Influence of dairy intake on odd-chain fatty acids and energy metabolism

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Glossary of Terms

Gene names presented in **Table 3**

3-HIB	3-Hydroxyisobutyrate
Ac-CoA	Acetyl-CoA
AKT	Protein Kinase B
AUC	Area under curve
BAT	Brown adipose tissue
BAIBA	β-aminoisobutyric acid
BCAA	Branched-chain amino acids
BW	Body weight
C15:0	Pentadecanoic acid
C16:0	Palmitic acid
C17:0	Heptadecanoic acid
C18:0	Stearic acid
C18:1n9	Oleic acid
cDNA	Complementary DNA
Ct	Cycle threshold
CVD	Cardiovascular disease
DEPC	Diethyl pyrocarbonate
DIO	Diet-induced obesity
ECFA	Even chain fatty acid
EE	Energy expenditure
eWAT	Epididymal white adipose tissue
FA	Fatty acid
FFA	Free fatty acid
H&E	Hematoxylin & eosin
HF	High-fat
HFC17	High-fat diet + 5% C17:0 supplementation
HFL	High-fat diet + 5% Leucine supplementation
HFMF	High-fat diet + 14% milk fat (butter) supplementation
HFMP	High-fat diet + 14% milk protein (casein) supplementation
HFV	High-fat diet + 5% Valine supplementation
Ile	Isoleucine
IR	Insulin resistance
ITT	Insulin tolerance test
LCFA	Long chain fatty acid
LDL	Low-density lipoprotein
Leu	Leucine
LF	Low-fat diet
MetS	Metabolic syndrome
MUFA	Mono-unsaturated fatty acid
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NMR	Nuclear magnetic resonance
OCFA	Odd chain fatty acids
oGTT	Oral glucose tolerance test
PL	Phospholipid
Pr-CoA	Propionyl-CoA
PUFA	Poly-unsaturated fatty acid
PVDF	Polyvinylidene difluoride
Quad	Quadriceps
RER	Respiratory exchange ratio
SCFA	Short chain fatty acids
SFA	Saturated fatty acids

sWAT	Subcutaneous white adipose tissue
T2DM	Type 2 Diabetes mellitus
TG	Triglycerides
Val	Valine

Abstract

As of late, epidemiological studies have highlighted a strong association of dairy intake with lower disease risk, and similarly with an increased amount of odd-chain fatty acids (OCFA). While the OCFA also demonstrate inverse associations with disease incidence, the direct dietary sources and mode of action of the OCFA remain poorly understood.

The overall aim of this thesis was to determine the impact of two main fractions of dairy, milk fat and milk protein, on OCFA levels and their influence on health outcomes under high-fat (HF) diet conditions. Both fractions represent viable sources of OCFA, as milk fats contain a significant amount of OCFA and milk proteins are high in branched chain amino acids (BCAA), namely valine (Val) and isoleucine (Ile), which can produce propionyl-CoA (Pr-CoA), a precursor for endogenous OCFA synthesis, while leucine (Leu) does not. Additionally, this project sought to clarify the specific metabolic effects of the OCFA heptadecanoic acid (C17:0).

Both short-term and long-term feeding studies were performed using male C57BL/6JRj mice fed HF diets supplemented with milk fat or C17:0, as well as milk protein or individual BCAA (Val; Leu) to determine their influences on OCFA and metabolic health. Short-term feeding revealed that both milk fractions induce OCFA *in vivo*, and the increases elicited by milk protein could be, in part, explained by Val intake. *In vitro* studies using primary hepatocytes further showed an induction of OCFA after Val treatment via *de novo* lipogenesis and increased α-oxidation. In the long-term studies, both milk fat and milk protein increased hepatic and circulating OCFA levels; however, only milk protein elicited protective effects on adiposity and hepatic fat accumulation—likely mediated by the anti-obesogenic effects of an increased Leu intake. In contrast, Val feeding did not increase OCFA levels nor improve obesity, but rather resulted in glucotoxicity-induced insulin resistance in skeletal muscle mediated by its metabolite 3-hydroxyisobutyrate (3-HIB). Finally, while OCFA levels correlated with improved health outcomes, C17:0 produced negligible effects in preventing HF-diet induced health impairments.

The results presented herein demonstrate that the beneficial health outcomes associated with dairy intake are likely mediated through the effects of milk protein, while OCFA levels are likely a mere association and do not play a significant causal role in metabolic health under HF conditions. Furthermore, the highly divergent metabolic effects of the two BCAA, Leu and Val, unraveled herein highlight the importance of protein quality.

