of FGF21 in the response to BCAA or isoleucine restriction. As FGF21 is essential for the metabolic benefits and lifespan extension of PR (Hill et al., 2022b; Laeger et al., 2014), and as BCAA and isoleucine addback to a PR diet blunts or eliminates these metabolic benefits (Yu et al., 2021a), it's logical to hypothesize that FGF21 is essential for the effects we observe in BCAA and isoleucine restriction, which is examined in **Chapter 4**. Several studies have examined the effect of BCAA restriction on FGF21 levels in mice. Two studies found that BCAA restriction did not increase blood levels of FGF21 (Fontana et al., 2016; Solon-Biet et al., 2019), while a third observed increased FGF21 expression in aged males fed a Low BCAA diet (Richardson et al., 2021). Another study observed that BCAA restriction study in the context of a Western diet temporarily

increased FGF21 (Cummings et al., 2018). Two studies of BCAA repletion in PR have presented divided results, with one reporting that BCAA addback did not blunt increases in FGF21 by PR (Maida et al., 2017), and the other showing a slight decrease (Mu et al., 2018).

The role of FGF21 in the response to specific restriction of isoleucine is a bit clearer; isoleucine restriction strongly raises FGF21 levels and induces *Fgf21* transcription in multiple tissues. Moreover, deletion of *Fgf21* blocks isoleucine-induced increases in food consumption and energy expenditure (Yu et al., 2021b). However, *Fgf21*$^{-/-}$ mice on a low isoleucine diet still displayed improved glucose tolerance and body composition (Yu et al., 2021b). Modifying hepatic AA sensing by deletion of *Gcn2* or *Tsc1* (Tuberous sclerosis complex 1—an upstream negative regulator of mTORC1) does not alter the metabolic response to isoleucine restriction (Yu et al., 2021b). Another study has shown that hepatic ATF4 can activate and increase FGF21 expression without GCN2 activity, though this process is delayed (Laeger et al., 2016). The molecular mechanisms by which FGF21 levels are increased by isoleucine restriction, and the molecular processes by which dietary isoleucine restriction promotes glucose tolerance, reduces adiposity, and impacts longevity remain to be determined, but are examined in **Chapter 4** of this thesis.

Interestingly, our lab recently discovered that the metabolic and molecular response to PR has sex- and strain-dependent effects (Green et al., 2022b). Though many benefits of PR diets are attributed to FGF21, these studies have primarily been conducted in C57BL/6J males. Surprisingly, we observed that while blood levels of FGF21 are strongly induced by PR in C57BL/6J males, this increase was more muted in C57BL/6J females, and not observed in DBA/2J or HET3 mice of either sex. Further, these results uncoupled increased energy expenditure on PR diets and metabolic health improvements, and in DBA females, EE negatively correlated with hepatic *Fgf21*. These results suggest that many PR outcomes may be independent of changes in FGF21. In **Chapter 3**, the role of FGF21 in response to isoleucine restriction in the context of a Western diet is evaluated in multiple strains and mice of both sexes.

Conclusions

There are still questions surrounding dietary AAs, metabolism, and longevity that remain unanswered by the current literature, especially in humans. The work discussed above raises new questions about how the amount and quality of protein intake influences health, and suggests that perhaps these dietary recommendations should be addressed at a personalized level with careful consideration of age, sex, and physical activity level. Further research into the areas specified in this review, and specifically the experiments discussed in this thesis, will aid our understanding of the molecular mechanisms which underlie the benefits of BCAA, isoleucine, and protein restriction. As nutrition and metabolism studies translate from the bench to patient bedside, specific protein recommendations may become personalized to one's circulating amino acid levels and genetics to promote optimal health and longevity.

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Figure 1.

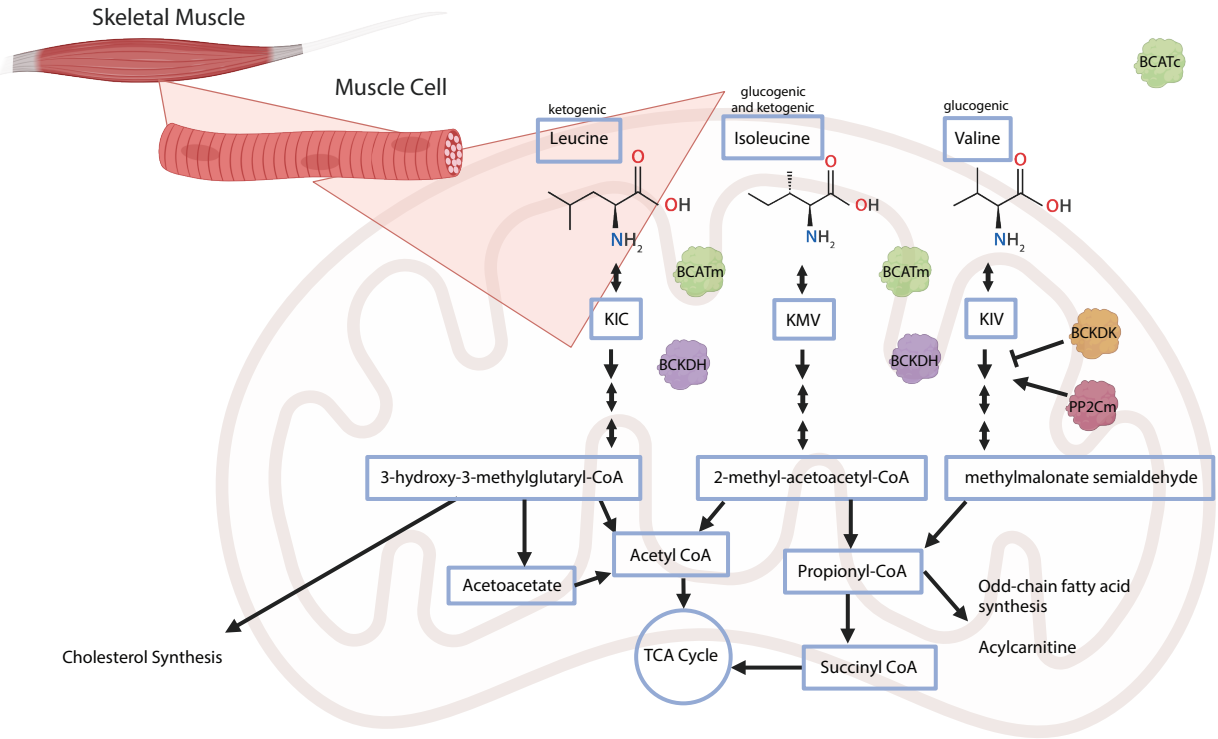
Skeletal Muscle
Muscle Cell
BCATc
ketogenic
Leucine
glucogenic and ketogenic
Isoleucine
glucogenic
Valine
BCATm
BCATm
KIC
KMV
KIV
BCKDH
BCKDH
BCKDK
PP2Cm
3-hydroxy-3-methylglutaryl-CoA
2-methyl-acetoacetyl-CoA
methylmalonate semialdehyde
Acetyl CoA
Acetoacetate
Propionyl-CoA
Odd-chain fatty acid synthesis
Acylcarnitine
Cholesterol Synthesis
TCA Cycle
Succinyl CoA

Figure Legends

Figure 1: BCAA catabolism.

Simplified outline of the process of BCAA catabolism into their respective ketogenic or glucogenic substrates by the skeletal muscle, though the first catabolic step, deamination, can also occur in many other tissues (but not in liver hepatocytes). KIC=α-ketoisocaproate, KMV=α-keto-methylvalerate, KIV=α-ketoisovalerate.

Figure 2.

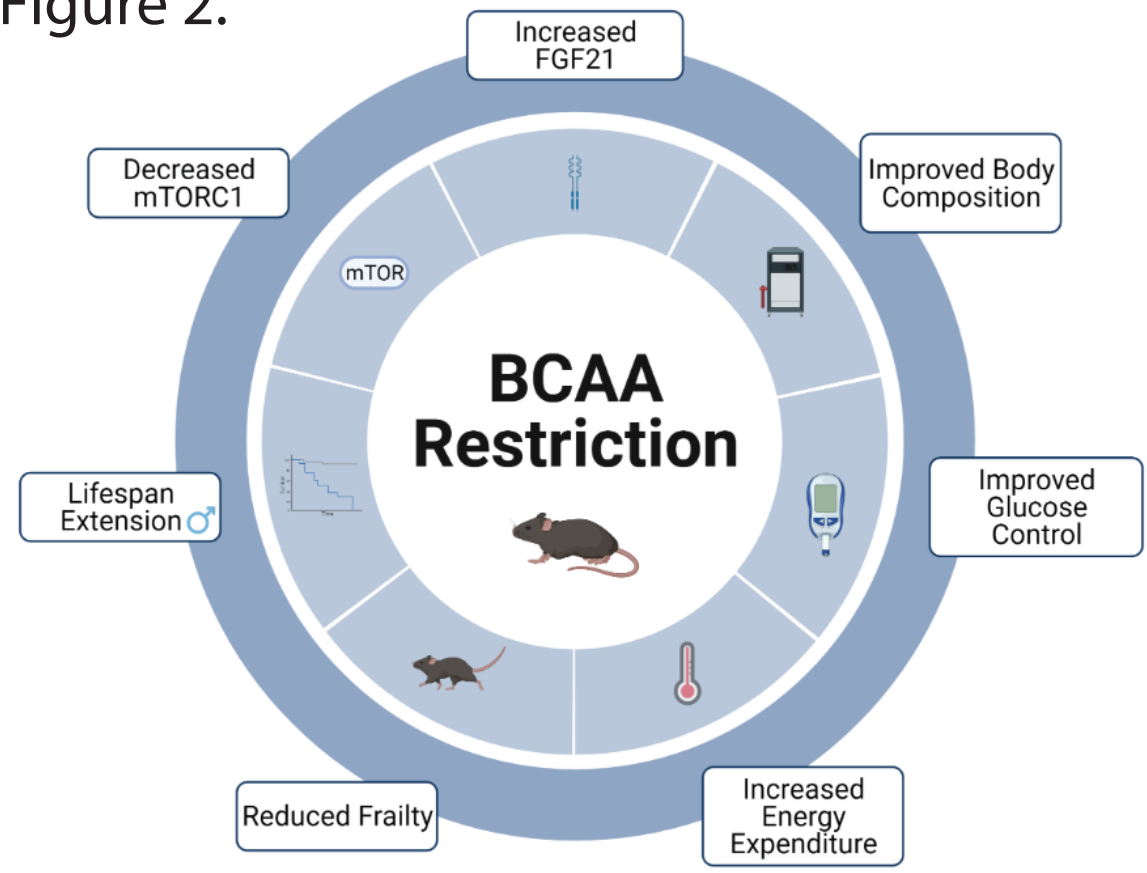
Increased FGF21
Improved Body Composition
Improved Glucose Control
Increased Energy Expenditure
Reduced Frailty
Lifespan Extension ♂
Decreased mTORC1
mTOR
BCAA Restriction

Figure 2. BCAA Restriction improves health and extends lifespan.

Visual summary of the effects of restricting dietary BCAAs on molecular signaling, healthspan and longevity.

Figure 3.

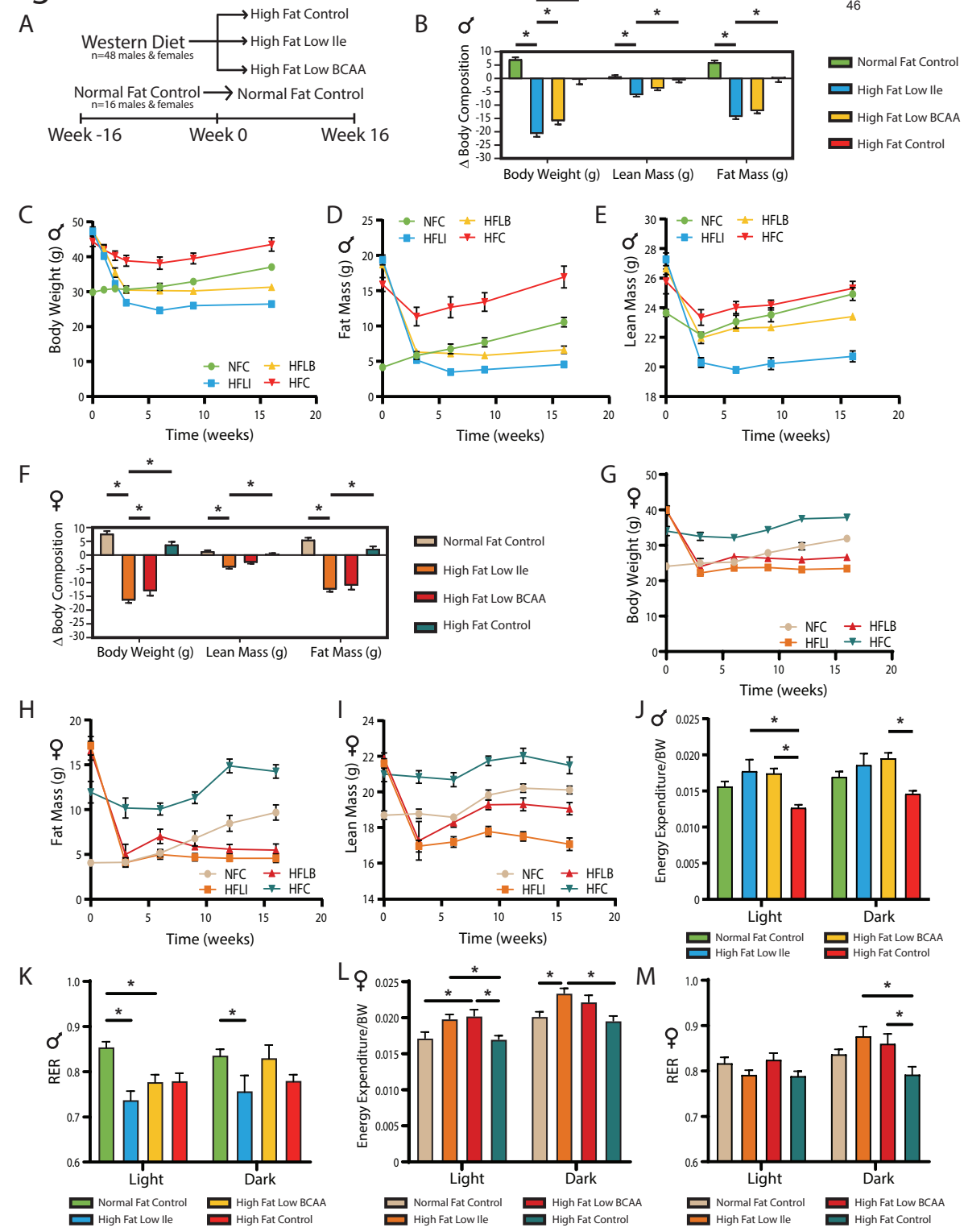
A
Western Diet
n=48 males & females
High Fat Control
High Fat Low Ile
High Fat Low BCAA
Normal Fat Control
n=16 males & females
Normal Fat Control
Week -16
Week 0
Week 16
B
♂
Δ Body Composition
Body Weight (g)
Lean Mass (g)
Fat Mass (g)
Normal Fat Control
High Fat Low Ile
High Fat Low BCAA
High Fat Control
C
Body Weight (g) ♂
Time (weeks)
NFC
HFLB
HFLI
HFC
D
Fat Mass (g) ♂
Time (weeks)
E
Lean Mass (g) ♂
Time (weeks)
F
♀
Δ Body Composition
Body Weight (g)
Lean Mass (g)
Fat Mass (g)
Normal Fat Control
High Fat Low Ile
High Fat Low BCAA
High Fat Control
G
Body Weight (g) ♀
Time (weeks)
H
Fat Mass (g) ♀
Time (weeks)
I
Lean Mass (g) ♀
Time (weeks)
J
♂
Energy Expenditure/BW
Light
Dark
Normal Fat Control
High Fat Low Ile
High Fat Low BCAA
High Fat Control
K
♂
RER
Light
Dark
Normal Fat Control
High Fat Low Ile
High Fat Low BCAA
High Fat Control
L
♀
Energy Expenditure/BW
Light
Dark
Normal Fat Control
High Fat Low Ile
High Fat Low BCAA
High Fat Control
M
♀
RER
Light
Dark
Normal Fat Control
High Fat Low Ile
High Fat Low BCAA
High Fat Control

Figure 3: FGF21 deletion reduces, but does not ablate, increased energy expenditure and food consumption by dietary isoleucine restriction.

(A) Experimental design.

(B) Change in male body composition from week 0 to week 16 in males.

(C-E) Male body composition over time. (C) Body weight, (D) fat mass, and (E) lean mass.

(F) Change in female body composition from week 0 to week 16 in males.

(G-I) Male body composition over time. (C) Body weight, (D) fat mass, and (E) lean mass.

(J-M) Male (J-K) and female (L-M) energy expenditure per BW (J, L) and RER (K, M).

n=8 per group. For body composition change, statistics for the overall effects of diet, body composition category, and the interaction represent the p value from a two-way RM ANOVA conducted individually for each sex. *$p<0.05$, Dunnett's post-test examining the effect of parameters identified as significant in the two-way ANOVA.

Chapter 2: Resistance exercise protects mice from protein-induced fat accretion

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Author Contributions

MET, TH, WZ, AK, CE, LB, and DWL conceived of and designed the experiments. MET, LB, EZ, MG, MS, CY, and CE performed the experiments. MET, CE, and DWL analyzed the data. MET and DWL secured funding and supervised personnel. MET and DWL wrote the manuscript.

Abstract

Low protein (LP) diets extend the lifespan of diverse species, and are associated with improved metabolic health in both rodents and humans. Paradoxically, many athletes and bodybuilders consume high protein (HP) diets and protein supplements, yet are both fit and metabolically healthy. Here, we examine this paradox using weight pulling, a validated progressive resistance exercise training regimen, in mice fed either an LP diet or an isocaloric HP diet. We find that despite having lower food consumption than the LP group, HP-fed mice gain significantly more fat mass than LP-fed mice when not exercising, while weight pulling protected HP-fed mice from this excess fat accretion. The HP diet augmented exercise-induced hypertrophy of the forearm flexor complex, and weight pulling ability increased more rapidly in the exercised HP-fed mice. Surprisingly, exercise did not protect from HP-induced changes in glycemic control. Our results confirm that HP diets can augment muscle hypertrophy and accelerate strength gain induced by resistance exercise without negative effects on fat mass, and also demonstrate that LP diets may be advantageous in the sedentary. Our results highlight the need to consider both dietary composition and activity, not simply calories, when taking a precision nutrition approach to health.

Introduction

In contrast to the long-standing idea that “a calorie is a calorie,” research over the last two decades has shown that calories from different macronutrient sources have distinct impacts on health (Hall et al., 2015). Dietary protein in particular has been shown to have a critical role in the regulation of metabolic health and longevity; low protein (LP) diets are associated with reduced rates of diabetes and other age related diseases in human longitudinal studies, and promote leanness and insulin sensitivity in human randomized clinical trials of metabolically unhealthy adults (Ferraz-Bannitz et al., 2022a; Fontana et al., 2016; Levine et al., 2014; Sluijs et al., 2010). In mice, low protein, high carbohydrate diets reduce adiposity, improve glucose tolerance, and extend lifespan (Hill et al., 2022b; Richardson et al., 2021; Solon-Biet et al., 2014; Solon-Biet et al., 2015a).

The idea that low protein diets are beneficial – and high protein diets are deleterious – challenges conventional wisdom. Several high-protein “fad” weight-loss diets have been popularized over the last two decades, and many researchers and medical professionals support consuming more protein to support healthy aging (Rodriguez, 2015). In addition to promoting satiety, some short-term studies have found that high protein intake improves glucose control in adults with type 2 diabetes (Dong et al., 2013; Gannon et al., 2003; Seino et al., 1983), and high protein diets are recommended for physically active individuals to support muscle growth and strength improvements (Andersen et al., 2005; Willoughby et al., 2007). Both exercise and amino acids activate the mechanistic target of TOR (mTOR) protein kinase, which stimulates the protein synthesis machinery needed to stimulate skeletal muscle hypertrophy (Schiaffino et al., 2021). To support this process, The Academy of Nutrition and Dietetics recommends consuming 1.2-2.0 grams of protein per kg of body weight (BW) per day in physically active individuals (Thomas et al., 2016). These protein estimations are substantially higher than the Recommended Daily Allowance (RDA) for sedentary people of 0.8 g/kg body

weight, an amount intended to provide sufficient nutrients in 97.5% of people, but it remains controversial if protein intake above this level is detrimental in this population.

If high dietary protein intake was always deleterious, the many individuals consuming high protein diets or taking widely sold protein or BCAA supplements to maximize muscle growth following resistance training would have higher adiposity and an increased risk of diabetes. However, there is no data available to support such a surprising conclusion, and indeed the opposite is true: exercise reduces the risk of developing type 2 diabetes (Grontved et al., 2012). We are therefore left with a paradox: while the human and animal data suggesting that increased dietary protein intake is detrimental for metabolic health and increases the risk for numerous age-related diseases is robust, some of the people consuming the highest levels of protein – athletes – are metabolically very healthy.

Here, we examine the possibility that exercise can protect mice from the deleterious metabolic effects of a high protein diet normally observed in sedentary mice. In order to evaluate the metabolic effects of exercise and dietary protein intake independent of weight loss, we utilized a recently validated method of progressive resistance exercise for mice that does not cause weight loss (Zhu et al., 2021b) to assess the interaction of dietary protein content and exercise in male C57BL/6J mice. We fed mice either a low protein (LP, 7% of calories from protein) or a high protein (HP, 36% of calories from protein) diet, and either pulled an increasing load of weight down a track 3x per week for 3 months, or pulled a sham unloaded cart. During the course of the experiment, we comprehensively assessed weight and body composition, metabolic parameters, and the fitness of the mice on these regimens. Finally, we euthanized the mice to determine the effect of both diet and exercise on tissue weight and muscle mass, muscle fiber type cross-sectional area and distribution, and muscle mitochondrial respiration.

In agreement with our hypothesis that HP diets impair metabolic health in sedentary mice, we found that HP-fed sham-exercised mice gained excess fat mass and increased in

adiposity relative to LP-fed mice. In sharp contrast, HP-fed mice that engaged in resistance exercise showed hypertrophy of specific muscles and were protected from fat accretion. However, exercise did not protect HP-fed mice from the effects of protein on blood sugar control. Interestingly, while exercising HP-fed mice gained strength more rapidly than exercising LP-fed mice, the difference in maximum weight that could be pulled by each mouse was not significant, but both the HP diet and weight training regimen increased muscle diameter and in general, size. Interestingly, neither diet nor exercise altered mitochondrial respiratory capacity. Our research shows that resistance exercise protects from HP-induced increases in adiposity in mice, and suggests that metabolically unhealthy sedentary individuals consuming a HP diet or protein supplements might benefit from either reducing their protein intake or beginning a resistance exercise program.

Results

Resistance exercise protects against high-protein induced fat and body weight gain

Seven-week-old C57BL/6J mice were randomized to groups of equal weight and body composition, and placed on either a Low Protein (LP, 7% of calories from protein) or High Protein (HP, 36% of calories from protein) diet. Of note, while the protein content of rodent chow varies, typically 17-24% of calories are derived from protein (Tuck et al., 2020). The two diets were isocaloric, as calories from fat were kept at 19%, and carbohydrates were reduced in the HP diet to compensate for the increased calories from amino acids. We have previously utilized this same LP diet, and the full composition of both diets can be found in **Table 1**. Throughout the course of the study, weight was collected weekly and body composition was analyzed every three weeks via EchoMRI (**Fig. 1A**). Starting after five weeks on the diets, we exercised half of

the mice on each diet using a recently validated mouse model of resistance exercise training, weight pulling (WP), in which mice pull progressively heavier weights down a track; the other half of the mice were sham-exercised by pulling an unloaded cart.

On both a per-animal and a weight-normalized basis, LP-diet fed mice ate more than HP-fed mice, consistent with the satiating effect of dietary protein (**Fig. 1B-C**). There was no effect of WP on food consumption (**Fig. 1B-C**). Despite consuming fewer calories than LP-fed mice – the two diets have the same caloric density – HP-fed mice gained more weight over the course of 18 weeks. By the end of the experiment, HP-fed WP mice had gained significantly less weight than HP-fed sham-exercised controls (**Fig. 1D**). As we expected based on previous studies investigating the effects of a LP diet as well as data demonstrating the beneficial effects of protein on muscle growth (Andersen et al., 2005; Willoughby et al., 2007), HP-fed mice gained significantly more lean mass than LP-fed mice, although surprisingly exercise did not affect this gain (**Fig. 1E**). In accordance with our previous results, we found that sham-exercised HP-fed mice gained substantially more body fat than their LP-fed counterparts; however, we found that WP was protective against these effects (**Fig. 1F**).

At the conclusion of the experiment, we euthanized the animals and measured the weight of several fat depots and the liver. We found that the inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) were significantly heavier in sham-exercised HP-fed mice than in WP HP-fed mice (**Figs. 1G-H**). While there was no statistically significant effect on the mass of brown adipose tissue (BAT), the weight of this depot also trended higher in sham-exercised HP-fed mice than in WP HP-fed mice (**Fig. 1I**). Finally, there was a statistically significant effect of diet on liver weight, with HP-fed mice having heavier livers, an effect that was larger in sham-exercised mice than in WP mice (**Fig. 1J**).

We collected portions of the liver and iWAT for Oil RedO and H&E staining, respectively, and quantified the size and number of hepatic lipid droplets and adipocytes. Despite the

increased liver weight of the HP groups, they did not have increased lipid accumulation; instead, there was a non-significant trend towards both larger and a greater number of liver lipid droplets in the LP-fed groups, with no effect of weight pulling (**Figs. 2A-C**). There was no difference in average iWAT adipocyte size or number (**Figs. 2D-F**).

Mice fed a low protein diet have better glycemic control and higher energy expenditure than high protein-fed mice

We and others have previously shown that consumption of a low protein diet improves glucose tolerance and insulin sensitivity, while the literature suggests that resistance exercise improves insulin sensitivity in mice and humans (Cui et al., 2020; Fontana et al., 2016; Green et al., 2022b; Kullmann et al., 2022; Laeger et al., 2014; McLeod et al., 2019; Westcott, 2012; Yu et al., 2021a). We performed glucose and insulin tolerance tests; in agreement with our previous findings, we found that LP-fed mice had improved glucose tolerance relative to HP-fed mice, and there was a trend ($p=0.0788$) towards a positive effect of WP on glucose tolerance (**Fig. 3A**). Similarly, while LP-fed mice were more insulin sensitive than HP-fed mice under sham conditions, we did not observe a difference between LP and HP-fed mice that performed WP (**Fig. 3B**).

In agreement with a model in which high dietary protein is deleterious to sedentary animals, we observed that HP-fed mice had higher fasting blood glucose than LP-fed mice under sham exercise conditions (**Fig. 3C**). Interestingly, WP did not correct this difference – instead, we observed that there was an overall effect of HP-feeding towards increased fasting blood glucose level in both sham-exercised and WP mice (**Fig. 3C**). A similar effect of diet was noted on fasting insulin levels, with HP-fed WP mice having significantly higher insulin levels than LP-fed WP mice (**Fig. 3D**). In agreement with the overall effect of diet on fasting glucose

and insulin levels, we also observed an overall effect of diet on insulin sensitivity calculated via the HOMA2-IR method, with LP WP mice having significantly better insulin sensitivity than HP-fed WP mice (**Fig. 3E**).

We and others have previously shown that a LP diet increases fasting FGF21 levels in C57BL/6J male mice (Green et al., 2022b; Hill et al., 2022b), and we observed that LP-fed sham mice had significantly higher levels of FGF21 in their blood compared to their HP-fed counterparts (**Fig 3F**). Interestingly, WP mice had a strong overall trend ($p=0.0751$) towards lower FGF21 levels, and LP-fed WP mice did not have significantly higher FGF21 levels than HP-fed WP mice (**Fig 3F**). FGF21 promotes ketogenesis, and in agreement with our FGF21 data we observed an overall effect of diet on fasting blood ketone levels, with LP-fed mice having higher ketone levels than HP-fed mice (**Fig. 3G**). Intriguingly, there was a significant interaction of diet and exercise, with WP lowering ketone levels in HP-fed mice.

Finally, we examined the effect of diet and exercise on blood lipids. Some studies have shown altered lipid levels in mice and humans fed a LP diet (Trevino-Villarreal et al., 2018), and we considered it likely that WP would promote a healthy lipid profile. Surprisingly, while diet had no effect on triglyceride or lipid levels, there was an overall increase of triglycerides in WP-fed mice, which was statistically significant in HP-fed mice (**Fig. 3H**). There was no effect of diet or exercise on blood levels of cholesterol (**Fig. 3I**).

In order to learn more about the effects of the HP diet on weight and adipose gain, we used metabolic chambers to evaluate multiple components of energy balance, including food consumption, spontaneous activity and energy expenditure. In agreement with our prior home-cage observations and the well-known satiating effect of dietary protein, we observed that HP-fed mice tended to consume less food than LP-fed mice, although this did not reach statistical significance. (**Supplemental Fig. 1A**). There was no overall effect of exercise on spontaneous

activity, but in the light phase there was an overall effect of diet, with LP-fed mice moving more (**Supplemental Fig. 1B**).

We examined respiratory exchange ratio (RER) by calculating the ratio of O_2 consumed and CO_2 produced; an RER of close to 1.0 indicates that carbohydrates are being preferentially utilized for energy production, while a value near 0.7 indicates that lipids are the predominant energy source (Bruss et al., 2010; Yu et al., 2019). As we anticipated, there was an overall effect of diet on RER, with mice consuming the LP diet having a higher RER during the dark cycle, reaching statistical significance in the WP groups (**Supplemental Figs. 1C-D**). In agreement with previous studies, we observed a significant effect of the LP diet on energy expenditure, both on a per animal basis and when weight was considered as a covariate (**Supplemental Figs. 1E-F**). Resistance exercise did not have an overall effect on either RER or energy expenditure.

Training while consuming a LP diet does not compromise maximum strength or coordination

Prior dogma suggests that in order to maximize strength and muscle hypertrophy from resistance exercise, consumption of a large quantity of high-quality protein is necessary (Phillips et al., 2005; Thomas et al., 2016). In agreement with this, we observed an significant overall effect of diet on the maximum load mice were able to pull during their training sessions as well as the corresponding area under the curve (AUC) (**Fig. 4A**), consistent with HP-fed mice being able to pull more weight. However, the difference was smaller than we had anticipated, and was statistically significant only during the third week of the training regimen; the maximum weight pulled by LP-fed and HP-fed mice appeared to converge after approximately 10 weeks of training (**Fig. 4A**). There was no overall effect of diet on the average number of sets per training bout (**Fig. 4B**) or the average number of stimulatory touches by the investigator (**Fig. 4C**).

The strength gains from WP did not translate into improved performance on two other tasks assessing muscle strength and function, inverted cling and rotarod (**Fig. 4D-G**). We observed an overall positive effect of a LP diet on inverted cling time, an effect that persisted (p=0.0597) in sham-exercised mice when we analyzed inverted cling performance with weight as a covariate (**Fig. 4D**). Although we observed no overall effect of either diet or training on raw rotarod performance (**Fig. 4F**), we observed a significant positive effect of a HP diet on the rotarod performance of sham-exercised mice when weight was considered as a covariate (**Figs. 4G**).

Weight pulling and high protein diet promote muscle hypertrophy without impacting mitochondrial respiration

At the conclusion of the weight training regimen and the *in vivo* experiments described above, we euthanized the animals and collected numerous tissues. As noted above, WP in HP-fed mice reduced the mass of iWAT, eWAT, and liver (**Figs. 1F-I**). We also collected a diverse array of skeletal muscles from different regions of the body. We have previously shown that weight pulling induces hypertrophy in the flexor digitorum longus (FDL) (Zhu et al., 2021b), and as we expected we found an overall positive effect of both a HP diet and weight pulling on FDL mass, both in absolute terms and when normalized to either body mass or tibia length (**Figs. 5A-C**). We observed the greatest muscle mass in mice that completed the WP regimen while consuming a HP diet, but HP diet consumption alone also had a positive effect on absolute FDL mass and FDL mass normalized to tibia length (**Figs. 5A-C**).

In humans, mitochondrial respiration is increased after both aerobic and resistance training regimens (Konopka et al., 2015; Konopka et al., 2019; McKenna et al., 2022; Robinson et al., 2017), perhaps due to the high energetic demands imposed by protein synthesis (Rolfe and Brown, 1997; Waterlow, 1984), which is upregulated following resistance training

(Ogasawara et al., 2016). However, the mitochondrial response to varying amounts of dietary protein with or without resistance training has not been examined, and we therefore performed high-resolution respirometry in permeabilized muscle fibers from the FDL. Using complex-I linked substrates pyruvate, glutamate, and malate we found no change to leak respiration (**Fig. 5D**). We next evaluated sub saturating and saturating complex-I driven respiration by providing ADP at 0.25mM, 0.5mM and 5.5mM; none of these parameters were different in any intervention group (**Figs. 5E-G**). Furthermore, we saw no difference in any group following succinate addition for complex I+II linked respiration (**Fig. 5H**).

We examined the effect of diet and weight pulling on the mass of the quadriceps, soleus, plantaris, and the forearm flexor complex, measuring mass in absolute terms (**Supplemental Figs. 2A-D**) and normalized to tibia length (**Supplemental Figs. 2E-H**). Overall, there was no positive effect of either resistance exercise or high protein diet on quadricep mass (**Supplemental Figs. 2A, E**), while the mass of the soleus and plantaris muscles were positively impacted by dietary protein but largely resistant to the benefits of WP (**Supplemental Figs. 2B-C, F-G**). However, there were strong effects of WP and a significant interaction between dietary protein and exercise on the mass of the forearm flexor complex, with the HP-fed WP mice having the greatest forearm flexor complex mass in raw weight and when normalized to tibia length (**Supplemental Figs. 2D, H**).

Finally, we assessed the maximum diameter of the bicep and forearm (**Fig. 6A**) and found that there were clear overall effects of both dietary protein and weight pulling on bicep and forearm diameter (**Fig. 6B-C**). Bicep diameter was maximized under high protein weight pulling conditions (**Fig. 6A-B**). There was a less potent but still significant effect on the forearm; HP-fed WP mice had the largest forearm diameter of all groups (**Fig. 6C**). Conversely, non-exercising LP-fed mice had the smallest bicep and forearm diameter (**Figs. 6A-C**).

Next, we further analyzed the FDL through staining (**Fig. 7A**) and quantification (**Fig. 7B-H**). There was no significant difference in mid-belly cross-sectional area (CSA) or the number of fibers per cross section (**Fig. 7B-C**), indicating that alterations in dietary protein or weight pulling might not induce hyperplasia of muscle fibers (Jorgenson and Hornberger, 2019). There was, however, an overall effect of diet on the CSA of muscle fibers, with individual fibers in HP-fed mice having greater CSA (**Fig. 7D**).

When we analyzed fiber type specific CSA, we observed an overall effect of diet on the size of type IIA, type IIB, and type IIX fibers, with HP diets increasing fiber size (**Figs. 7F-H**). We also saw an effect of resistance exercise on the size of type IIA and type IIX fibers, with WP inducing fiber hypertrophy (**Figs. 7F, 7H**). Finally, we observed a significant interaction between dietary protein and WP on the size of type I fibers, with the greatest hypertrophy occurring in HP-fed WP mice (**Figs. 7E**).

Discussion

Dietary protein is a critical regulator of a wide variety of biological processes that determine metabolic health and lifespan in diverse species (Trautman et al., 2022). Consumption of protein is generally thought of as good, promoting muscle growth and strength particularly in combination with exercise, and skeletal muscle function and mass is associated with a host of health benefits including a reduced risk for diabetes and frailty (Liao et al., 2019; Phillips, 2007). Paradoxically though, in sedentary humans increased consumption of dietary protein is associated with cancer, cardiovascular disease, and diabetes as well as increased mortality (Lagiou et al., 2007; Levine et al., 2014; Mittendorfer et al., 2020; Sluijs et al., 2010; Zhang et al., 2020). In agreement with the potential for dietary protein to negatively impact health, low protein diets and diets with reduced levels of specific essential amino acids promote

healthspan and lifespan in flies and rodents (Flores et al., 2022; Juricic et al., 2020; Lee et al., 2014; Lees et al., 2014; Orentreich et al., 1993; Richardson et al., 2021; Solon-Biet et al., 2014), and short-term protein restriction improves the metabolic health of metabolically unhealthy adult humans (Ferraz-Bannitz et al., 2022a; Fontana et al., 2016).

We know that many of the humans deliberately consuming high protein diets or consuming protein supplements to support their exercise regimen are not metabolically unhealthy – indeed, many of these individuals have commendable metabolic health (Antonio et al., 2015; Antonio et al., 2016; Grontved et al., 2012). We considered that a potential solution to this paradox is that exercise itself is protective from the effects of high protein diets; our and others previous studies examined sedentary mice and largely sedentary humans. Here, we investigated this hypothesis by feeding isocaloric diets with either high or low levels of dietary protein to mice and subjecting them to a recently validated mouse model of progressive resistance exercise, weight pulling (WP) or sham control exercise. Importantly, unlike aerobic exercise models like treadmill running, WP does not result in weight loss, allowing us to study the metabolic impact of exercise without confounding changes in body weight. We also took advantage of this opportunity to rigorously test long-standing assumptions regarding the effects of dietary protein and resistance exercise on muscle mass and function.

In agreement with previous studies by our lab and others, we found that in sham-exercised mice, LP-fed mice were metabolically healthier than HP-fed animals, remaining leaner and having better glycemic control; conversely, HP-fed animals accumulated fat mass and had worse glycemic control. However, when HP-fed mice were subjected to WP, the negative effects of the HP diet on overall fat mass gain as well as on individual fat depots were completely blocked. Surprisingly, although WP did further lower fasting blood glucose in LP-fed mice and these mice had a tendency towards improved glucose tolerance and lower fasting

insulin, the negative effect of the HP diet on glucose tolerance and insulin sensitivity was not blocked by WP.

While HP-feeding did, as we expected, support muscle mass gain and strength gain more than an LP diet, the superiority of the HP diet in the maximum weight pulled group was transitory; by the end of the training period, LP-fed mice could pull just as much weight as HP-fed mice. There was a modest effect of diet on performance of mice on inverted cling and rotarod tasks, with HP-fed sham mice performing better on the rotarod than LP-fed sham mice when weight was considered as a covariate, but this effect was not observed in WP groups. As rotarod and inverted cling outcomes can be improved by voluntary aerobic exercise (wheel running) (Graber et al., 2015), this could be the result of either the type of exercise or the precise muscles strengthened by WP vs. aerobic exercise. Somewhat surprisingly, mitochondrial respiration outcomes were also independent of diet and exercise in the FDL, but we did not examine mitochondrial function in other tissues.

Limitations of study

Our study was subject to a number of limitations. We examined only young inbred C57BL/6J male mice, and we have previously shown that sex, genetic background, and age impact the metabolic response to dietary protein (Green et al., 2022b). We consider it likely that these factors may also influence the response to exercise, and examining the interaction between diet and WP in females, older mice, and different genetic backgrounds should be a high priority for future studies. Further, as the metabolic response to dietary protein is responsive to the exact level of protein in the diet, examining additional levels of dietary protein between the LP and HP diets we examined here might provide greater insight. This would also allow the study of dietary protein levels within the Acceptable Macronutrient Distribution Range

(AMDR) for humans, which is 10-35% of calories from protein. Finally, to keep our diets isocaloric, isonitrogenous and consistent in fat content, we had to reduce carbohydrates in the HP diet, which might play a role in the results found here.

Many people who eat high protein diets or take protein supplements also engage in aerobic exercise, which we did not examine in the present study. Here, we have not examined the effects of dietary protein and exercise on molecular changes and have not explained how WP inhibits HP-induced gains in adiposity. The hormone FGF21, which is induced by a LP diet, is responsive to exercise in mice and humans (Haghighi et al., 2022; Jin et al., 2022; Li et al., 2022), but we did not observe increased FGF21 in the LP weight trained group, suggesting another mechanism could be at play. As for the improved body composition of HP-fed WP mice, we hypothesize that these interventions may activate beiging in iWAT or BAT. Other possibilities include changes in mitochondrial respiration in muscles other than the FDL, or exercise-induced alterations in lipogenesis or lipolysis. Lastly, we have not explored the role of individual amino acids in the response; many athletes and body builders take supplements enriched in BCAAs, and future work should determine if there is a specific role for these or other dietary amino acids in the metabolic and physiological response to resistance exercise.