Zusammenfassung

In den letzten Jahren haben epidemiologische Studien einen Zusammenhang zwischen dem Verzehr von Milchprodukten und einem geringeren Krankheitsrisiko sowie einem erhöhten Gehalt an ungeradzahligen Fettsäuren (OCFA) aufgezeigt. Während die OCFA ebenfalls mit einem verminderten Krankheitsrisiko assoziiert sind, ist über die direkten diätetischen Quellen und die physiologische Rolle der OCFA noch wenig bekannt.

Das Hauptziel dieser Arbeit war die Untersuchung der Bedeutung der beiden Hauptfraktionen von Milchprodukten, Milchfett und Milchprotein, für den OCFA-Gehalt und ihren Einfluss auf die Gesundheit unter den Bedingungen einer fettreichen Ernährung (HF). Beide Fraktionen sind mögliche OCFA-Quellen, da Milchfette selber signifikante Mengen an OCFA enthalten und Milchproteine einen hohen Anteil an verzweigtkettigen Aminosäuren (BCAA) haben, nämlich Valin (Val) und Isoleucin (Ile), aus denen Propionyl-CoA (Pr-CoA), eine Vorstufe für die endogene OCFA-Synthese, gebildet werden kann, während das für Leucin (Leu) nicht der Fall ist. Außerdem sollten in diesem Projekt die spezifischen metabolischen Auswirkungen der OCFA Heptadecansäure (C17:0) geklärt werden.

Dazu wurden Kurzzeit- und Langzeit-Fütterungsstudien mit männlichen C57BL/6JRj-Mäusen durchgeführt, die mit HF-Diäten gefüttert wurden, die mit Milchfett oder C17:0 sowie mit Milchprotein oder einzelnen BCAA (Val; Leu) supplementiert wurden, um deren Einfluss auf die OCFA und die metabolische Gesundheit zu untersuchen. Kurzzeitstudien zeigten, dass beide Milchfraktionen OCFA induzieren, wobei die Erhöhungen durch Milchprotein teilweise durch die Val-Aufnahme erklärt werden konnten. Studien mit primären Hepatozyten zeigten außerdem eine Induktion von OCFA nach Val-Behandlung durch *de-novo*-Lipogenese und eine erhöhte α-Oxidation. In den Langzeitstudien erhöhten Milchfett und Milchprotein die hepatischen und zirkulierenden OCFA-Spiegel; allerdings hatte nur Milchprotein eine schützende Wirkung auf Adipositas und hepatische Fettansammlung, wahrscheinlich vermittelt durch eine erhöhte Leu-Aufnahme. Im Gegensatz dazu hatte die Val-Supplementierung keinen Einfluss auf die OCFA-Spiegel oder die Entwicklung von Adipositas, führte jedoch zu einer durch Glukotoxizität induzierten Insulinresistenz im Skelettmuskel, vermittelt durch den Val-Metaboliten 3-Hydroxyisobutyrat (3-HIB). Die C17:0-Supplementierung schließlich hatte keine Auswirkungen auf die HF-Diät-induzierte Adipositas und assoziierten Gesundheitsbeeinträchtigungen.

Die hier vorgestellten Ergebnisse zeigen, dass die mit dem Verzehr von Milchprodukten verbundenen positiven gesundheitlichen Auswirkungen wahrscheinlich durch die Wirkung von Milchprotein vermittelt werden, während der OCFA-Gehalt wahrscheinlich nur eine Assoziation darstellt und keine signifikante kausale Rolle für die metabolische Gesundheit unter HF-Bedingungen spielt. Die hier aufgeklärten deutlich unterschiedlichen metabolischen Auswirkungen der beiden BCAA Leu und Val unterstreichen zudem die Bedeutung der Proteinqualität.

1 Introduction

1.1 Metabolic syndrome

At present, the incidence of the metabolic syndrome (MetS) is rapidly increasing and represents one of the major, preventable health challenges of the modern world. Multiple definitions for MetS exist; however, overall it has been defined as a pathologic condition that is characterized by abdominal obesity, insulin resistance (IR), dyslipidemia and hypertension (Eckel, Alberti et al. 2010). The co-occurrence of these conditions ultimately leads to the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). Etiological mechanisms for MetS are complex and still remain to be fully elucidated, although evidence suggests that visceral adiposity is the initial causative factor driving the pathogenesis of these conditions. Importantly, the contribution of visceral fat accumulation, as opposed to subcutaneous fat mass, is key to the etiology of MetS, due to the fact that lipolysis in the visceral fat leads to an increased supply of free fatty acids (FFA) to the liver. In the liver, high concentrations of FFA increase triglyceride (TG) synthesis and cholesterol esters, which lead to production of TG-rich very low-density lipoprotein (LDL) driving the hallmark dyslipidemia of MetS (Adiels, Olofsson et al. 2008). While not a defining feature of MetS, non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of obesity and insulin resistance (Williams 2015).

In healthy individuals in response to increasing glucose and other metabolites, post-prandially for example, insulin increases glucose uptake in muscle and liver while simultaneously inhibiting adipose tissue lipolysis and hepatic gluconeogenesis (Petersen and Shulman 2018). Chronic energy excess promotes hyperinsulinemia, which in turn leads to decreased insulin sensitivity (i.e. insulin resistance). Aberrant lipolysis occurs when insulin resistance develops in fat tissue and the increased dyslipidemia plays a role in hypertension onset in part due to the decreased action of insulin in vasodilation and FFA-induced vasoconstriction—a consequence of oxidative stress (Tripathy, Mohanty et al. 2003).

Overall, these increases in abdominal adiposity and subsequent sequelae are generally due to a positive energy balance, where energy intake exceeds that of energy consumption. This is a manifestation of certain modifiable lifestyle factors including an excess caloric intake and inactivity, as well as certain non-modifiable predispositions,

including specific genetic and epigenetic influences (**Figure 1**) (for review see (Fahed, Aoun et al. 2022)).

Easily modifiable lifestyle factors are good targets for treatment of MetS including certain nutritional interventions in order to also prevent associated comorbidities. As global diets are shifting from high-fiber low-fat towards low-fiber high-fat (HF) diets (Ogden, Yanovski et al. 2007), fat intake has been suggested as a major contributor to development of adiposity. This has likely been suggested due to the fact that fat is an energy-dense macronutrient (37.3 kJ per gram) when compared to carbohydrates or protein (16.7 kJ per gram); however, evidence suggests that high-fat diets alone are not sufficient to explain the rising rates of obesity (Jéquier 2002). Thus, understanding the interplay of macronutrients, as well as certain micronutrients, is crucial in determining preventative and therapeutic concepts for obesity.

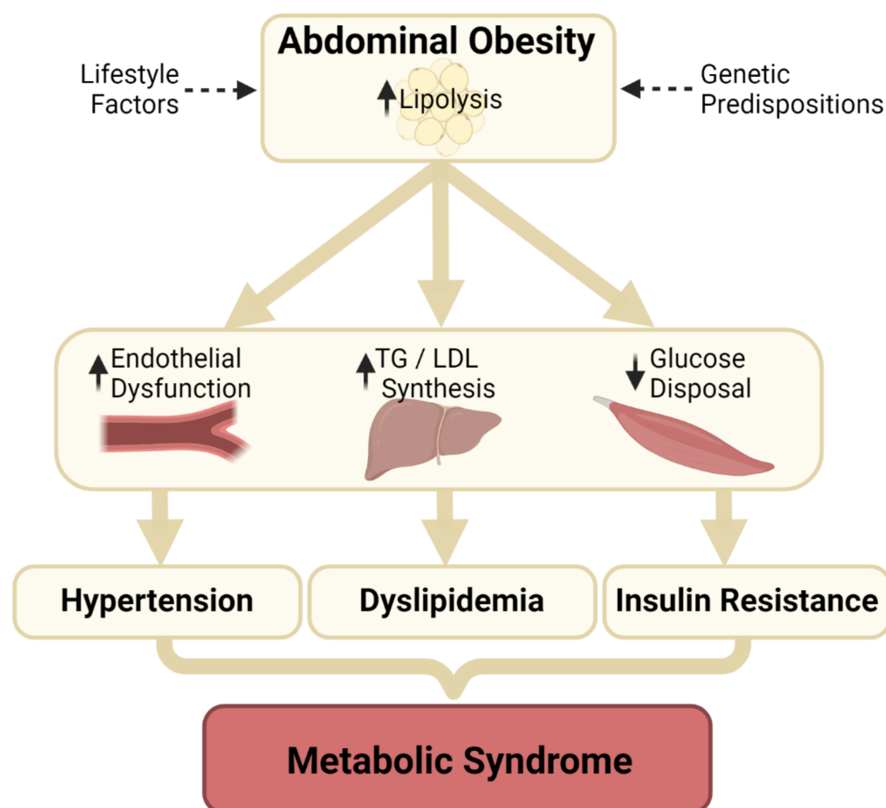


Figure 1. Pathogenesis of Metabolic Syndrome. Certain modifiable lifestyle factors and genetic predispositions can drive abdominal obesity, which results in an increased lipolysis and release of free fatty acids (FFA). Increased circulating levels of FFAs lead to impairments in endothelial function, feed into triglyceride (TG) and low density lipoprotein (LDL) synthesis in the liver and impact glucose disposal in skeletal muscle. These metabolic disturbances result in hypertension, dyslipidemia and insulin resistance, all defining factors of the metabolic syndrome. Figure created with BioRender.com.