Finally, when broken down to individual muscle fiber types, we saw an increase in type 1 muscle fiber size in the FDL of the HP + WP group, which indicates that both high protein and resistance training are necessary for inducing hypertrophy of slow-twitch, oxidative fiber types. However, as three of our HP + WP samples had no detectable type 1 fibers, future research should evaluate this effect in muscles with an abundance of type 1 fibers. These findings are consistent with specific fiber type hypertrophy previously observed in the FDL in response to WP (Zhu et al., 2021b). We saw a similar induction of growth by both diet and training in type IIA fibers, which are fast-twitch, oxidative and glycolytic. Here, HP feeding resulted in larger fibers than LP in the context of both sham training and weight pulling, which is consistent with human

recommendations that increased dietary protein supports muscle hypertrophy (Thomas et al., 2016). Surprisingly, there was no effect of training observed in fast-twitch, glycolytic type IIB fibers, but there was an effect of diet, with HP feedings inducing hypertrophy. Finally, fast-twitch type IIX fibers demonstrated a similar trend as type IIA fibers, with both a HP diet and WP inducing growth. A striking takeaway observed here is that there was no difference in hypertrophy of Type IIX and IIA fibers in the FDL induced by WP when fed a low or high protein diet. Though these changes occurred in just one muscle, and effects on other muscles may vary, our results indicate that both resistance training and high protein diets individually and in combination optimize growth of muscle fibers.

In summary, dietary protein and progressive resistance exercise interact to regulate metabolic health as well as strength and muscle mass. Specifically increasing protein consumption has – as we anticipated based on both animal and human data – negative effects on the metabolic health of mice that are not exercising. However, the negative effects of a HP diet on body composition are eliminated in mice performing progressive resistance exercise training. While mice consuming a HP diet gained strength during weight pulling more quickly than LP-fed mice, the ultimate max load the HP and LP-fed mice could pull by the end of twelve weeks was indistinguishable, despite clear differences in muscle mass. Future research could investigate this apparent paradox by examining the effect of the diet and WP regimens on muscle mass and fiber type at earlier time points than those examined in this study. However, our results are consistent with studies in healthy, resistance-trained individuals that show high protein diets can be consumed without negative effects on body composition (Antonio et al., 2015; Antonio et al., 2016). We hypothesize that further studies will find that sedentary individuals would be metabolically healthier if consuming a low protein diet. Finally, our results suggest that a precision nutrition approach to metabolic health must consider not only diet, sex, and genetic variation, but also take into account activity level.

Methods

Animal care, housing and diet

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital. Male C57BL/6J mice were procured from the Jackson Laboratory (000664) at six weeks of age. All studies were performed on animals or on tissues collected from animals. All mice were acclimated to the animal research facility for at least one week before entering studies. All animals were housed 2 per cage in static microisolator cages in a specific pathogen-free mouse facility with a 12:12 h light–dark cycle, maintained at approximately 22 °C. At the start of the experiment, mice were randomized to receive either the 7% (Low Protein, TD.140712) or 36% (High Protein, TD.220097) amino acid defined diets, and assigned to the sham or weight pulling groups; all diets were obtained from Envigo. In the High Protein diet, carbohydrates were reduced and amino acids increased relative to the low protein diet in order to keep the diets isocaloric, while calories from fat were held fixed. Full diet descriptions, compositions and item numbers are provided in **Table 1**.

Weight training paradigm

The procedures of this weight pulling regimen (acclimation, weighted paradigm, and unweighted paradigm) were described previously (Zhu et al., 2021b).

Metabolic Phenotyping

Glucose and insulin tolerance tests were performed following a 16 hour overnight or 4 hour fast, respectively and then injecting either glucose (1g/kg) or insulin (0.75U/kg) intraperitoneally

(Bellantuono et al., 2020; Yu et al., 2019). Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips. Blood ketone measurements were taken using a KetoBM ketone meter and test strips that detect beta-hydroxybutyrate, the most abundant ketone in the body and proxy for ketone level. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. For assays of multiple metabolic parameters (O_2, CO_2, food consumption, and activity tracking), mice were acclimatized to housing in a Columbus Instruments Oxymax/CLAMS-HC metabolic chamber system for approximately 24 hours, and data from a continuous 24-hour period was then recorded and analyzed.

Tissue collection

Terminal collections were performed 60–96 h after the final training session and after a 3 hour morning fast. During this procedure, all potentially identifiable information (e.g., tail markings, etc.) was masked, and then the animals were weighed and subsequently anesthetized with isoflurane. The plantaris, flexor digitorum longus (FDL), forearm flexor complex (FF) (which consisted of the flexor carpi ulnaris, flexor carpi radialis, flexor digitorum superficialis, and all three heads of the flexor digitorum profundus), and soleus (SOL) muscles from both the left and right hindlimb were then weighed. The left and right plantaris and right FDL were submerged in optimal cutting temperature compound (OCT, Tissue-Tek; Sakura Finetek, The Netherlands) at resting length, and frozen in liquid nitrogen-chilled isopentane. Left FDL was prepared for mitochondrial analysis. At this point, the mice were euthanized by cervical dislocation, a photograph that included both a scale bar and the musculature of the left forelimb was obtained, and then quadriceps muscles from both the left and right side of the body were collected. In addition to skeletal muscles, the inguinal white adipose tissue (iWAT), brown adipose tissue (BAT), epididymal white adipose tissue (eWAT), liver, as well as the right tibia bone, were collected post-mortem and frozen in liquid nitrogen or prepared for histology (OCT or formalin, to then be incubated in ethanol). Oil Red O staining of liver samples and H&E staining of iWAT

samples were conducted by UW Carbone Cancer Center Experimental Animal Pathology Lab (UWCCC EAPL) on a fee-for-service basis. Muscle photographs were taken with a ruler for scale, and precise maximum muscle diameter was measured using ImageJ. All of the collection procedures were performed by investigators blinded to treatment and diet group.

Immunohistochemistry and image analysis

Fiber type staining and analysis was performed as previously described (Zhu et al., 2021b). ImageJ software was utilized to quantify hepatic lipid droplets and the ImageJ Adiposoft plugin was used to quantify iWAT adipocytes.

High Resolution Respirometry

FDL muscle was excised, weighed, and cut in half. The distal half was snap frozen in liquid nitrogen while proximal half was placed in ice cold (4°C) Buffer X containing (in mM) 7.23 K2EGTA, 2.77 CaK2EGTA, 20 imidazole, 20 taurine, 5.7 ATP, 14.3 phosphocreatine, 6.56 MgCl2·6H2O, and 50 K-MES (pH 7.1). Muscle was mechanically permeabilized with fine tip forceps removing connective and adipose tissue. Fiber bundles were chemically permeabilized in Buffer X with saponin (50 µg/mL) for 30 min on ice. Permeabilized muscle fiber bundles were rinsed with MiR05 containing (in mM) 0.5 mM EGTA, 3 MgCl2, 60 K-lactobionate, 20 taurine, 10 KH2PO4, 20 HEPES, 110 sucrose, 1 g/L BSA essentially fatty acid free, (pH 7.1) and 25 µM blebbistatin. Fibers were then blotted on filter paper to remove excess buffer and weighed on a microscale. Fibers bundles (1.5-2.5 mg) were then placed into chambers of the Oxygraph-2k (O2K; OROBOROS INSTRUMENTS, Innsbruck, Austria) containing MiR05 plus 12.5 µM blebbistatin, a myosin ATPase inhibitor, at 37°C. Chambers were hyperoxygenated to ~425uM and mitochondrial respiration was supported by complex-I linked substrates 5mM pyruvate,

10mM glutamate, and 0.5mM malate. ADP was provided at 0.25mM, 0.5mM and 5.5mM to evaluate sub saturating and saturating complex-I driven mitochondrial respiration. Mitochondrial membrane integrity was evaluated using 10μM cytochrome c. A >15% change in oxygen consumption was considered a loss of mitochondrial membrane integrity and 3 samples were removed from final analysis. 10mM succinate was provided to evaluate complex I and II driven respiration. Data is normalized to muscle fiber bundle wet weight.

Statistics

Data are presented as the mean ± SEM unless otherwise specified. Statistical analyses were performed using one-way, two-way ANOVA, two-way RM ANOVA, or a mixed-effects model (Restricted Maximum Likelihood (REML)) followed by an appropriate posttest as specified in the figure legends. In all figures, n represents the number of biologically independent animals, except in figure 5C, where n represents the number of weeks that touches were determined for across all animals. Outliers were determined using GraphPad Prism Grubbs' calculator (https://www.graphpad.com/quickcalcs/grubbs1/). Sample sizes were chosen based on our previously published experimental results with the effects of dietary interventions plus consideration of the training time required by personnel (Cummings et al., 2018; Fontana et al., 2016; Yu et al., 2021a; Yu et al., 2018). Data distribution was assumed to be normal, but this was not formally tested.

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Chapter 2. Table 1.

Amino Acid Defined Diets	**Low Protein**	**High Protein**
Teklad Diet Name	7% Protein Calories	36% Protein Calories
Teklad Diet Number	TD.140712	TD.220097
Colour	Blue	Green
Formula	**g/kg**	**g/kg**
Sucrose	291.248	214.867
Corn Starch	232.4	110.7
Maltodextrin	232.4	110.7
Corn Oil	52.0	52.0
Olive Oil	29.0	29.0
Cellulose	30.0	30.0
Mineral Mix, AIN-93G-MX (94046)	35.0	35.0
Calcium Phosphate, dibasic	8.2	8.2
Vitamin Mix, Teklad (40060)	10.0	10.0
% kcal from		
Protein	7.1	36.4
Carbohydrate	74.4	44.7
Fat	18.5	18.9
Kcal/g	3.9	3.9
Amino Acid Profile	**g/kg**	**g/kg**
L-Lysine HCl	6.64	33.308
L-Methionine	2.18	10.95
L-Cystine	2.34	11.767
L-Arginine	2.05	10.296
L-Phenylalanine	2.15	10.787
L-Tyrosine	2.25	11.277
L-Histidine HCl, monohydrate	1.15	7.518
L-Isoleucine	2.54	12.748
L-Leucine	8.27	41.512
L-Threonine	3.16	15.853
L-Tryptophan	1.1	5.557
L-Valine	2.735	13.729
L-Aspartic Acid	6.7	33.634
L-Glutamic Acid	9.43	47.347
L-Alanine	3.05	15.33
Glycine	0.96	4.838
L-Proline	2.41	12.111
L-Serine	2.41	12.111

Figure 1.

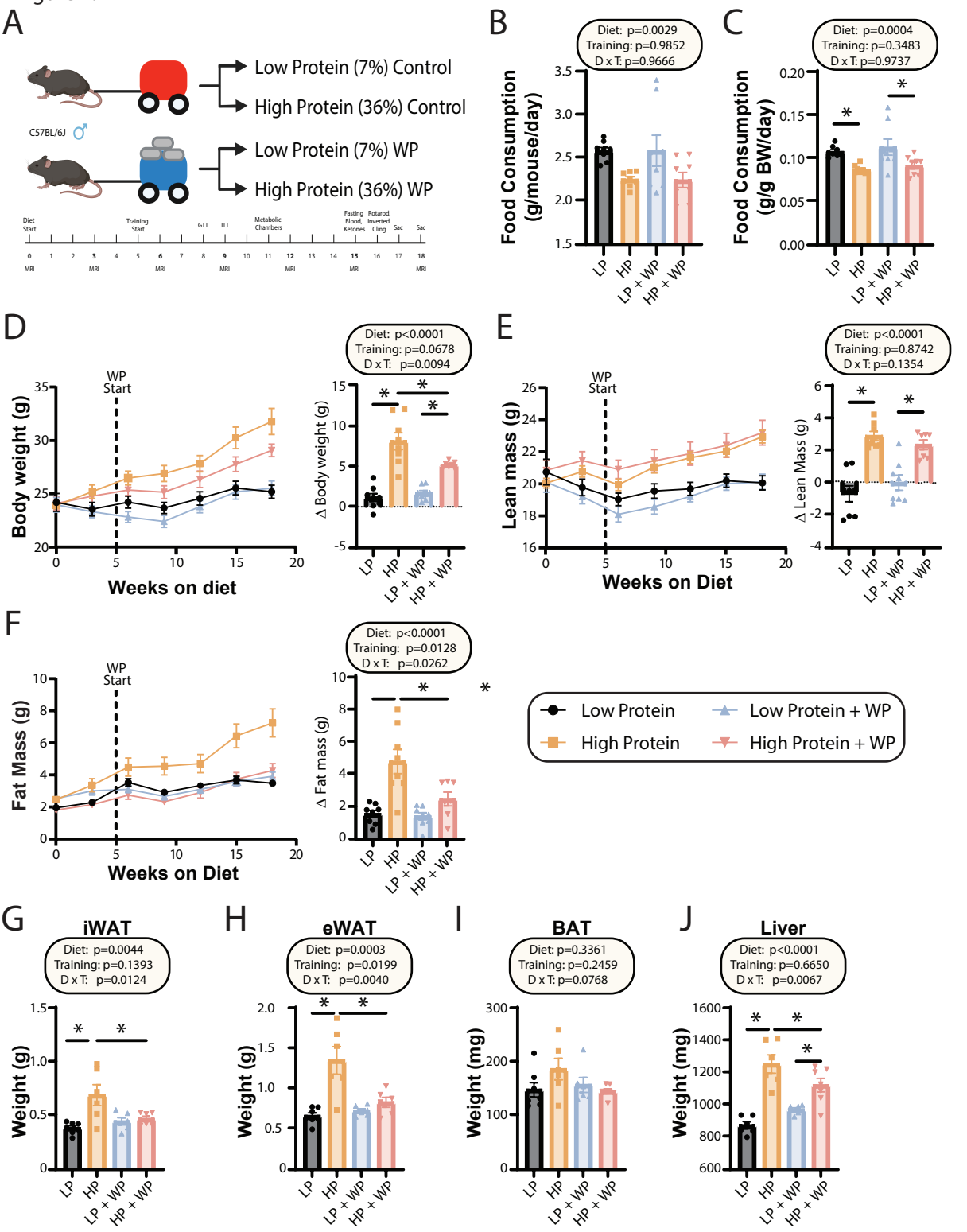
A
Low Protein (7%) Control
High Protein (36%) Control
C57BL/6J ♂
Low Protein (7%) WP
High Protein (36%) WP
Diet Start
Training Start
GTT
ITT
Metabolic Chambers
Fasting Blood, Ketones
Rotarod, Inverted Cling
Sac
Sac
MRI
B
Diet: p=0.0029
Training: p=0.9852
D x T: p=0.9666
Food Consumption (g/mouse/day)
C
Diet: p=0.0004
Training: p=0.3483
D x T: p=0.9737
Food Consumption (g/g BW/day)
LP
HP
LP + WP
HP + WP
D
WP Start
Body weight (g)
Weeks on diet
Diet: p<0.0001
Training: p=0.0678
D x T: p=0.0094
Δ Body weight (g)
E
WP Start
Lean mass (g)
Weeks on Diet
Diet: p<0.0001
Training: p=0.8742
D x T: p=0.1354
Δ Lean Mass (g)
F
WP Start
Fat Mass (g)
Weeks on Diet
Diet: p<0.0001
Training: p=0.0128
D x T: p=0.0262
Δ Fat mass (g)
Low Protein
High Protein
Low Protein + WP
High Protein + WP
G
iWAT
Diet: p=0.0044
Training: p=0.1393
D x T: p=0.0124
Weight (g)
H
eWAT
Diet: p=0.0003
Training: p=0.0199
D x T: p=0.0040
Weight (g)
I
BAT
Diet: p=0.3361
Training: p=0.2459
D x T: p=0.0768
Weight (mg)
J
Liver
Diet: p<0.0001
Training: p=0.6650
D x T: p=0.0067
Weight (mg)

Figure 1. Weight pulling protects from high protein diet induced weight and fat gain.

(A) Experimental design. (B-C) Food consumption per mouse (B) or normalized to body weight (C) after ~6 weeks on the indicated diets. n=8/group. (D-F) Body weight (D), lean mass (E) and fat mass (F) over time, and change (Δ) from the beginning to end of study. n=7-8 mice/group. (G-J) Weight of the iWAT (G), eWAT (H), BAT (I), and liver (J) at the conclusion of the study. n=6-7 mice per group. (B-J) Statistics for the overall effects of diet, training, and the interaction represent the p value from a two-way ANOVA; *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 2.

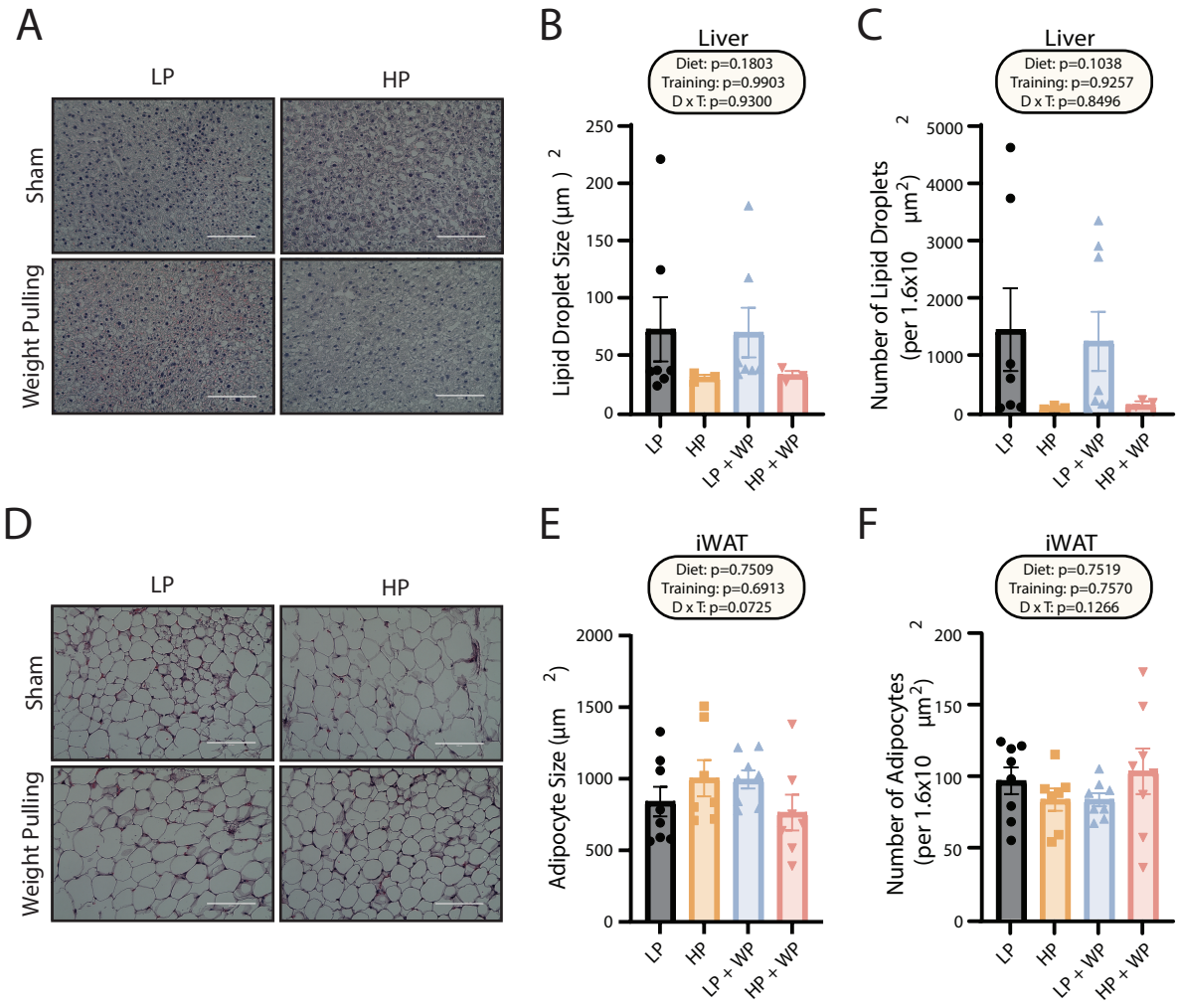
A
LP
HP
Sham
Weight Pulling
B
Liver
Diet: p=0.1803
Training: p=0.9903
D x T: p=0.9300
Lipid Droplet Size (µm 2)
250
200
150
100
50
0
LP
HP
LP + WP
HP + WP
C
Liver
Diet: p=0.1038
Training: p=0.9257
D x T: p=0.8496
Number of Lipid Droplets (per 1.6x10 µm2)
5000
4000
3000
2000
1000
0
LP
HP
LP + WP
HP + WP
D
LP
HP
Sham
Weight Pulling
E
iWAT
Diet: p=0.7509
Training: p=0.6913
D x T: p=0.0725
Adipocyte Size (µm 2)
2000
1500
1000
500
0
LP
HP
LP + WP
HP + WP
F
iWAT
Diet: p=0.7519
Training: p=0.7570
D x T: p=0.1266
Number of Adipocytes (per 1.6x10 µm2)
200
150
100
50
0
LP
HP
LP + WP
HP + WP

Figure 2. Dietary protein content and resistance training did not significantly impact liver lipid droplet or inguinal white adipocyte size.

(A-C) Representative Oil-Red-O stained liver sections from mice in the indicated groups, with quantification of average lipid droplet size (B) and number (C). n=3-7/group. (D-F) Representative H&E stained iWAT sections from mice in the indicated groups, with quantification of average lipid droplet size (E) and number (F). n = 7-8/group. (B-C, E-F) Statistics for the overall effects of diet, training, and the interaction represent the p value from a two-way ANOVA; *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Scale bar = 100μM. Data represented as mean ± SEM.

Figure 3.

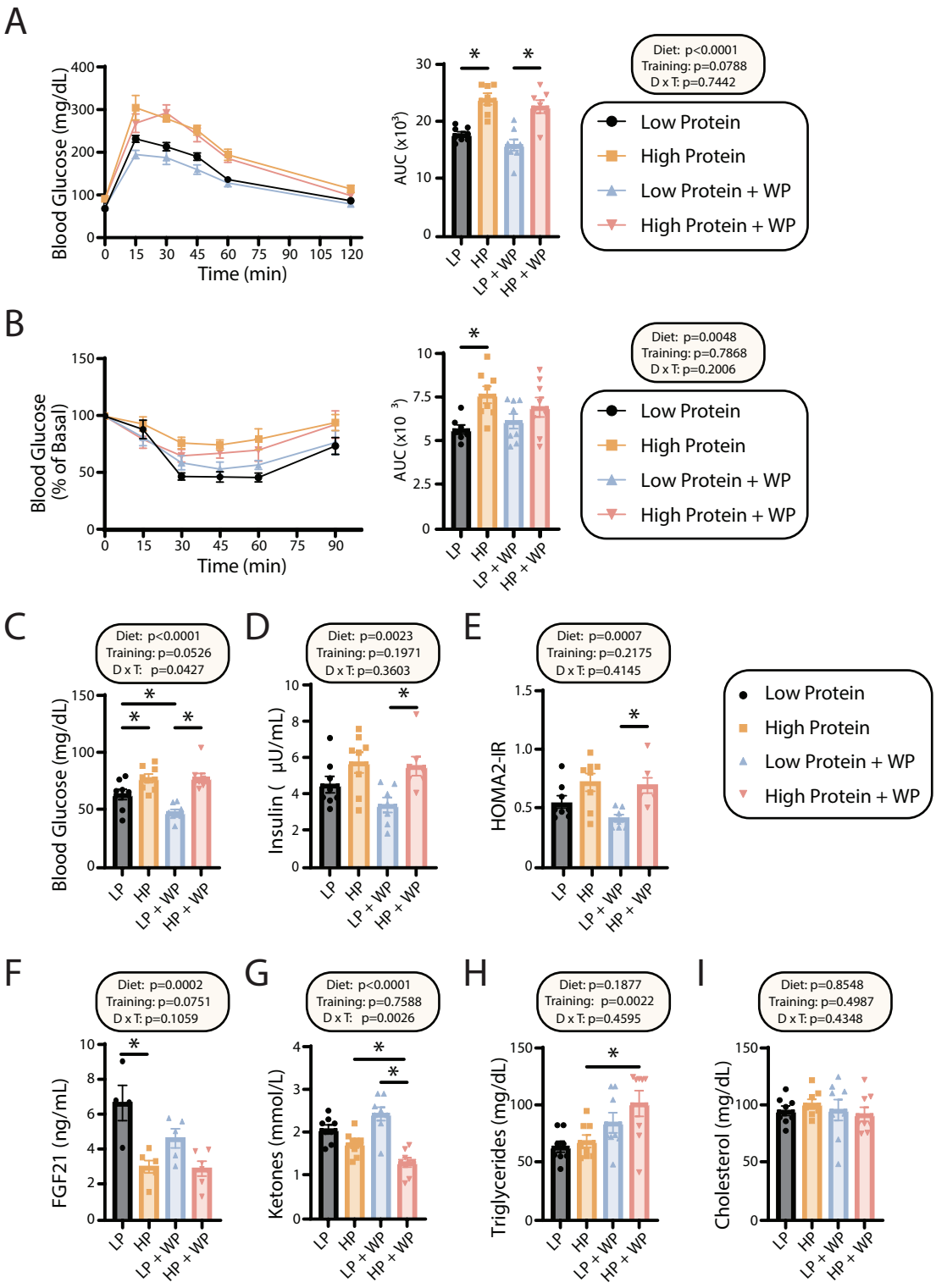
A
Blood Glucose (mg/dL)
Time (min)
AUC (x10³)
Diet: p<0.0001
Training: p=0.0788
D x T: p=0.7442
Low Protein
High Protein
Low Protein + WP
High Protein + WP
LP
HP
LP + WP
HP + WP
B
Blood Glucose (% of Basal)
Time (min)
AUC (x10 ³)
Diet: p=0.0048
Training: p=0.7868
D x T: p=0.2006
C
Blood Glucose (mg/dL)
Diet: p<0.0001
Training: p=0.0526
D x T: p=0.0427
D
Insulin (µU/mL)
Diet: p=0.0023
Training: p=0.1971
D x T: p=0.3603
E
HOMA2-IR
Diet: p=0.0007
Training: p=0.2175
D x T: p=0.4145
F
FGF21 (ng/mL)
Diet: p=0.0002
Training: p=0.0751
D x T: p=0.1059
G
Ketones (mmol/L)
Diet: p<0.0001
Training: p=0.7588
D x T: p=0.0026
H
Triglycerides (mg/dL)
Diet: p=0.1877
Training: p=0.0022
D x T: p=0.4595
I
Cholesterol (mg/dL)
Diet: p=0.8548
Training: p=0.4987
D x T: p=0.4348

Figure 3. Effect of diet and exercise on glycemic control and blood metabolites.

(A-B) Glucose (A) and Insulin (B) tolerance tests were performed after 9-10 weeks on the diet, respectively, and area under the curve (AUC) was calculated. n=7-8 mice/group. (C-E) Blood was collected from animals after a 16-hour overnight fast; fasting blood glucose (C) and insulin (D) were determined and used to calculate HOMA2-IR (E). n=7-8 mice/group. (F-I) Blood was collected from animals after a 16-hour overnight fast after 16 weeks on the diets. Fasting FGF21 (F), ketones (G), triglycerides (H) and cholesterol (I) were determined. n=4-8 mice/group. (A-I) statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 3 Supplement.

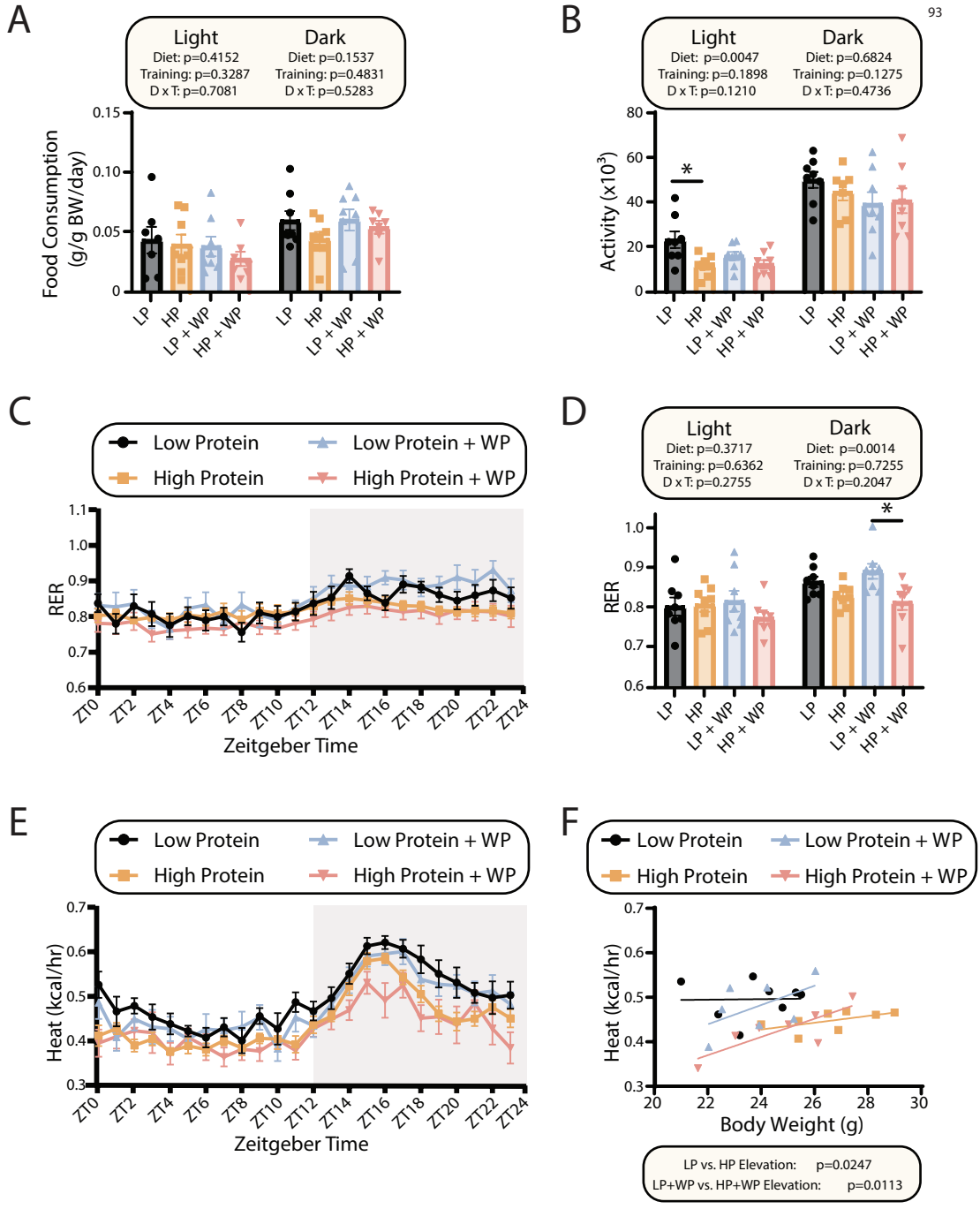

Figure 3 Supplement. Low protein fed animals have increased energy expenditure regardless of training regimen.

(A-F) Metabolic chambers were used to examine metabolic parameters after mice were fed the indicated diets for 8-9 weeks. These included food consumption (A), spontaneous activity (B), Respiratory Exchange Ratio (RER) (C-D), and energy expenditure (E) over a 24 hour period. (F) Energy expenditure (EE) as a function of body weight was calculated (data for each individual mouse are plotted, and slopes and intercepts were calculated using ANCOVA). n=7-8 mice/group. (A, B, D) Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA conducted separately for the light and dark cycles; *$p<0.05$ from a Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 4.

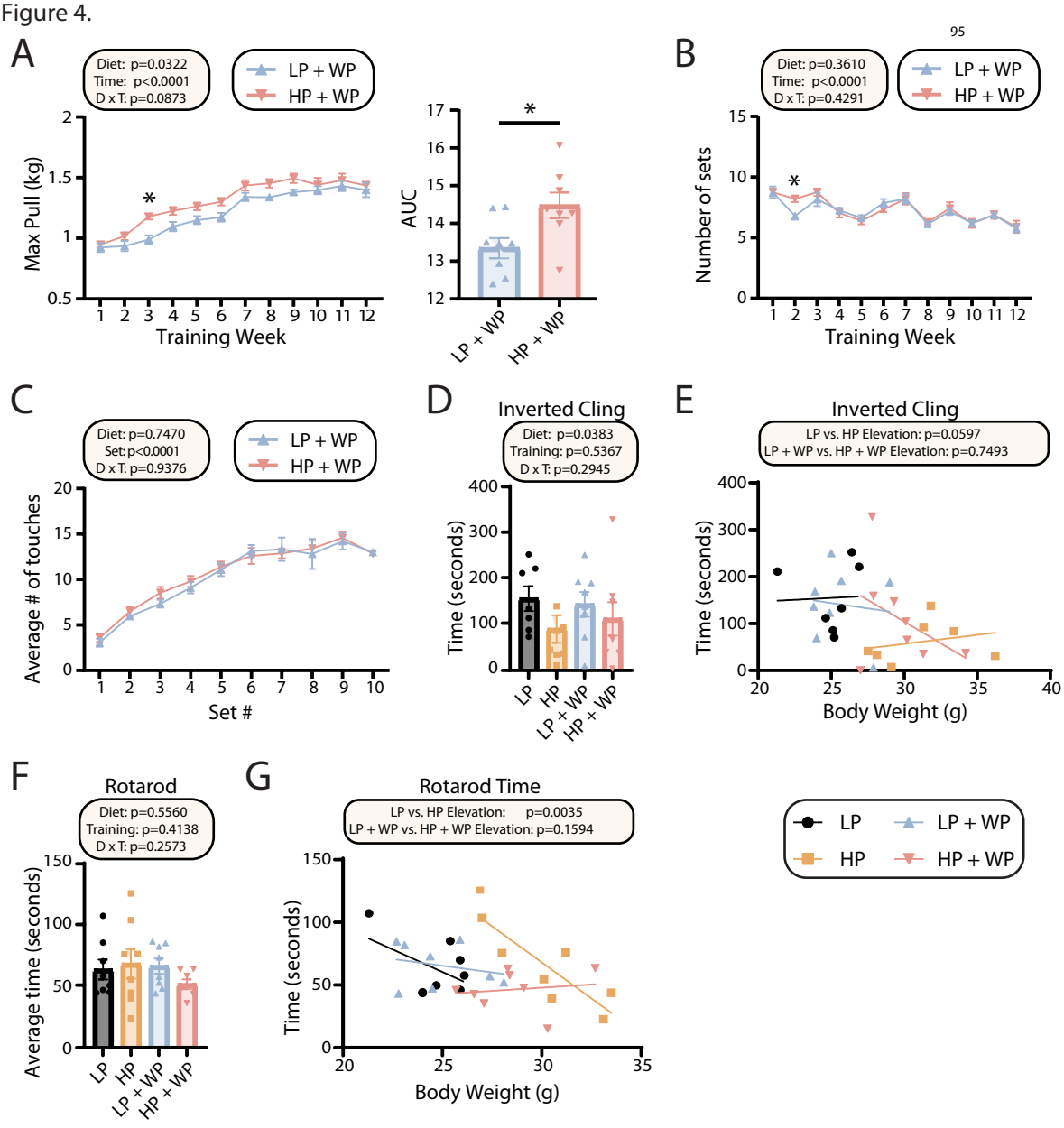
A
Diet: p=0.0322
Time: p<0.0001
D x T: p=0.0873
LP + WP
HP + WP
Max Pull (kg)
Training Week
AUC
B
Diet: p=0.3610
Time: p<0.0001
D x T: p=0.4291
Number of sets
Training Week
C
Diet: p=0.7470
Set: p<0.0001
D x T: p=0.9376
Average # of touches
Set #
D
Inverted Cling
Diet: p=0.0383
Training: p=0.5367
D x T: p=0.2945
Time (seconds)
LP
HP
LP + WP
HP + WP
E
Inverted Cling
LP vs. HP Elevation: p=0.0597
LP + WP vs. HP + WP Elevation: p=0.7493
Time (seconds)
Body Weight (g)
F
Rotarod
Diet: p=0.5560
Training: p=0.4138
D x T: p=0.2573
Average time (seconds)
G
Rotarod Time
LP vs. HP Elevation: p=0.0035
LP + WP vs. HP + WP Elevation: p=0.1594
Time (seconds)
Body Weight (g)
LP
HP
LP + WP
HP + WP

Figure 4. Strength and muscle growth is maximized by high protein and progressive resistance exercise.

(A-C) Weight pulling was performed three times per week for 12 weeks. The average maximum weight pulled each week with area under the curve (AUC) (A), and the number of sets achieved (B) is shown. Similarly, the number of stimulatory touches (C) by the investigator on all mice was averaged through multiple weeks of training and plotted per set. (A-C) n=8 mice/group (A-B); for (C) values for 8 mice/group were assayed over n=5 weeks. Statistics for the overall effects of diet, time or set, and the interaction represent the p value from a two-way RM ANOVA or REML; *p<0.05 from a Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. (D-G) Physical performance was assessed by an inverted cling test (D-E) or rotarod test (F-G). (E, G) Cling time (E) or rotarod time (G) as a function of body weight was calculated (data for each individual mouse are plotted, and slopes and intercepts were calculated using ANCOVA). n=7-8 mice/group. Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *p<0.05, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 5.

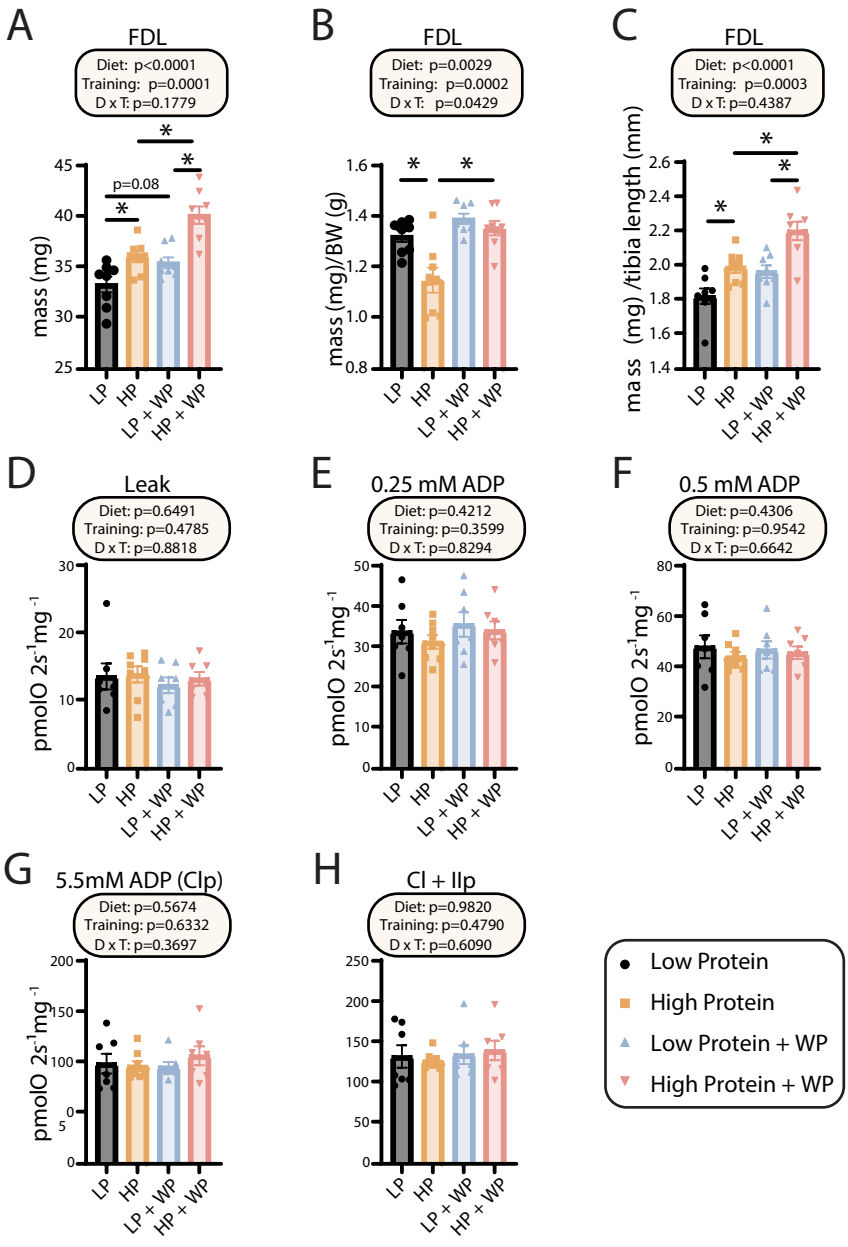
A
FDL
Diet: p<0.0001
Training: p=0.0001
D x T: p=0.1779
p=0.08
mass (mg)
B
FDL
Diet: p=0.0029
Training: p=0.0002
D x T: p=0.0429
mass (mg)/BW (g)
C
FDL
Diet: p<0.0001
Training: p=0.0003
D x T: p=0.4387
mass (mg) /tibia length (mm)
D
Leak
Diet: p=0.6491
Training: p=0.4785
D x T: p=0.8818
pmolO 2s-1mg-1
E
0.25 mM ADP
Diet: p=0.4212
Training: p=0.3599
D x T: p=0.8294
pmolO 2s-1mg-1
F
0.5 mM ADP
Diet: p=0.4306
Training: p=0.9542
D x T: p=0.6642
pmolO 2s-1mg-1
G
5.5mM ADP (Clp)
Diet: p=0.5674
Training: p=0.6332
D x T: p=0.3697
pmolO 2s-1mg-1
H
Cl + IIp
Diet: p=0.9820
Training: p=0.4790
D x T: p=0.6090
LP
HP
LP + WP
HP + WP
Low Protein
High Protein
Low Protein + WP
High Protein + WP

Figure 5. Weight pulling and high protein diet increased FDL mass but not mitochondrial respiration.