1.2 Odd-chain fatty acids & metabolic health

While saturated fatty acids (SFA) as a whole have typically been thought to drive certain unfavorable health risks and a reduction of intake has been suggested (Eyre, Kahn et al. 2004), recent studies have illuminated that not all SFA are alike. As even chain fatty acids (ECFA) represent >99% of total SFA (Hodson, Skeaff et al. 2008), research into odd-chain fatty acids (OCFA) was relatively limited. However, in human tissue there are detectable amounts of four OCFA, including: pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1) and tricosanoic acid (C23:0) (Phillips and Dodge 1967, Coker, de Klerk et al. 1996). As of late, C15:0 and C17:0 have gained interest with regards to human health, initially as biomarkers of milk fat (Vlaeminck, Fievez et al. 2006, Matthan, Ooi et al. 2014), though more recently, prospective studies demonstrated that, while ECFA were associated with increased CVD risk, the OCFA showed significant inverse associations with disease risk (Khaw, Friesen et al. 2012). Despite being previously thought to have little biological relevance, (Mead and Levis 1963) as these FA only represent roughly 0.5% and 1 % (C15:0 and C17:0, respectively) of total long chain FA, these initial studies furthered the interest in OCFA and metabolic health. It was later demonstrated that phospholipid (PL) OCFA levels in humans are inversely associated with MetS risk (Yang, Chen et al. 2018), as well as certain aspects or consequences of MetS, such as adiposity (Aglago, Biessy et al. 2017), insulin resistance and T2DM (Kröger, Zietemann et al. 2010, Forouhi, Koulman et al. 2014), and NAFLD (Chowdhury, Steur et al. 2016, Yoo, Gjuka et al. 2017, Sawh, Wallace et al. 2021). Together these studies highlighted a possible role for OCFA in metabolic health, and that SFA biological relevance is not homogenous.

1.2.1 Proposed sources of OCFA

Previously, it was hypothesized that OCFA were not endogenously produced, but rather derived from the diet. This reasoning was based on the fact that OCFA are abundant in dairy fat, where these FA are derived from rumen microbial fermentation of fiber and subsequent *de novo* lipogenesis (Vlaeminck, Fievez et al. 2006). OCFA are formed by rumen bacteria (Laliotis, Bizelis et al. 2010) and are then taken up by the animal and utilized by the mammary gland in milk fat production (Stefanov, Baeten et al. 2013). Data from the European Prospective Investigation into Cancer and Nutrition Study (EPIC)-InterAct study (Langenberg, Sharp et al. 2011) demonstrated that while ECFA were negatively associated with fruits and vegetables, OCFA were positively associated with intake of dairy products (i.e. butter, cheese, milk, yoghurt, etc.) as well as fruits,

vegetables, nuts and seeds (Forouhi, Koulman et al. 2014), indicating that specific foods have a direct correlation between intake and OCFA levels. Dietary fibers and dairy products are among the strongest associations, suggesting that both contribute to OCFA levels *in vivo*.

Despite previously being suggested merely as a biomarker for dairy fat intake (Wolk, Furuheim et al. 2001), there is an apparent inconsistency between the levels found in milk fat versus those found in circulation. Milk fat typically has a ratio of C15:0 to C17:0 that is roughly 2 to 1 (Vlaeminck, Fievez et al. 2006, Dewhurst, Moorby et al. 2007), whereas the plasma levels in humans are approximately 1 to 2 (Quehenberger, Armando et al. 2010), stressing that milk fat is likely not the only source of OCFA. Recently, it has been explored that dietary fiber intake can increase OCFA levels in both mice and humans (Weitkunat, Schumann et al. 2017, Weitkunat, Stuhlmann et al. 2017). These studies have shown the role of short-chain fatty acids (SCFA), primarily propionate, in producing OCFA through direct *de novo* lipogenesis in the liver. The involvement of bacterial composition on hepatic OCFA formation in mice is, however, rather inconsistent and remains to be fully clarified (Jenkins, Seyssel et al. 2017, Weitkunat, Bishop et al. 2021). Meanwhile, others have revealed the involvement of the process of α-oxidation, the single carbon removal from fatty acids, in C17:0 production alone (Jenkins, de Schryver et al. 2017). Together, these recent studies suggest that, while milk fat intake does influence OCFA levels, there are certain endogenous synthetic pathways for OCFA *in vivo*.

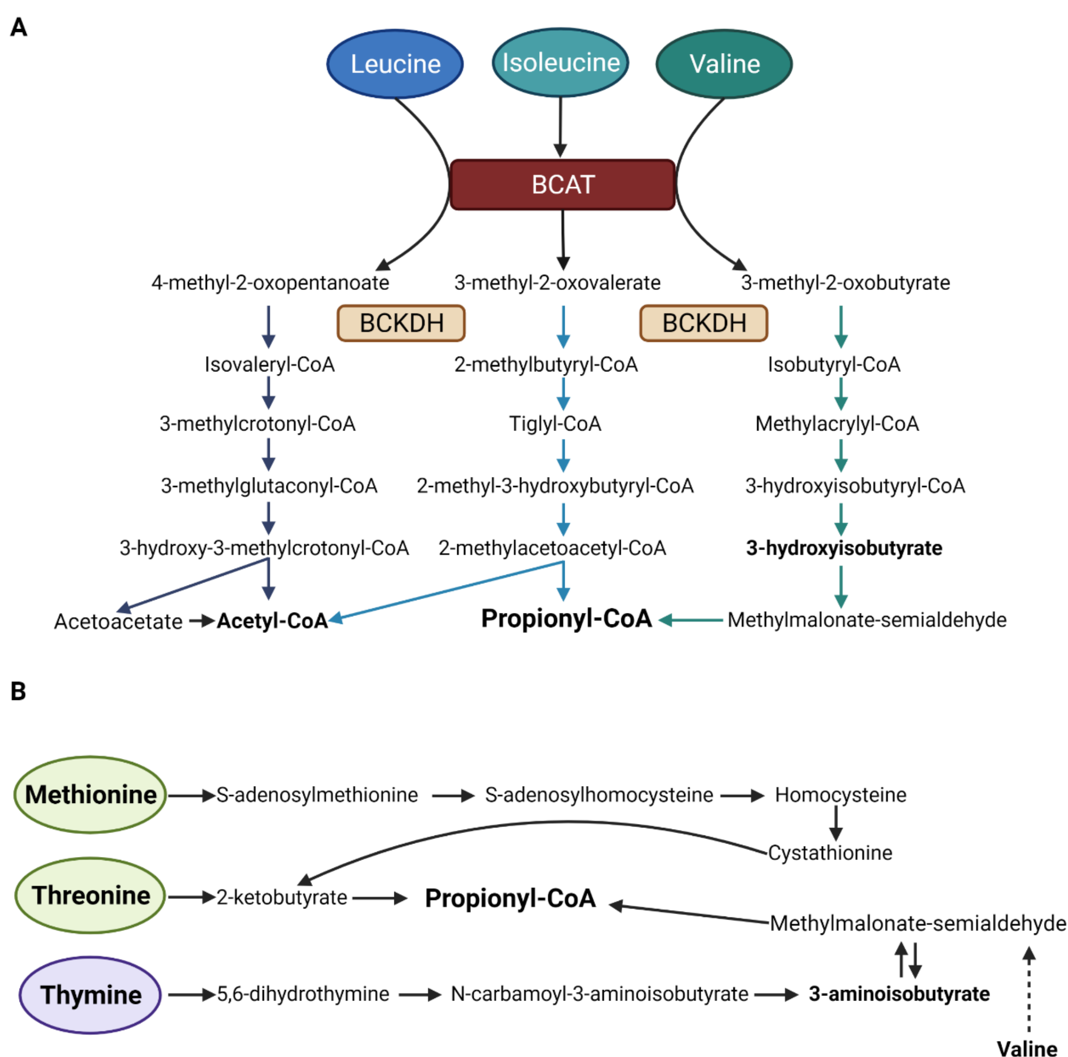


Figure 2. Amino Acid Catabolic Pathway. (A) Branched-chain amino acid catabolic pathway feeds into propionyl-CoA (Pr-CoA) pool via isoleucine (Ile) and Val (Val), while leucine (Leu) and Ile feed into acetyl-CoA (Ac-CoA) pools. **(B)** Additional metabolic pathways involving the degradation of methionine, threonine and thymine feed into the formation of Pr-CoA as well. BCAT, branched-chain amino acid aminotransferase; BCKDH, branched-chain keto acid dehydrogenase. Figure created with BioRender.com.