(A-C) The muscle mass of the FDL in absolute mass (A), normalized to body weight (B), and (C) normalized to tibia length. n=8/group. (D-H) Mitochondrial respiration parameters as measured in the FDL, including mitochondrial leak (D), and following addition of 0.25 mM (E), 0.5 mM (F), and 5.5 mM ADP (G). (H) 10mM succinate was provided to evaluate complex I and II driven respiration. n=7-8 mice/group. (A-H) Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 5 Supplement.

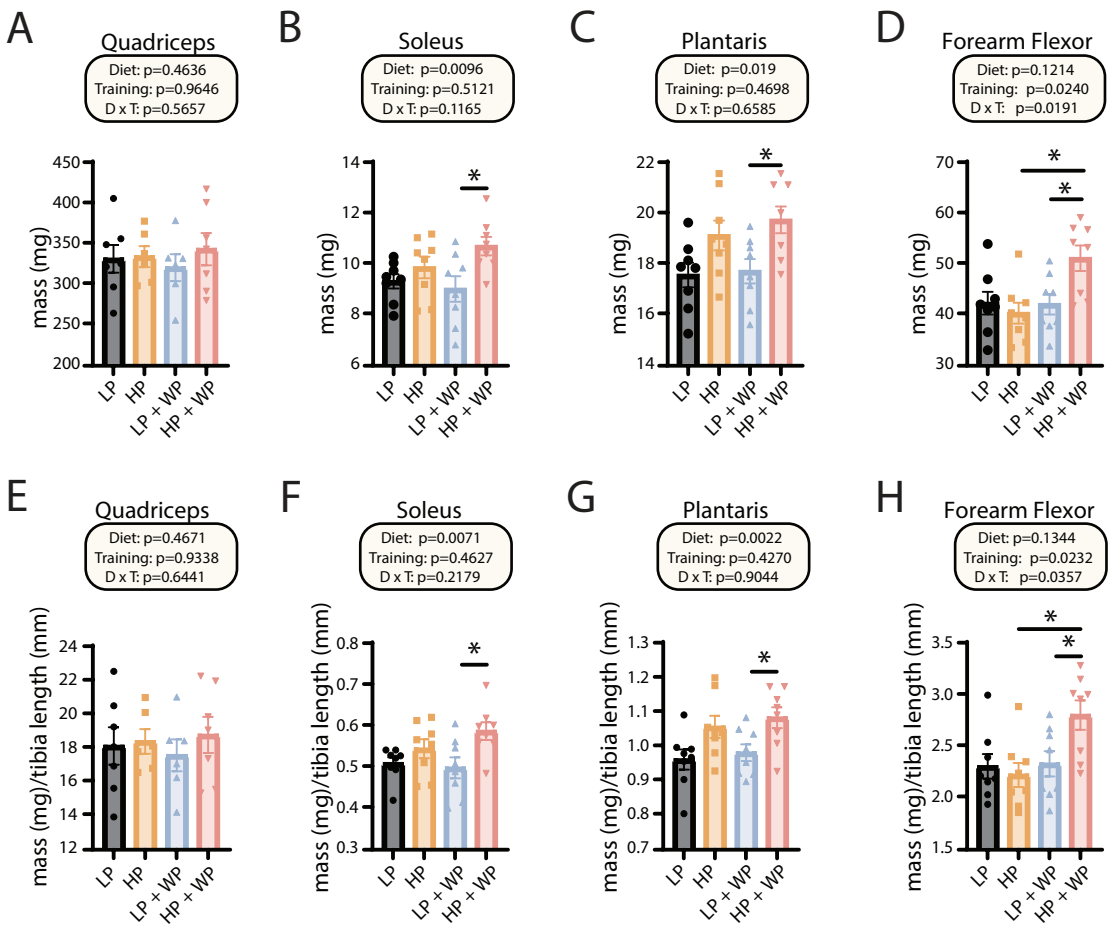

Figure 5 Supplement. Weight pulling and high protein diet increase the mass of specific muscles.

(A-L) The mass of muscles was measured in absolute terms (A-D) and normalized to tibia length (E-H). The muscles measured were the quadriceps (A, E), soleus (B, F), plantaris (C, G) and forearm flexor complex (D, H). n=6-8 mice/group. Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 6.

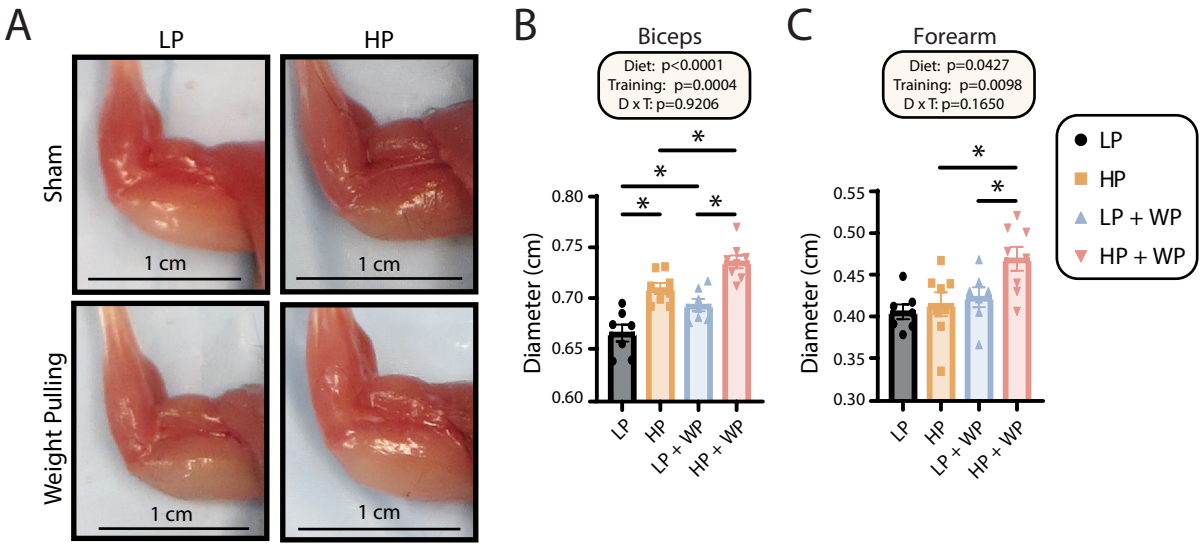
A
LP
HP
Sham
Weight Pulling
1 cm
1 cm
1 cm
1 cm
B
Biceps
Diet: p<0.0001
Training: p=0.0004
D x T: p=0.9206
Diameter (cm)
0.80
0.75
0.70
0.65
0.60
LP
HP
LP + WP
HP + WP
C
Forearm
Diet: p=0.0427
Training: p=0.0098
D x T: p=0.1650
Diameter (cm)
0.55
0.50
0.45
0.40
0.35
0.30
LP
HP
LP + WP
HP + WP

Figure 6. Bicep and forearm hypertrophy is maximized by HP diets and WP.

(A-C) Representative images of arm musculature (A) with quantification of biceps (B) and forearm (C) diameter. (B-C) n=7-8 mice per group. Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *p<0.05, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Figure 7.

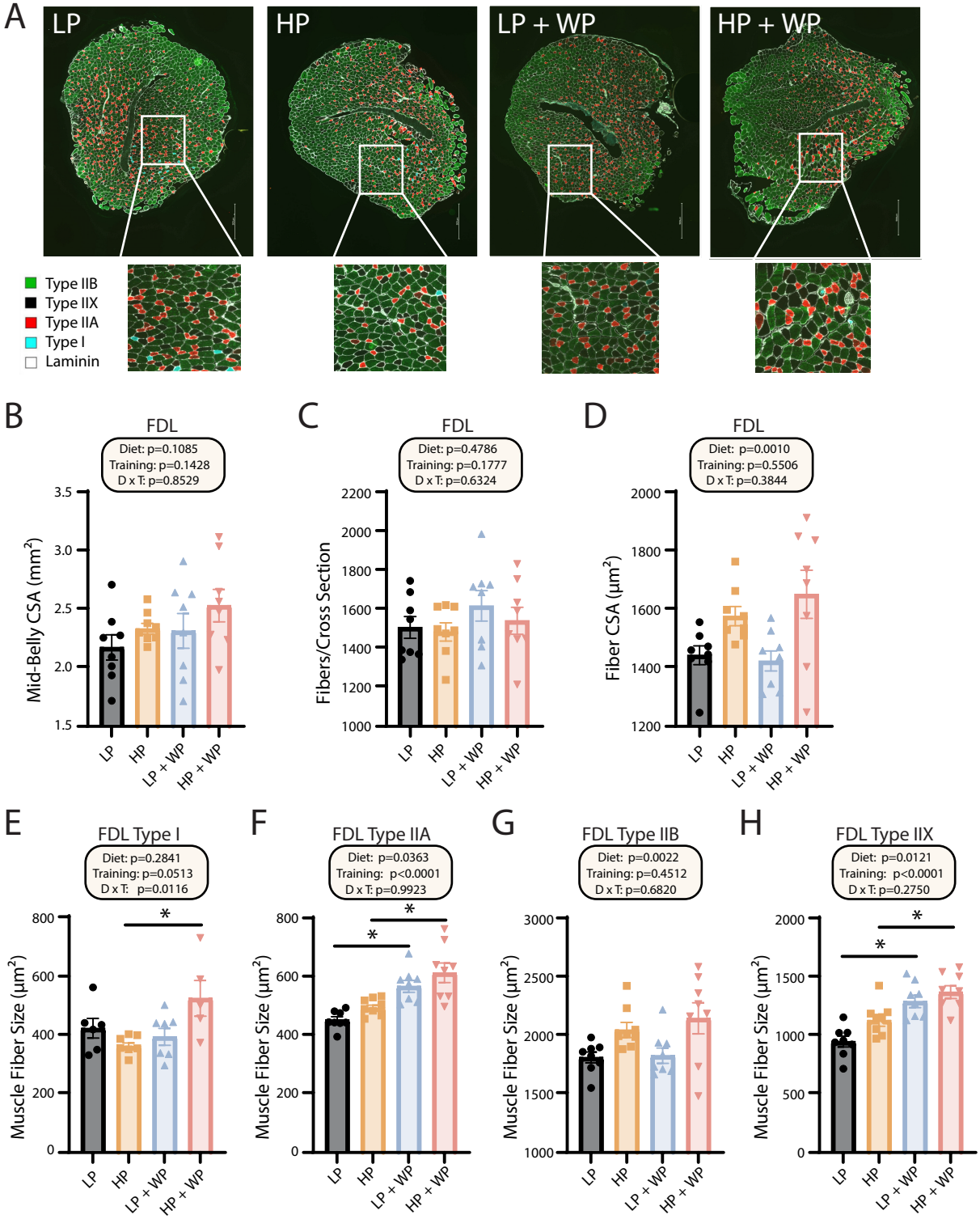
A
LP
HP
LP + WP
HP + WP
Type IIB
Type IIX
Type IIA
Type I
Laminin
B
FDL
Diet: p=0.1085
Training: p=0.1428
D x T: p=0.8529
Mid-Belly CSA (mm²)
C
FDL
Diet: p=0.4786
Training: p=0.1777
D x T: p=0.6324
Fibers/Cross Section
D
FDL
Diet: p=0.0010
Training: p=0.5506
D x T: p=0.3844
Fiber CSA (µm²)
E
FDL Type I
Diet: p=0.2841
Training: p=0.0513
D x T: p=0.0116
Muscle Fiber Size (µm²)
F
FDL Type IIA
Diet: p=0.0363
Training: p<0.0001
D x T: p=0.9923
Muscle Fiber Size (µm²)
G
FDL Type IIB
Diet: p=0.0022
Training: p=0.4512
D x T: p=0.6820
Muscle Fiber Size (µm²)
H
FDL Type IIX
Diet: p=0.0121
Training: p<0.0001
D x T: p=0.2750
Muscle Fiber Size (µm²)
LP
HP
LP + WP
HP + WP

Figure 7. FDL fiber type hypertrophy is maximized by high protein diets and weight pulling.

(A-H) Representative images of the FDL and fiber type with quantification of mid-belly CSA (B), fibers per cross section (C), fiber CSA (D), and individual muscle fiber type size: type I (E), type IIA (F), type IIB (G) and type IIX (H).n=5-8 mice per group. Statistics for the overall effects of diet, training and the interaction represent the p value from a two-way ANOVA, *$p<0.05$, Sidak's post-test examining the effect of parameters identified as significant in the 2-way ANOVA. Data represented as mean ± SEM.

Chapter 3: Dietary isoleucine content defines the metabolic and molecular response to a Western diet

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Author Contributions

MET and DWL conceived of and designed the experiments. MET, CLG, MRM, YAH, RB, IJ, MG, EZ, MG, MMS, C-YY, RNM and VF performed the experiments. MET, CLG, MRM, KC, YHA, JAS, IMO, CJ and DWL analyzed the data. MET, CLG, CJ, JS, IMO, and DWL secured funding and supervised personnel. MET, MM, IMO, CJ, KAM, and DWL wrote the manuscript.

Abstract

The amino acid composition of the diet has recently emerged as a critical regulator of metabolic health. Consumption of the branched-chain amino acid isoleucine is positively correlated with body mass index in humans, and reducing dietary levels of isoleucine rapidly improves the metabolic health of diet-induced obese male C57BL/6J mice. However, it is unknown how sex, strain, and dietary isoleucine intake may interact to impact the response to a Western Diet (WD). Here, we find that although the magnitude of the effect varies by sex and strain, reducing dietary levels of isoleucine protects C57BL/6J and DBA/2J mice of both sexes from the deleterious metabolic effects of a WD, while increasing dietary levels of isoleucine impairs aspects of metabolic health. Despite broadly positive responses across all sexes and strains to reduced isoleucine, the molecular response of each sex and strain is highly distinctive. Using a multi-omics approach, we identify a core sex- and strain- independent molecular response to dietary isoleucine, and identify mega-clusters of differentially expressed hepatic genes, metabolites, and lipids associated with each phenotype. Intriguingly, the metabolic effects of reduced isoleucine in mice are not associated with FGF21 – and we find that in humans, plasma FGF21 levels are likewise not associated with dietary levels of isoleucine. Finally, we find that foods contain a range of isoleucine levels, and that consumption of dietary isoleucine is lower in humans with healthy eating habits. Our results demonstrate that the dietary level of isoleucine is critical in the metabolic and molecular response to a WD, and suggest that lowering dietary levels of isoleucine may be an innovative and translatable strategy to protect from the negative metabolic consequences of a WD.

Keywords: Isoleucine, Branched-chain amino acids, Western diet, metabolic health, adiposity, insulin resistance

Introduction

Around the world, the prevalence of obesity has grown dramatically over the last few decades. In the United States, the age-adjusted percentage of adults who are either overweight or obese is now greater than 70% (NIDDK, 2021). Exposure to unhealthy “Western” diets begins early, and childhood obesity has steadily risen for about 50 years; about one-third of children in the United States are now overweight or obese (NIDDK, 2021). The health consequences of these effects are significant, as obesity is a risk factor not just for type 2 diabetes, but also for Alzheimer’s disease, cancer, cardiovascular disease, and hypertension (2020). While losing weight is a highly effective method to improve metabolic health, few individuals can consistently adhere to a reduced calorie diet; and while glucagon-like peptide-1 (GLP-1) agonists have recently been wildly successful at inducing weight loss through appetite suppression, they are expensive, have a wide-range of side effects, and their long-term consequences are unknown.

While flying in the face of conventional wisdom, over the past decade it has gradually become clear that calories derived from all macronutrient sources are not equivalent (Mihaylova et al., 2023). Accumulating evidence now supports a critical role for dietary protein in metabolic health. Dietary protein is often thought of as beneficial, promoting satiety, muscle growth, and blood sugar control, a view supported by some short-term human trials and studies of the elderly (Dong et al., 2013; Gannon et al., 2003; Kuzuya, 2022; Ribeiro et al., 2019; Seino et al., 1983). However, multiple prospective and retrospective cohort analyses suggest that consumption of dietary protein increases the risk of developing diabetes (Akter et al., 2021; Huang et al., 2020; Lagiou et al., 2007; Levine et al., 2014; Sluijs et al., 2010). Supporting this latter view of high dietary protein consumption as detrimental, two randomized clinical trials of protein restriction (PR) have found that PR reduces weight and adiposity and improves multiple aspects of glycemic control (Ferraz-Bannitz et al., 2022a; Fontana et al., 2016). Research in mice, where PR has significant benefits for metabolic health (Maida et al., 2016; Solon-Biet et al., 2014; Solon-Biet et

al., 2015a), suggests that these seemingly paradoxical findings regarding the metabolic effects of dietary protein may be due to the ability of exercise to protect from the negative effects of dietary protein observed in sedentary rodents and the majority of humans (Trautman et al., 2023).

We hypothesized that the benefits of PR might results from decreased intake of specific dietary essential amino acids. Blood levels of the three branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) have long been associated with diabetes, insulin resistance and obesity in humans and rodents (Batch et al., 2013; Connelly et al., 2017; Felig et al., 1969; Newgard et al., 2009), and we found that that dietary restriction of the BCAAs is sufficient to promote glycemic control and reduce adiposity in both lean and diet-induced obese mice without restricting calorie intake (Cummings et al., 2018; Fontana et al., 2016; Richardson et al., 2021). BCAA-restricted mice had increased food intake that was more than offset by increased energy expenditure; similar beneficial results of restricting BCAAs have been observed in rats (White et al., 2016). Two short-term randomized clinical trials have observed metabolic benefits from BCAA restriction, including improved insulin resistance and white adipose tissue metabolism (Karusheva et al., 2019; Ramzan et al., 2020).

We recently determined that the metabolic benefits of BCAA restriction are driven specifically by reduced levels of isoleucine. Restriction of isoleucine is sufficient to promote metabolic health in lean mice, improving glucose tolerance through increased hepatic insulin sensitivity and decreasing adiposity by promoting the beiging of inguinal white adipose tissue (Yu et al., 2021a). Further, the restriction of isoleucine is necessary for the full metabolic benefits of PR (Yu et al., 2021a), and can robustly extend the healthspan and lifespan of adult male mice (Green et al., 2023a). In diet-induced obese mice consuming a high-fat, high-sucrose Western diet (WD), the benefits of isoleucine restriction are even more profound, with mice consuming a diet with reduced levels of isoleucine rapidly losing excess adipose mass, becoming glucose tolerant and insulin sensitive, and a reversal of fatty liver (Yu et al., 2021a). These results point to

a key role of dietary isoleucine in the metabolic response to diet – and in particular, suggest that the level of dietary isoleucine may play a key role in determining if a WD is deleterious.

We therefore comprehensively examined the impact of dietary isoleucine level on the metabolic and molecular response to an unhealthy high-fat, high-sucrose WD. As sex and genetic background play an important role in the response to a variety of dietary interventions (Barrington et al., 2018; Browning et al., 2012; De Groef et al., 2021; Green et al., 2022b; Mitchell et al., 2016; Roy et al., 2021), we challenged male and female mice from two different inbred strains of mice, C57BL/6J and DBA/2J, with Western diets containing one of three different levels of isoleucine. Surprisingly, unlike the highly sex- and strain-specific metabolic responses of mice to PR (Green et al., 2022b), we found that reducing dietary levels of isoleucine improved aspects of metabolic health such as body weight, energy expenditure, and glucose tolerance across all sexes and strains, albeit to different degrees, while aspects of metabolic health was impaired in all groups when dietary levels of isoleucine were elevated. Surprisingly, despite the similar effects of isoleucine on aspects of metabolic health, dietary isoleucine had diverse sex-specific and strain-specific effects on molecular pathways. Using a multi-omics approach, we identified a conserved sex and strain-independent molecular signature of isoleucine restriction in the liver of mice; we also identified gene-metabolite-lipid mega-clusters that correlate with key phenotypes, finding that the expression of the hormone FGF21 does not group with energy expenditure. While in humans BMI is positively associated with dietary isoleucine levels (Yu et al., 2021a), we find that plasma FGF21 in humans in not significantly associated with dietary isoleucine. Finally, we find that animal-derived protein contains more isoleucine than plant-derived protein, and that there is diversity in BCAA levels between individual foods in different category types. Finally, we find that individuals surveyed in the National Health and Nutrition Examination Survey (NHANES) eating the healthiest diets consumed the lowest amount of isoleucine relative to their intake of dietary protein.

We conclude that isoleucine is a potent regulator of metabolic health across sexes and strains of mice, and that the dietary level of isoleucine is critical in determining the metabolic impact of a WD. Our results further suggest that consuming less isoleucine, which can be achieved in the context of a natural human diet through attentive food choices, may be an easy and translatable way to protect individuals from the seemingly ubiquitous exposure to Western diets.

Results

Dietary isoleucine levels are associated with WD-induced increases in body weight and fat mass

To examine the specific role of dietary isoleucine on the metabolic response to a WD, we designed a series of diets based on an amino acid-defined Western diet (WD Control) that we have previously utilized (Cummings et al., 2018; Yu et al., 2021a), and which closely matches the macronutrient profile of the widely used natural source high-fat high-sucrose Western diet TD.88137. We designed diets with three different levels of isoleucine: the WD Control, a diet with a 67% reduction in isoleucine (WD Low Ile) relative to the WD Control, and a diet in which isoleucine is increased three-fold (WD High Ile) relative to the WD Control diet. These three diets are isocaloric with identical levels and sources of fat and carbohydrates, and the percentage of calories derived from AAs was kept constant by proportionally adjusting the amount of non-essential AAs. We also utilized a fourth diet group, which was fed an AA-defined diet with a composition similar to that of a natural chow diet (Ctrl AA); the amino acid profile of the WD Control AA and Ctrl AA diet are matched. The full composition of these diets can be found in **Table S1**.

We fed these diets to both male and female C57BL/6J (B6) and DBA/2J (DBA) mice for 3 months starting at 6 weeks of age (**Fig. 1A**). We saw an increase in food intake on WD Low Ile diets relative to the WD Control fed mice in all sex-strain groups, which was statistically significant

in B6 and DBA males and trended higher in DBA females (p = 0.0638) and B6 females (**Fig. 1B**). Food consumption was not significantly altered in any group of mice by the WD High Ile diet (**Fig. 1B**).

We tracked the weight and body composition of all groups for 12 weeks (**Figs. 1C-F**). Despite the increased food consumption of WD Low Ile-fed mice, all groups of mice consuming the WD Low Ile diet gained less body weight than WD Control-fed mice during the course of the experiment (**Figs. 1C, F**). In most groups, this was due to reduced accretion of both fat mass and lean mass by WD Low Ile-fed mice, although B6 females showed a net decrease of fat mass (**Figs. 1D-F**). The overall effect was that the WD Low Ile-fed mice of all groups had the smallest increase in adiposity during the course of the experiment, with B6 females showing an overall decline in adiposity after 12 weeks (**Supplemental Figs. 1A-D**). Conversely, B6 males fed a WD High Ile diet gained significant additional weight and accreted significant additional fat mass relative to WD Control-fed males; and an overall increase in adiposity was seen in WD High Ile-fed B6 males and females (**Fig. 1D and Supplemental Figs. 1A-B**). Altogether, mice eating diets with lower levels of Ile had reduced body weight, fat mass gain, and adiposity relative to mice eating diets with higher levels of isoleucine, while mice eating WD High Ile diets showed strain and sex-specific increases in weight and fat mass relative to WD Control-fed mice (**Fig. 1F**).

Dietary isoleucine restriction protects from the negative effects of a WD on glycemic control

During the course of the experiment, we assessed the effect of isoleucine on glucose homeostasis of WD-fed mice. After three weeks on diet, we found that Ile restriction improved glucose tolerance in all mice, with WD Low Ile-fed mice having significantly reduced area under the curve (AUC) relative to WD Control-fed mice in all groups except DBA females (p=0.0543) (**Supplemental Fig. 2A**). After 9 weeks, all sexes and strains consuming the WD Low Ile diet had

significantly improved glucose tolerance relative to WD Control-fed mice (**Fig. 2A**). Insulin sensitivity, as assessed by I.P. administration of insulin, was unaffected by dietary Ile in both male and female B6 mice; in male and female DBA mice, lower levels of Ile were associated with improved insulin sensitivity (**Fig. 2B and Supplemental Fig. 2B**).

We also collected blood to measure fasting blood glucose and insulin levels and performed a homeostasis model assessment for insulin resistance (HOMA2-IR). In almost all groups, fasted blood glucose following an overnight fast was significantly lower in WD Low Ile-fed mice relative to mice fed either WD Control or WD High Ile diets (**Fig. 2C**), with smaller differences between groups observed following a 4-hour daytime fast (**Supplemental Fig. 2C**). Across all sexes and strains, fasting insulin levels were correlated with dietary Ile levels, with the highest levels generally observed in WD High Ile fed mice and the lowest in WD Low Ile-fed mice, though these differences did not reach statistical significance in DBA females (**Fig. 2D**). Using these fasting glucose and insulin values, we determined insulin resistance using the HOMA2 method (Geloneze et al., 2009; Mather, 2009). In B6 mice, increased dietary levels of Ile was associated with higher insulin resistance as assessed by HOMA2-IR, while there was a trend toward lower HOMA2-IR in WD Low Ile-fed B6 females and a significantly lower HOMA2-IR in DBA males (**Figs. 2E-F**). We also calculated HOMA2-%B, which indicates pancreatic beta cell function; we observed an overall decrease in beta cell function in WD High Ile-fed males, which was statistically significant in B6 males (**Supplemental Fig. 2D**). Finally, we visualized the impact of dietary Ile levels on body composition and glucose homeostasis using heatmaps. As shown in **Fig. 2F**, reducing dietary isoleucine generally promoted metabolic health, improving parameters related to both body composition and glycemic control across sexes and strains, while increasing dietary isoleucine levels conversely impaired metabolic health, especially in B6 males and females.

A low isoleucine diet increases energy expenditure, with strain and sex-dependent effects on the FGF21-UCP1 axis

We hypothesized that the effects of dietary isoleucine on weight and body composition were likely mediated by changes in energy balance. We utilized metabolic chambers to evaluate energy balance, analyzing energy expenditure and spontaneous activity. Based on our previous experiments with isoleucine restriction in diet-induced obese B6 males, we expected that the WD Low Ile diet would increase energy expenditure; in agreement with our prediction, we observed a robust increase in energy expenditure in B6 males and females during both the light and dark cycles (**Fig. 3A** and **Supplemental Fig. 3A**). Energy expenditure was also increased in the WD Low Ile-fed DBA mice, reaching statistical significance as compared to WD Control-fed mice during the dark cycle in DBA females; in DBA males, WD Low Ile-fed males had higher energy expenditure than WD High Ile-fed males during the dark cycle (**Fig. 3A** and **Supplemental Fig. 3A**). In both the light and dark phases, there was a strong effect of diet on energy expenditure. In the light phase, there were significant interactions between diet and strain as well as sex and strain on energy expenditure; in the dark phase, there was a significant effect of sex (**Fig. 3A**).

Ile restriction has previously been shown to increase the respiratory exchange ratio (RER) of B6 males on a low-fat diet, suggesting an increased utilization of carbohydrates. Surprisingly, here we observed that RER decreases in WD Low Ile-fed B6 males relative to WD Control-fed mice, suggesting an increase in fat oxidation, which reached statistical significance during the light cycle; we also observed a similar effect of Ile restriction in DBA males, reaching statistical significance during the dark cycle (**Supplemental Fig. 3B**). In contrast, the RER of B6 and DBA females decreased as dietary Ile increased, which was statistically significant in B6 females in the dark cycle (**Supplemental Fig. 3B**). Overall, in the light phase we observed significant effects of diet, sex, and strain, as well as a significant interaction between diet and sex on RER, and found that there was a significant effect of diet on RER in the dark phase (**Supplemental Fig. 3B**).

The increased energy expenditure of B6 males on a PR diet is mediated by fibroblast growth factor 21 (FGF21), a key energy balance hormone that is induced by PR in both humans and rodents, and which promotes the beiging of white adipose tissue (Flippo et al., 2020; Hill et al., 2019; Laeger et al., 2014). Ile restriction similarly induces FGF21 and induces the beiging of white adipose tissue in B6 males consuming a low-fat diet, increasing energy expenditure via a partially *Fgf21*-dependent pathway (Yu et al., 2021a). However, we recently found that the effect of PR on FGF21 is dependent upon sex and strain, and that FGF21 may not be required for the metabolic response to PR in females (Green et al., 2022b).

To better understand the role of FGF21 in the response to dietary isoleucine, we examined the effect of dietary Ile level on the FGF21-UCP1 axis across sexes and strains. In B6 males, we observed that FGF21 levels are significantly lower in WD High Ile-fed B6 males than in B6 males fed either the WD Control or WD Low Ile diets (**Fig. 3B**). However, this association with Ile level was not observed in other sexes and strains; in B6 and DBA females, the highest level was found in WD Control-fed animals, while in DBA males, FGF21 was elevated in both WD Low Ile-fed and WD High Ile-fed mice relative to WD Control-fed mice (**Fig. 3B**).

Next, we examined relative *Fgf21* and thermogenic and lipolytic gene expression in inguinal white adipose tissue (iWAT). We were surprised to see that unlike active FGF21 in circulation, relative *Fgf21* expression in iWAT was largely unchanged in all groups, except for DBA females (**Supplemental Fig. 3C**). Both high and low Ile-fed DBA females displayed significantly reduced *Fgf21* compared to the WD Control (**Supplemental Fig. 3C**).

In the context of a low-fat diet, we have previously observed that isoleucine restriction increases the expression of Uncoupling protein 1 (*Ucp1)* and other thermogenic and lipolytic genes, including cell death-inducing DNA fragmentation factor alpha-like effector A (*Cidea*), elongation of very long chain fatty acids protein 3 (*Elovl3*), Peroxisome proliferator-activated receptor gamma (*Pparg*), hormone-sensitive lipase (*Lipe*) and fatty acid synthase (*Fasn*) (Yu et

al., 2021a) (**Supplemental Fig. 3C, Table S10**). Here we show only B6 males displayed a robust increase in *Ucp1, Pparg, Fasn, Lipe and Cidea*, showing clear sex and strain-dependent FGF21-UCP1 signaling when consuming a WD. (**Supplemental Fig. 3C, Table S10**). Intriguingly, DBA males under the WD Control exhibited increased signaling of these genes compared to both high and low Ile diets (**Supplemental Fig. 3C, Table S10**). These data suggest that isoleucine levels regulate energy expenditure largely independently of the FGF21-UCP1 axis and iWAT beiging.

In addition to energy expenditure parameters, we examined how dietary isoleucine levels impacted cholesterol and triglycerides. Despite evidence that in response to a Western style diet, B6 mice display a greater rise in circulating cholesterol and triglycerides than DBA animals (Zhu et al., 2009), we observed roughly the same levels of plasma cholesterol in all groups, regardless of diet (**Supplemental Fig. 4A**). WD Low Ile-fed mice had significantly lower plasma triglycerides in DBA males, and we observed a similar effect in B6 females (p=0.0677) (**Supplemental Fig. 4B**).

Metabolic phenotypes and hepatic -omics highlight sex and strain-dependent differences in the response to dietary Ile

We next used multivariate analysis to comprehensively identify sex- and strain-dependent responses to dietary Ile. We correlated the Ile intake of each individual mouse on the WD Low Ile, WD Control, and WD High Ile diets with 33 phenotypic and molecular measurements obtained from each animal for each strain and sex, and plotted these in a clustered heatmap (**Fig. 3C**). Overall, there was high similarity in the response to altered isoleucine intake across sexes and strains, with isoleucine intake positively correlating with body weight, fat mass, adiposity, and AUC during a glucose tolerance test, and Ile intake correlating negatively with calorie intake and energy expenditure. In the middle of the plot (black outline, **Fig. 3A**) are phenotypes which tend

to vary the most in response to sex and strain; these include insulin sensitivity, activity, and RER as well as calories of carbohydrate consumed.

We performed a principal component analysis (PCA) with the phenotypic data, plotting males and females separately (**Supplemental Figs. 4C-D**). We observed a significant overlap between WD Low Ile-fed B6 and DBA males, indicating a strong effect of diet over strain in Ile restriction. In contrast, B6 WD Control-fed males largely overlapped with WD High Ile-fed males, and DBA WD Control-fed males overlapped with WD High Ile-fed males, suggesting that strain was more responsible for the variation between groups than diet when Ile content is normal or high (**Supplemental Fig. 4C**). This pattern was not as strong in females, with only partial overlap of B6 and DBA females fed the WD Low Ile diet, and the groups appearing to cluster primarily by strain (**Supplemental Fig. 4D**). The phenotypes with the greatest contribution to the spread of the PCA in males were fasting blood glucose, final fat mass/adiposity, and circulating FGF21 (**Supplemental Fig. 4E**), while in females the main drivers included energy expenditure, HOMA2-IR, RER, and food consumption (**Supplemental Fig. 4F**).

Due to the central role of the liver in maintaining metabolic homeostasis, we performed a detailed molecular analysis of the livers of WD Low Ile, WD Control, and WD High Ile-fed mice of both sexes and strains, performing transcriptional profiling as well as metabolomic and lipidomic analysis. We first analyzed the effects of dietary Ile on each type of data separately.

At the transcriptional level, we saw many sex- and strain-dependent effects on gene transcription (**Table S5**) and we identified significantly altered pathways using KEGG pathway enrichment analysis. As shown in **Figure 4A**, there are clear effects of sex and strain on the molecular response to dietary Ile. Comparing the WD Low Ile diet to the WD Control diet, we found two significantly downregulated and 16 significantly upregulated pathways in B6 males, many of metabolic interest, such as “Metabolic pathways,” “Glutathione metabolism,” “Lysosome,” and “Terpenoid backbone biosynthesis” (**Fig. 4A**). However, many more and different pathways

were altered in B6 females and DBA males, while very few pathways were significantly altered in DBA females (**Fig. 4A**). Comparing the WD High Ile diet and the WD Control diet, robust changes were observed only in B6 males, while neither B6 nor DBA females had significant changes in KEGG pathways in response to a WD High Ile diet (**Supplemental Fig. 5A**).

Both of these outcomes were in agreement with what we observed during our metabolic phenotyping; that is, strong changes in males, especially of the B6 background, and muted responses in females, especially DBAs. Intriguingly, insulin resistance and the insulin signaling pathway were the only pathways that were significantly increased in DBA males in response to a WD High Ile diet (**Supplemental Fig. 5A**), which is consistent with our ITT results, but not HOMA-IR findings (**Figs. 2B, 2E**). There were no overlapping genes between WD High Ile-fed B6 and DBA male mice (**Supplemental Fig. 5C**).

Next, we analyzed the hepatic metabolome. Though there were clear patterns present between the diet groups within the same sex and strain, the significant metabolites altered in different sexes and strains were quite different (**Fig. 4B** and **Table S6)**. In B6 animals, the impact of the WD High Ile diet on metabolites was limited to males, as additional dietary Ile did not make much of an impact on liver metabolites in females (**Fig. 4B and Supplemental Fig. 6A**). In DBA mice, the WD High Ile diet had the strongest effect on hepatic metabolites changes in males, while in females, different metabolites were increased by isoleucine restriction or supplementation (**Supplemental Figs. 6A-C**).

We also identified sex- and strain- dependent changes at the lipid level, with significant changes in pathways altered by both reduction and supplementation of dietary isoleucine (**Supplemental Fig. 6D**). Consistent with our transcriptomics and metabolomics results, there was little conservation of lipid species and pathways that were significantly altered between sex and strains; lipidomic analysis revealed that no single pathway was simultaneously altered in all four groups in response to either diet (**Supplemental Fig. 6D**). Some trends did emerge, such as

the shared downregulation of polyunsaturated fatty acids, glycerophosphoglycerols, and fatty acids with more than 3 double bonds in B6 males and females in the WD Control vs WD Low Ile analysis. There were also instances of two groups sharing a significantly altered pathway in the WD High Ile analysis, but overall there were far fewer lipids and lipid pathways changed in response to elevated Ile (**Supplemental Fig. 6D**).

Multiomic identification of a conserved molecular signature of isoleucine restriction

In order to identify a conserved molecular response to dietary isoleucine, we generated Venn diagrams of significantly up- and downregulated genes, lipids, and metabolites in response to WD Low Ile (**Fig. 5A-F, Table S8**). and the genes of WD High Ile diets (**Supplemental Fig. 5B-C**). In response to a WD Low Ile diet, we observed increased expression in all sexes and strains of genes related to the circadian CLOCK-BMAL transcription complex (**Fig. 5A**), and decreased expression in all sexes are strains of genes involved in nitrogen metabolism, metabolic pathways, and protein processing in the endoplasmic reticulum (ER) (**Fig. 5B**).

There were no conserved upregulated lipids, but there was one lipid downregulated in all groups, a phosphatidylinositol (PI 18:0_20:3) (**Fig. 5C-D**). Lastly, there were no conserved upregulated metabolites, but B6 females and DBA males and females all had significantly lower levels of glycerylphosphorylethanolamine (**Fig. 5E-F**).To further evaluate a conserved molecular hepatic signature by Ile restriction, we combined the genes, metabolites and lipids conserved in **Fig. 5A-F** and used Metaboanalyst to identify significantly up- and downregulated pathways (**Fig. 5G-H**). We find that in response to a WD Low Ile diet, circadian rhythm and purine metabolism pathways are strongly upregulated in the liver, as well as ABC transporters and vitamin digestion and absorption (**Fig. 5G**). Conversely, nitrogen metabolism, protein processing in the ER,

necroptosis, ether lipid metabolism, galactose metabolism, glycolysis or gluconeogenesis, and apoptosis pathways are all downregulated (**Fig. 5H**).

Using the conserved significantly up- and downregulated genes from our transcriptomics data, we utilized the MAGIC transcription factor prediction database (Roopra, 2020), where we identified 4 significantly enriched transcription factors (**Fig. 5I**). Based on our transcriptomics data, the majority of these genes change expression less than 2 fold – suggesting that any changes driven by these TFs are mediated by post-translational modifications, nuclear localization, and/or changes in DNA binding (**Fig. 5J**).

Multi-omics analysis reveals that dietary Ile is a potent driver of molecular change

To examine the relationship between molecular changes and whole-organism physiology and metabolism induced by changes in dietary Ile, we constructed a Spearman's Rank Order Correlation matrix using phenotypic data as well as hepatic transcriptomic and metabolomic data, a technique we have previously utilized to gain insight into the effects of dietary macronutrients (Green et al., 2022b; Green et al., 2023a). Statistically significant changes in gene expression, lipids and metabolites for each group of mice were concatenated with phenotypic data, and Spearman's correlation was used to calculate coefficients that were then rendered by hierarchical clustering (based on 1-correlation coefficient between all molecules) to produce 9 distinct clusters (**Table S9**).

We identified KEGG-enriched pathways in each cluster and related these to the corresponding cluster phenotypes. While these results are based on correlations and are therefore not necessarily causative, they are consistent with a critical role for Ile, and link specific molecular and metabolic processes in the liver directly to the robust phenotypic response to changes in the level of dietary Ile. In cluster 1, we saw that energy expenditure relative to body

weight correlated with “Isoleucine biosynthesis”, but not circulating FGF21, which presented in cluster 8 (**Fig. 6A, clusters 1, 8**). This again suggests that the increased energy expenditure of WD Low Ile-fed mice may be due to a non-canonical and FGF21-independent pathway. Indeed, FGF21 did not cluster with other phenotypes or KEGG terms related to metabolism beyond “metabolic process.” (**Fig. 6C, cluster 8**).

Not surprisingly based on our glucose homeostasis in vivo data, BCAA metabolism clustered with glucose tolerance, as well as autophagy, glycolysis, and fatty acid synthesis (**Fig. 6A, cluster 4**). Interestingly, the KEGG term “Lysosome” is associated with insulin sensitivity in cluster 7, suggesting that isoleucine may act to regulate blood sugar in part via lysosomal signaling. While not a conventional pathway, this possibility is consistent with emerging evidence for a role of the lysosome as a key regulator of glucose metabolism (Mancini et al., 2023) (**Fig. 6A, cluster 7**). Mitophagy, NAD+ and mitochondrial protein processing are also present in this cluster (**Fig. 6C, cluster 7**).