Given that propionyl-CoA (Pr-CoA) levels are modified by the catabolism of multiple different metabolites, the endogenous production of OCFA is likely occurring through the production of propionate via fermentation of dietary fibers and further influenced by other pathways as well. Studies looking into disturbed Pr-CoA metabolism showed that in-born errors in catabolism result in increased OCFA levels (Wendel, Baumgartner et al. 1991, Coker, de Klerk et al. 1996, Sperl, Murr et al. 2000). These deficiencies present a strong phenotype with metabolic dysfunction (Schwab, Sauer et al. 2006, Gallego-Villar, Rivera-Barahona et al. 2016) that can be reasonably well treated with certain dietary restrictions. These dietary restrictions include decreased intake of certain amino acids, including the branched-chain amino acids (BCAA) valine (Val) and isoleucine (Ile), as well as the amino acids methionine (Met) and threonine (Thr)—catabolism of these amino acids

feed into Pr-CoA pathway (**Figure 2**). The production of OCFA from the BCAA has been shown once before in an adipocyte cell culture model (Green, Wallace et al. 2016); however, it has yet to be explored in detail the influence of dietary proteins on OCFA levels *in vivo*.

1.3 Dairy intake

Dietary recommendations generally suggest a reduced dairy intake (i.e. butter, milk, etc.) because its high SFA content could theoretically drive cholesterol synthesis, leading towards an increased dyslipidemia. However, epidemiological studies demonstrated an inverse association of milk intake with MetS. These studies showed that between 1 and 3 servings of dairy products per day were generally associated with a lower prevalence of MetS and related comorbidities compared to the lowest dairy intake (van Meijl, Vrolix et al. 2008). A cross-sectional study by Beydoun *et al* further revealed that these inverse associations varied dependent on the type of dairy intake (Beydoun, Gary et al. 2008). For example, cheese consumption positively correlated with prevalence of MetS, while yoghurt intake resulted in a negative association. Several studies found no associations between dairy consumption and MetS; while others found no significant associations after differentiating between low-fat and high-fat dairy products. Overall, the driving factors for metabolic health with regards to dairy intake are yet to be fully defined, the input from milk fat versus milk protein requires further research, as well as the influence of the many micronutrients present in dairy, such as calcium (for review see (van Meijl, Vrolix et al. 2008, Jakobsen, Trolle et al. 2021)).

1.3.1 Dairy fat intake

To date, a western diet contains approximately 90g of fat per day, of which a substantial portion is derived from dairy fat (Heuer, Krems et al. 2015). The role of dairy fat in metabolic health is, however, rather controversial. As stated before, dairy fat is rich in SFA, especially palmitic (C16:0) and stearic (C18:0) acids (German and Dillard 2006) which account for approximately 65% of total FA. While C18:0 has little effect on serum lipid profiles, C16:0 is known to increase LDL-cholesterol concentrations (Weinberg, Berner et al. 2004, de Oliveira Otto, Mozaffarian et al. 2012). The so called “diet-heart hypothesis” suggested that diets high in SFA drive CVD (Page, Allen et al. 1961) and has since influenced the dietary guidelines. Mounting evidence indicates that this hypothesis is rather an oversimplification, and that the impact of SFA is very dependent on food source and matrix. Several studies have shown a positive impact of habitual dairy product consumption on metabolic disease risk—even demonstrating that high-fat

dairy intake may have protective properties in attenuating MetS in adults (for review see (Unger, Torres-Gonzalez et al. 2019)). This review suggests that data from cross-sectional studies describe either a protective or neutral effect of dairy-derived SFA in development of MetS and related disorders. However, these studies generally are limited mechanistically, and individual nutrient studies are required to fully understand the input from dairy fat. Of the SFA present in dairy fat, specific ones have been shown to elicit beneficial effects in rodent feeding studies. Butyrate (C4:0) supplementation for example can induce positive effects with regards to adiposity and glucose tolerance (Unger, Torres-Gonzalez et al. 2019). Mechanistic studies for the influence of OCFA are limited; however, it remains a possibility that these elicit beneficial effects with regards to metabolic health and energy metabolism.

1.3.2 Dairy protein intake

Apart from milk fat, multiple components of milk have been posited as the drivers of the beneficial associations attributed to dairy intake. These components include the protein fraction of milk, namely casein and whey (Rice, Cifelli et al. 2011). Dairy protein, predominantly derived from cow's milk, is composed of approximately 80% casein and 20% whey proteins, which have been both classified as high-quality proteins, based on human amino acid requirements (McGregor and Poppitt 2013). Currently, there is accumulating evidence that dietary protein plays an important role in the prevention of MetS development, as several human and animal studies have revealed that dietary protein is able to reduce disease incidence (Paddon-Jones, Westman et al. 2008, Layman, Evans et al. 2009, Westerterp-Plantenga, Nieuwenhuizen et al. 2009). The positive effects attributed to protein intake are explained via multiple mechanisms.

First, protein has generally been shown to increase satiety far greater than carbohydrates or fat, facilitating a reduced energy consumption (Paddon-Jones, Westman et al. 2008). High-protein intake has been shown to reduce food intake (Westerterp-Plantenga, Nieuwenhuizen et al. 2009) by increasing satiety which is apparently mediated through multiple pathways. Some have suggested the action of protein in inducing secretion of anorexigenic gut hormones (i.e. cholecystokinin (CCK) or glucagon-like peptide 1 (GLP-1)) (Blom, Lluch et al. 2006, Bowen, Noakes et al. 2006) or a decrease in ghrelin, an orexigenic hormone (Lejeune, Westerterp et al. 2006, Tannous dit El Khoury, Obeid et al. 2006), however this was not always found to be the case (Moran, Luscombe-Marsh et al. 2005). Another anorexigenic hormone, peptide YY (PYY), has also been suggested to play a role in the satiating effect of increased protein intake (Batterham, Heffron et al. 2006). Moreover, amino acids themselves may be

acting directly to regulate food intake, as several studies have shown the importance of circulating amino acids on satiety signals via the hypothalamus (Cota, Proulx et al. 2006).

Certain studies have also acknowledged that high-protein diets can increase energy expenditure through both increased diet-induced thermogenesis and non-shivering thermogenesis (Madsen, Myrmel et al. 2018). The first is essentially the thermic effect of food, referring to the energy required for digestion, absorption and disposal of nutrients (Halton and Hu 2004). However, heat may be produced as well by non-shivering thermogenesis via the induction of uncoupling in the mitochondria by uncoupling proteins (UCP). High-protein diets have been shown in some cases, but not all, to induce UCP1 expression in brown adipose tissue, which may lead to elevated energy expenditure (Madsen, Myrmel et al. 2018). Together, these represent possible mechanisms by which dietary protein may be driving the positive effects which have been described in multiple mouse studies, especially under HF conditions (Shertzer, Woods et al. 2011, Freudenberg, Petzke et al. 2012, Freudenberg, Petzke et al. 2013). Notably, the studies have highlighted the importance of protein source. Data on dairy protein supplementations have generally used whey as opposed to casein. (Bortolotti, Maiolo et al. 2011, Freudenberg, Petzke et al. 2012, Ahmadi-Kani Golzar, Fathi et al. 2019), though casein represents roughly 80% of the protein found in milk—thus it is important to understand the source and matrix in which the protein is ingested. Moreover, while high-protein diets may be important in body-weight management, data is lacking to fully clarify the contribution to T2DM, NAFLD and CVD.

While intervention studies have demonstrated a positive anabolic effect of high protein intake, epidemiological studies show an association of low protein with improved metabolic health—this has been coined the protein paradox (Klaus, Pfeiffer et al. 2018). Certain studies demonstrated that reduced protein intake, also in the form of altered protein to carbohydrate ratios, played an important role in metabolic health and longevity (for review see (Kitada, Ogura et al. 2019)). Solon-Biet et al. highlighted that protein and carbohydrates primarily regulate food intake and that this ratio is key in regulating metabolic health and longevity, not the caloric intake (Solon-Biet, McMahon et al. 2014). Further studies suggested that metabolic improvements seen after protein restriction could in fact be due to restriction of the BCAA (Fontana, Cummings et al. 2016, Solon-Biet, Cogger et al. 2019). Interestingly, whey protein contains a higher amount of total BCAA compared to casein; however, the ratios of the BCAA differ between the two, with casein having a higher amount of valine (Hall, Millward et al. 2003).

1.3.2.1 Branched chain amino acids (BCAA) in metabolic health

In line with this previously described protein paradox, the BCAA appear to have a rather conflicting role in metabolic health—both detrimental and positive effects have been described, though this is heavily dependent on the context. The BCAA are essential amino acids that comprise approximately 15-25% of total protein intake (Tournissac, Vandal et al. 2018) and clearly play an important role in health and disease.

In humans, increased circulating BCAA have been described to be a hallmark of disease in obese and T2DM patients (Chevalier, Marliss et al. 2005, Um, D'Alessio et al. 2006, Tremblay, Lavigne et al. 2007, Newgard, An et al. 2009) which has been deemed the “BCAA signature”. Furthermore, impaired BCAA metabolism has been linked to a causal role in insulin resistance development (Lotta, Scott et al. 2016). Similarly, genetic mouse models of obesity display this signature as well, where BCAA catabolism is highly impacted by obesity (Zhou, Shao et al. 2019). Studies supplementing increasing doses of BCAA on a normal fat background could demonstrate a negative impact on body weight development, due in part to increased energy intake, as well as a shortened lifespan in a mouse feeding study (Solon-Biet, Cogger et al. 2019). Further research has also revealed that the BCAA may be a driving force in IR, specifically under HF conditions. When supplemented into a HF diet, BCAA appeared to drive IR, while having either no impact on body weight or fat mass (Newgard, An et al. 2009) or a protective effect against HF-induced weight gain—primarily due to aberrant lipolysis (Zhang, Zhao et al. 2016). Restriction of the individual BCAA appear to protect mice from metabolic impairments (Xiao, Huang et al. 2011, Xiao, Yu et al. 2014, White, Lapworth et al. 2016, McGarrah, Zhang et al. 2020) and activation of the BCAA catabolic pathway elicits a similar effect (Zhou, Shao et al. 2019). The suggested mechanism for BCAA-induced IR generally involves increased circulating levels of BCAA via impaired adipose and liver catabolic capacity finally resulting in skeletal muscle lipotoxicity (White and Newgard 2019). Though, whether the increased BCAA levels in humans are a consequence or cause for IR still remains inconclusive, as insulin signaling is crucial for BCAA catabolism.