As we have previously described, when we examined associations between estimated isoleucine intake relative to total protein and BMI (kg/m^2) among a randomly selected, cross-sectional population-based sample of 788 adults, we found that after adjustment for confounding factors, an increase in the intake of dietary isoleucine relative to total protein of a single percentage point—for example, from 4% to 5% of protein—is associated with a 2.46 unit increase in BMI ($p = 0.046$) (**Fig. 6B,** (Yu et al., 2021a). To determine if FGF21 levels in humans are correlated with isoleucine, we examined stored plasma from 140 of these same subjects. Here we see that consistent with our mouse findings across sexes and strain, human plasma levels of FGF21 when adjusted for confounding factors are not associated with dietary levels of isoleucine relative to total protein ($p=0.27$) (**Fig. 6C**).

Healthy human diet patterns are lower in relative Ile

We next investigated the contribution of dietary isoleucine to human nutrition and health using food item databases and cross-sectional dietary reporting data from the National Health and Nutrition Examination Survey (NHANES). We first looked at relative proportions of isoleucine across food item groups using the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 28. When we compared the amino acid composition of plant vs. animal-based food items, we observed that animal-based items have higher proportional isoleucine content (**Fig. 7A**). While the isoleucine content of animal-based foods followed a largely unimodal distribution, plant-based items had a proportionally lower isoleucine content but showed greater variability, following a bimodal distribution with a small high proportion sub-group (**Fig. 7A**). Further sub-division of food items revealed that several animal-based food groups had the highest proportional abundance of isoleucine, including beef, fish, pork and poultry (**Fig. 7B**). Interestingly, fruits had the lowest proportional isoleucine abundance followed by nuts and seeds and cereal grains, while legumes tended to have the highest relative isoleucine abundance among plant-based items (**Fig. 7B**). The same patterns in food items were observed when we examined the other two BCAAs, leucine and valine, except that isoleucine tended to have lower relative abundance in cereal grains (**Supplemental Fig. 7A**).

We next looked at isoleucine consumption across dietary patterns using NHANES data. When stratifying individuals by decile of protein energy intake (% of kcals), a clear trend emerged with lower protein consumers eating a lower relative proportion of isoleucine (**Fig. 7C**). Interestingly, this trend was not evident with respect to leucine and valine, and we considered the possibility that the effect of isoleucine was driven by the lower relative abundance of isoleucine in grain-based food items (**Supplemental Fig. 7B**). Identifying how dietary food groups varied with protein intake, we find that lower protein consumers obtained a larger relative amount of isoleucine from items including baked goods and grain, while the highest protein consumers

obtained a larger relative amount of their daily isoleucine from poultry, beef and beverages (including powdered protein drinks) (**Fig. 7D**).

Based on this initial data, we hypothesized that there would also be significant variation in the isoleucine content of individual foods in each category. As substitutions within a category – e.g., one vegetable for another – may be easier to incorporate into standard diets, we analyzed individual foods for the relative percentage of their isoleucine content (**Fig. 7E, Supplemental Fig. 7C**). Within the category of Meats, we found that emu and turkey had some of the lowest isoleucine content (**Fig. 7E**), while within Fruits, grapes and grapefruits were significantly lower than the other fruits (**Supplemental Fig. 7C**), and within Nuts, almonds and peanuts contained the lowest relative amount of isoleucine (**Fig. 7E**). Although there were no specific vegetables that were significantly lower in isoleucine, several individual vegetables, including carrots, chard, and lima beans had very high levels of isoleucine (**Supplemental Fig. 7C**).

Finally, we stratified participants based on indices of dietary health. Interestingly, when participants were stratified by healthy eating index (HEI) score, we found that despite participants in the highest HEI quintile having the greatest protein intake, they also had the lowest proportional intake of isoleucine, which may be due to relatively higher intake of foods with lower isoleucine levels (**Fig. 7F**).

Discussion

Obesity is a growing problem throughout the world; not only are the majority of adults overweight or obese, so are an increasing percentage of children and teenagers. In the United States, 19.7% of those 2-19 years old are obese, with many additional people in this age range likely overweight (2021). While a number of causes contribute to the early onset of obesity, lifelong consumption of an unhealthy Western diet is likely a critical factor. Intervening in the context of

already established diet-induced obesity is clearly important; perhaps even more critical, however, is understanding how to prevent the development of obesity in the first place.

Based on emerging evidence for the regulation of metabolic healthy by the dietary branched-chain amino acid (BCAA) isoleucine (Green et al., 2023a; Mihaylova et al., 2023; Solon-Biet et al., 2014; Yu et al., 2021a), and a growing awareness that sex and genetic background impact the response to diet (Barrington et al., 2018; Green et al., 2022b), we examined here the impact of sex and strain on the response to dietary levels of isoleucine. We find that lower levels of isoleucine consumption in the context of a Western diet promote metabolic health in all groups, including both sexes of C57BL/6J and DBA/2J mice. However, the size of this effect varied across groups; the greatest metabolic benefits of reduced isoleucine intake were observed in C57BL/6J males, with some of the smallest effects in DBA/2J females. We show that restriction of isoleucine has sex- and strain-dependent and independent effects on hepatic metabolism, and identify a conserved molecular signature of isoleucine restriction. Finally, we show that blood levels of FGF21 are not associated with dietary isoleucine levels in mice or humans, and examine how dietary intake of isoleucine varies with food source and dietary patterns.

Our studies here were conducted in mice consuming a high-fat, high-sucrose WD as they transition from adolescents (6 weeks old) to young adults (18 weeks old) – precisely aligning with the period in humans where obesity often begins (Flurkey et al., 2007). Surprisingly, our findings suggest that the deleterious effects of a WD on metabolism are strongly dependent upon the dietary level of isoleucine. Irrespective of sex and strain, mice consuming a WD with a low level of isoleucine are protected from what we normally think of as the inevitable consequences of consuming large quantities of a high-fat, high-sucrose diet, including weight gain and impaired glycemic control. Conversely, a diet with high levels of isoleucine potentiates the negative effects of a WD, with animals gaining more weight and adiposity and becoming more insulin resistance when consuming a WD. These effects are broadly in alignment in studies which have shown that

increased consumption of all three BCAAs promotes obesity and insulin resistance (Cummings et al., 2018; Newgard et al., 2009; Solon-Biet et al., 2019), but importantly the negative effects of isoleucine we observed are not coupled to any increase in calorie intake. Indeed, we find that the opposite is true: as levels of dietary isoleucine fall, calorie consumption rises even as metabolic health improves.

This aligns well with the molecular changes we observed, with DBA/2J females having the fewest number of hepatic pathways and metabolites altered. Increasing isoleucine levels impaired metabolic health primarily in C57BL/6J mice, where the WD High Ile diet promoted adiposity in males and increased fasting insulin and HOMA2-IR in both sexes. Increased dietary isoleucine altered hepatic transcriptional pathways almost exclusively in C57BL/6J males.

We and others have puzzled over the role of the energy balance hormone FGF21 in the response to dietary protein, with data from C57BL/6J males supporting a model in which induction of FGF21 by PR acts to increase energy expenditure via activation of sympathetic signaling and induction of *Ucp1* in iWAT (Hill et al., 2017; Hill et al., 2019). However, we recently suggested that this might be sex and strain-specific, as PR increases blood levels of FGF21 only in C57BL/6J males and not in females or DBA/2J mice (Green et al., 2022b). Across all groups, we find that energy expenditure is negatively associated with isoleucine content, and is highest in WD Low Ile-fed mice. However, there is little support for the idea that this increase is mediated by activation of a FGF21-UCP1 axis; dietary levels of isoleucine are negatively correlated with FGF21 levels in C57BL/6J males, but show a U-shaped response curve in DBA/2J males and an inverted U-shaped response curve in females of both strains. FGF21 levels likewise generally do not correlate with *Ucp1* expression, or the expression of other thermogenic and lipolytic genes besides in B6 males. As further evidence that the increased energy expenditure in WD Low Ile-fed mice is not mediated by FGF21, blood levels of FGF21 do not cluster with energy expenditure

or amino acid metabolism pathways in our multi-omics analysis; similarly, there is no correlation between circulating FGF21 and dietary Ile levels in humans.

While all protein-containing foods necessarily contain isoleucine, studies of the amino acid methionine have previously suggested that different classes of foods – e.g. plant-derived and animal-derived – may have different amino acid profiles (MacArthur et al., 2021; McCarty et al., 2009). In food item databases we observed that animal items had the highest proportional abundance of isoleucine, although plant-based food items had substantially greater variability in relative isoleucine abundance. Interestingly, investigating human intake data using NHANES led to the observation that lower protein consumers – who as a previous analysis has shown have lower rates of diabetes (Levine et al., 2014) – had lower proportional isoleucine intake. Finally, while those in the highest quintile of the HEI had the highest percentage of their diet coming from protein, surprisingly they had the lowest relative consumption of isoleucine. These data are consistent with our findings here and previous studies on isoleucine in humans that support a model in which lower dietary levels of isoleucine promote metabolic health (Deelen et al., 2019; Yu et al., 2021a).

While drastically reducing dietary levels of isoleucine across the board would require significant changes in the food supply, our analysis within food categories suggests that specific foods in each category may be unusually low in isoleucine content. Similarly, grains and baked goods tended to have lower levels of isoleucine than other food categories. Thus, substituting specific healthy low isoleucine foods may effectively lower dietary levels of isoleucine, and could be a way of rapidly applying these findings to humans. However, such dietary modifications would inevitably alter the intake of many macro- and micro- nutrients, and thus would need to be explicitly tested.

In summary, our results demonstrate that dietary levels of isoleucine strongly regulate the metabolic response to a Western diet across genetic backgrounds and in both male and female

mice. Reducing dietary isoleucine broadly reduces adiposity, improves the regulation of blood sugar, and increases energy expenditure. These effects are likely independent of the FGF21-UCP1 axis, as the correlation of FGF21 and *Ucp1* with dietary isoleucine levels is highly dependent upon sex and strain. Exposure to a Western diet containing high isoleucine results in strain-specific detriments in fat mass and insulin resistance. We also find that diet levels of isoleucine as a percentage of protein varies by type of food as well as specific items, and that humans with the highest healthy index scores – diets that most closely align with the Dietary Guidelines for Americans – naturally consume the lowest relative level of dietary isoleucine. Preventing obesity and metabolic syndrome in the face of the cheap high-calorie and high-fat foods ubiquitously available to adolescents is of the highest importance for preventing lifelong obesity and increased risk of many age-related diseases, and our results suggest that reducing dietary isoleucine may be a novel and translatable way to protect adolescents and young adults from the harmful effects of a Western diet on metabolic health.

Limitations of study

Limitations of our work include the relatively short length of our studies, as well as the use of only two different strains of young mice. Additionally, all of our studies were conducted in the context of a single high-fat, high-sucrose Western diet, and the effects of dietary isoleucine could potentially be different on the background of other unhealthy diets as has been seen for dietary protein (Wali et al., 2021). Additionally, this study did not consider physical activity or age as variables that alter metabolism and dietary responses. Recent data shows that resistance exercise can protect mice from fat accretion observed on a high protein diet (Trautman et al., 2023), but how altering isoleucine content would impact the response to exercise, particularly in the context of a Western diet, is unknown. Finally, skeletal muscle retention is vitally important in older adults, and the studies performed here were examined only in young animals.

Our molecular analysis was limited to the liver, and branched-chain amino acids including isoleucine are metabolized in tissues throughout the body (Neinast et al., 2019). Although the phenotypic and molecular correlations we have identified make strong biological sense and align with many of our previous findings, additional work will be required to determine which if any of the findings here are causative. This is true not just with respect to our mouse studies, but also to the human nutrition data analyzed here. Finally, while it appears feasible to construct a diet from natural foods that reduces isoleucine levels, it will require further research to determine if such a diet can improve metabolic health and protect from exposure to an otherwise Western diet.

Methods

Animal care, housing and diet

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital. Male and female DBA/2J and C57BL/6J mice were procured from the Jackson Laboratory (000664) at six weeks of age. All studies were performed on animals or on tissues collected from animals. All mice were acclimated to the animal research facility for at least one week before entering studies. All animals were housed 2 per cage in static microisolator cages in a specific pathogen-free mouse facility with a 12:12 h light–dark cycle, maintained at approximately 22 °C. At the start of the experiment, mice were randomized to receive the control diet (AA Chow, TD. 140711), 33% isoleucine western diet (WD Low Ile, TD. 170484), 100% isoleucine Western diet (WD Control, TD. 160186), or the 300% isoleucine Western (WD High Ile, TD. 200416) amino acid defined diets; all diets were obtained from Envigo. In the low and high isoleucine diets, nonessential amino acids were adjusted up or down to maintain the same

level of dietary nitrogen as the WD Control. Full diet descriptions, compositions and item numbers are provided in **Table 1**.

In vivo procedures and metabolic phenotyping

Average food consumption was measured over three days in the third week on diet by calculating the difference in food weight between the food put into the cage and that remaining. Food consumption was normalized to weight and lean mass determined at the same time food consumption was measured. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. Glucose and insulin tolerance tests were performed following a 16 hour overnight or 4 hour fast, respectively, and then injecting either glucose (1g/kg) or insulin (0.75U/kg) intraperitoneally (Bellantuono et al., 2020; Yu et al., 2019). Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips. For assays of multiple metabolic parameters (O2, CO2, food consumption, and activity tracking), mice were acclimatized to housing in a Columbus Instruments Oxymax/CLAMS-HC metabolic chamber system for approximately 24 hours, and data from a continuous 24-hour period was then recorded and analyzed.

Tissue collection for molecular analysis

Terminal submandibular blood was collected on the morning of euthanasia following a 16 hour overnight fast and was then separated into serum. Tissues were rapidly collected and flash frozen in liquid nitrogen, then, with the serum, stored at -80 degrees Celsius until utilized for molecular analysis.

Assays and kits

Serum insulin was quantified using an ultrasensitive mouse insulin ELISA kit (90080) from Crystal Chem (Elk Grove Village, IL, USA) and blood FGF21 levels were assayed by a mouse/rat FGF21 quantikine ELISA kit (MF2100) from R&D Systems (Minneapolis, MN, USA).

Quantitative real-time Polymerase Chain Reaction (PCR)

RNA was extracted from iWAT using TRIzol Reagent according to the manufacturer's protocol (Thermo Fisher Scientific). The concentration and purity of RNA were determined by absorbance at 260/280 nm using Nanodrop (Thermo Fisher Scientific). 1 µg of RNA was used to generate cDNA (Superscript III; Invitrogen, Carlsbad, CA, USA). Oligo dT primers and primers for real-time PCR were obtained from Integrated DNA Technologies (Coralville, IA, USA); sequences are in **Table S2**. Reactions were run on a StepOne Plus machine (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Invitrogen). Actin was used to normalize the results from gene-specific reactions.

Hepatic RNA extraction and –omics analyses

RNA was extracted from liver using TRIzol Reagent according to the manufacturer's protocol (Thermo Fisher Scientific). The concentration and purity of RNA were determined by absorbance at 260/280 nm using Nanodrop (Thermo Fisher Scientific) and samples were diluted to roughly 1000 ng/µl. RNA was submitted to the University of Wisconsin-Madison Biotechnology Center Gene Expression Center (*RRID:SCR_017757)* & DNA Sequencing Facility *(RRID:SCR_017759).* Each RNA sample was assayed on an Agilent RNA NanoChip to assess its integrity. RNA samples that met Illumina's TruSeq Stranded Total RNA Reference Guide (#1000000040499 v00) (Illumina, San Diego, CA) quality criteria were prepared for

sequencing following the protocol as recommended using a 250ng total RNA input. Libraries were multiplexed and sequenced on the NovaSeq6000 sequencer. Reads were aligned to the mouse (*Mus musculus*) with genome-build GRCm38.p5 of accession NCBI:GCA_000001635.7 and expected counts were generated with ensembl gene IDs (Zerbino et al., 2018).

Analysis of significantly differentially expressed genes (DEGs) was completed in R version 3.4.3 (Team, 2017) using *edgeR* (Robinson et al., 2010) and *limma* (Ritchie et al., 2015). Gene names were converted to gene symbol and Entrez ID formats using the *mygene* package. Genes with too many missing values were removed, if genes were present in less than one diet/strain/sex group they were removed. To reduce the impact of external factors not of biological interest that may affect expression, data was normalized to ensure the expression distributions of each sample are within a similar range. We normalized using the trimmed mean of M-values (TMM), which scales to library size. Heteroscedasticity was accounted for using the voom function, DEGs were identified using an empirical Bayes moderated linear model, and log coefficients and Benjamini-Hochberg (BH) adjusted p-values were generated for each comparison of interest (Benjamini and Hochberg, 2018). DEGs were used to identify enriched pathways, both Gene Ontology (for Biological Processes) and KEGG enriched pathways were determined for each contrast, enriched significantly differentially expressed genes (FDR cutoff=0.1). All genes, $\log_2$ fold-changes and corresponding unadjusted and Benjamini-Hochberg adjusted p-values can be found in **Tables S3** and **S4**.

Lipids were extracted from liver using a method modified from Matayash et al (2008). Approximately 40mg of liver tissue was added to a bead tube (Qiagen # 13113-50) with 250uL LCMS-grade water and 215uL LCMS-grade MeOH containing 10uL SPLASH lipidomix standard (Avanti #330707) and homogenized in a TissueLyzer (Qiagen) at 4C. 750uL methyl tert-butyl ether was added to each tube, and the tubes were mixed again and allowed to sit on ice for 15 minutes. Then, the samples were centrifuged for 5 minutes at 16100xG at 4C and 450uL of the

top organic layer was removed into a new tube. This phase was dried using a speedvac, and lipids were resuspended in 150uL LCMS-grade IPA.

Global lipidomics analysis was performed in positive ion mode on 3uL of lipid extract and in negative ion mode on 5uL of lipid extract. Lipids were separated on a VanGuard BEH C18 precolumn (Waters 18003975) attached to an Acquity BEH C18 column (2.1x100mm, 1.7uM, Waters 186009453) kept at 50C on an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6545 Q-TOF MS dual AJS ESI mass spectrometer. Mobile phase A consisted of 600:400 Acetonitrile:H2O containing 1mL of formic acid (Fisher A11710X1-AMP) and 0.63g ammonium formate (Honeywell 55674). Mobile phase B consisted of 900:90:10 IPA:Acetonitrile:H2O with 1mL of formic acid and 0.63g ammonium formate. The flow rate was 0.5mL per minute. For both positive and negative mode, the gradient began with 15% mobile phase B, increased to 30% B until 2.4 minutes, and then increased to 48% B until 3 minutes. The gradient increased to 82% B until 13.2 min, and then to 99% B until 13.8 minutes, when it stayed at 99% B until 15.4 minutes, when re-equilibration began and the gradient decreased to 15% B until 20 min.

For both positive and negative ionization mode, the gas temperature was 250C, the flow rate was 12L/min, VCap was 4000V, skimmer was 75V, fragmentor was 190V, and Octapole RF peak was 750V. For positive mode, the sheath gas temperature was 300C, the sheath gas flow rate was 11L/min, and the nebulizer was 35 psig. Reference masses used in positive mode were 121.05 and 922.00 m/z. In negative mode, the sheath gas temperature was 375C, the sheath gas flow rate was 12L/min, and the nebulizer was 30 psig. Reference masses used in negative mode were 112.98 and 966.00 m/z. In both ionization modes, the collision energy for tandem MS was fixed at 25V.

For liver metabolomics, tissue powder was weighed (~20 mg) on dry ice. The extraction was done by adding 20C methanol:acetonitrile:water (40:40:20) mixture to the powder, followed by vortexing and centrifugation at 16,000 x g for 10 min at 4C. The volume of the extraction

solution (mL) was 40 x the weight of tissue (mg) to make an extract of 25 mg tissue per mL solvent. The supernatant (3 mL) was loaded to LC-MS. Samples were analyzed using a quadrupole-orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, San Jose, CA) operating in negative or positive ion modes, coupled to hydrophilic interaction chromatography via electrospray ionization and used to scan from m/z 70 to 1000 at 1 Hz and 140,000 resolution. LC separation was on a XBridge BEH Amide column (2.1 mm x 150 mm, 2.5 mm particle size, 130A° pore size) using a gradient of solvent A (20mM ammonium acetate, 20mM ammonium hydroxide in 95:5 water: acetonitrile, pH 9.45) and solvent B (acetonitrile). Flow rate was 150 ml/min. The LC gradient was: 0 min, 85% B; 2 min, 85% B; 3 min, 80% B; 5 min, 80% B; 6 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 16 min, 25% B; 18 min, 0% B; 23 min, 0% B; 24 min, 85% B; 30 min, 85% B. Autosampler temperature was 4°C.

Multi-omics analyses

Four data types (metabolomics, transcriptomics, lipidomics, phenotypic outcomes) were obtained from experiments with three factors (strain (B6, DBA), sex, and diet (WD Low Ile, WD Control, and WD High Ile) from 72 mice (6 per diet group of each sex and strain). The data consisted of 534 metabolites, 15636 probes, 344 lipids, and 29 phenotypes.

To identify molecules of interest in each of the -omics datasets, significantly differentially expressed molecules between WD Control and WD High/Low Ile Groups were identified using an empirical Baye's moderated linear model. The Benjamini-Hochberg method was applied to control false discovery rate, selecting those with adjusted p-value < 0.05 (Benjamini and Hochberg 1995). Transcriptomics, metabolomics and lipidomics data were preprocessed, log2 transformed, z-scale normalized across molecules and samples for each data type individually. Phenotypic outcome data were similarly z-scale normalized just across phenotypes. The data consisted of

5903 inputs across individual mice which had no missing data points including 65 metabolites, 5688 transcripts, 121 lipids, and 29 phenotypes.

To integrate the data, all four datatypes were concatenated for each comparison. Correlations were performed between the 5903 data points using Spearman's rank (5903 × 5903 = 34,845,409 correlations). Complete hierarchical clustering was used to reorder molecules based on 1 – Spearman correlation between all molecules. The number of clusters were determined by silhouette scores (Rousseeuw, 1987).

All analyses were performed in R (v. 4.2.1) using emmeans (Lenth, 2023) (v. 1.8.4), ComplexHeatmap (Gu et al., 2016) (v. 2.14.0), cluster (Maechler, 2022) (v. 2.1.4). For each cluster, the over representation of KEGG pathways (Kanehisa et al., 2017) from genes were determined using kegga and the gene ontology terms were determined using goana from limma (Ritchie et al., 2015) (v. 3.54.2).

Transcription factor analysis

Significantly up- and downregulated conserved genes were entered into the Mining Algorithm for Genetic Controllers (MAGIC) algorithm (Roopra, 2020). Significant predicted readouts were selected and cross referenced with our hepatic expression analysis to generate a heatmap.

SHOW study

We analyzed the association between dietary isoleucine intake and FGF21 levels in blood samples collected from 2016−2017 SHOW participants. SHOW is an ongoing population-based health examination survey of non-institutionalized residents of Wisconsin. Detailed survey methods have been previously described (Malecki et al., 2022; Nieto et al., 2010).

Survey components relevant to the current analysis included an in-home interview accompanied by measurements of weight and height, and a self-administered questionnaire including the National Cancer Institute's Diet History Questionnaire, from which specific dietary intake variables were derived.

The study population included 788 individuals who completed all parts of the survey including diet history, blood draw and exam visit. Demographic characteristics of the population have been described previously (Flores et al.). All study protocols were approved by the University of Wisconsin Health Sciences Institutional Review Board, and all participants provided written informed consent as part of the initial home visit. The intake of isoleucine was estimated from the Diet History Questionnaire II (National Cancer Institute) using Diet*Calc software (National Cancer Institute). The estimated levels of isoleucine are expressed as a percentage (%) of the total protein.

FGF21 was quantified from 140 subjects using the previously described ELISA kit and plasma isoleucine determined was determined by the Wisconsin State Laboratory of Hygiene. Multiple linear regression was performed using STATA 17.0 (STATA Corp LLC, College Station, TX, USA) with FGF21 as the outcome and percentage of total protein from isoleucine as the predictor of interest. In the model we also adjusted for age, sex, education, income, total caloric intake and physical activity.

Diet analyses

For food item analyses, the USDA Food and Nutrient Database SR-28 (NDB) was downloaded. Food items were categorized based on food group codes including dairy and eggs (100), poultry (500), pork (1000), beef (1300), finfish and shellfish (1500), lamb, veal and game (1700), fruits

(900), vegetables (1100), nuts and seeds (1200), legumes (1600), cereal grains and pasta (2000). Amino acid analyses in the USDA NDB were performed using three methods for tryptophan, sulfur-containing amino acids and all other amino acids. Tryptophan was measured by alkaline hydrolysis followed by HPLC. Sulfur-containing amino acids were measured by oxidation with performic acid followed by HPLC. All other amino acids were measured by acid hydrolysis followed by HPLC. Values in the USDA NDB are presented as grams of amino acid per 100 grams of food item. Full details on methodology, calculations and data organization are available in the USDA NDB SR28 documentation.

For dietary intake data, data from the National Health and Nutrition Examination Survey (NHANES) was used. Records obtained from the CDC NHANES FTP were read into R using the sasxport.get function from the Hmisc package. Dietary data included two 24-hour dietary recalls conducted by trained dietary interviewers. To assess amino acid composition across dietary records, amino acid data from the USDA NBD SR28 were linked to NHANES food items using SR28 link codes from the Food and Nutrition Database for Dietary Studies (FNDDS). Records were obtained from survey rounds conducted from 2005 to 2012, including four rounds of data release. Individuals who were not pregnant, were over the age of 18 and had two complete 24-hour recalls were included in the analysis, for a total of 23,245 participants. For all nutrient variables including amino acids and total protein, the mean value across two recalls was computed and used for analyses. Healthy Eating Index scores were calculated using the HEI2015_NHANES_FPED function from the dietaryindex R package (v. 1.0.3) using the same set of records described above.

Statistical analyses

Statistical analysis was conducted using Prism, version 9 (GraphPad Software Inc., San Diego, CA, USA). Tests involving repeated measurements were analyzed with two-way repeated-measures ANOVA, followed by a Tukey–Kramer or Dunnett's post hoc test as specified. All other comparisons of three or more means were analyzed by one-way ANOVA followed by a Dunnett's or Tukey–Kramer post hoc test as specified where appropriate. Additional comparisons, if any, were corrected for multiple comparisons using the Bonferroni method. Outliers were determined using GraphPad Prism Grubbs' calculator (https://www.graphpad.com/quickcalcs/grubbs1/). PCA plots were created using the *"FactoMineR"* package in R. Male and female phenotypic data contained 30 different measures variables and were plotted separately in PCA plots for clarity.

DECLARATION OF INTERESTS

DWL has received funding from, and is a scientific advisory board member of, Aeovian Pharmaceuticals, which seeks to develop novel, selective mTOR inhibitors for the treatment of various diseases.

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Chapter 3. Table 1.

	AA Control	WD Low Ile	WD Control	WD High Ile
Teklad Diet Number	TD.140711	TD.170484	TD.160186	TD.200416
Color	Red	Pink	Blue	Purple
Formula	g/kg	g/kg	g/kg	g/kg
Sucrose	291.248	341.46	291.248	341.46
Corn Starch	150	50.2436	232.4	47.8125
Maltodextrin	150	50.2436	232.4	47.8125
Corn Oil	52	0	52	0
Olive Oil	29	0	29	0
Anhydrous Milkfat	0	210	210	210
Cholesterol	0	1.5	1.5	1.5
Cellulose	30	50	50	50
Mineral Mix, AIN -93G -MX (94046)	35	35	35	35
Calcium Phosphate, monobasic, monohydrate	8.2	8.2	8.2	8.2
Food Color	0.1	0.1	0.1	Red 0.067, Blue 0.033
TBHQ (antioxidant)	0.012	0.04	0.04	0.04
Vitamin Mix, Teklad (40060)	10	10	10	10
% kcal from	%	%	%	%
Protein	22	20.7	20.7	19
Carbohydrate	59.4	38.5	38.5	39.1
Fat	18.6	40.9	40.9	41.9
Kcal/g	3.9	4.6	4.6	4.5
Amino Acid Profile	g/kg	g/kg	g/kg	g/kg
L-Alanine	9.38	9.8267	9.38	8.055
L-Arginine	6.3	6.3	6.3	6.3
L-Asparagine	20.58	20.9113	20.58	19.6
L-Aspartic Acid	20.58	21.2475	20.58	18.6
L-Cystine	7.2	7.2	7.2	7.2
L-Glutamic Acid	28.97	29.7077	28.97	26.78
L-Glutamine	33.77	34.1395	33.77	32.67
Glycine	2.96	3.3363	2.96	1.84
L-Histidine HCl, monohydrate	4.6	4.6	4.6	4.6
L-Isoleucine	7.8	2.54	7.8	23.4
L-Leucine	25.4	25.4	25.4	25.4
L-Lysine HCl	20.38	20.38	20.38	20.38
L-Methionine	6.7	6.7	6.7	6.7
L-Phenylalanine	6.6	6.6	6.6	6.6
L-Proline	7.41	7.987	7.41	5.7
L-Serine	7.41	7.9368	7.41	5.85
L-Threonine	9.7	9.7	9.7	9.7
L-Tryptophan	3.4	3.4	3.4	3.4
L-Tyrosine	6.9	6.9	6.9	6.9
L-Valine	8.4	8.4	8.4	8.4

Figure 1

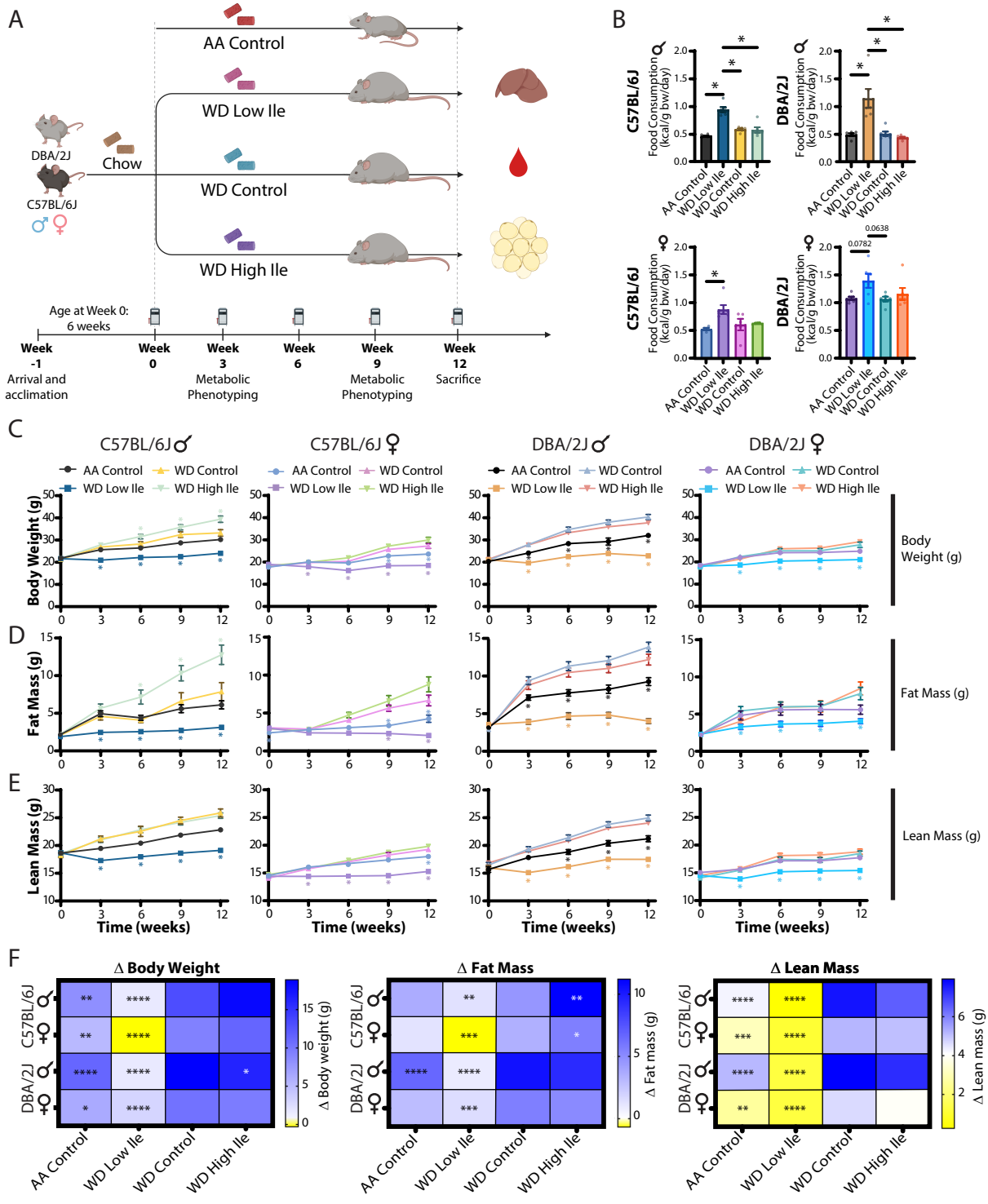

A
AA Control
WD Low Ile
WD Control
WD High Ile
DBA/2J
C57BL/6J
Chow
Age at Week 0:
6 weeks
Week -1
Arrival and acclimation
Week 0
Week 3
Metabolic Phenotyping
Week 6
Week 9
Metabolic Phenotyping
Week 12
Sacrifice
B
C57BL/6J
DBA/2J
Food Consumption (kcal/g bw/day)
AA Control
WD Low Ile
WD Control
WD High Ile
0.0638
0.0782
C
C57BL/6J♂
C57BL/6J♀
DBA/2J♂
DBA/2J♀
Body Weight (g)
Fat Mass (g)
Lean Mass (g)
Time (weeks)
D
E
F
Δ Body Weight
Δ Fat Mass
Δ Lean Mass
Δ Body weight (g)
Δ Fat mass (g)
Δ Lean mass (g)

Figure Legends

Figure 1. Reduced dietary isoleucine protects from WD-induced weight and fat gain.

(A) Experimental design.

(B) Food consumption normalized to body weight after 3 or 8 weeks on the indicated diets.

(C-F) Body weight (C), fat mass (D) and lean mass (E) was tracking longitudinally, and change (Δ) from the beginning to end of study was calculated (F).

(B) n=2-12 mice per group. Statistics for the overall effects of diet represent the p value from a one-way ANOVA when compared to the WD Control diet; *p<0.05, Sidak's post-test examining the effect of parameters identified as significant in the one-way ANOVA.

(C-F) For longitudinal studies, statistics for the overall effects of time, diet, and the interaction represent the p value from a two-way RM ANOVA or residual maximum likelihood (REML) analysis conducted individually for each sex and strain. Each diet was compared to the WD Control diet; *p<0.05, Dunnett's post-test examining the effect of parameters identified as significant in the two-way ANOVA.

Data represented as mean ± SEM.

Figure 2.

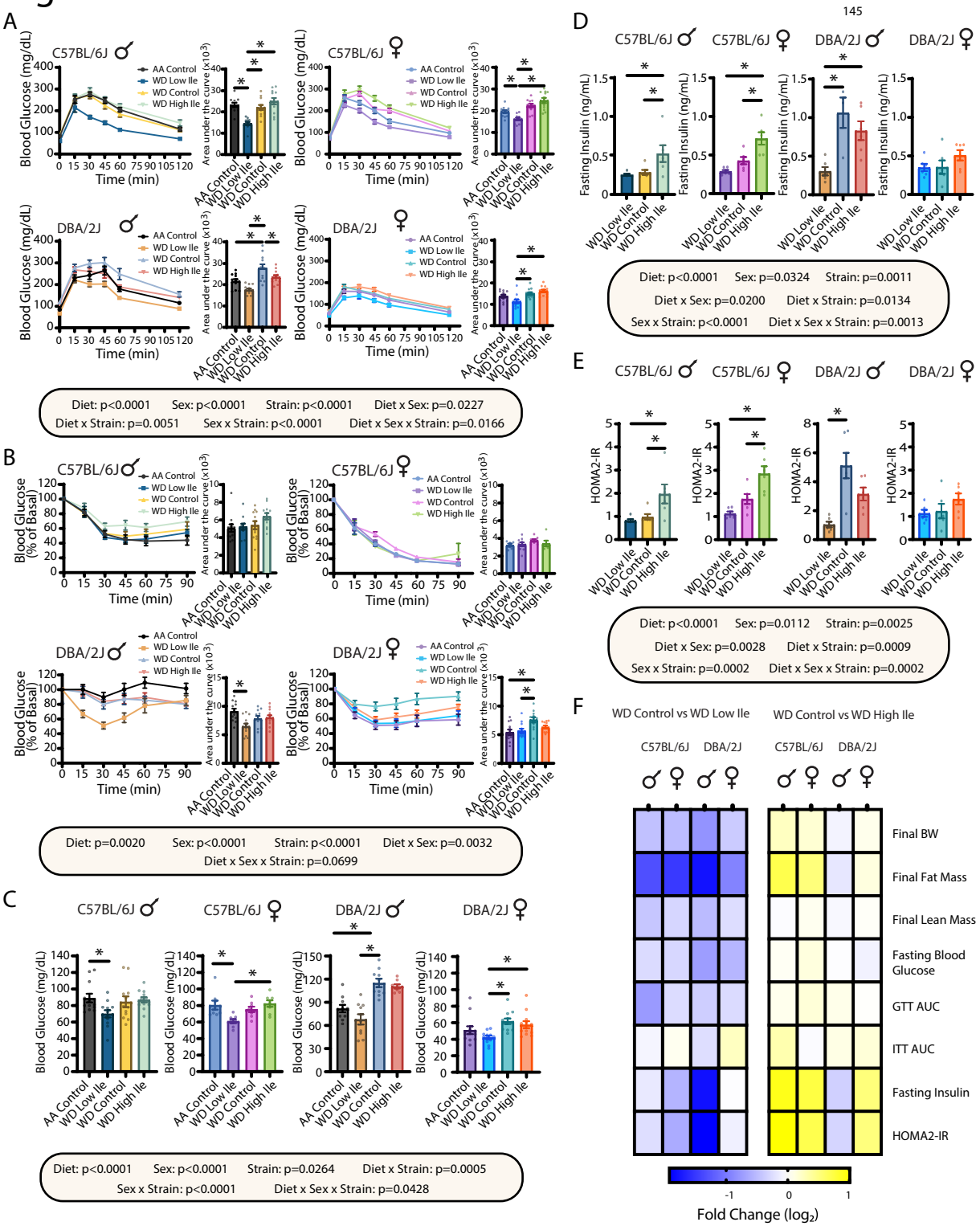

A
C57BL/6J ♂
C57BL/6J ♀
DBA/2J ♂
DBA/2J ♀
AA Control
WD Low Ile
WD Control
WD High Ile
Blood Glucose (mg/dL)
Time (min)
Area under the curve (x10³)
Diet: p<0.0001 Sex: p<0.0001 Strain: p<0.0001 Diet x Sex: p=0.0227
Diet x Strain: p=0.0051 Sex x Strain: p<0.0001 Diet x Sex x Strain: p=0.0166
B
Blood Glucose (% of Basal)
Diet: p=0.0020 Sex: p<0.0001 Strain: p<0.0001 Diet x Sex: p=0.0032
Diet x Sex x Strain: p=0.0699
C
Diet: p<0.0001 Sex: p<0.0001 Strain: p=0.0264 Diet x Strain: p=0.0005
Sex x Strain: p<0.0001 Diet x Sex x Strain: p=0.0428
D
Fasting Insulin (ng/mL)
Diet: p<0.0001 Sex: p=0.0324 Strain: p=0.0011
Diet x Sex: p=0.0200 Diet x Strain: p=0.0134
Sex x Strain: p<0.0001 Diet x Sex x Strain: p=0.0013
E
HOMA2-IR
Diet: p<0.0001 Sex: p=0.0112 Strain: p=0.0025
Diet x Sex: p=0.0028 Diet x Strain: p=0.0009
Sex x Strain: p=0.0002 Diet x Sex x Strain: p=0.0002
F
WD Control vs WD Low Ile
WD Control vs WD High Ile
Final BW
Final Fat Mass
Final Lean Mass
Fasting Blood Glucose
GTT AUC
ITT AUC
Fasting Insulin
HOMA2-IR
Fold Change (log2)

Figure 2. Dietary isoleucine is negatively associated with glycemic control in WD-fed mice.

(A-B) Glucose (A) and insulin (B) tolerance tests in mice fed the indicated diets for 9 weeks and 10 weeks, respectively, with quantified area under the curve (AUC).

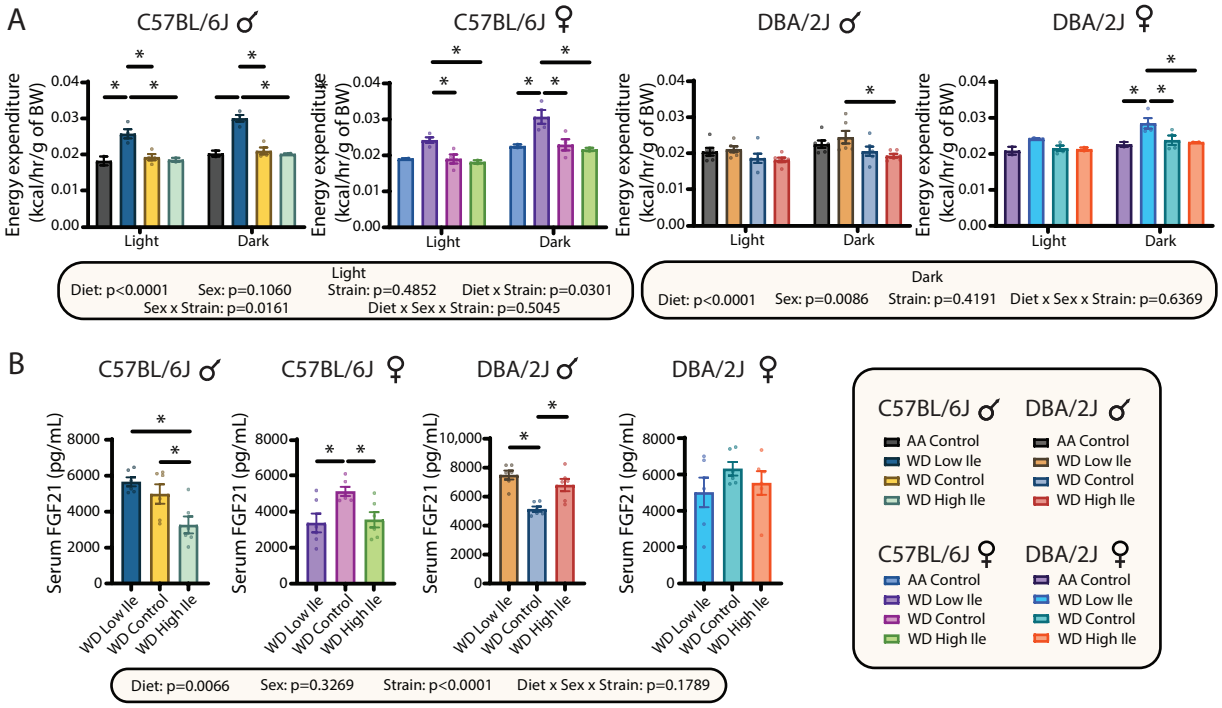
(C-E) Fasting blood glucose (C) and insulin (D) levels were determined and HOMA2-IR (E) was calculated after 12 weeks on the indicated diets.

(F) Heatmap of the effect of isoleucine on body composition and glucose homeostasis.

(B-F) n=6-12 mice per group. Statistics for the overall effects of diet, sex, and strain represent the p value from a three-way ANOVA; *$p<0.05$, Tukey's test post ANOVA for each sex/strain group shown.

Data represented as mean ± SEM.

Figure 3.


A
C57BL/6J ♂
C57BL/6J ♀
DBA/2J ♂
DBA/2J ♀
Energy expenditure (kcal/hr/g of BW)
Light
Dark
Light
Diet: p<0.0001 Sex: p=0.1060 Strain: p=0.4852 Diet x Strain: p=0.0301
Sex x Strain: p=0.0161 Diet x Sex x Strain: p=0.5045
Dark
Diet: p<0.0001 Sex: p=0.0086 Strain: p=0.4191 Diet x Sex x Strain: p=0.6369
B
C57BL/6J ♂
C57BL/6J ♀
DBA/2J ♂
DBA/2J ♀
Serum FGF21 (pg/mL)
WD Low Ile
WD Control
WD High Ile
Diet: p=0.0066 Sex: p=0.3269 Strain: p<0.0001 Diet x Sex x Strain: p=0.1789
C57BL/6J ♂
DBA/2J ♂
AA Control
WD Low Ile
WD Control
WD High Ile
C57BL/6J ♀
DBA/2J ♀
AA Control
WD Low Ile
WD Control
WD High Ile

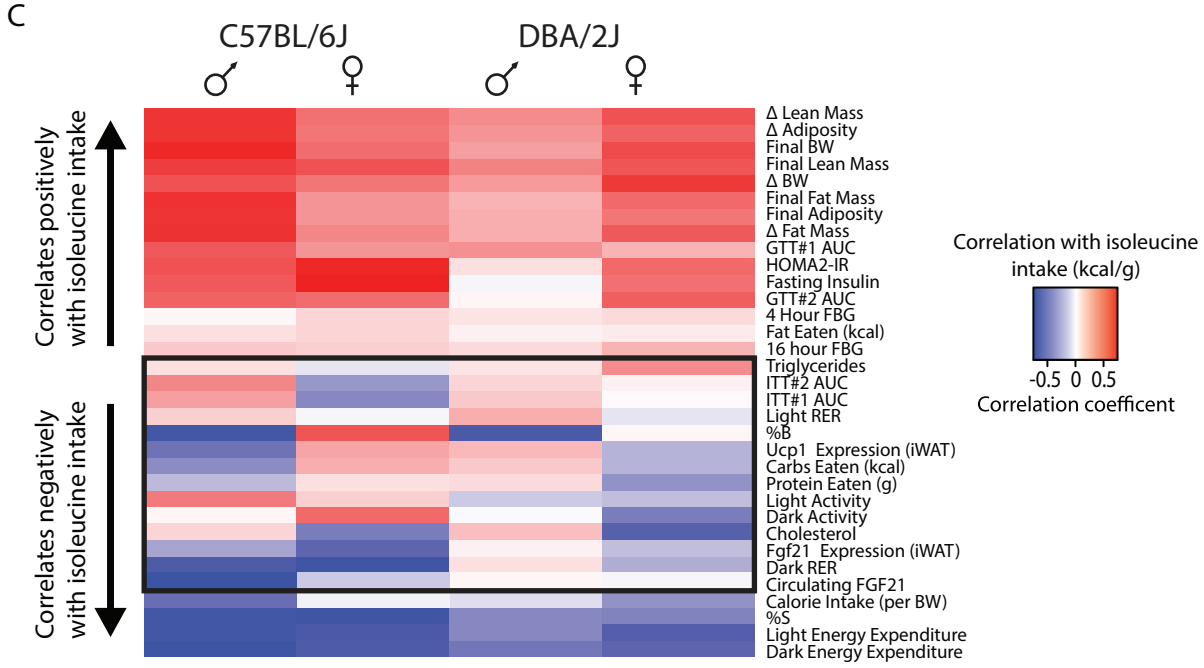

C
C57BL/6J
DBA/2J
♂
♀
Correlates positively with isoleucine intake
Correlates negatively with isoleucine intake
Δ Lean Mass
Δ Adiposity
Final BW
Final Lean Mass
Δ BW
Final Fat Mass
Final Adiposity
Δ Fat Mass
GTT#1 AUC
HOMA2-IR
Fasting Insulin
GTT#2 AUC
4 Hour FBG
Fat Eaten (kcal)
16 hour FBG
Triglycerides
ITT#2 AUC
ITT#1 AUC
Light RER
%B
Ucp1 Expression (iWAT)
Carbs Eaten (kcal)
Protein Eaten (g)
Light Activity
Dark Activity
Cholesterol
Fgf21 Expression (iWAT)
Dark RER
Circulating FGF21
Calorie Intake (per BW)
%S
Light Energy Expenditure
Dark Energy Expenditure
Correlation with isoleucine intake (kcal/g)
-0.5 0 0.5
Correlation coefficent

Figure 3. Dietary isoleucine is negatively associated with energy expenditure, but not FGF21, in WD-fed mice.

(A) Energy expenditure per gram of body weight.

(B) Circulating FGF21.

(C) Phenotypic and molecular measurements correlated with consumption of isoleucine (kcals) in each mouse (Pearson's correlation) and clustered (hierarchical clustering). Phenotypic measurements that do not cluster as well appear in the middle of the correlation plot (black outline). n=3-7 mice per group. Statistics for the overall effects of diet, sex, and strain represent the p value from a three-way ANOVA; *$p<0.05$, Tukey post-test examining the effect of parameters identified as significant in the one-way ANOVA.

Data represented as mean ± SEM.

Figure 4.


A
Significant Pathways - WD Control vs WD Low Ile
Direction
Up
Down
NS
Pathway Names
Terpenoid backbone biosynthesis
Steroid biosynthesis
Asthma
Intestinal immune network for IgA production
2–Oxocarboxylic acid metabolism
Sulfur metabolism
Vitamin B6 metabolism
Ribosome
Fc gamma R–mediated phagocytosis
Phagosome
Neutrophil extracellular trap formation
Epstein–Barr virus infection
Toll–like receptor signaling pathway
Carbon metabolism
Ribosome biogenesis in eukaryotes
Inflammatory bowel disease
Leukocyte transendothelial migration
Graft–versus–host disease
Allograft rejection
Autoimmune thyroid disease
Osteoclast differentiation
Fc epsilon RI signaling pathway
Natural killer cell mediated cytotoxicity
Staphylococcus aureus infection
B cell receptor signaling pathway
Coronavirus disease – COVID–19
AGE–RAGE signaling pathway in diabetic complications
Cysteine and methionine metabolism
Measles
One carbon pool by folate
Arginine biosynthesis
Leishmaniasis
Viral myocarditis
Type I diabetes mellitus
Tuberculosis
Necroptosis
Kaposi sarcoma–associated herpesvirus infection
RIG–I–like receptor signaling pathway
Nicotine addiction
Biosynthesis of amino acids
Pentose phosphate pathway
Pertussis
Signaling pathways regulating pluripotency of stem cells
Antifolate resistance
Systemic lupus erythematosus
Antigen processing and presentation
Influenza A
Human immunodeficiency virus 1 infection
JAK–STAT signaling pathway
C–type lectin receptor signaling pathway
mRNA surveillance pathway
Complement and coagulation cascades
Hepatitis C
Spliceosome
NOD–like receptor signaling pathway
Peroxisome
Toxoplasmosis
Lipid and atherosclerosis
Nucleocytoplasmic transport
D–Amino acid metabolism
Glyoxylate and dicarboxylate metabolism
Amino sugar and nucleotide sugar metabolism
Rheumatoid arthritis
Purine metabolism
Ascorbate and aldarate metabolism
NF–kappa B signaling pathway
Pyruvate metabolism
Glycine, serine and threonine metabolism
Valine, leucine and isoleucine degradation
Pentose and glucuronate interconversions
Histidine metabolism
Arginine and proline metabolism
Chemokine signaling pathway
Tryptophan metabolism
Steroid hormone biosynthesis
Alanine, aspartate and glutamate metabolism
Lysosome
Fat digestion and absorption
Biosynthesis of cofactors
Other glycan degradation
Sphingolipid metabolism
Viral protein interaction with cytokine and cytokine receptor
Hepatocellular carcinoma
Cytokine–cytokine receptor interaction
Fluid shear stress and atherosclerosis
Drug metabolism – cytochrome P450
Metabolism of xenobiotics by cytochrome P450
Platinum drug resistance
Chemical carcinogenesis – DNA adducts
Drug metabolism – other enzymes
Glutathione metabolism
Metabolic pathways
C57BL/6J
DBA/2J
B
C57BL/6J
WD Low Ile
WD Control
WD High Ile
2 1 0 -1 -2
WD Low Ile
WD Control
WD High Ile
*
4–aminobenzoate
cortisol 21–acetate
Ethyl 3–Ureidopropropionate
3–hydroxybutyrate
Dioxoheptanoic acid
Cholesteryl sulfate
2–Keto–6–Acetamidocaproate
N–acetyl–gln
3–Methylglutaconate
Encelin isomer 1
Cresol sulfate
Thymidine
N–Lauroylglycine
N–Nonanoylglycine
Thiamine Pyrophosphate
Hypotaurine
LysoPC(18:0)
Asparagine
LysoPC(15:0)
LPE 20:0
LysoPC(22:4)
Lysyl–Tyrosine
Trans–Cinnamate
Phenylalanine
phenol
Furazolidone
4–Coumarate
Tyrosine
LPE 20:1
Indoleacrylic acid
1,5–Naphthalenediamine
LysoPC (20:3)
LysoPC(20:4)
LysoPC(22:6)
LysoPC(20:0)
Gonyautoxin I
SM d18:1/14:0
LysoPE(22:1)
LysoPE(22:0)
Ascorbate
**
LysoPC (20:2)
3–amino–4–hydroxybenxoate
***
Indole
Quinoline
N–Methylglutamate
benzaldehyde
Glycodehydrocholic acid
*Bis–tert–butoxycarbonyl-amino-oxy-acetic acid isomer
**(4–amino–1,2,5–oxadiazol-3-yl)(1-pyrrolidinyl) methanone oxime
***N1–Methyl–4–pyridone-3-carboxamide isomer 1

Figure 4. Altering the isoleucine content of a WD has sex and strain-specific effects on the hepatic transcriptome and metabolome.

(A) KEGG pathway analysis of significantly altered genes in WD Low Ile-fed mice relative to WD Control-fed mice in each sex and strain.

(B) Hepatic metabolites significantly altered in response to dietary isoleucine in C57BL/6J male mice consuming a Western diet.

Figure 5.

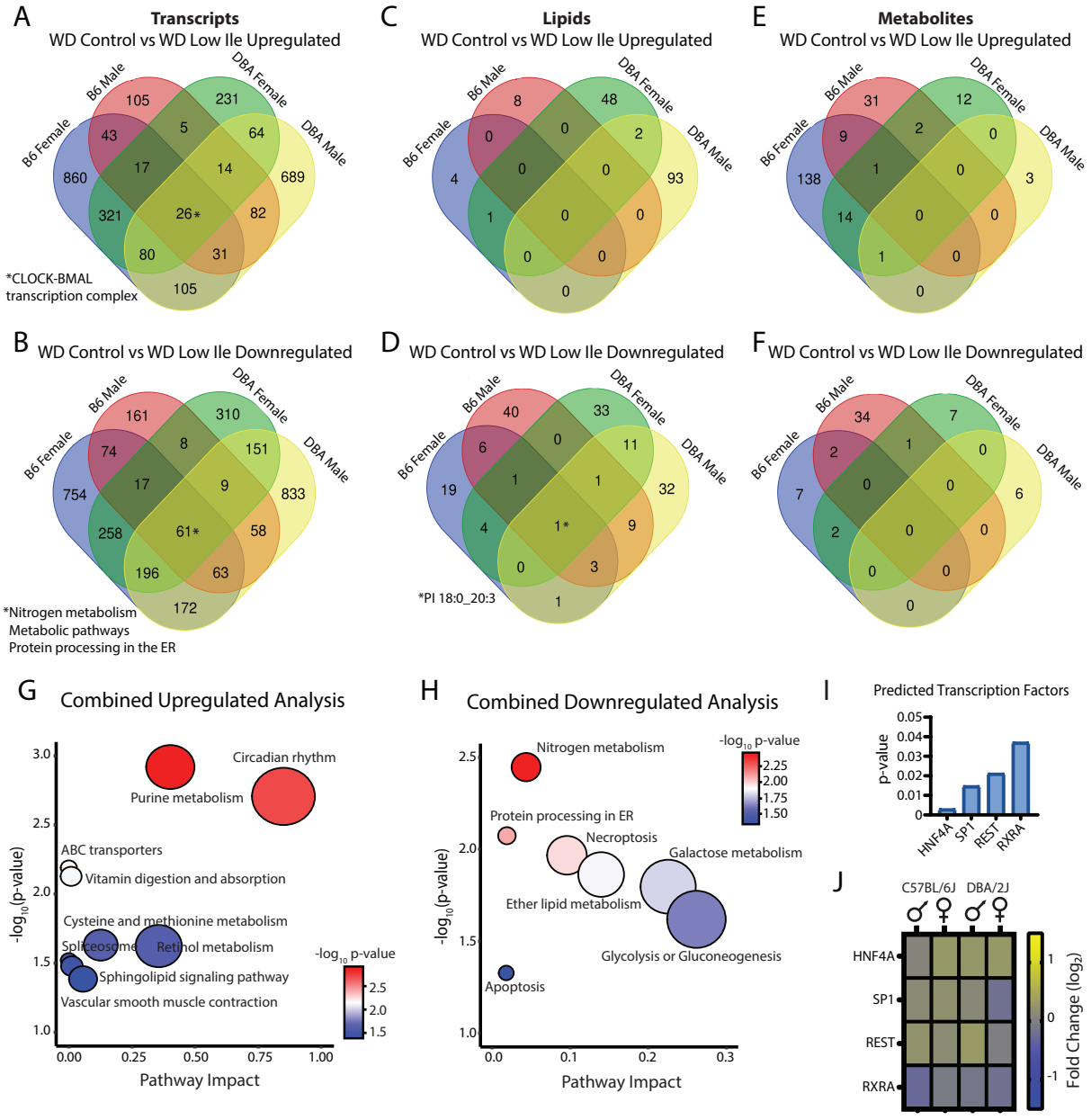

A
Transcripts
WD Control vs WD Low Ile Upregulated
B6 Male
DBA Female
B6 Female
DBA Male
105
231
5
43
64
17
14
860
689
321
26*
82
80
31
105
*CLOCK-BMAL
transcription complex
C
Lipids
WD Control vs WD Low Ile Upregulated
8
48
0
0
2
4
0
0
93
1
0
0
0
0
0
E
Metabolites
WD Control vs WD Low Ile Upregulated
31
12
9
2
0
138
1
0
3
14
0
0
1
0
0
B
WD Control vs WD Low Ile Downregulated
161
310
8
74
151
17
9
754
833
258
61*
58
196
63
172
*Nitrogen metabolism
Metabolic pathways
Protein processing in the ER
D
WD Control vs WD Low Ile Downregulated
40
33
0
6
11
1
1
19
32
4
1*
9
0
3
1
*PI 18:0_20:3
F
WD Control vs WD Low Ile Downregulated
34
7
1
2
0
0
7
6
2
0
0
0
0
0
G
Combined Upregulated Analysis
Circadian rhythm
Purine metabolism
ABC transporters
Vitamin digestion and absorption
Cysteine and methionine metabolism
Retinol metabolism
Sphingolipid signaling pathway
Vascular smooth muscle contraction
-log10 p-value
2.5
2.0
1.5
-log10(p-value)
3.0
2.5
2.0
1.5
1.0
0.00
0.25
0.50
0.75
1.00
Pathway Impact
H
Combined Downregulated Analysis
Nitrogen metabolism
-log10 p-value
2.25
2.00
1.75
1.50
Protein processing in ER
Necroptosis
Galactose metabolism
Ether lipid metabolism
Glycolysis or Gluconeogenesis
Apoptosis
-log10(p-value)
2.5
2.0
1.5
1.0
0.0
0.1
0.2
0.3
Pathway Impact
I
Predicted Transcription Factors
p-value
0.05
0.04
0.03
0.02
0.01
0
HNF4A
SP1
REST
RXRA
J
C57BL/6J
DBA/2J
HNF4A
SP1
REST
RXRA
Fold Change (log2)
1
0
-1

Figure 5. Analysis across sexes and strains identifies a conserved molecular response of the liver to restriction of dietary isoleucine.

(A-F) Venn diagrams of the genes (A-B), lipids (C-D), and metabolites (E-F) significantly altered by a WD Low Ile diet in the livers of mice of the indicated strain and sex.

(G-H) Metaboanlyst was used to identify pathways altered in the shared upregulated (G) and downregulated (H) genes and lipids identified in A-F.

(I-J) Predicted transcription factors driving the genes altered in all sexes and strains (I) and their corresponding expression level (J).

Figure 6.

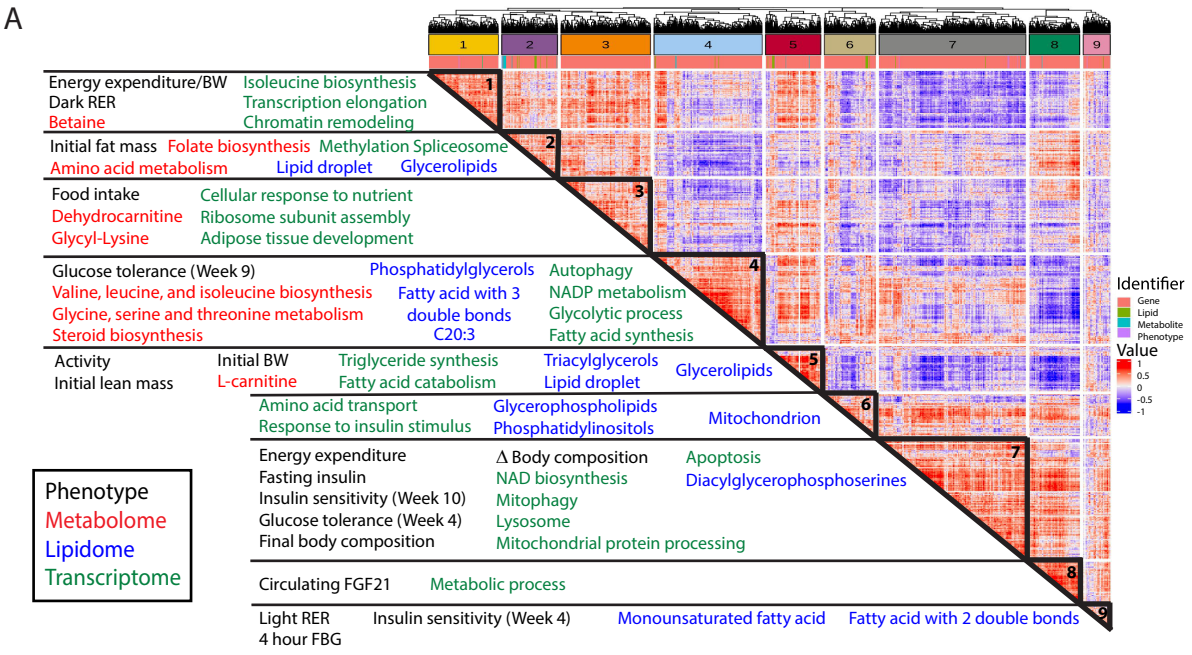

A
1
2
3
4
5
6
7
8
9
Energy expenditure/BW
Dark RER
Betaine
Isoleucine biosynthesis
Transcription elongation
Chromatin remodeling
Initial fat mass
Amino acid metabolism
Folate biosynthesis
Methylation
Spliceosome
Lipid droplet
Glycerolipids
Food intake
Dehydrocarnitine
Glycyl-Lysine
Cellular response to nutrient
Ribosome subunit assembly
Adipose tissue development
Glucose tolerance (Week 9)
Valine, leucine, and isoleucine biosynthesis
Glycine, serine and threonine metabolism
Steroid biosynthesis
Phosphatidylglycerols
Fatty acid with 3 double bonds
C20:3
Autophagy
NADP metabolism
Glycolytic process
Fatty acid synthesis
Activity
Initial lean mass
Initial BW
L-carnitine
Triglyceride synthesis
Fatty acid catabolism
Triacylglycerols
Lipid droplet
Glycerolipids
Amino acid transport
Response to insulin stimulus
Glycerophospholipids
Phosphatidylinositols
Mitochondrion
Energy expenditure
Fasting insulin
Insulin sensitivity (Week 10)
Glucose tolerance (Week 4)
Final body composition
Δ Body composition
NAD biosynthesis
Mitophagy
Lysosome
Mitochondrial protein processing
Apoptosis
Diacylglycerophosphoserines
Circulating FGF21
Metabolic process
Light RER
4 hour FBG
Insulin sensitivity (Week 4)
Monounsaturated fatty acid
Fatty acid with 2 double bonds
Phenotype
Metabolome
Lipidome
Transcriptome
Identifier
Gene
Lipid
Metabolite
Phenotype
Value
1
0.5
0
-0.5
-1
B
β=2.46
p=0.046
BMI
70
60
50
40
30
20
2.5
3
3.5
4
4.5
5
Isoleucine (% of total protein)
C
β=-102.15
p=0.27
FGF21 level (pg/mL)
1500
1000
500
0
2.5
3
3.5
4
4.5
5
Isoleucine (% of total protein)

Figure 6. Hepatic omic analyses reveal correlation clusters.

(A) Spearman's Rank Order Correlation matrix utilizing hepatic metabolomics, lipidomics, and transcriptomics data to correlate with phenotypes. n=6 per group.

Isoleucine restriction produces changes across phenotypes that correlate with genes, metabolites, and lipids. Spearman's rank order correlation matrix of significant observations, including phenotypic (black), transcriptomic (green), metabolomics (red), and lipidomic (blue) changes between WD Control and WD Low Ile diet groups. Hierarchical clustering identified 9 mega-clusters (outlined in black; **Table S9**). Enriched pathways listed in **Table S5**; phenotypes and pathways of interest in each cluster are highlighted.

(B) Association between BMI and percent of total protein from Ile from the SHOW study (n = 788, shaded area represents 95% CI).

(C) Association between circulating FGF21 and dietary isoleucine intake (n = 788, shaded area represents 95% CI).

Figure 7.

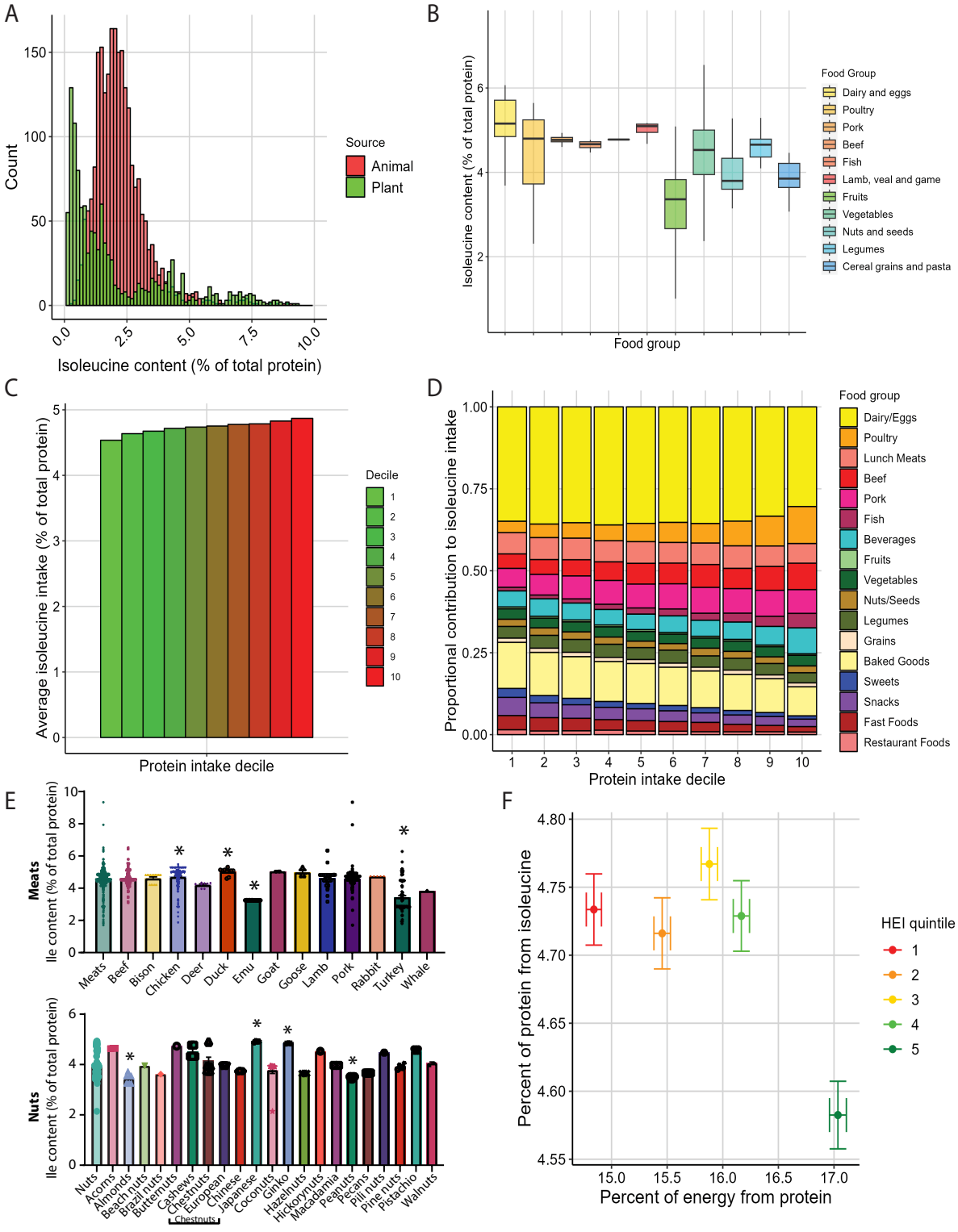

A
Count
Isoleucine content (% of total protein)
Source
Animal
Plant
B
Isoleucine content (% of total protein)
Food group
Food Group
Dairy and eggs
Poultry
Pork
Beef
Fish
Lamb, veal and game
Fruits
Vegetables
Nuts and seeds
Legumes
Cereal grains and pasta
C
Average isoleucine intake (% of total protein)
Protein intake decile
Decile
D
Proportional contribution to isoleucine intake
Protein intake decile
Food group
Dairy/Eggs
Poultry
Lunch Meats
Beef
Pork
Fish
Beverages
Fruits
Vegetables
Nuts/Seeds
Legumes
Grains
Baked Goods
Sweets
Snacks
Fast Foods
Restaurant Foods
E
Meats
Ile content (% of total protein)
Meats
Beef
Bison
Chicken
Deer
Duck
Emu
Goat
Goose
Lamb
Pork
Rabbit
Turkey
Whale
Nuts
Ile content (% of total protein)
Nuts
Acorns
Almonds
Beach nuts
Brazil nuts
Butternuts
Cashews
Chestnuts
European
Chinese
Japanese
Coconuts
Ginko
Hazelnuts
Hickorynuts
Macadamia
Peanuts
Pecans
Pili nuts
Pine nuts
Pistachio
Walnuts
Chestnuts
F
Percent of protein from isoleucine
Percent of energy from protein
HEI quintile
1
2
3
4
5

Figure 7. People eating the healthiest diets consume relatively less isoleucine.

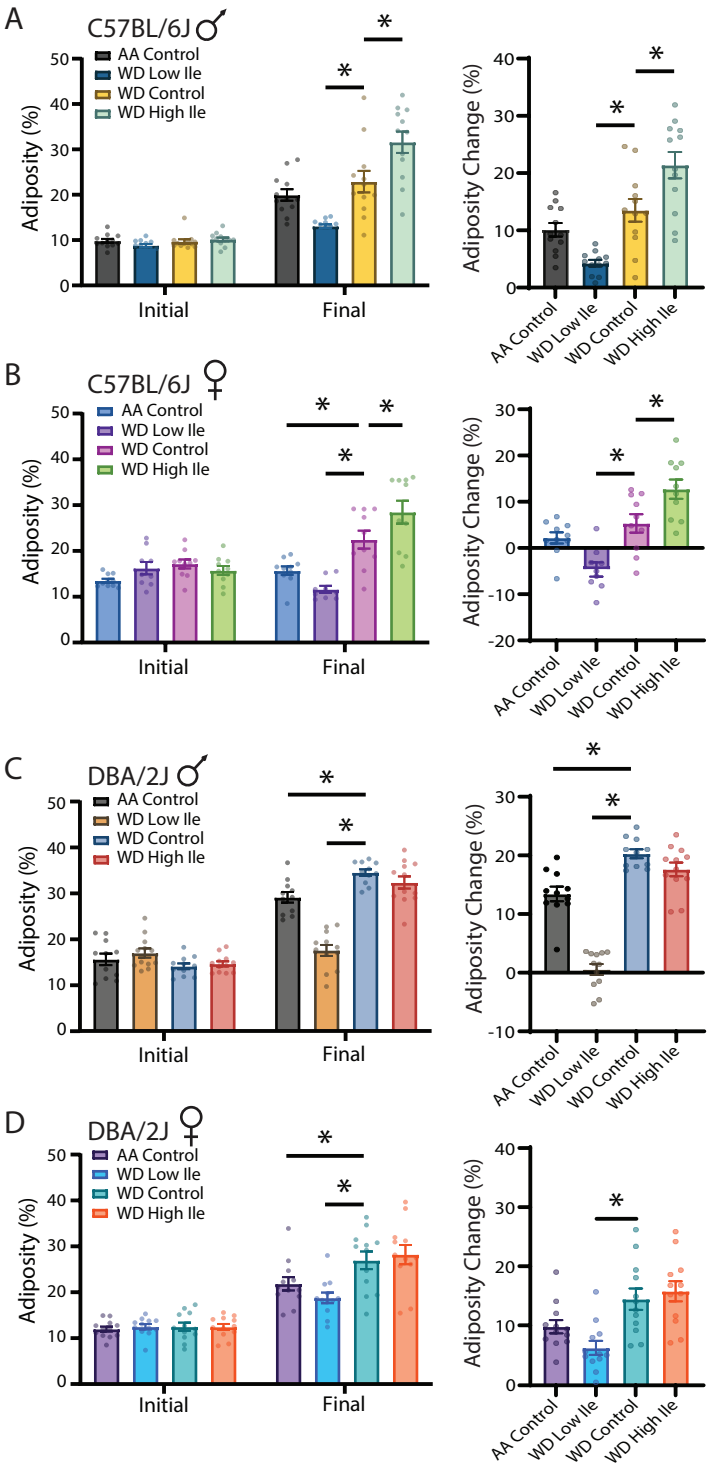
(A-C) Relative isoleucine content in animal versus plant foods (A), by food group (B), and per decile of protein intake (C).

(D) Proportional contribution of isoleucine intake per decile of protein intake by food group.

(E) Relative isoleucine content in commonly consumed meats and nuts. Statistics for the overall effects of diet, sex, and strain represent the p value from a one-way ANOVA; *$p<0.05$, Dunnett's post-test examining the effect of parameters identified as significant in the one-way. Data represented as mean ± SEM.

(F) Relative isoleucine per protein intake and HEI quintile scores.

Supplemental Figure 1.

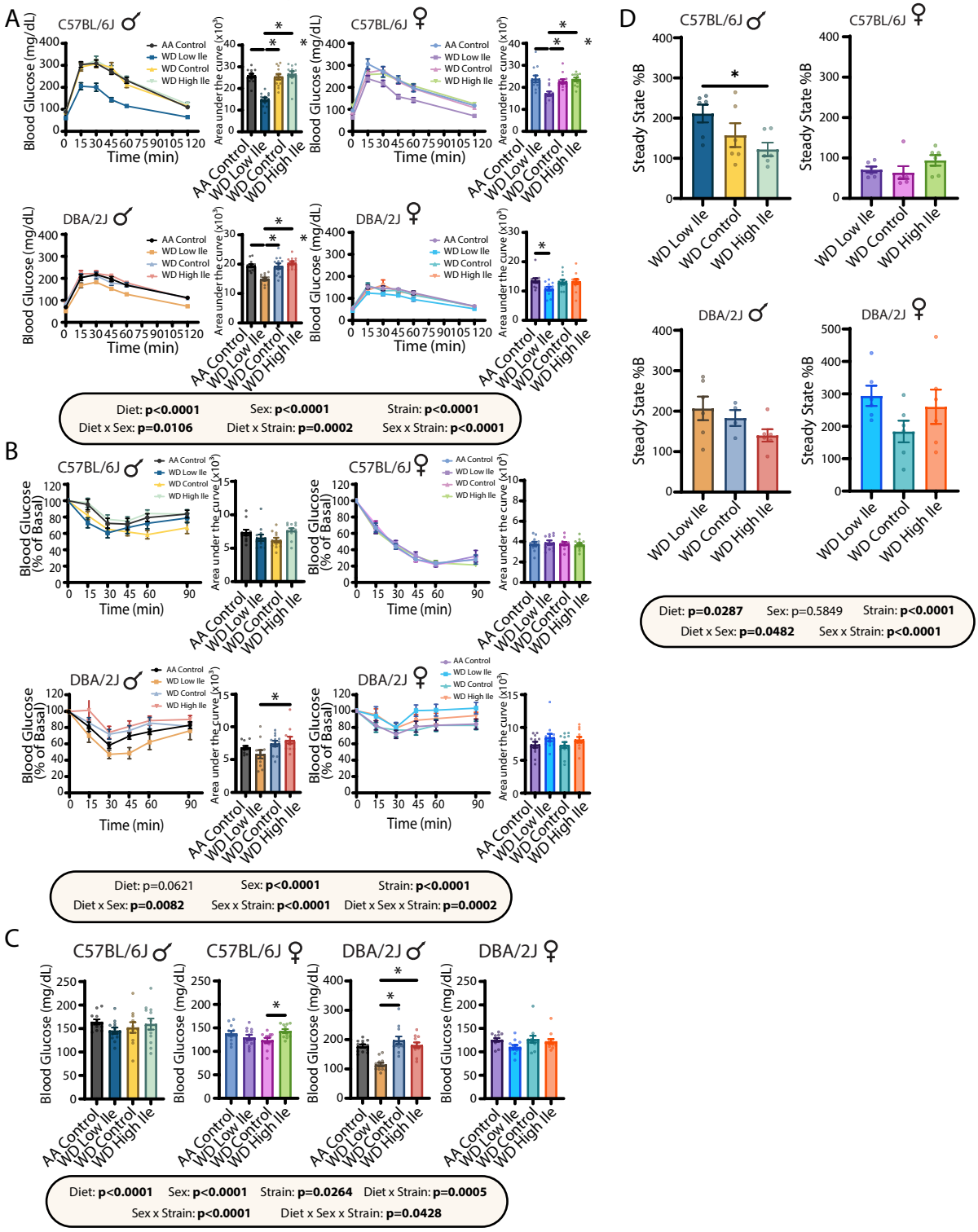


Supplemental Figures

Supplemental Figure 1. Limiting isoleucine reduces adiposity on a Western Diet.

(A-E) Adiposity percent and percent change from week 0 to week 12 in (A) B6 males, (B) B6 females, (C) DBA males, and (D) DBA females. n=12 mice per group. Statistics for the overall effect of diet and time represent the p value from a two-way ANOVA; *p<0.05, Sidak's post-test examining the effect of parameters identified as significant in the two-way ANOVA. Statistics for the overall effect of diet on adiposity change represent the p value from a one-way ANOVA; *p<0.05, Dunnett's post-test examining the effect of parameters identified as significant in the one-way. Data represented as mean ± SEM.

Supplemental Figure 2.



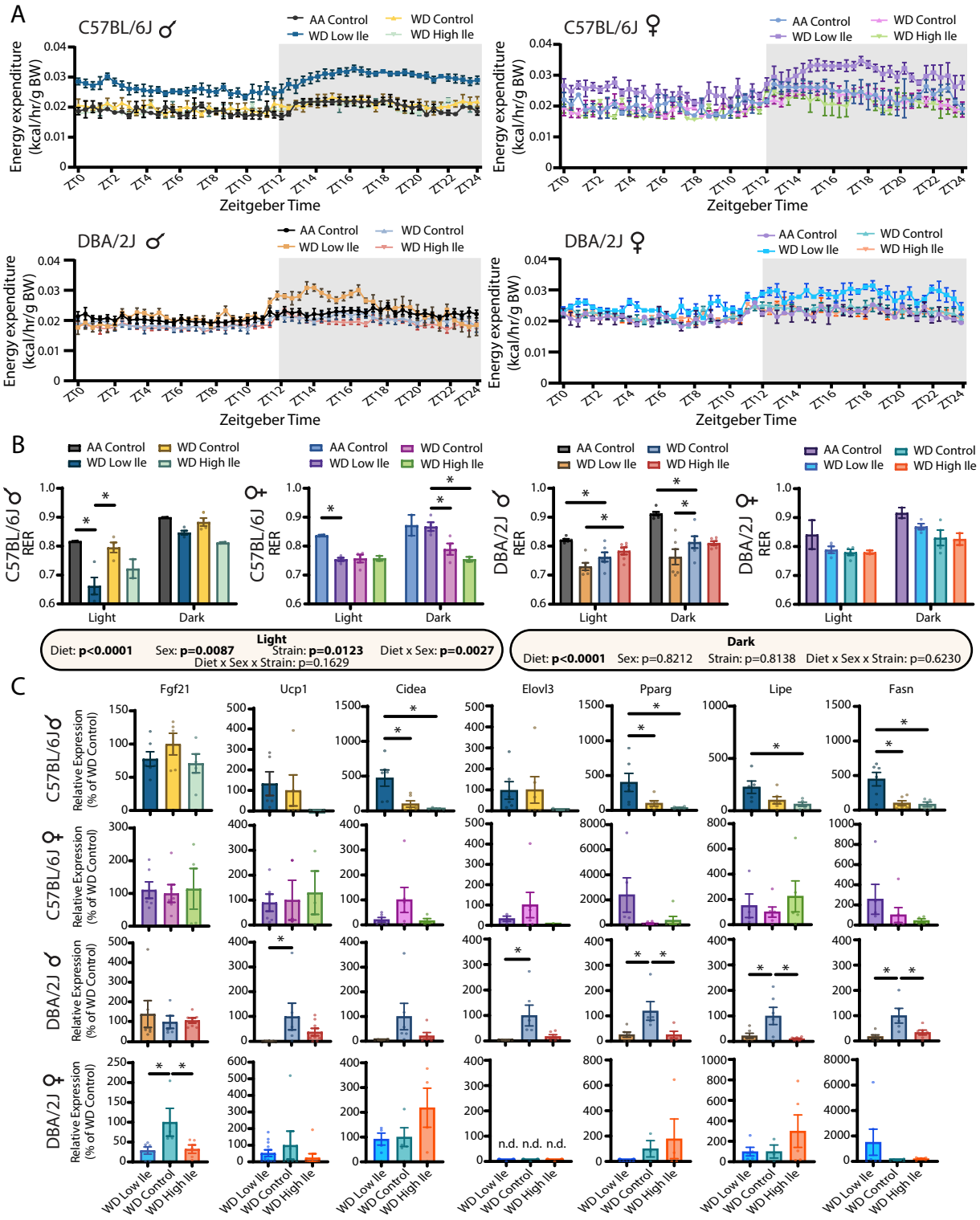
Supplemental Figure 2. Glucose homeostasis improvements are present by 3 weeks on diet.

(A-B) I.P. glucose tolerance test and (B) insulin tolerance test with quantified AUC.

(C) 4 hour fasting blood glucose levels.

(D) Steady state %B calculated from HOMA2-IR parameters. n=5-12 mice per group. Statistics for the overall effects of diet, sex, and strain represent the p value from a three-way ANOVA; *$p<0.05$. Data represented as mean ± SEM.

Supplemental Figure 3.



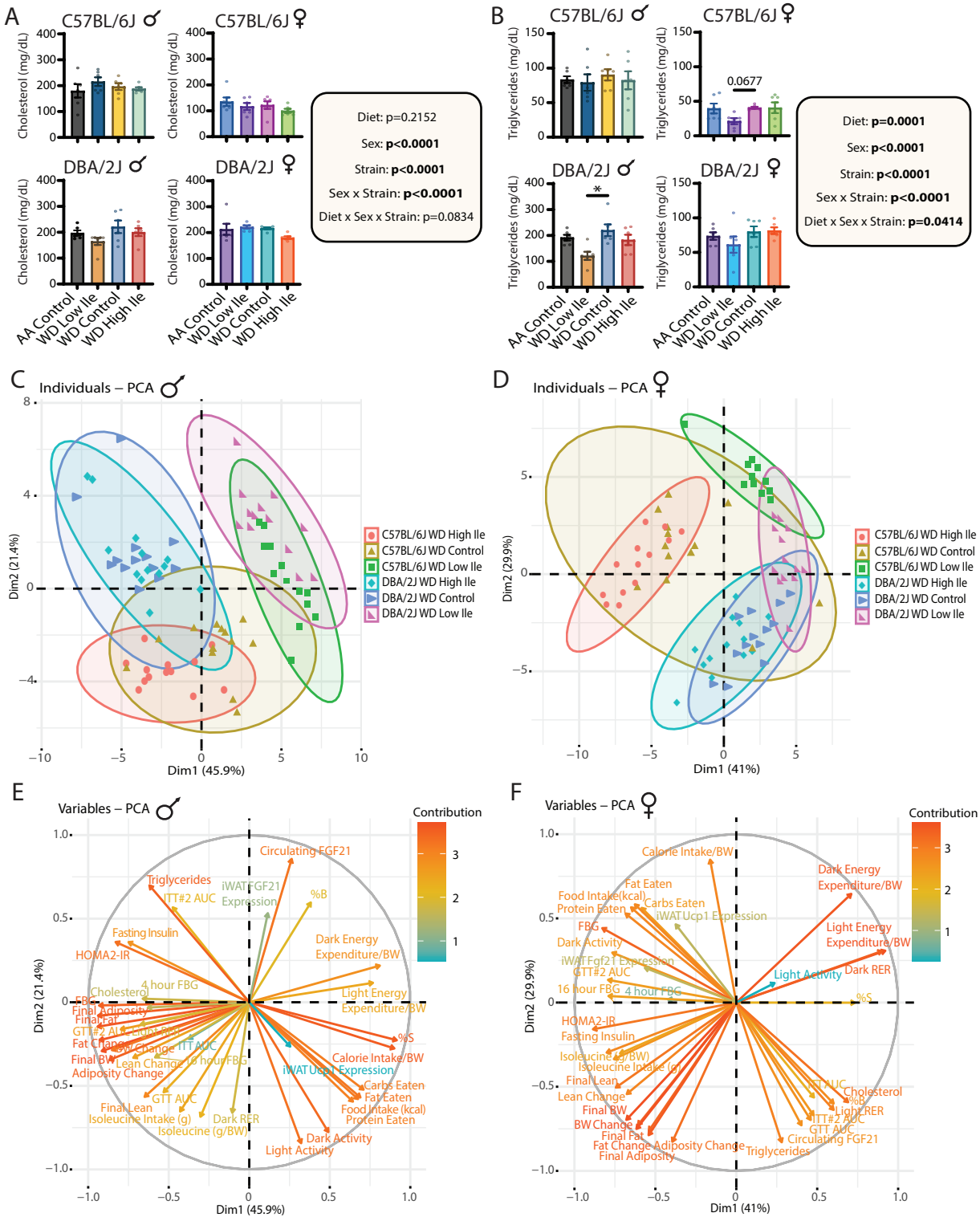
Supplemental Figure 3. Ile alters respiration.

(A) 24-hour metabolic chamber analysis of energy expenditure per body weight.

(B) Light and dark average respiratory exchange ratio values.

(C) Inguinal white adipose tissue gene expression. n=2-12 mice per group. Statistics for the overall effects of diet, sex, and strain represent the p value from a three-way ANOVA; *$p<0.05$. Data represented as mean ± SEM.

Supplemental Figure 4.



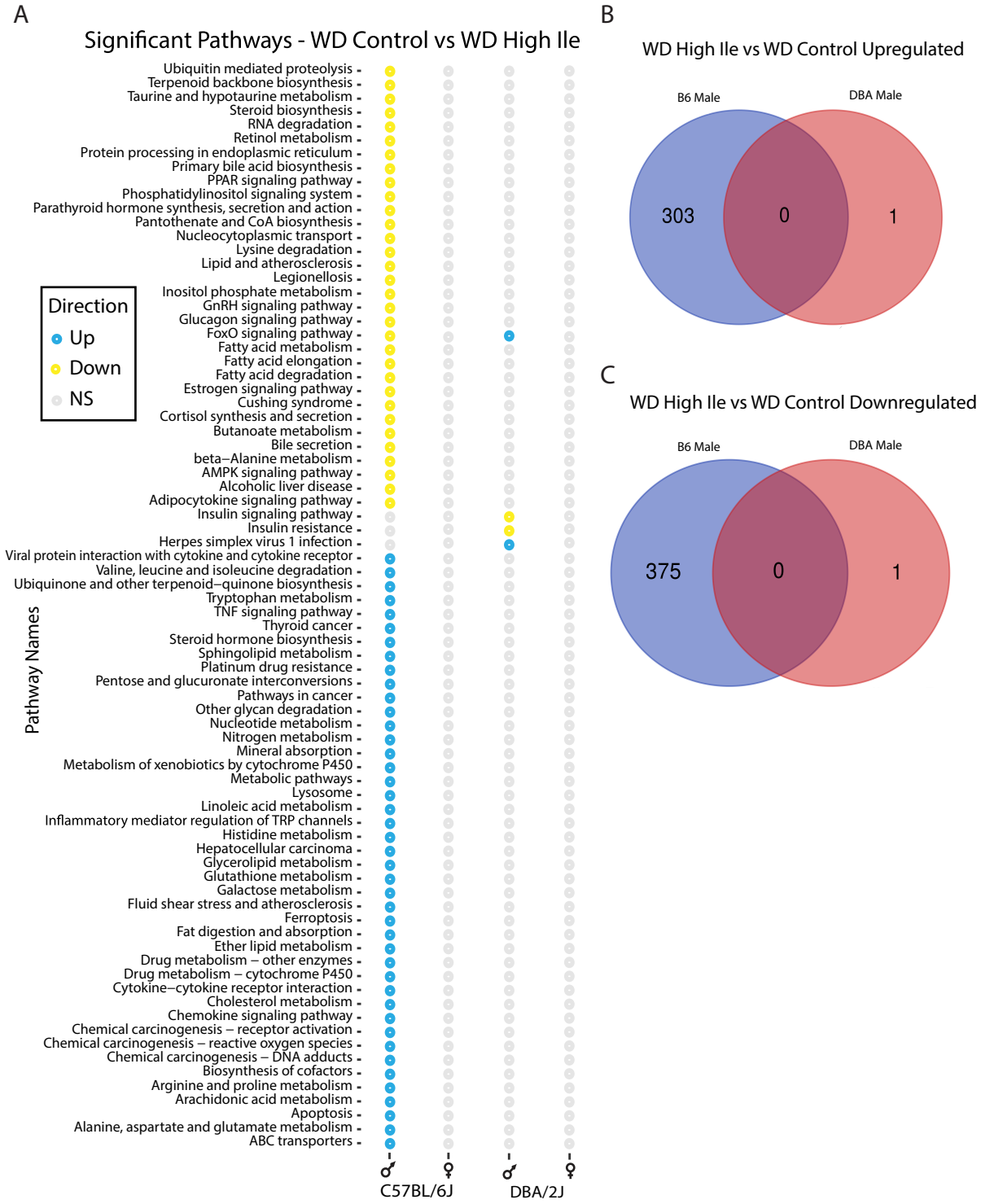
Supplemental Figure 4. Circulating lipids are largely unchanged by dietary isoleucine.

(A-B) Circulating fasting (A) cholesterol and (B) triglycerides.

(C-D) Principle component analysis of phenotypes in (C) males and (D) females, and

(E-F) the variables contributing to the PCA spread. n=6 mice per group. Statistics for the overall effects of diet, sex, and strain represent the p value from a three-way ANOVA; *$p<0.05$. Data represented as mean ± SEM.

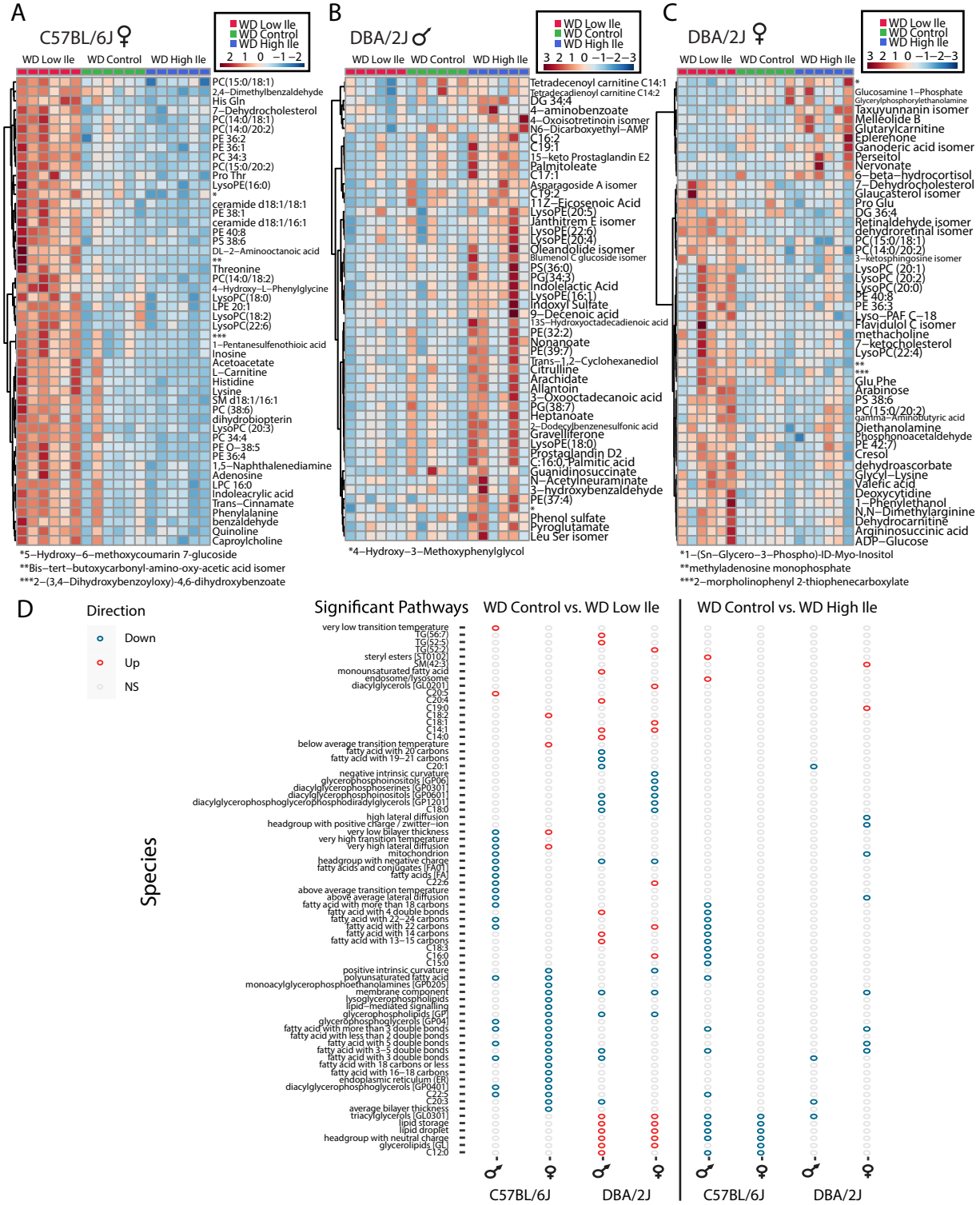
Supplemental Figure 5.



Supplemental Figure 5. A high ile diet does not alter transcripts in females.

(A-C) Transcriptomics and (A) corresponding pathway analysis of the WD Control and WD High Ile diets, plus shared (B) upregulated and (C) downregulated genes.

Supplemental Figure 6.

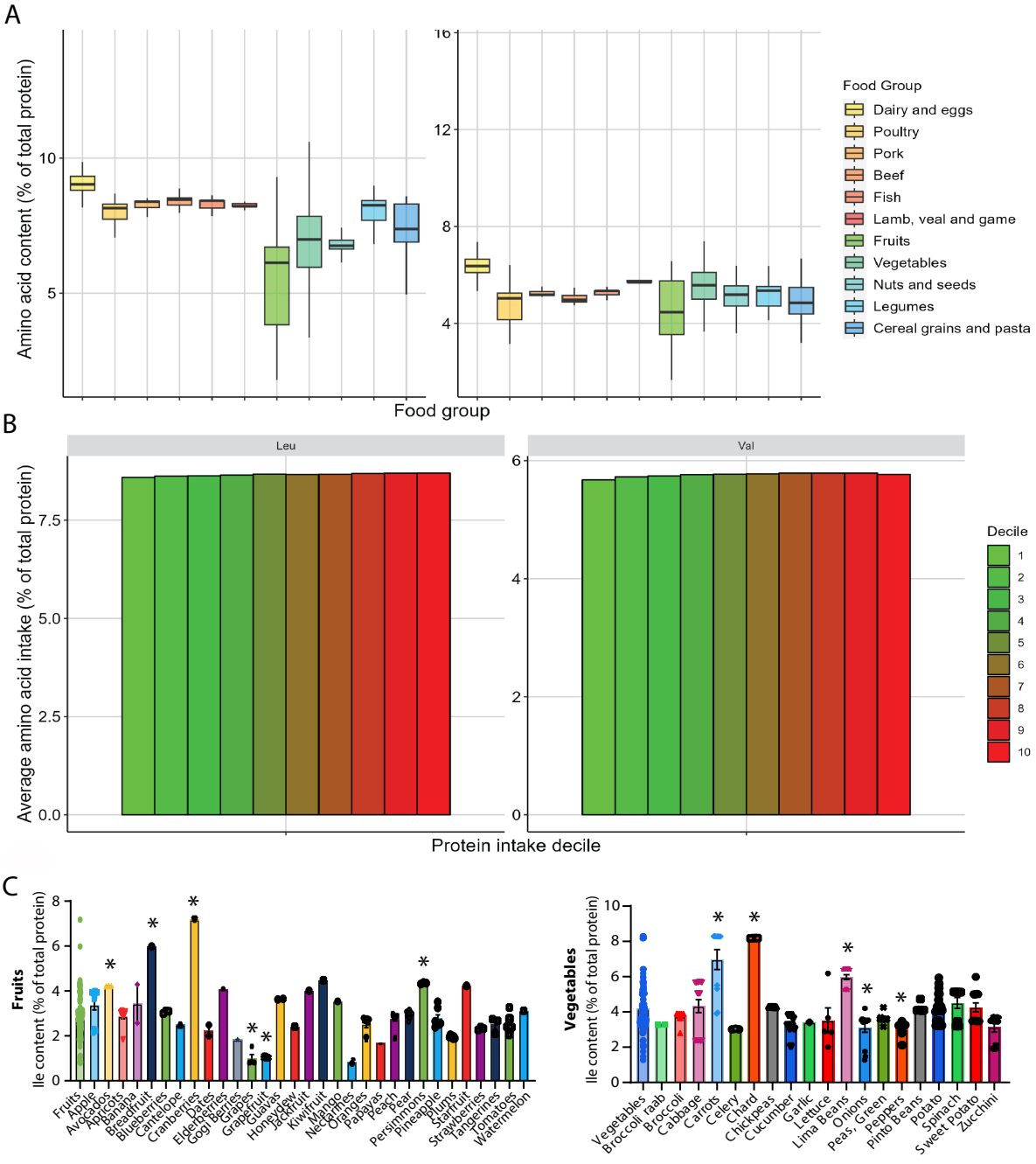


Supplemental Figure 6. Metabolites and lipids altered by isoleucine.

(A-C) Metabolomics analysis of (A) B6 females, (B) B6 males, and (C) DBA females.

(D-E) Significant lipids altered in the (D) WD Low Ile and (E) the WD High Ile diet.

Supplemental Figure 7.



Supplemental Figure 7. NHANES Diet analyses.

(A) Leucine and valine as a percentage of total protein across food groups.

(B) Relative leucine and valine content per decile of protein intake from NHANES dietary data.

(C) Relative isoleucine content in commonly consumed fruits and vegetables.

Chapter 4: FGF21 is dispensable for low isoleucine induced metabolic health improvements

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Author Contributions

MET and DWL conceived of and designed the experiments. MET, RB, IG, EZ, MG, AR, MS, BU, CLG, CYY, MC, and MMS performed the experiments. MET and DWL analyzed the data. MET and DWL secured funding and supervised personnel. MET and DWL wrote the manuscript.

Abstract

While calorie restriction (CR) is the gold standard for interventions that prolong mammalian lifespan and healthspan, adherence to these diets is difficult for humans. Recent findings by our lab and others have shown that protein restriction (PR) promotes health and longevity in mice, and that lower consumption of dietary protein is associated with increased longevity and health in humans. Our lab has found that the key mediators of the metabolic health and longevity benefits seen on PR are the branched-chain amino acids (BCAAs). Restriction of all three BCAAs, or specific restriction of isoleucine (Ile), promotes metabolic health and fitness in mice, due in part to upregulated expression of FGF21 in various tissues and levels of the hepatokine in circulation. We hypothesized that FGF21 is indispensable for lifespan extension and metabolic improvements observed in mice, perhaps more potently in males. Surprisingly, we find that whole-body deletion of FGF21 is largely dispensable for the metabolic, physical, and behavioral benefits that an isoleucine restricted diet yields in mice. Specifically, there was little effect of FGF21 on male and female body weight, fat mass, glucose and insulin tolerance, Rotarod performance and in males, and novel object recognition. Further work is needed to determine the mechanistic cause of such improvements.

Introduction

Though calorie restriction (CR) has traditionally been credited as the optimal diet for improving body composition, metabolic health, and aging parameters in the laboratory, this type of eating regimen is notoriously difficult for human adherence. As such, alternatives have recently been examined, especially as rates of obesity, cardiovascular disease, and other metabolic conditions increase in prevalence. As thoroughly outlined in **Chapter 1**, protein restriction (PR) is a promising substitute for CR in mice, resulting in reduced body weight, improved glucose homeostasis, and more without limiting caloric intake (Mirzaei et al., 2014; Solon-Biet et al., 2015a; Trautman et al., 2022). Human clinical trials of low protein diets, though short in nature due, show similar improvements, reducing body weight, hemoglobin A1C, circulating lipids, blood pressure, and more (Ferraz-Bannitz et al., 2022b; Fontana et al., 2016).

Mechanistically, these outcomes are theorized to be due to an increase in the liver-derived hormone FGF21; this was in fact observed in mice (Laeger et al., 2014) and in human blood samples from a 6-week trial of 7-9% PR (Fontana et al., 2016). Conversely, FGF21 is generally dispensable for the outcomes of a calorie-restricted diet and was actually lower in WT, CR fed males than their *ad libitum*-fed counterparts (Calubag et al., 2022). FGF21 yields a variety of systemic metabolic shifts, including increased energy expenditure via increased white adipose tissue browning and *Ucp1* expression and increased insulin sensitivity (Trautman et al., 2022). PR has been shown to increase energy expenditure in an FGF21-dependent manner (Hill et al., 2017; Hill et al., 2019), and transgenic overexpression of *Fgf21* has been found to extend lifespan (Zhang et al., 2012).

Isoleucine (Ile) restriction demonstrates similar metabolic health improvements as PR while also increasing FGF21 in circulation and expression of various tissues (Yeh et al.,

2024; Yu et al., 2021b). However, in a study of heterogeneous UM-HET3 male and female mice, there was surprisingly no effect of a low Ile diet on circulating FGF21 (Green et al., 2023b), though this was tested at 4 months of age. Here, both young and old male and female mice were fed a low Ile diet from 6 months until their natural deaths, unless sacrificed for molecular analysis. In both the young and old cohorts, the phenotypic outcomes were largely similar. It was determined that Ile intake positively correlates with worse glycemic control, body weight, fat mass and lean mass and negatively correlated with RER and energy expenditure.

This study also demonstrated for the first time that an Ile-restricted diet can significantly extend lifespan when begun in early adulthood (6 months of age); the average median lifespan was extended by 33% in males and 7% in females (Green et al., 2023b). Beyond lifespan, limited Ile leads to reduced frailty in both males and females at multiple time-points throughout the life course. Finally, there was a significant reduction in cancer-related deaths by a Low Ile diet.

Age of diet initiation is an important and often overlooked factor when evaluating diet outcomes. Even when a Low Ile diet is initiated at 20 months of age in C57BL/6J.Nia mice, adiposity is reduced, glucose tolerance and components of frailty are improved, and molecular enhancements in the heart, liver, and indices of aging are observed (Yeh et al., 2024). Clearly, a Low Ile diet is expected to improve metabolic health and lifespan.

Though the data in mouse models is strong, there is less available data in humans examining Ile and health outcomes. Deelen and colleagues demonstrated that blood levels of Ile, but not amounts of leucine or valine in circulation, are positively associated with mortality (Deelen et al., 2019). We have also shown similar outcomes in terms of body mass; there is a positive association between relative Ile intake and BMI (Yu et al., 2021b). A recent clinical trial utilized medical-grade food and supplements to recapitulate a low BCAA diet in humans

and this diet did in fact reduce circulating BCAAs by 50% (Ramzan et al., 2020). More work is needed to determine if a similar Low Ile human diet can be developed and utilized to improve health.

Given the evidence that Ile restriction significantly extends life expectancy in HET3 animals and increases FGF21 in male C57BL/6J mice, we developed a study in which male and female wild type (WT) and whole-body FGF21 knockout (KO) mice were fed either a controlor 33% Ile-restricted diet upon weaning. Here, we were surprised to see that in both males and females, a low-Ile diet improves body weight and composition, improves glucose homeostasis, some physical parameters, and frailty in males in an FGF21-independent manner. In females, there was also improved frailty by a Low Ile diet, but FGF21 KO animals had significantly worse frailty on both diets. This negative genotype effect in the KOs regardless of diet was also observed in other parameters, including female short-term memory, male and female lean mass, and insulin and glucose tolerance in males at 6 and 12 months on diet, respectively.

Results

Diet, not FGF21, improves metabolic health

To assess whether FGF21 is essential for lifespan extension and metabolic health improvements, we separated male and female WT and FGF21 KO mice onto either a Control or Low Ile diet at weaning (roughly 21 days of age), analogous to human childhood (**Fig. 1A, B**). Though there was a strong improvement throughout adulthood on body composition by the Low Ile diet; neither sex experienced a genotype-dependent improvement in body weight or fat mass (**Fig. 1C, D**). Low Ile-fed animals had less lean mass than the Controls, plus there was a genotype effect in males and females, with the KO Controls having significantly

less lean mass than WT Controls (**Fig. 1C, D**).

Using the glucose homeostasis parameters detailed on chapter 2 and 3, we conducted intraperitoneal glucose and insulin tolerance tests at multiple points throughout the lifespan. In males, there was a strong improvement in glucose tolerance as quantified by area under the curve (AUC) in Low Ile-fed mice that had been on diet for 3 and 6 months, regardless of genotype (**Fig. 2A, C**). This trend continued at 12 months on diet, but here we also saw a worsening of glucose tolerance in the KO Control-fed mice when compared to WT Controls (**Fig. 2E**). This effect disappeared by 18 months on diet (**Fig. 2G**). Any significant improvement in WT glucose tolerance between males on the Control and Low Ile diet vanished by 18 months on diet as well, leaving just improved AUC in the KO Low Ile compared to KO Controls (**Fig. 2G**). When GTT AUCs were plotted over time, it is clear that there was little effect of genotype but strong effect of diet in improving glucose tolerance over the lifespan, besides perhaps at 12 months on diet (**Fig. 2J**).

In females, glucose tolerance adaptations to the diet took longer to develop; there was no change in any diet group at 3 months on diet (**Fig. 2B**). By 6 months of age, we saw that like the males at this time point, there was a significant effect of the Low Ile diet improving glucose tolerance over the Control-fed mice, regardless of genotype (**Fig. 2D**). The same trend was present after 12 months on diet (**Fig. 2F**), but the significance disappeared by 18 months on diet, though the trend was the same (**Fig. 2H**). Like in the males, analysis of GTT AUCs over time show that in general, there was little effect of genotype but strong effect of diet in improving glucose tolerance over the lifespan in females (**Fig. 2J**).

We next utilized an intraperitoneal insulin tolerance test conducted after a 4 hour fast. Here, we saw that there was no significant effect of diet or genotype on insulin sensitivity in

females at any time point (**Fig. 3B, D, F, G, H**), though perhaps a trend in improved insulin sensitivity in the KO Low Ile diet group at 12 months on diet (**Fig. 3F**) and in the WT Low Ile-fed mice at 18 months on diet (**Fig 3H**).

In males, the insulin tolerance test results were far more variable. After 3 months on diet, there was a significant interaction between diet and genotype; the WT Low Ile group had improved glucose tolerance relative to the KO Low Ile group, but not in comparison to the WT Control like we expected (**Fig. 3A**). By 6 months on diet, we also did not see improvement in insulin sensitivity by the Low Ile diet, but there was a genotype effect (**Fig. 3C**). The KO Control mice had significantly worse insulin sensitivity than the WT Control animals, and the same trend was seen on the WT and KO Low Ile-fed animals (**Fig. 3C**). By 12 months on diet, this effect was no longer present and instead, there was a significant interaction between diet and genotype (**Fig. 3E**). Here, there was an improvement in insulin tolerance by the Low Ile diet in WT mice only, consistent with our initial hypothesis (**Fig. 3E**). Lastly, by 18 months on diet there was a worsening of insulin sensitivity in the KOs regardless of diet, though this did not reach statistical significance (**Fig. 3G**). These results show that except for at one time point in males, our hypothesis that a Low Ile diet improves insulin sensitivity in an FGF21-dependent manner is not supported. This data also demonstrates not only the importance of timing and age in any metabolic assay, but also highlight that sexual dimorphisms often present and both sexes should be evaluated.

Low Ile diets increase energy expenditure

After 6 and 12 months on diet, we evaluated both Ile and FGF21's impact on metabolic response, as previously described. Overall, metabolic analysis of Respiratory Exchange Ratio (RER), which indicates fuel source utilization, energy expenditure, and

spontaneous activity were consistent throughout the time points tested. RER was unchanged in males at both 6 and 12 months (**Fig. 4A, H**), while there was a significant increase in RER in KO Low Ile-fed females at both time points (**Fig. 4D, K**). In males, there was a significant increase in energy expenditure by the Low Ile diet, with no effect of FGF21 (**Fig. 4B, H**). However, in females, there were significant effects of both diet and genotype at both time points, with the WT Control-fed mice having increased energy expenditure over the KO Controls (**Fig. 4E, L**). Additionally, there was no significant increase by a Low Ile diet in the WTs, but there was in the KOs (**Fig. 4E, L**).

Finally, there was no difference in spontaneous activity in any group of males or females at either time point (**Fig. 4C, F, J, M**). Overall, this data concludes that consistent with previous data, we observed a significant increase in energy expenditure per BW in males, but this was surprisingly not ablated by *Fgf21* deletion. In females, there was only increased energy expenditure per BW in the KOs, but FGF21 KO lead to reduced energy expenditure in Control- fed mice. There was no difference in activity in any group of either sex, or in RER in males.

Low Ile diet improves frailty in males, not females

To assess the influence of both dietary isoleucine and FGF21 on physical function, we utilized a validated, 28-point frailty assessment at multiple points throughout the lifespan (Whitehead et al., 2014). The resulting frailty index represents the accumulation of a myriad of age-related deficits, including body condition, coat quality, gait disorders, vision loss, mouse grimace score, and more. Each item is scored 0 for no deficit, 0.5 for moderate deficit, or 1 for severe deficit; item scores are added together and divided by the number of items tallied. Here, blinded undergraduate researchers conducted these assessments monthly beginning at 18 months on diet, or late adulthood.

Though there was some variation in scores depending on the personnel conducting the assay, generally the WT and KO Control-fed male mice had worse frailty than the WT and KO Low Ile-fed animals (**Fig. 5A, B**). Consistent with other results in this study, there was no effect of FGF21 in males. Conversely, in females, there was a worsening of frailty in the KOs when compared to the corresponding WT diet, but the improvement in frailty based on diet alone did not reach statistical significance (**Fig. 5C, D**).

Inverted cling is improved by a Low Ile diet, with sexual dimorphic influence of FGF21

We next assessed physical endurance and forelimb strength with an inverted cling assay at both 18 and 24 months on diet. In males, there was an improvement in cling time by the Low Ile diet at 18 and 24 months (**Fig. 6A**). This trend was very similar in females, with the Low Ile diet improving cling time regardless of genotype, but failing to reach significance in the WTs at 18 months (**Fig. 6B**). Curiously, there was also increased cling time based on genotype at 18 months, with an increase the KO Low Ile-fed animals over their WT counterparts (**Fig. 6B**). This effect disappeared by 24 months on diet (**Fig. 6B**).

We also evaluated motor coordination, balance, and grip strength with an accelerated Rotarod test after 6 months on diet. There was no effect of diet or genotype in male mice both in absolute terms and when you analyze body weight as a covariate (**Fig. 6C, D**). Surprisingly, we saw worsened Rotarod performance in absolute terms in the Low Ile-fed females, reaching statistical significance in the WTs (**Fig. 6E**). When we assessed Rotarod using body weight as a covariate, the elevations between the KO Control and the KO Low Ile diets were significant, with the Low Ile-fed mice having greater Rotarod time as BW and vice versa in the Controls (**Fig. 6F**). Generally, there was an improvement by the Low Ile diet with no effect of FGF21 on male cling performance, while there was also a genotype effects in females.

Memory is unaffected by Ile regardless of genotype in males

After 24 months on the respective diets, we assessed both short- and long-term memory with the Novel Object Recognition assay. Here, a discrimination index (DI) of 0.2-1 indicates that the mouse was able to discriminate between the two objects and preferred the novel object while a DI of -0.2 to -1.0 means that the mouse preferred the familiar object. A DI of -0.2 to 0.2 indicates that the mouse could not discriminate between the two objects. In males, there were no trends of diet or genotype altering either short- or long-term memory (**Fig. 7A, B**). A similar trend was observed in female short-term memory (**Fig. 7C**), but surprisingly, there was a positive effect of diet and genotype in long-term memory (**Fig. 7D**). Here, female WT animals fed a Low Ile diet preferred the novel object and this was ablated in the KO animals (**Fig. 7D**). Overall, this indicates that FGF21 may be required for long-term memory improvement by a Low Ile diet in a sexually dimorphic manner.

Discussion

Dietary macronutrient composition has been largely overlooked as a determinant for healthy metabolism and aging. Multiple studies have recently demonstrated that dietary protein is a key regulator of these processes, and that limiting protein is generally beneficial (Ferraz- Bannitz et al., 2022b; Sluijs et al., 2010; Solon-Biet et al., 2015a; Wu et al., 2022). Restriction of specific amino acids can confer similar benefits as total protein dilution, specifically the branched-chain amino acids (Richardson et al., 2021). Isoleucine has been shown to be the main driver of the benefits of the BCAAs (Yu et al., 2021b) and has been demonstrated to extend lifespan and reduce morbidity in mice (Green et al., 2023b).

A downstream effect of protein and amino acid restriction is upregulation of the liver-derived hormone FGF21, which increases preference for dietary protein (Hill et al., 2020). In addition to protein sensing, FGF21 senses other nutritional imbalances such as high sucrose

intake or starvation (Fazeli et al., 2015; Maekawa et al., 2017). FGF21 then results in a variety of metabolic alterations, such as increased energy expenditure, ketogenesis, and increased fatty acid oxidation (Laeger et al., 2014; Zhang et al., 2012). Pharmacologic administration of FGF21 promotes weight loss in diet-induced obese mice; just one dose lowers blood sugar by 50% and increases insulin sensitivity (Xu et al., 2009). Also, transgenic overexpression of FGF21 extends lifespan (Zhang et al., 2012) and is required for PR-induced lifespan extension and metabolic improvements (Hill et al., 2022a; Hill et al., 2017). We set out to determine whether FGF21 is also essential for metabolic health improvements of a Low Ile diet; we hypothesized that it is.

We utilized a variety of metabolic phenotyping, cognition, and physical function assays to assess the overall effect of diet and genotype on the health and life course of male and female mice. We found that in both sexes, the Low Ile diet dramatically improves body weight and reduces fat mass independently of genotype. Lean mass, on the other hand, was greater in the Control-fed mice and in the FGF21 WT genotype of both sexes.

When we assessed glucose homeostasis, there was a clear improvement in glucose tolerance by a Low Ile diet in males and females regardless of genotype. At one time point in males, there was a worsening of glucose tolerance in the KO Control compared to the WT Controls. Insulin sensitivity was unchanged by diet, genotype, and time in females, while in males, there was a significant interaction between diet and genotype at 12 months on diet in alignment with our hypothesis.

We also utilized metabolic chambers to analyze fuel source utilization, energy expenditure, and activity level. There was no change in activity level in males or females, or in RER in males. In females, there was an increase towards carbohydrate oxidation in the KO Low Ile group. Lastly, as expected, there was increased energy expenditure in males by a Low Ile diet, but we were surprised to see that this was not dependent on FGF21. In females, there was a significant increase in EE in the KO Low Ile group but not in the WT Low Ile

group.

When assessing physical performance, we expected to see a significant interaction between diet and genotype, with improvements by a Low Ile diet disappears in FGF21 deletion. Frailty was lower in the Low Ile groups with no genotype effect in males, but in females the KO Controls had worse frailty than the WT Controls group. Inverted cling was generally improved by a Low Ile diet, while there was no effect on Rotarod performance in males. Finally, there was no impact of diet or genotype on long- and short-term memory in males. In females, there was a genotype effect in the short-term, with FGF21 WTs having improved short-term memory and preference for the novel object. WT Low Ile-fed females also had significantly improved long- term memory.

Limitations of study

These experiments were subject to a variety of limitations. First, there were multiple blinded undergraduates conducting frailty scoring throughout the study. Each had been trained using the same protocols, but some variation likely occurred between individuals when assigning scores. Another limitation to this study was the age of which we assigned diets.

Weaning was possibly too early in the murine developmental stage for an Ile-restricted diet, which was surprising as we have successfully initiated amino acid-restricted diets at this time in previous works (Richardson et al., 2021). As a result, a subset of our mice experienced seizures, some of which were lethal; we excluded any mice that died as a result of seizures from subsequent analysis.

Methods

Animal care, housing and diet

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital. Male and female FGF21 WT and KO breeding pairs were procured from University of Wisconsin's Biomedical Research Model Services Core. Offspring were then separated into cages of the same sex and genotype upon weaning and initiated upon their experimental diet. All studies were performed on animals or tissues (blood, ear punches) collected from animals. Breeding mice were acclimated to the animal research facility for at least one week before entering studies. All animals were housed 2-3 per cage in static microisolator cages in a specific pathogen-free mouse facility with a 12:12 h light–dark cycle, maintained at approximately 22 °C. At the start of the experiment, mice were randomized to receive the control diet (Control, TD. 140711) or the 33% isoleucine diet (Low Ile, TD.160734), both of which are amino acid defined; all diets were obtained from Envigo. In the Low Ile diet, nonessential amino acids were adjusted to maintain the same level of dietary nitrogen as the WD Control. Full diet descriptions, compositions and item numbers are provided in **Table 1**.

In vivo procedures and metabolic phenotyping

Average food consumption was measured from the scales within metabolic chambers. Food consumption was normalized to body weight from the same time food consumption was measured. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. Glucose and insulin tolerance tests were performed following a 16 hour overnight or 4 hour fast, respectively, and then injecting either glucose (1g/kg) or insulin (0.75U/kg) intraperitoneally (Bellantuono et al., 2020; Yu et al., 2019). Glucose measurements were

taken using a Bayer Contour blood glucose meter and test strips. For assays of multiple metabolic parameters (O2, CO2, food consumption, and activity tracking), mice were acclimatized to housing in a Columbus Instruments Oxymax/CLAMS-HC metabolic chamber system for roughly 24-72 hours, and data from a continuous period was then recorded and analyzed.

Frailty assessment

Frailty was assessed longitudinally in a subset of mice using a list of 28 frailty measures based on the procedures outlined by Whitehead et al (Whitehead et al., 2014). This frailty index reflects an accumulation of deficits associated with aging, akin to Rockwood's frailty index in humans (Mitnitski et al., 2001). The items scored are scored 0 (no deficit), 0.5 (mild deficit) or 1 (severe deficit) and included alopecia, loss of fur color, dermatitis, loss of whiskers, coat condition, tumors, distended abdomen, kyphosis, tail stiffening, gait disorders, tremor, body condition score, vestibular disturbance, cataracts, corneal opacity, eye discharge/swelling, microphthalmia, vision loss, menace reflex, nasal discharge, malocclusions, rectal prolapse, vaginal/uterine/penile prolapse, diarrhea, breathing rate/depth, mouse grimace score, and piloerection. Scores for all items are added together, and then divided by the total number of items scored.

Inverted cling and rotarod assays

For inverted cling tests, mice were placed on a wire frame which was carefully inverted until mice were hanging upside down, a timer was started, and the time until the mouse fell was recorded. The average time of two rounds of testing conducted at least 30 min apart was calculated. For Rotarod testing, mice were trained at a constant speed of 4rpm the day before testing. On the day of testing, mice were put on the Rotarod for two rounds, at least 30 min

apart, and the average time spent on the Rotarod and max speed were recorded. For testing, the Rotarod started at a speed of 4rpm with an acceleration of 0.5 rpm/s up to a max of 40rpm.

Assays and kits

Blood for circulating FGF21 analysis was obtained following an overnight fast. Blood FGF21 levels were assayed by a mouse/rat FGF-21 quantikine ELISA kit (MF2100) from R&D Systems (Minneapolis, MN, USA).

Novel object recognition

The full protocol for NOR analysis can be described in greater detail elsewhere (Antunes and Biala, 2012). Briefly, after an acclimation period, the acquisition trail with two of the same objects (object A and object A) is conducted. Short-term memory test (STM) is executed 1 hour after the acquisition trial (object A and object B), and the long-term memory test is carried out 24 hours from the acquisition trial (object A and object C). The habituation, acquisition, short-, and long- term memory test are 5 min each.

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Chapter 4. Table 1.

	Control	Low Ile
Teklad Diet Number	TD.140711	TD. 160734
Color	Red	Orange
Formula	g/kg	g/kg
Sucrose	291.248	291.248
Corn Starch	150	150.6136
Maltodextrin	150	150.6136
Corn Oil	52	52
Olive Oil	29	29
Cellulose	30	30
Mineral Mix, AIN-93G-MX (94046)	35	35
Calcium Phosphate, monobasic, monohydrate	8.2	8.2
Food Color	0.1	0.1
TBHQ (antioxidant)	0.012	0.012
Vitamin Mix, Teklad (40060)	10	10
% kcal from	%	%
Protein	22	22
Carbohydrate	59.4	59.4
Fat	18.6	18.6
Kcal/g	3.9	3.9
Amino Acid Profile	g/kg	g/kg
L-Alanine	9.38	9.8267
L-Arginine	6.3	6.3
L-Asparagine	20.58	20.9113
L-Aspartic Acid	20.58	21.2475
L-Cystine	7.2	7.2
L-Glutamic Acid	28.97	29.7077
L-Glutamine	33.77	34.1395
Glycine	2.96	3.3363
L-Histidine HCl, monohydrate	4.6	4.6
L-Isoleucine	7.8	2.54
L-Leucine	25.4	25.4
L-Lysine HCl	20.38	20.38
L-Methionine	6.7	6.7
L-Phenylalanine	6.6	6.6
L-Proline	7.41	7.987
L-Serine	7.41	7.9368
L-Threonine	9.7	9.7
L-Tryptophan	3.4	3.4
L-Tyrosine	6.9	6.9
L-Valine	8.4	8.4

Figure 1.

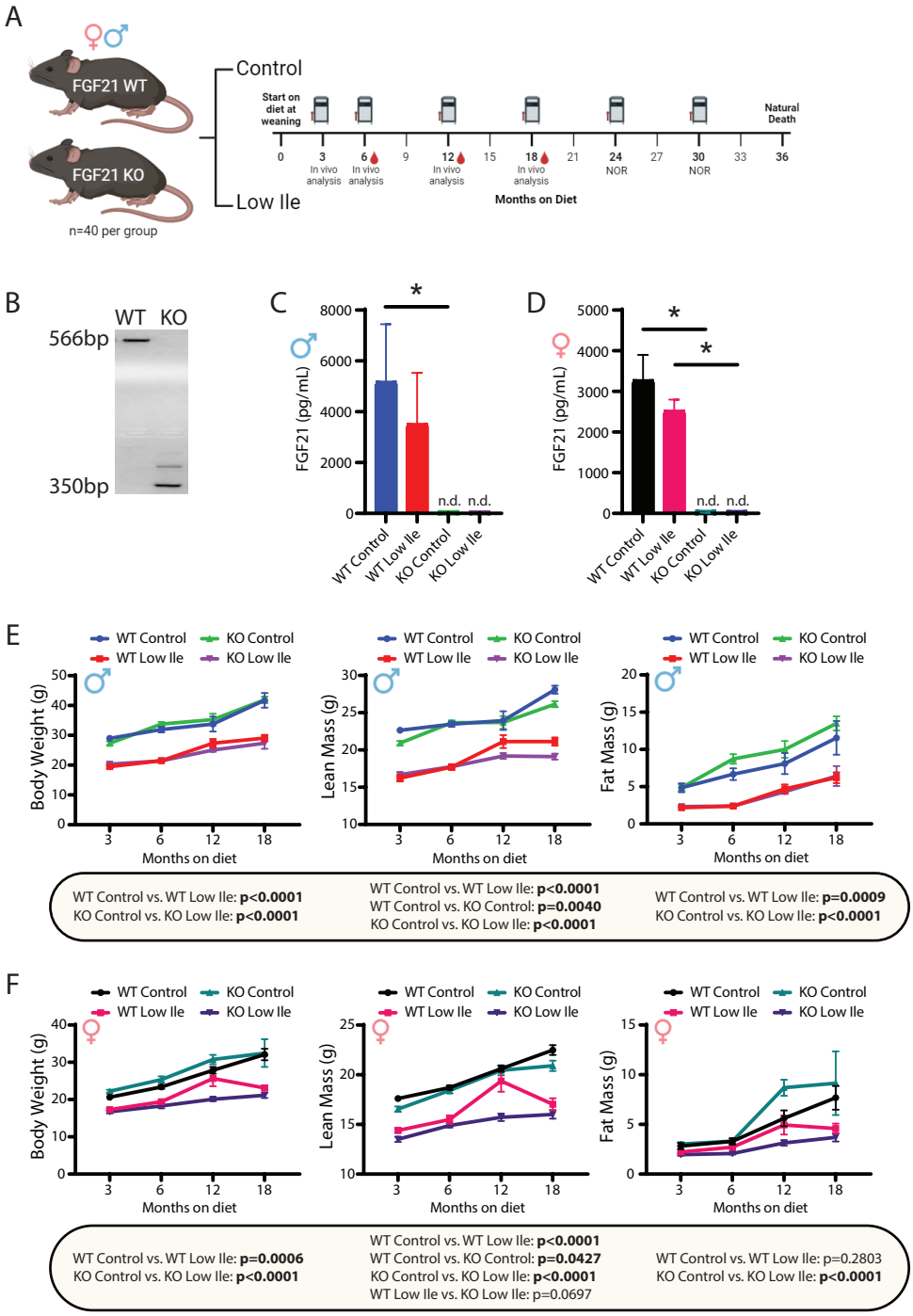

A
FGF21 WT
FGF21 KO
n=40 per group
Control
Low Ile
Start on diet at weaning
Natural Death
0
3
In vivo analysis
6
In vivo analysis
9
12
In vivo analysis
15
18
In vivo analysis
21
24
NOR
27
30
NOR
33
36
Months on Diet
B
WT
KO
566bp
350bp
C
FGF21 (pg/mL)
n.d.
n.d.
WT Control
WT Low Ile
KO Control
KO Low Ile
D
FGF21 (pg/mL)
n.d.
n.d.
WT Control
WT Low Ile
KO Control
KO Low Ile
E
WT Control
KO Control
WT Low Ile
KO Low Ile
Body Weight (g)
Months on diet
Lean Mass (g)
Months on diet
Fat Mass (g)
Months on diet
WT Control vs. WT Low Ile: p<0.0001
KO Control vs. KO Low Ile: p<0.0001
WT Control vs. WT Low Ile: p<0.0001
WT Control vs. KO Control: p=0.0040
KO Control vs. KO Low Ile: p<0.0001
WT Control vs. WT Low Ile: p=0.0009
KO Control vs. KO Low Ile: p<0.0001
F
WT Control
KO Control
WT Low Ile
KO Low Ile
Body Weight (g)
Months on diet
Lean Mass (g)
Months on diet
Fat Mass (g)
Months on diet
WT Control vs. WT Low Ile: p=0.0006
KO Control vs. KO Low Ile: p<0.0001
WT Control vs. WT Low Ile: p<0.0001
WT Control vs. KO Control: p=0.0427
KO Control vs. KO Low Ile: p<0.0001
WT Low Ile vs. KO Low Ile: p=0.0697
WT Control vs. WT Low Ile: p=0.2803
KO Control vs. KO Low Ile: p<0.0001

Figure 1. A Low Ile diet reduces fat mass and body weight independently of FGF21.

(A) Experimental design.

(B) WT and KO genotyping.

(C-D) Circulating FGF21 in males (C) and females (D).

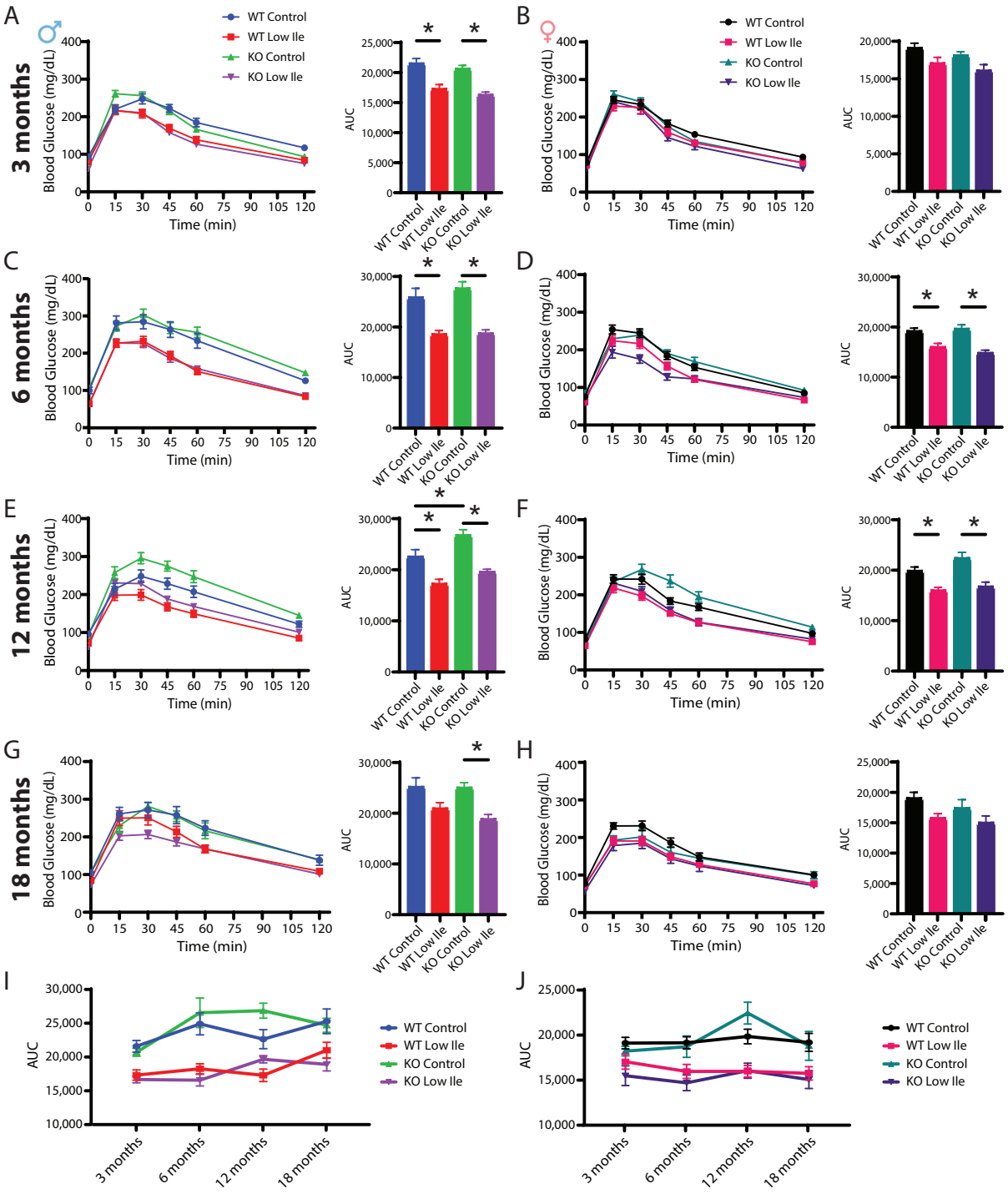
(E) Male body composition.

(F) Female body composition.

n=4-30 per group. Statistics for the overall effects of genotype, diet, and the interaction on circulating FGF21 represent the p value from a two-way RM ANOVA; *p<0.05, Sidak's test post ANOVA for each sex shown. For longitudinal assessment of body composition, statistics for the overall effects of genotype, diet, and the interaction represent the p value from a residual maximum likelihood (REML) analysis conducted individually for each diet and genotype. *p<0.05.

Data represented as mean ± SEM.

Figure 2.



	3 months	6 months	12 months	18 months
WT Control vs. WT Low Ile	**p=0.0033**	**p=0.0059**	**p=0.0142**	p=0.2218
KO Control vs. KO Low Ile	**p<0.0001**	**p=0.0044**	**p<0.0001**	**p=0.0020**

	3 months	6 months	12 months	18 months
WT Control vs. WT Low Ile	p=0.2134	**p=0.0168**	**p=0.0029**	**p=0.0414**
KO Control vs. KO Low Ile	p=0.1400	p=0.0513	**p=0.0006**	p=0.2275

Figure 2. Glucose tolerance is improved by a Low Ile diet, largely independent of FGF21.

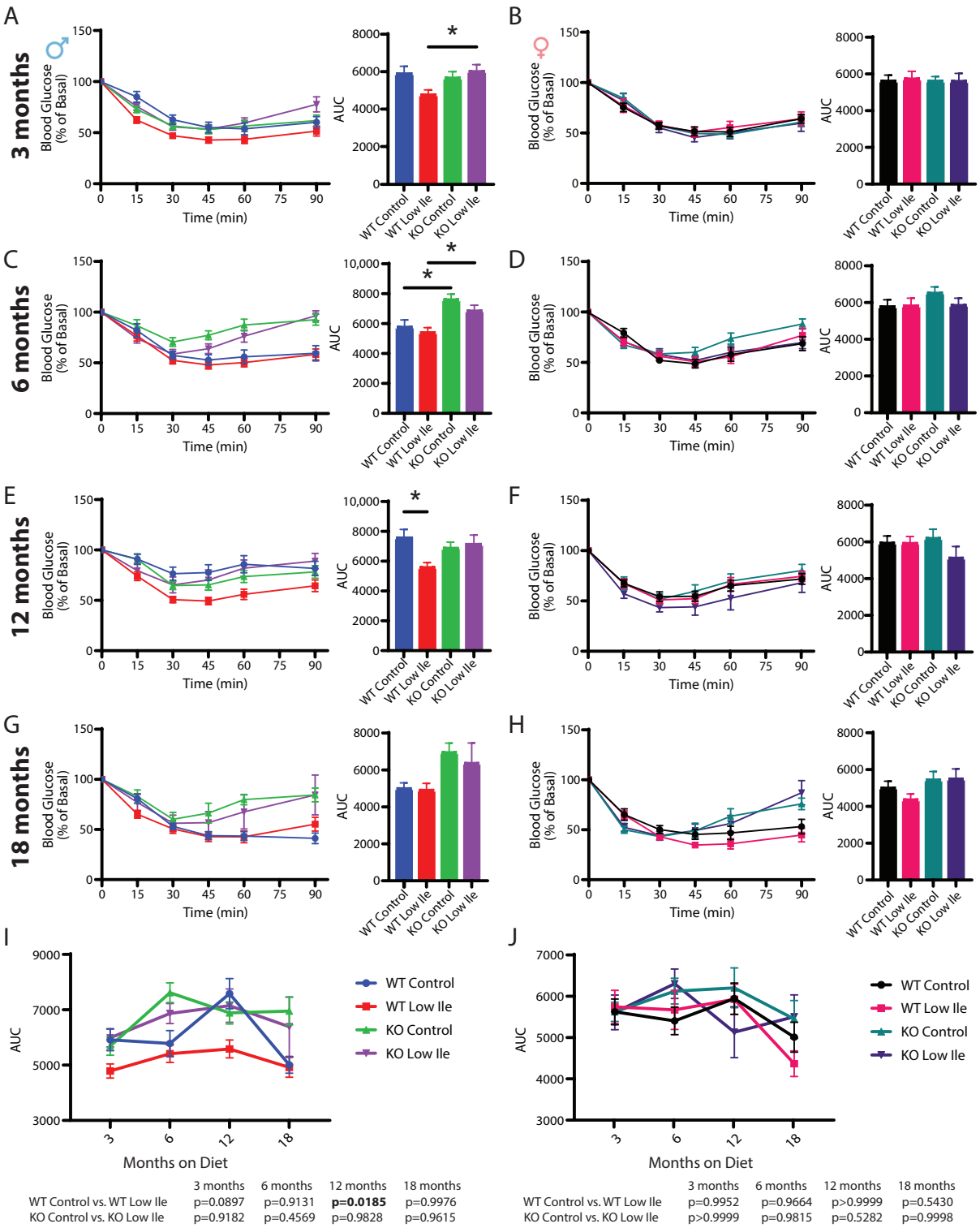
(A-H) Male (A, C, E, G) and female (B, D, F, H) glucose tolerance and corresponding area under the curve (AUC) as assessed by an intraperitoneal glucose tolerance test (1 g/kg bw) after 3 (A, B), 6 (C, D), 12 (E, F), and 18 (G, H) months on diet.

(I-J) Male (I) and female (J) glucose tolerance AUC over time.

n=17-35 per group. For longitudinal assessment of GTT AUC, statistics for the overall effects of genotype, diet, and the interaction represent the p value from a two-way RM ANOVA or a residual maximum likelihood (REML) analysis conducted individually for each diet and genotype. *$p<0.05$. Tukey post-test examining the effect of parameters identified as significant in the two-way ANOVA.

Data represented as mean $\pm$ SEM.

Figure 3.



	3 months	6 months	12 months	18 months
WT Control vs. WT Low Ile	p=0.0897	p=0.9131	**p=0.0185**	p=0.9976
KO Control vs. KO Low Ile	p=0.9182	p=0.4569	p=0.9828	p=0.9615

	3 months	6 months	12 months	18 months
WT Control vs. WT Low Ile	p=0.9952	p=0.9664	p>0.9999	p=0.5430
KO Control vs. KO Low Ile	p>0.9999	p=0.9815	p=0.5282	p=0.9998

Figure 3. Insulin sensitivity is worse in male FGF21 KOs and unchanged in females.

(A-H) Male (A, C, E, G) and female (B, D, F, H) insulin sensitivity and corresponding area under the curve (AUC) as assessed by an intraperitoneal insulin tolerance test (0.75u insulin/kg bw) after 3 (A, B), 6 (C, D), 12 (E, F), and 18 (G, H) months on diet.

(I-J) Male (I) and female (J) glucose tolerance AUC over time.

n=8-22 per group. For longitudinal assessment of ITT AUC, statistics for the overall effects of genotype, diet, and the interaction represent the p value from a two-way RM ANOVA or a residual maximum likelihood (REML) analysis conducted individually for each diet and genotype. *p<0.05. Tukey post-test examining the effect of parameters identified as signficant in the two-way ANOVA.

Data represented as mean ± SEM.

Figure 4.

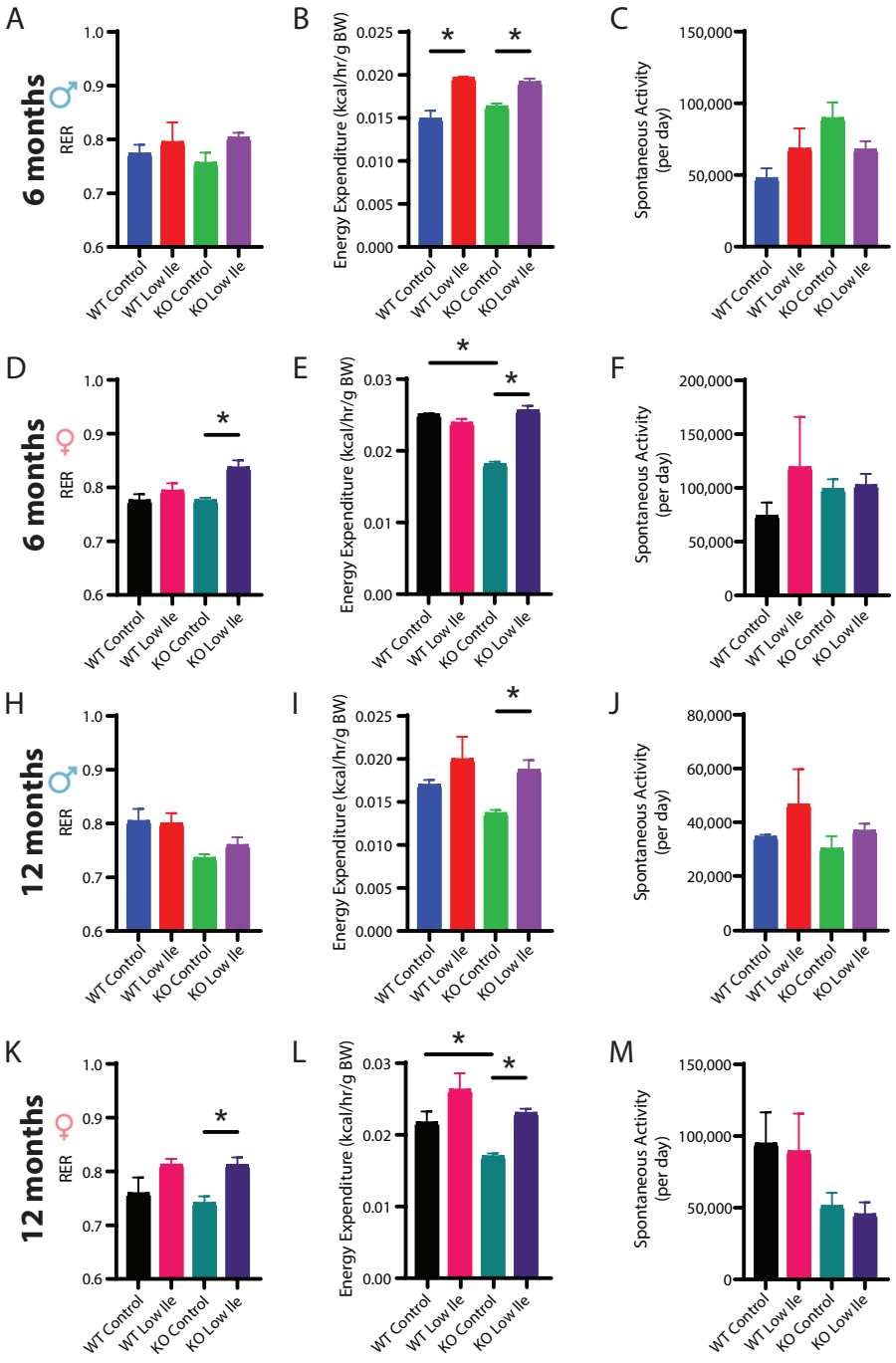

A
6 months ♂
RER
1.0
0.9
0.8
0.7
0.6
WT Control
WT Low Ile
KO Control
KO Low Ile
B
Energy Expenditure (kcal/hr/g BW)
0.025
0.020
0.015
0.010
0.005
0.000
*
*
C
Spontaneous Activity (per day)
150,000
100,000
50,000
0
D
6 months ♀
E
F
200,000
H
12 months ♂
I
J
80,000
60,000
40,000
20,000
K
12 months ♀
L
M

Figure 4. A Low Ile diet increases energy expenditure.

(A-F) Metabolic chambers conducted at 6 months (A-F) and at 12 months (H-M) in males (A-C, H-J) and females (D-F, K-M). 24-averages of RER (A, D, H, I), energy expenditure normalized to body weight (B, E, I, L), and spontaneous energy (C, F, J, M).

n=3-8 per group. Statistics for the overall effects of genotype, diet, and the interaction represent the p value from a two-way RM ANOVA; *p<0.05, Sidak's test post ANOVA for each sex shown.

Data represented as mean ± SEM.

Figure 5.

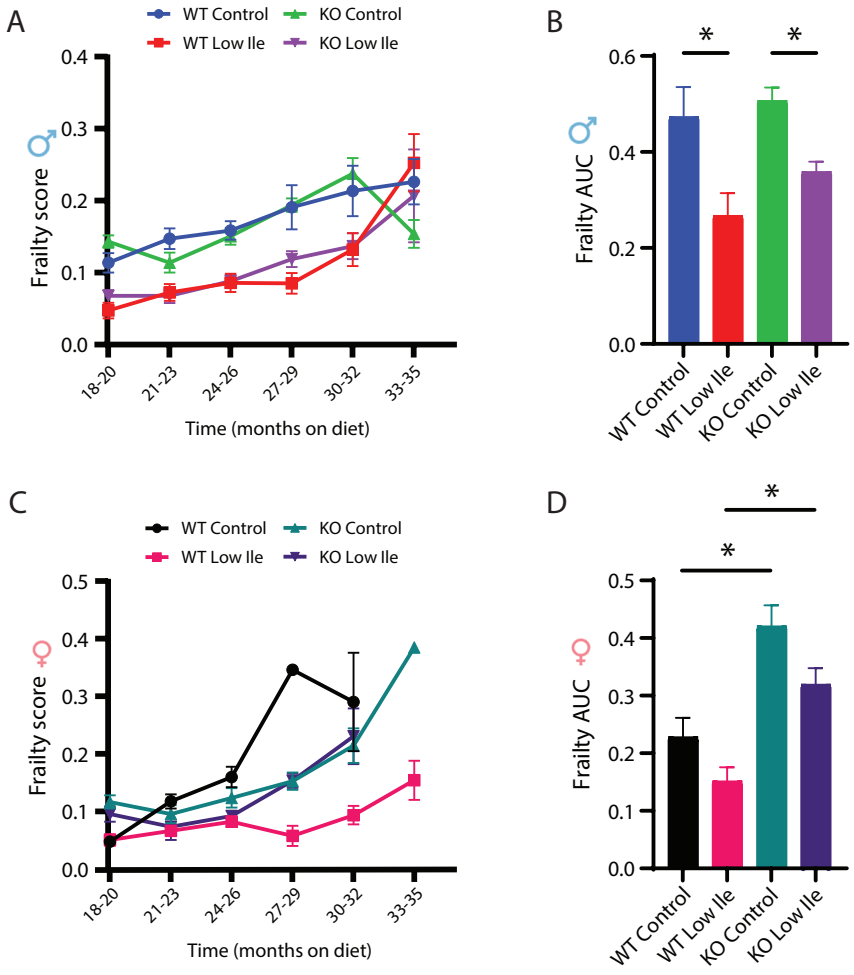

A
WT Control
KO Control
WT Low Ile
KO Low Ile
Frailty score
♂
0.0
0.1
0.2
0.3
0.4
18-20
21-23
24-26
27-29
30-32
33-35
Time (months on diet)
B
Frailty AUC
♂
0.0
0.2
0.4
0.6
*
*
WT Control
WT Low Ile
KO Control
KO Low Ile
C
WT Control
KO Control
WT Low Ile
KO Low Ile
Frailty score
♀
0.0
0.1
0.2
0.3
0.4
0.5
18-20
21-23
24-26
27-29
30-32
33-35
Time (months on diet)
D
Frailty AUC
♀
0.0
0.1
0.2
0.3
0.4
0.5
*
*
WT Control
WT Low Ile
KO Control
KO Low Ile

Figure 5. Low Ile diets improve frailty.

(A-B) Frailty in males over time (A) and AUC (B).

(C-D) Frailty in females over time (C) and AUC (D).

n=7-45 per group. Statistics for the overall effects of diet group represent the p value from a one-way ANOVA; *p<0.05, Tukey's test post ANOVA for each sex shown.

Data represented as mean ± SEM.

Figure 6.

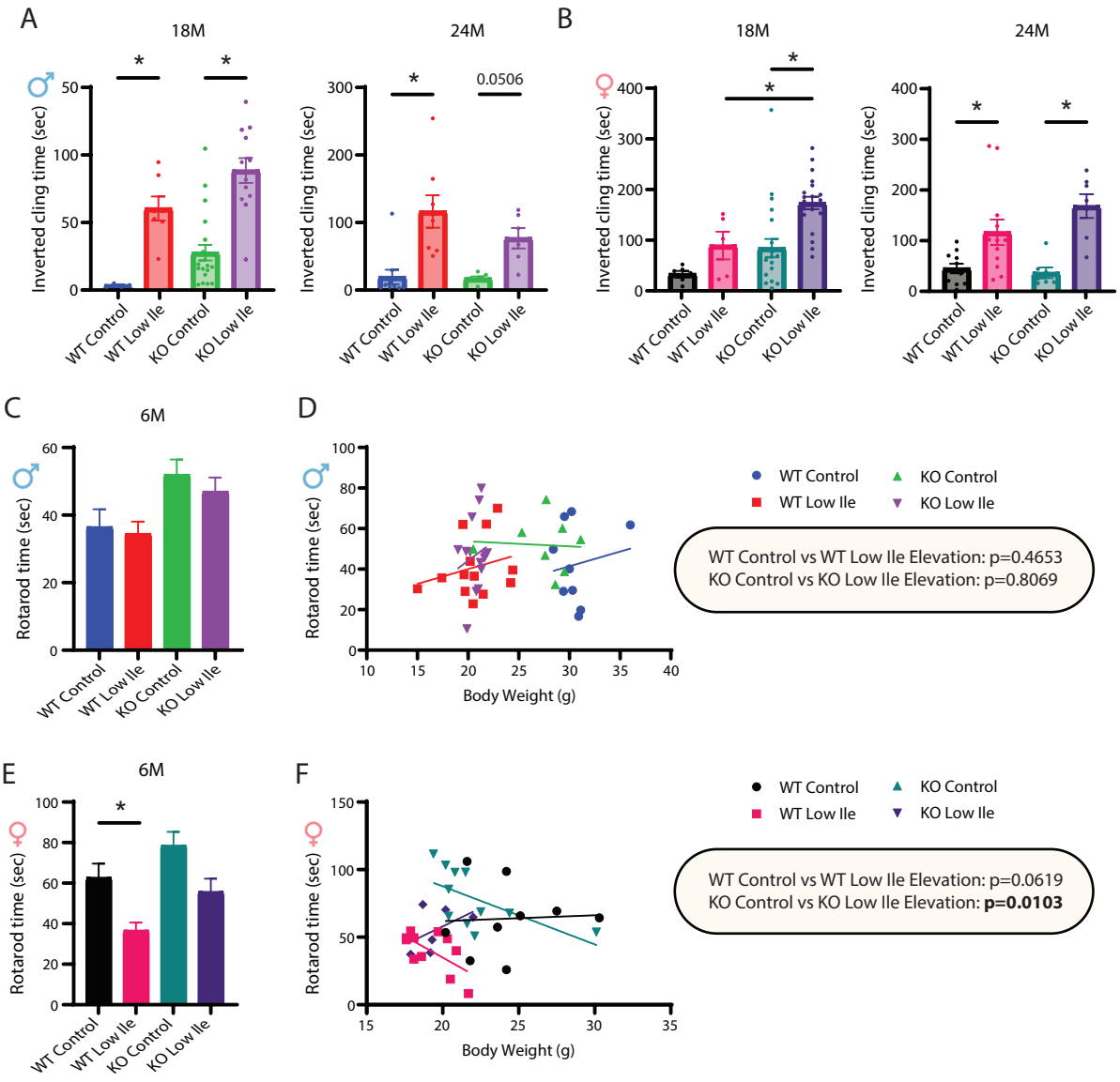

A
18M
24M
Inverted cling time (sec)
WT Control
WT Low Ile
KO Control
KO Low Ile
0.0506
B
18M
24M
C
6M
Rotarod time (sec)
D
Body Weight (g)
WT Control vs WT Low Ile Elevation: p=0.4653
KO Control vs KO Low Ile Elevation: p=0.8069
E
6M
F
Body Weight (g)
WT Control vs WT Low Ile Elevation: p=0.0619
KO Control vs KO Low Ile Elevation: p=0.0103

Figure 6. Coordination but not endurance is improved by a Low Ile diet.

(A-B) Male (A) and female (B) inverted cling hang time at 18 and 24 months on diet.

(C-F) Male (C-D) and female (E-F) rotarod time (C, E) and with body weight as a covariate (D, F).

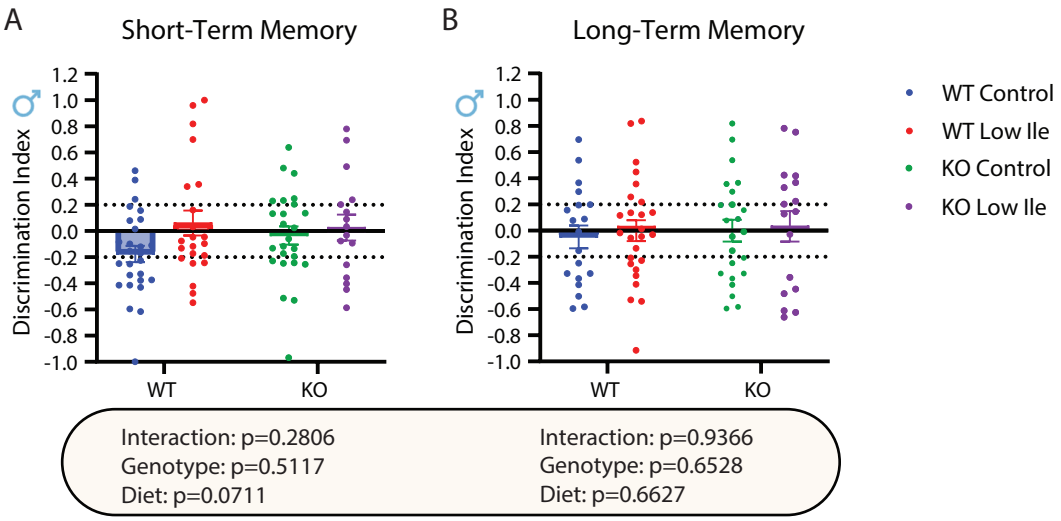
n=4-21 mice per group.

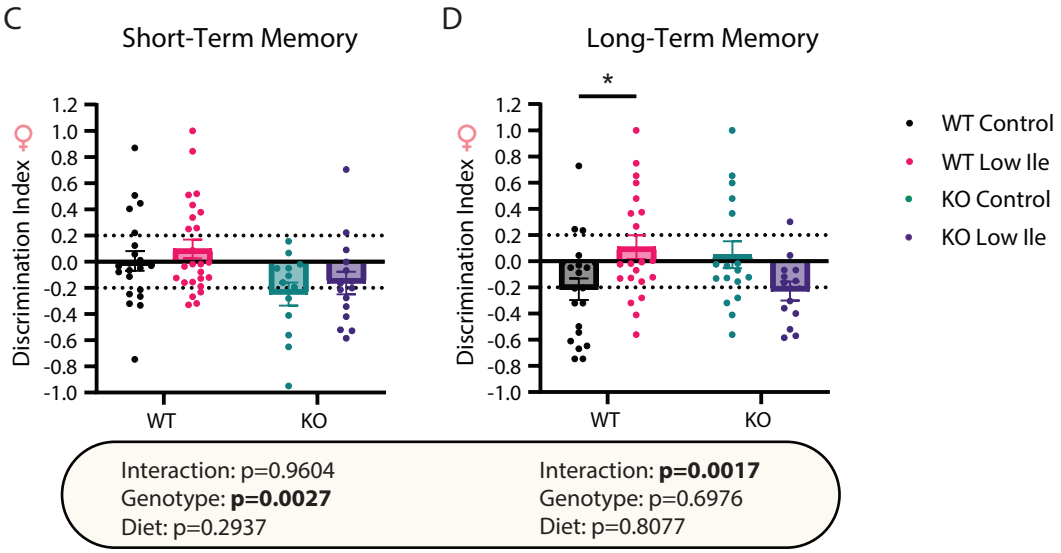
(A, B, C, E) Statistics for the overall effects of diet and genotype represent the p value from a two-way ANOVA; *$p<0.05$, Tukey's test (A, B) or Sidak's test post ANOVA (C, E) for each sex shown.

(D,F) Rotarod time as a function of body weight was calculated (data for each individual mouse are plotted, and slopes and intercepts were calculated using ANCOVA).

Data represented as mean $\pm$ SEM.

Figure 7.


A
Short-Term Memory
B
Long-Term Memory
♂
Discrimination Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
WT
KO
WT Control
WT Low Ile
KO Control
KO Low Ile
Interaction: p=0.2806
Genotype: p=0.5117
Diet: p=0.0711
Interaction: p=0.9366
Genotype: p=0.6528
Diet: p=0.6627


C
Short-Term Memory
D
Long-Term Memory
*
♀
Discrimination Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
WT
KO
WT Control
WT Low Ile
KO Control
KO Low Ile
Interaction: p=0.9604
Genotype: **p=0.0027**
Diet: p=0.2937
Interaction: **p=0.0017**
Genotype: p=0.6976
Diet: p=0.8077

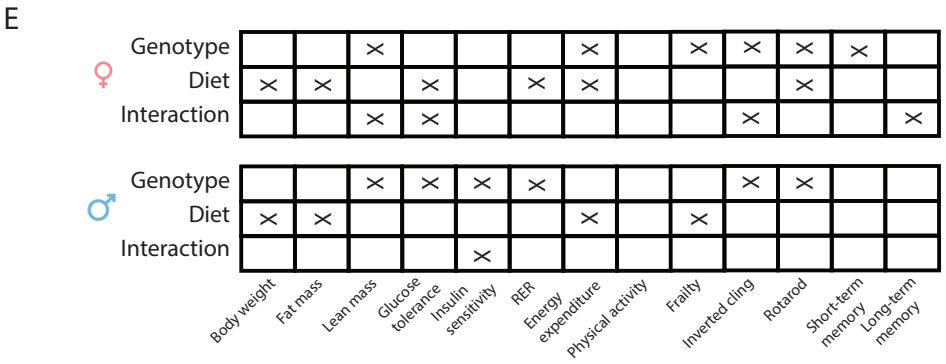

E
♀
Genotype
Diet
Interaction
♂
Genotype
Diet
Interaction
Body weight
Fat mass
Lean mass
Glucose tolerance
Insulin sensitivity
RER
Energy expenditure
Physical activity
Frailty
Inverted cling
Rotarod
Short-term memory
Long-term memory

Figure 7. Long-term memory is improved in females by a Low Ile diet in a genotype-dependent manner.

(A-D) Short- (A) and long-term memory (B) in males and females (C, D, respectively) assessed by novel object recognition.

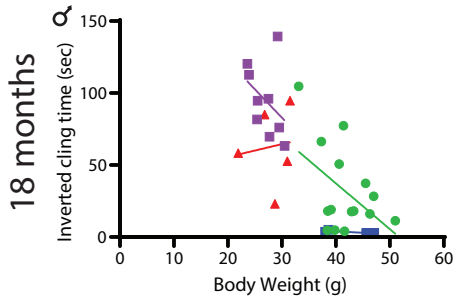
n=13-25 mice per group. Statistics for the overall effects of diet and genotype represent the p value from a two-way ANOVA; *p<0.05, Tukey's test post ANOVA for each sex shown.

(E) Schematic showing the overall effects of diet, genotype and their interaction on the parameters of this study in males and females.

Data represented as mean ± SEM.

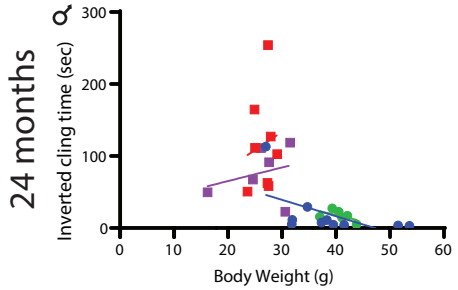
Supplemental Figure 1.

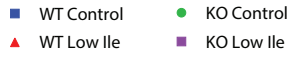
A



WT Control vs WT Low Ile Elevation: p=0.1036
KO Control vs KO Low Ile Elevation: p=0.5504

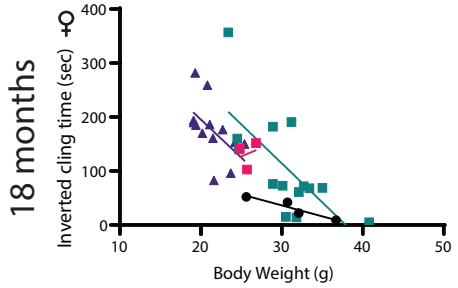
B

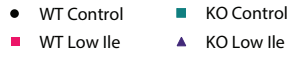




WT Control vs WT Low Ile Elevation: p=0.0515
KO Control vs KO Low Ile Elevation: **p=0.0449**

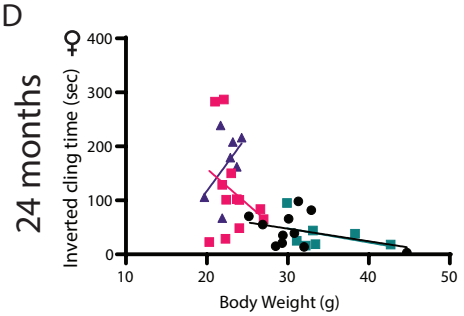
C

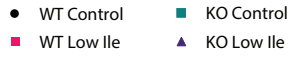




WT Control vs WT Low Ile Elevation: **p=0.0172**
KO Control vs KO Low Ile Elevation: p=0.1130

D





WT Control vs WT Low Ile Elevation: p=0.3321
KO Control vs KO Low Ile Elevation: **p=0.0474**

Supplemental Figure 1. Inverted cling time with body weight as a covariate.

(A-D) Inverted cling time per body weight in males and females at 18 (A, C) and 24 months (B, D).

Chapter 5: Conclusions and Future Directions

Conclusions

Though modern humans are indeed living longer than our ancestors, too often we are plagued with chronic disease in older age. In fact, the average American can expect to live until age 79 (Crimmins, 2015), though the last 16 of those years will be spent battling at least one chronic illness. As diet therapies and medicines are developing to increase both lifespan and healthspan, it is becoming clearer that a calorie is not just a calorie. Dietary protein is essential to sustain life, but its limitation improves many aspects of health and extends lifespan in a variety of model organisms (Hill et al., 2022a).

In a resistance training model, we were surprised to see that even then, a low protein diet yields benefits over the high protein-fed mice. Though the HP weight pulling mice gained more lean mass and didn't gain significantly more fat mass like their sham-pulling counterparts, the LP diet was still superior in improving glucose homeostasis. Further, by the end of the experiments, the LP and HP weight pulling animals that were just as strong (**Chapter 2**); in fact, when normalized to body weight, the LP-fed mice were actually stronger (data not shown). It is important to note that these mice were young, and evidence suggests that older humans benefit from greater protein intake to stave off sarcopenia (Liao et al., 2019). Regardless, a low protein diet is a powerful tool in improving health.

Similarly, limitation of the branched-chain amino acids yields positive effects on healthspan and lifespan (Richardson et al., 2021). We have shown that the when we restrict each of the three BCAAs individually, isoleucine limitation is the main driver of the benefits of BCAA restriction and recapitulates many of the outcomes of total protein restriction (Yu et al., 2021b). In the context of a high-fat, high-sucrose Western diet, a diet low in Ile improves body weight, glucose homeostasis, energy expenditure, and alters hepatic metabolism in males and females of two inbred strains (**Chapter 3**). Finally, many of these Low-Ile induced benefits occur

independently of FGF21 in mice (**Chapter 4**). Future work is needed to conclusively determine if a Low Ile diet requires FGF21 for lifespan extension and to examine whether a Low Ile diet is beneficial in humans.

Future Directions

Identified avenues of future study

Though the work here has made strides in identifying the root mechanisms through which a Low Ile diet improves health status and the process of aging, there is still more work to be done. In young C57BL/6J male mice, a Low Ile diet results in improvements independently of hepatic mTORC1 activity and GCN2 (Yu et al., 2021b). As demonstrated in **Chapter 4**, FGF21 is largely dispensable for metabolic effects of a Low Ile diet in both males and females, at least when started on diet at weaning. It is curious that these major amino acid sensing pathways are not required for the beneficial outcomes of Ile restriction, but we have identified other potential mechanisms to explore.

We determined a variety of both up- and downregulated hepatic mechanisms using our combined omics analyses in **Chapter 3**. Circadian rhythm, purine metabolism, cysteine and methionine metabolism, splicesome, and retinol metabolism are just a few of the upregulated hepatic mechanisms while nitrogen metabolism, protein processing, necroptosis, glycolysis and gluconeogenesis were downregulated. Further examination of these pathways in the context of a Low Ile diet is warranted, both in model organisms and in human clinical trials. Additionally, examination of extra-hepatic tissues may also reveal additional pathways of interest. Molecular evaluation of skeletal muscle in male and female mice under a Low Ile could be of particular interest, as that is the primary site of Ile catabolism.

Also in **Chapter 3**, we identified four predicted significant transcription factors that require examination based on our combined molecular omics analyses: Hepatocyte nuclear factor 4 alpha (HNF4A), specificity protein 1 (SP1), RE1-silencing transcription factor (REST),

and retinoid X receptor alpha (RXRA). HNF4A is a transcription factor that regulates genes that play a role in beta cell development; as such, HNF4A has been implicated in diabetes, specifically maturity onset diabetes of the young (MODY) (Warncke et al., 2019). Perhaps Ile plays a role in HNF4A's ability to regulate pancreatic insulin secretion. HNF4A is required for the development of the colon and liver (Garrison et al., 2006), so perhaps a murine inducible or knockdown model could be utilized to examine Low Ile diet outcomes. Deep exploration of these transcription factors as well as their potentnial mechanicstic alterations – potst-translational modificiations, nuclear localization, and/or changes in DNA binding – are waiting to be discovered. Clearly, identification of hepatic mechanisms and transcription factors will lead to development of a variety of future hypotheses and experiments.

Hallmarks of aging

Previous works have shown that a Low Ile diet reduces the burden of conserved molecular indicators of aging in 24 month old mice, including phosphorylated S6 and AKT S473, readouts of mTORC1 and mTORC2 activity in the liver, respectively (Yeh et al., 2024). There is also evidence that a Low Ile diet reduces cancer risk throughout the lifespan, though the potential mechanisms behind this reduction remain unidentified and untested (Green et al., 2023b). Further exploration of Ile's influence on other hallmarks of aging, including senescence, epigenetic alterations, and loss of proteostasis is warranted (López-Otín et al., 2013). For example, consumption of high amounts of dietary protein has been shown to exacerbate senescence and SASP in the liver (Nehme et al., 2021).

Human Clinical Trials

Clinical trials examining protein restriction have shown improvements in body composition, glucose homeostasis, and reduction in disease severity, even in the short term (Ferraz-Bannitz et al., 2022b; Fontana et al., 2016). Similarly, reducing BCAAs in human diets with the use of medical foods and supplements for MSUD does in fact decrease the circulating

BCAAs by 50% (Ramzan et al., 2020), which have been associated with insulin resistant obesity (Lynch and Adams, 2014). A four-week study of BCAA limitation in patients with well-controlled Type 2 Diabetes Mellitus (T2DM) lowered postprandial insulin secretion (Karusheva et al., 2019). Our diet data analysis also reveals that the healthiest human diets are naturally lowest in dietary Ile (**Chapter 3, Fig. 7F**). Therefore, a clinical trial examining whether a Low Ile diet improves the metabolic health of unhealthy individuals is necessary.

It would also be interesting to determine if the development of medical-grade foods that are limited in just isoleucine instead of all three BCAAs is possible; this would make a Low Ile diet regimen possible. Further work is also necessary to determine if Low Ile cycling in mice or humans recapitulates the benefits of chronic Ile limitation.

Gut microbiota

There is evidence that FGF21's adaptive stress response to dietary protein limitation is mediated by gut microbiota (Martin et al., 2021). Similarly, there was a shift in the fecal microbiome in the clinical trial of dietary BCAA restriction, with a depletion of Firmicutes and an enrichment of Bacteroidetes (Karusheva et al., 2019). As isoleucine is one of the BCAAs, its limitation stimulates FGF21, and gut microbiota can and do metabolize the BCAAs and their related metabolites, and it seems plausible that gut microbiota would be altered by a Low Ile diet. This experiment could be conducted by profiling banked cecal samples from our Low Ile studies, and confirmed by testing the Low Ile diet in germ free mice.

Other considerations

Protein restriction has been tested in organisms ranging from simple yeast all the way to complex humans. Examination of Ile restriction in models outside of mice would give insight into the conservation of such mechanisms and benefits. In a similar vein, investigation of the efficacy of a Low Ile diet in improving metabolic health in mice of different genetic backgrounds would be beneficial. One study of heterogeneous HET3 mice has been conducted (Green et al., 2023b), but it would be fascinating to examine outcomes in Diversity Outbred mice, that contain chromosomes from 8 founder strains. Finally, deeper evaluation into the age at which a Low Ile diet is initiated as well as time of diet duration is necessary. Nutrition need change throughout the lifespan, and protein is required in higher amounts during development, pregnancy, and older age.

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Appendix I.

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Article

Folic Acid Fortification and Neural Tube Defect Risk: Analysis of the Food Fortification Initiative Dataset

Michaela E. Murphy and Cara J. Westmark

Special Issue

Nutrition Status and Health

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nutrients

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Article

Folic Acid Fortification and Neural Tube Defect Risk: Analysis of the Food Fortification Initiative Dataset

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Abstract: The United States implemented mandatory fortification of cereal grains with folic acid in 1998 to prevent neural tube defects (NTDs) during pregnancy. The health benefits of folate (vitamin B9) are well documented; however, there are potential risks of exceeding the upper tolerable limit, particularly in vulnerable populations. We conducted a population-based analysis of the Food Fortification Initiative dataset to determine the strength of the evidence regarding reports of decreased NTDs at the national level in response to mandatory folic acid fortification of cereal grains. We found a very weak correlation between NTD prevalence and the level of folic acid fortification, irrespective of the cereal grain fortified (wheat, maize or rice). Stratification of the data based on socioeconomic status (SES) indicated a strong linear relationship between reduced NTDs and better SES. We conclude that national fortification with folic acid is not associated with a significant decrease in the prevalence of NTDs at the population level.

Keywords: folate; Food Fortification Initiative; methylenetetrahydrofolate reductase (MTHFR); national fortification; neural tube defect; vitamin B9; vitamin B12

1. Introduction

Neural tube defects (NTDs) are a heterogeneous group of structural birth defects that arise from a complex array of genetic and environmental factors and adversely affect the structure and function of the brain and spinal cord [1]. The United States of America (USA) was the first country to mandate a national folic acid food fortification program to prevent NTDs including spina bifida. Currently, more than 80 other countries fortify cereal grains with folic acid. Based on rates from the Centers for Disease Control and Prevention (CDC) Birth Defects Monitoring Program from 1980 through 1987, an estimated 13,600 infants born in the USA had spina bifida without anencephaly [2]. Of these, approximately 3800 died as a result of their defects [2].

Periconceptional intake of folic acid reduces a women's risk of having an infant affected by an NTD [3]. Folic acid intervention studies in pregnant women with prior NTD-affected pregnancies showed a 60–100% reduction in NTD risk with a later pregnancy. Observation studies of folic acid efficacy in preventing NTD in pregnant women without a prior NTD-affected pregnancy showed a 0–75% reduction in risk [4]. Thus, in 1992, the USA Public Health Service (PHS) recommended that all women of childbearing age consume 400 µg of folic acid daily to prevent NTDs [4]. Specifically, they stated, "All women of childbearing age in the United States who are capable of becoming pregnant should consume 0.4 mg of folic acid per day for the purpose of reducing their risk of having a pregnancy affected with spina bifida or other NTDs. Because the effects of higher intakes are not well known but include complicating the diagnosis of vitamin B12 deficiency, care should be taken to keep total folate consumption at <1 mg per day, except under the supervision of a physician." [4].

Despite the publicized guidelines, there was still concern that women of childbearing age were not consuming enough folate and were at risk of NTD-affected pregnancies. To promote compliance, the USA Food and Drug Administration (FDA, Silver Spring, MD, USA) issued a regulation in 1996 requiring that all enriched cereal grain products be fortified with folic acid by January of 1998 [5,6]. Cereal grains were required to be fortified with folic acid at levels ranging from 0.43 mg to 1.4 mg per pound. At the time, the federal agencies recognized that high folate intake was masking vitamin B12 deficiency-associated anemia, which leads to neurological damage if untreated. There was also uncertainty regarding the lack of data on the minimal level of folate needed to significantly reduce NTDs. Despite these concerns, the PHS found the evidence sufficiently consistent to make its recommendation because 50% of pregnancies are unplanned and the neural tube closes early in embryonic development (gestation day 28); thus, folic acid fortification should commence prior to becoming pregnant. They concluded that "the intakes that are likely to result from the level of fortification established in their final rule do not present a health concern to the general population", and it was estimated that there would be a 50% reduction in NTDs in the USA.

The efficacy of national folic acid fortification programs in reducing NTDs is debated, and there has been an increase in the prevalence of individuals exceeding the tolerable upper limit (UL) for folic acid intake (1000 µg/day) [7,8]. The UL for folic acid was designated as one-fifth of the lowest observed dose associated with a potential adverse outcome (5000 µg/day). The aim of this study was to retrospectively address the question of whether folic acid fortification improves NTDs at the population level by extracting and analyzing relevant data from the Food Fortification Initiative (FFI, Atlanta, GA, USA) dataset.

2. Materials and Methods

We utilized the FFI database found online at ffinetwork.org to extract data on folic acid fortification levels and the prevalence of NTDs per 10,000 births as a function of country. The FFI is a global partnership composed of public, private, and civic members that was founded in 2002 to promote fortification of industrially milled flours (wheat, maize, and rice). Members include the CDC's National Center for Chronic Disease Prevention and Health Promotion, Emory University, and the International Federation for Spina Bifida Hydrocephalus. Funding partners include the Bill & Melinda Gates Foundation (Seattle, WA, USA), Australian Department of Foreign Affairs and Trade (Barton, Australia), Global Alliance for Improved Nutrition (Geneva, Switzerland), Micronutrient Initiative (Ottawa, Ontario, Canada), United Nations International Children's Emergency Fund (UNICEF, New York, NY, USA), Cargill, Inc. (Minneapolis, MN, USA), GiveWell (Oakland, CA, USA), The Amit J. & Vicky L. Patel Foundation (Atlanta, GA, USA), and Nutrition International (Ottawa, Ontario, Canada). Under the website link for country profiles, the FFI database lists which nutrients including folic acid are added to grains via fortification in units of parts per million (ppm) based on data from the Food and Agriculture Organization of the United Nations (FAO, Roma, Italy) using 2013 data, which was the last year with all data available in March 2018. The FFI attained NTD data from a combination of sources [9–13]. Thus, the FFI dataset contains a single value for folic acid fortification levels for each country and a single value for NTD prevalence.

There was a total of 236 countries monitored by the FFI. Of those countries, data on NTDs were available for 194 countries. Inclusion data consisted of all countries with available data on both folic acid fortification levels and NTD prevalence (186 countries). Eight countries were excluded that were listed as fortifying with folic acid at 0 ppm (Antigua and Barbuda, Bahamas, Barbados, Dominica, Guyana, Philippines, Suriname, and Tajikistan). The study compared countries with national folic acid fortification versus countries without national folic acid fortification with the rationale that a national fortification policy would directly affect the vast majority of inhabitants of a particular country despite the import of grain products from other countries. The null hypothesis was that there is no association between national folic acid fortification and the prevalence of NTDs. The alternative hypothesis was that national folic acid fortification alters the prevalence of NTDs. The primary endpoint of

interest examined was prevalence of NTDs. The primary predictor variable was folic acid fortification. To calculate the average prevalence of NTDs per 10,000 births as a function of folic acid fortification, countries were binned into groups based on which cereal grain was fortified and NTD prevalence was summed and divided by the number of countries for each fortification group. Potential confounders included variations in consumption of folic acid, genetic variants, and overall nutritional status at the individual subject level, the accuracy of data reporting agencies, voluntary fortification with folic acid, periconception folic acid supplementation, and variable implementation timing of mandatory folic acid programs. These confounders could not be addressed with the available data. Study size was dependent on the available data in the FFI database.

For analysis of socioeconomic status, UNICEF economic indicator data were merged with the FFI dataset. The percentage of the gross domestic product (GDP) spent on health, education, and social protection were summed, ranked, and divided into quintiles. The UNICEF government expenditure data were based on the most recent year available between 2010–2018. Five countries (Cook Islands, North Korea, Libya, Niue, Syrian Arab Republic) were excluded from the socioeconomic status (SES) analysis because there were no data available on health, education or social protection government expenditures.

Data were analyzed in accordance with STROBE guidelines (https://strobe-statement.org/index.php?id = available-checklists). Means, standard deviations, regression coefficients, and 95% confidence intervals (CI) were computed to describe the results. To statistically test for differences in NTD rates as a function of folic acid fortification, the Student's t-test was used. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Description of Data Utilized from the Food Fortification Initiative (FFI)

There are discrepant reports regarding the efficacy of national folic acid fortification. We conducted a retrospective analysis of the FFI dataset. The FFI tracks food fortification levels for 236 countries. Of these countries, data on both the prevalence of NTDs and the level of folic acid fortification were available for 186 countries (Table 1). There are two countries that fortify wheat, maize, and rice (United States and Costa Rica). There are 14 countries that fortify wheat and maize but not rice, and six countries that fortify wheat and rice but not maize. All the countries that fortify maize also fortify wheat, except for Rwanda, and all of the countries that fortify rice also fortify wheat, except for Bangladesh.

Table 1. Fortification levels of folic acid as a function of cereal grain.

Cereal Grain Fortified	Number of Countries	Average Fortification Level (ppm) ± Standard Deviation [1]	Range of Fortification (ppm) [2]
Wheat	68	1.94 (0.75)	0.1–5.11
Maize	17	1.52 (0.50)	0.5–2.6
Rice	9	1.25 (0.63)	0.1–2.31
None	116	*n*/a	*n*/a

[1] Data extracted from the Food Fortification Initiative dataset at www.ffinetwork.org. Average fortification levels in parts per million (ppm) were calculated by summing country folic acid fortification levels as a function of the cereal grain fortified and dividing by the number of countries in that cohort. Standard deviation is presented in parentheses following the average. [2] The range of fortification is the low and high values for each cereal grain cohort in ppm.

3.2. Prevalence of NTDs as a Function of Folic Acid Fortification in the FFI Dataset

The average prevalence of NTDs per 10,000 births in countries that do not fortify any cereal grains with folic acid was 13.32 (SD: 5.50, $n = 116$ countries), and the average prevalence of NTDs in countries with at least one cereal grain fortified with folic acid was 13.30 (SD: 6.13, $n = 70$). Stratification of the data based on which cereal grain was fortified with folic acid indicated no statistically significant differences

in the prevalence of NTDs comparing wheat, maize, and rice fortification versus no fortification, albeit there was a trend for reduced NTDs with folic acid fortification of maize ($p = 0.065$) (Table 2, Figures 1–4). There was a very weak correlation between NTD prevalence and the level of folic acid fortification irrespective of the cereal grain fortified: wheat (Figure 1), maize (Figure 2), and rice (Figure 3).

Table 2. Prevalence of neural tube defects (NTDs) as a function of folic acid fortification.

Cereal Grain Fortified	*n*	Average Number of NTDs per 10,000 Births [1]	*p* [2]	Regression Coefficient [3]	95% CI [4]
Wheat	68	13.07 (5.76)	0.78	−1.07	−1.44–1.94
Maize	17	10.76 (3.44)	0.065	−1.15	−0.16–5.28
Rice	9	13.44 (10.67)	0.95	−6.57	−4.21–3.97
None	116	13.32 (5.50)	*n*/a	*n*/a	*n*/a

[1] Data extracted from the Food Fortification Initiative dataset at www.ffinetwork.org. Average number of NTDs per 10,000 births was calculated by summing NTD prevalence as a function of fortified cereal grain and dividing by the number of countries in that cohort. Standard deviation is presented in parentheses following the average. [2] The *p*-value is the Student's t-test result comparing the indicated cereal grain with the non-fortified cohort. [3] The regression coefficient is the constant (a) from the regression line $y = ax + b$ that represents the rate of change of NTDs (y) as a function of folic acid fortification (x). [4] CI is the 95% confidence interval for the difference in means compared with the non-fortified cohort.

There was a trend for about 20% reduced NTDs when maize was fortified with folic acid compared to no fortification ($p = 0.065$) although the level of fortification did not correlate with the prevalence of NTDs. Seventeen countries fortified maize with folic acid at a range of 0.5–2.6 ppm, with the United States at 1.87 ppm. The lower prevalence of NTDs with maize fortification was due to the absence of greater than 15 NTDs per 10,000 births in all 17 countries in the maize cohort. The top quintile for prevalence of NTDs in the wheat and rice cohorts was >15 per 10,000 births with the highest reported prevalence of 32 per 10,000 births for both cohorts.

It should be noted that India and Guatemala had the lowest reported fortification levels of wheat and rice at 0.1 ppm and 0.4 ppm folic acid, respectively, which are more than two standard deviations lower than the mean. If India and Guatemala are deleted from the analysis, then the regression coefficients are −0.16 and 2.06, respectively.

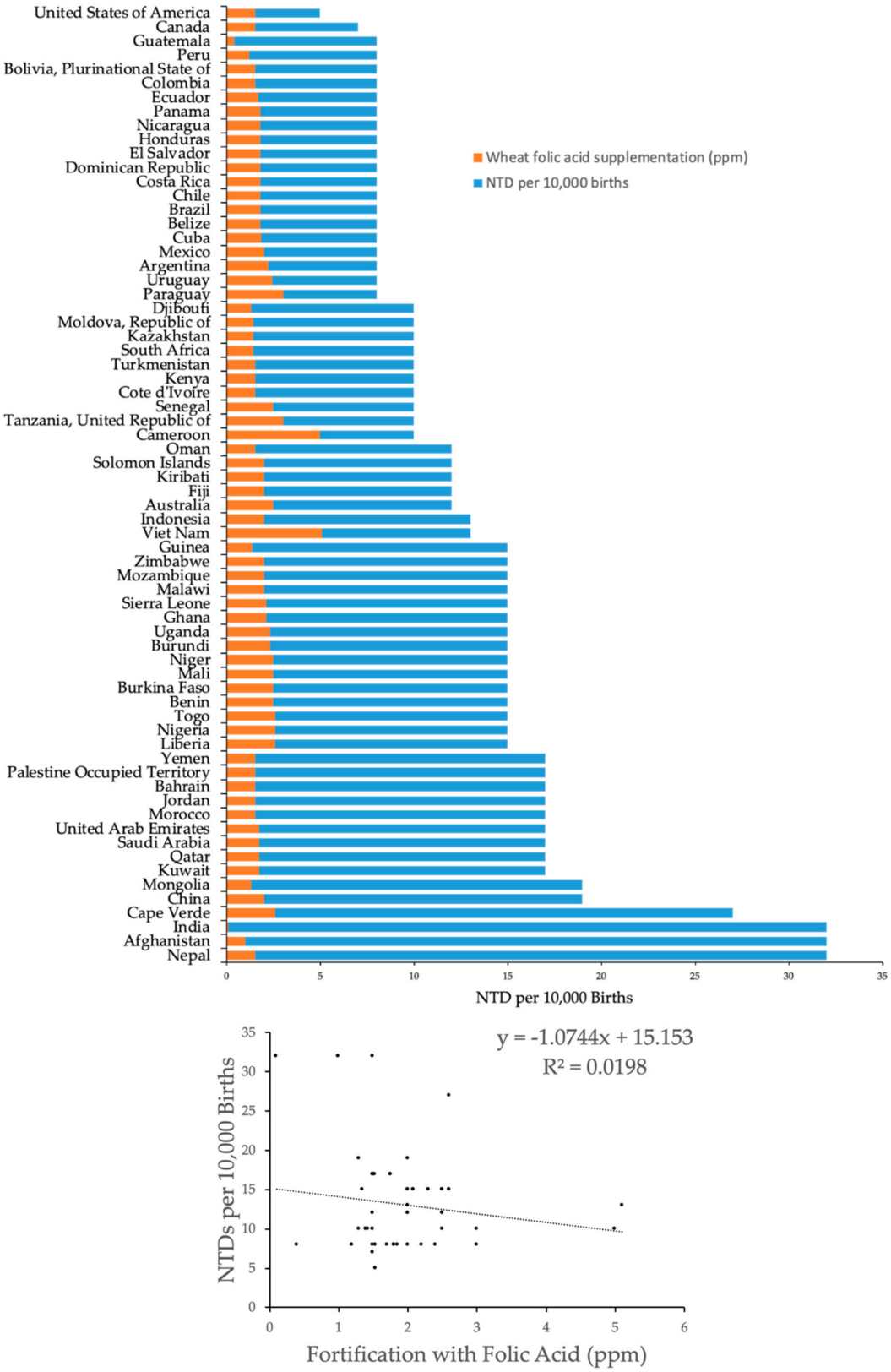

Figure 1. Prevalence of NTDs as a function of folic acid fortification levels in wheat. The number of NTDs per 10,000 births was plotted (blue bars) versus country (n = 68). Folic acid fortification levels of wheat in ppm (orange bars) were superimposed on NTD prevalence. Linear regression analysis indicates a regression coefficient (a) of −1.07.

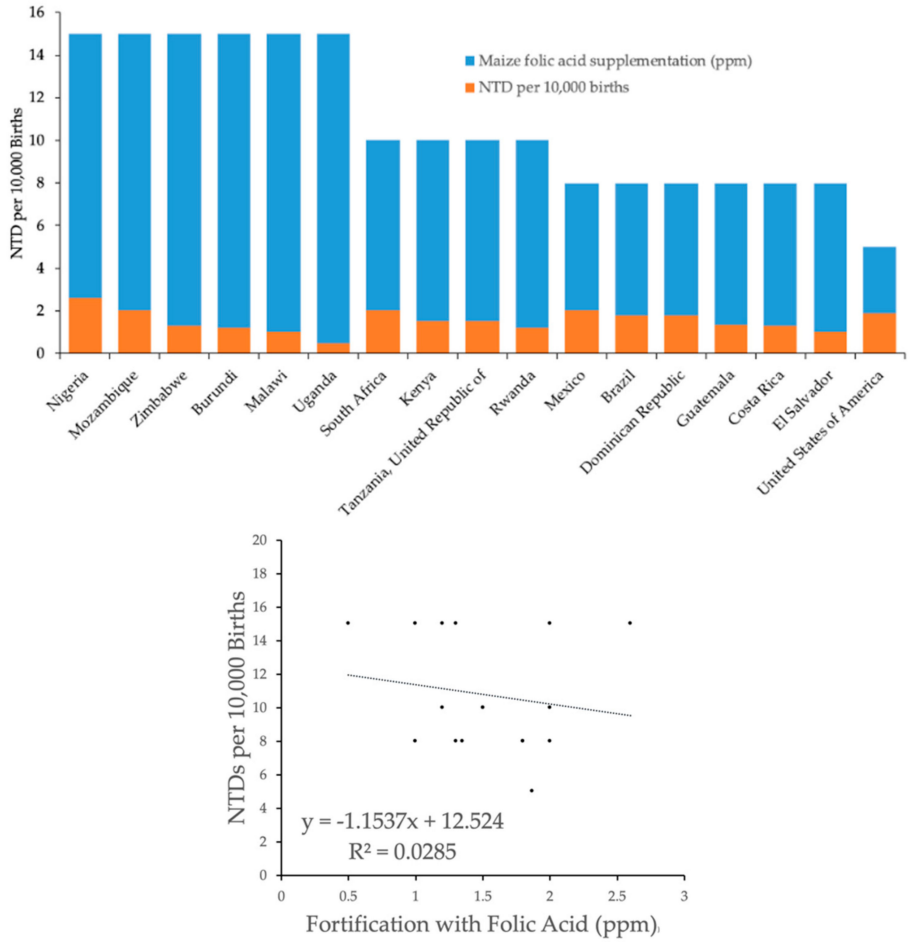

Figure 2. Prevalence of NTDs as a function of folic acid fortification levels in maize. The number of NTDs per 10,000 births was plotted (blue bars) versus country (n = 17). Folic acid fortification levels of maize in ppm (orange bars) were superimposed on NTD prevalence. Linear regression analysis indicates a regression coefficient (a) of −1.15.

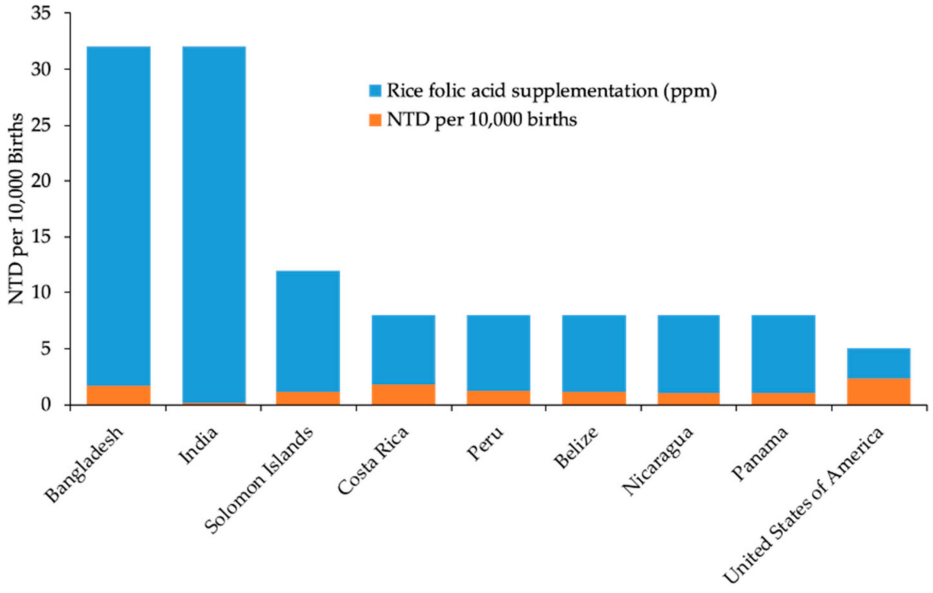

Figure 3. *Cont*.

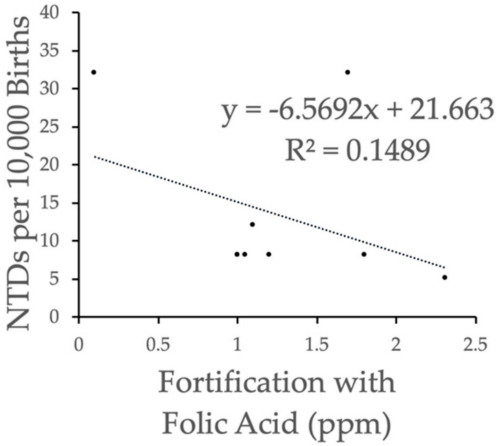

Figure 3. Prevalence of NTDs as a function of folic acid fortification levels in rice. The number of NTDs per 10,000 births was plotted (blue bars) versus country (n = 17). Folic acid fortification levels of rice in ppm (orange bars) were superimposed on NTD prevalence. Linear regression analysis indicates a regression coefficient (a) of −6.57.

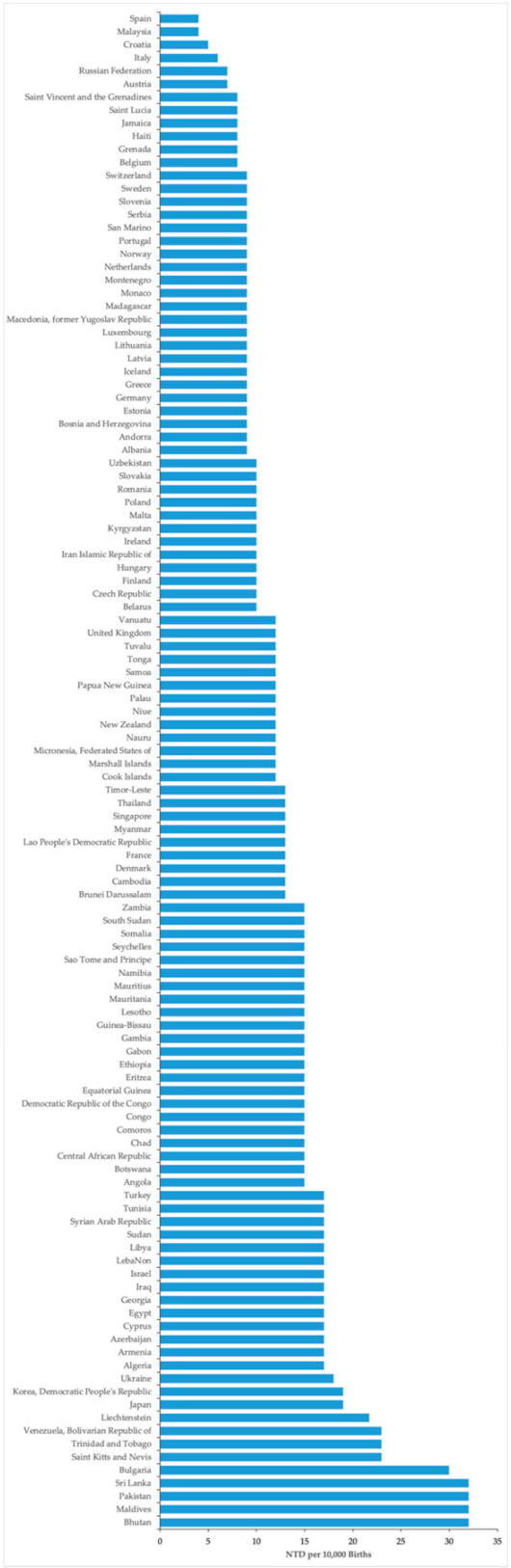

Figure 4. Prevalence of NTDs in the absence of national folic acid fortification. The number of NTDs per 10,000 births was plotted (blue bars) versus country (n = 116).

3.3. Prevalence of NTDs after Stratification of the Data Based on Socioeconomic Status

An indicator of SES is the percent of GDP spent on healthcare, education, and social protection. Stratification of the FFI NTD data based on these national economic indicators produces a strong linear correlation of reduced NTDs with higher SES (a = 1.43; R^2 = 0.85). The average prevalence of NTDs in quintile 1 (highest SES) was 10.97 (4.83) versus quintile 5 at 16.11 (6.47) (p = 0.0003; greater than 30% reduced prevalence). Binning the countries by folic acid fortification status gives a near perfect linear relationship between improved SES and reduced prevalence of NTDs when cereal grains are fortified with folic acid (a = 2.05; R^2 = 0.996), and a moderate correlation between SES and NTDs with the non-fortification cohort (a = 1.19; R^2 = 0.55) (Table 3). Interestingly, the quintile 3 countries had a higher average prevalence of NTDs with folic acid fortification ($p < 0.03$). In total, these data strongly suggest that improved SES contributes to reduced prevalence of NTDs.

Table 3. Prevalence of NTDs as a function of socioeconomic status (SES) and folic acid fortification.

SES Quintile [1]	N	Average NTDs With Fortification [2,3]	N	Average NTDs Without Fortification [2,3]	p [4]
1	10	8.90 (2.81)	27	11.74 (5.22)	0.11
2	15	10.87 (5.15)	22	12.45 (5.64)	0.39
3	19	13.11 (3.33)	18	10.50 (3.59)	0.03
4	16	15.38 (7.14)	21	16.19 (5.69)	0.70
5	9	16.90 (6.74)	24	15.82 (6.49)	0.68

[1] Data extracted from United Nations International Children's Emergency Fund (UNICEF) dataset at https://data.unicef.org/resources/dataset/sowc-2019-statistical-tables/. [2] Data extracted from the Food Fortification Initiative dataset at www.ffinetwork.org. [3] Average number of NTDs per 10,000 births was calculated by summing NTD prevalence of countries, as a function of SES quintile, and dividing by the number of countries in that quintile. Standard deviation is presented in parentheses following the average. [4] The p-value is the Student's t-test result comparing the fortified and non-fortified countries within the quintile.

4. Discussion

Mandatory folic acid fortification programs in the USA, Canada, Costa Rica, Chile, and South Africa are associated with significant increases in blood folate concentrations and declines of 25%–50% in the prevalence of NTD-affected pregnancies [14–19]. Reported NTDs in the USA decreased from 10.8/10,000 births in 1995–1996 to 6.9/10,000 births in 2006 [14]. A systematic review (179 studies) and meta-analysis (123 studies) covering the prevalence of spina bifida in response to folic acid fortification status, geographic region, and study population indicate a lower prevalence of spina bifida in geographic regions with mandatory folic acid fortification (33.86 per 100,000 live births) versus voluntary fortification (48.35 per 100,000 live births) [20,21]. Based on 59 countries meeting the criteria of mandatory folic acid fortification of at least 1.0 ppm, it is estimated that 50,270 spina bifida and anencephaly births were prevented out of a possible 280,500 [22]. Overall, these reports suggest that national folic acid fortification protects against NTDs. However, two important confounding issues with these studies are that they do not take into account that NTD rates were declining prior to folic acid fortification and do not include comparison to non-fortification control groups during the same time period.

The annual numbers of NTD-births in the USA and the United Kingdom declined without mandatory fortification [2,23,24]. In the USA, the CDC analyzed data from 16 states for the prevalence of spina bifida at birth between the years 1983–1990 and found 4.6 cases of spina bifida per 10,000 births. The peak of 5.9 cases per 10,000 births in 1984 declined to 3.2 cases per 10,000 births in 1990. Rates varied substantially by state and racial/ethnic groups with the lowest prevalence for Asians/Pacific Islanders and the highest prevalence for Hispanics although the rate for Hispanics declined substantially from 1983–1990. Thus, in the absence of mandatory folic acid fortification, the prevalence of NTDs was substantially decreasing in the USA. It is not possible to discern if that trend would have continued in the absence of national mandatory folic acid fortification.

Health Canada mandated national folic acid fortification by early 1998 consistent with the FDA deadline, which allowed the export of Canadian flour to the USA [25]. Retrospective cross-sectional studies indicate increased folic acid status in both young and older women and an approximately 50% reduction in NTDs. There were no federal or provincially designated studies implemented to prospectively monitor blood folate levels or the prevalence of NTDs. Over one million obstetrical deliveries of all liveborn or stillborn infants were studied in a retrospective population-based longitudinal study between April 1990 and March 2000 to assess whether the Canadian folic acid fortification program was associated with a decline in the rate of pre-eclampsia (PET) and all hypertensive disorders of pregnancy [26]. No significant decline in the monthly rate of either PET or all hypertensive disorders of pregnancy was found after fortification, albeit here was a slight increase in the risk of all hypertensive disorders of pregnancy [26]. While a decline in open NTDs was observed after fortification, the prevalence of NTDs was declining before fortification [26].

To address the confounding issue of a lack of a control group in observational folic acid fortification studies, we analyzed the whole FFI dataset at the population level comparing countries that fortified cereal grains with folic acid to countries not fortifying. We did not find an association between national folic acid fortification and decreased prevalence of NTDs, 13.32 (5.50) per 10,000 births (no fortification) versus 13.30 (6.13) per 10,000 births (plus fortification). There are many reasons why NTDs could decrease over time irrespective of folic acid fortification; for example, improved health care or socioeconomic conditions. We found a strong linear relationship between reduced NTDs and increased GDP spent on socioeconomic indicators ($a = 1.43$; $R^2 = 0.85$). Our findings suggest that improved NTD outcomes are not associated with mandatory folic acid fortification at the population level but rather with SES as indicated by >30% reduced prevalence of NTDs between the lowest and highest SES quintiles. Thus, increased GDP spent on healthcare and education would be expected to improve NTDs. It remains to be determined if improved NTD outcomes as a function of SES are due to periconception folic acid supplementation.

A Cochrane systematic review assessed the efficacy of folic acid fortification of wheat and maize flour on health outcomes in the overall population [27]. The review included 10 studies (five randomized control clinical trials (RCTs), three non-RCTs, and two interrupted time series (ITS) studies) conducted in upper-middle-income countries (China, Mexico, South Africa), a lower-middle-income country (Bangladesh), and a high-income country (Canada). The duration of the intervention studies ranged from 2 weeks to 36 months. The ITS studies included post-fortification periods for up to seven years. The authors concluded that, "Fortification of wheat flour with folic acid may reduce the risk of neural tube defects; however, this outcome was only reported in one non-RCT. Fortification of wheat or maize flour with folic acid (i.e., alone or with other micronutrients) may increase erythrocyte and serum/plasma folate concentrations. Evidence is limited for the effects of folic acid-fortified wheat or maize flour on haemoglobin levels or anaemia. The effects of folic acid fortification of wheat or maize flour on other primary outcomes assessed in this review is not known. No studies reported on the occurrence of adverse effects. Limitations of this review were the small number of studies and participants, limitations in study design, and low-certainty of evidence due to how included studies were designed and reported." The Cochrane Database of Systematic Reviews (CDSR) is the leading journal and database for systematic reviews in health care, and a contributing author on the review was from the FFI, which tracks global progress in grain fortification. Thus, evidence at the population level is "very low certainty" regarding the efficacy of folic acid fortification in improving NTD outcomes.

While the USA and Canada mandated folic acid fortification in 1998, the United Kingdom, Ireland, and the European Union did not because of the uncertain risk of folic acid fortification to ageing populations who are at increased risk of vitamin B12 deficiency as well as the ethical issue of mandating food fortification when there is a possible risk that fortification might cause risk to a group of the population different from that receiving the benefits [24,28]. In the absence of prospective monitoring of fortification programs, it is not possible to establish a cause and effect relationship on the risks and benefits of national folic acid fortification. Legislation and monitoring guidelines on food fortification

for 72 countries as of 31 January 2015 indicate that there is sufficient documentation in terms of establishing mandatory programs but a lack of documentation concerning product compliance to national standards [29]. Thus, surveillance systems are in place to monitor national fortification policies, but not actual exposure and downstream biological effects [30]. Mandatory folic acid fortification in the USA was projected to increase the average folic acid intake by 100 μg/day; however, the mean increase was approximately twice as large as projected [6]. The prevalence of individuals that exceed the UL for folic acid intake is 10% in the subset of the USA population that consumes folic acid supplements [5].

There are several vulnerable populations that may be affected from national folic acid fortification. There is a general risk of nervous system damage in persons with low or insufficient status of B12 who are exposed to high intake of folic acid [7,24]. Low vitamin B12 levels in the elderly, in conjunction with higher serum folate, are associated with anemia and cognitive impairment [5,31–35]. Elderly persons are more likely to take medications such as proton pump inhibitors (PPIs) for heartburn, which are associated with vitamin B12 deficiency, and elderly persons with a generic variant in the transcobalamin gene TCN2 (C776G) who consume twice the recommended daily allowance (RDA) of folate are seven times more likely to have neuropathy [36]. In addition to masking vitamin B12 deficiency, other potential consequences of mandatory folic acid fortification include exceeding the UL of folic acid consumption and folic acid interaction with genetic mutations such as the methylenetetrahydrofolate reductase (MTHFR) polymorphism.

The limitations of this study include: (1) it is based on retrospective data regarding folic acid fortification and the prevalence of NTDs; (2) the level of folic acid fortification is declarative; (3) there are only single data points available for folic acid fortification levels and NTD prevalence (year 2013); and (4) there is a lack of data regarding confounding issues such as voluntary fortification with folic acid, national recommendations for preconception and first trimester folic acid intake, and the implementation timing of mandatory folic acid programs. Despite these limitations, we would expect the main result of the study to be similar for the years before and after 2013. However, without prospective monitoring of folic acid intake and the prevalence of NTDs while taking into account confounding issue such as SES and genetic variations, one cannot definitely prove the findings.

5. Conclusions

In conclusion, we did not find reduced prevalence of NTDs at the population level in response to national folic acid fortification, but rather stratification of the data based on SES indicated a strong linear relationship between reduced NTDs and better SES.

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