As discussed before, however, high protein diets are key in promoting weight loss in animals and humans (Halton and Hu 2004, Westerterp-Plantenga, Nieuwenhuizen et al. 2009). These beneficial effects seem to be clear under a HF context, where protein supplementations elicit an anti-obesogenic effect. It has been posited that the beneficial effects of a higher protein intake are mediated, at least in part by leucine (Leu) (Zhang,

Guo et al. 2007). This was demonstrated when it was supplemented in HF diets; Leu supplementation appeared to protect mice from diet-induced obesity, while also protecting from HF-induced impairments in glucose and insulin homeostasis (Freudenberg, Petzke et al. 2012, Brunetta, de Camargo et al. 2018). These studies demonstrated that Leu led to increases in energy expenditure and reductions in food intake, despite leading to increased levels of Leu in the circulation. Leu was suggested to elicit these effects through several mechanisms. On one hand, Leu has been shown to be a strong activator of mammalian target of rapamycin (mTOR), a key kinase that regulates cell proliferation, protein synthesis, autophagy and transcription (Szwed, Kim et al. 2021). While in tissues like the liver, mTOR signaling has been suggested to play a part in IR development (Newgard, An et al. 2009), hypothalamic mTOR activation by Leu was proposed as a possible explanation for the regulation of food intake (Cota, Proulx et al. 2006). Freudenberg *et al*. highlighted the role of Leu in protecting mice from ectopic lipid accumulation in the liver, which occurred through both the down regulation of FA transporters as well as key genes of *de novo* lipogenesis. Recent studies have also demonstrated a similar role for Ile in the prevention of HF diet induced obesity (Ma, Zhou et al. 2020).

Taken together, these contrasting data demonstrate that further research is required into the function of BCAA in obesity and IR development. While studies have previously focused on the role of Leu, with some studies also including Ile, the contribution of Val to metabolic health is rather limited, especially in a HF context. Val deprivation from the diet does have a beneficial effect (Yu, Richardson et al. 2021) and one study demonstrated that the Val-derived catabolite 3-hydroxyisobutyrate (3-HIB) is a major player in the BCAA-induced IR (Jang, Oh et al. 2016). This metabolite is also a strong marker for IR in humans (Nilsen, Jersin et al. 2020), however, understanding the molecular mechanisms of an increased Val intake on disease is key in fleshing out these rather conflicting data.

2 Aim of study

Epidemiological studies, as of late, have demonstrated a strong correlation between OCFA and beneficial health outcomes, however, direct sources and their relationship to these metabolic health outcomes are poorly understood. While the metabolic effects of dietary fiber and their digestion products (SCFA) have been described in relation to OCFA, the role of dairy intake (both dairy protein and dairy fat) and its relation to OCFA is rather lacking in description. Milk intake has also been demonstrated to have an inverse association with poor metabolic health outcomes. Thus, the primary objectives of this PhD project were to determine the impact of dairy fat and dairy protein intake on OCFA levels and the long-term effects of these supplementations in diet-induced obesity. Initial short-term feeding studies were performed to determine the impact of milk fat versus milk protein on OCFA formation using male C57BL/6JRj mice. The influence of Val supplementation on OCFA levels was then also tested, with further molecular characterization of its influence on OCFA levels *in vitro*.

To address the long-term metabolic effects of milk fat and milk protein, in-depth phenotyping of mice fed HF diets with these supplementations was performed, including indirect calorimetry and glucose tolerance assessments. These studies were also established as a means to compare the long-term effects of individual BCAA in metabolic health, primarily Leu and Val, to further elucidate their roles and address the contradicting data found in literature.

Furthermore, this project sought to clarify the specific metabolic effects of the OCFA, more specifically heptadecanoic acid (C17:0) by analyzing the influence of C17:0 supplementation on whole-body energy metabolism and specific molecular pathways.

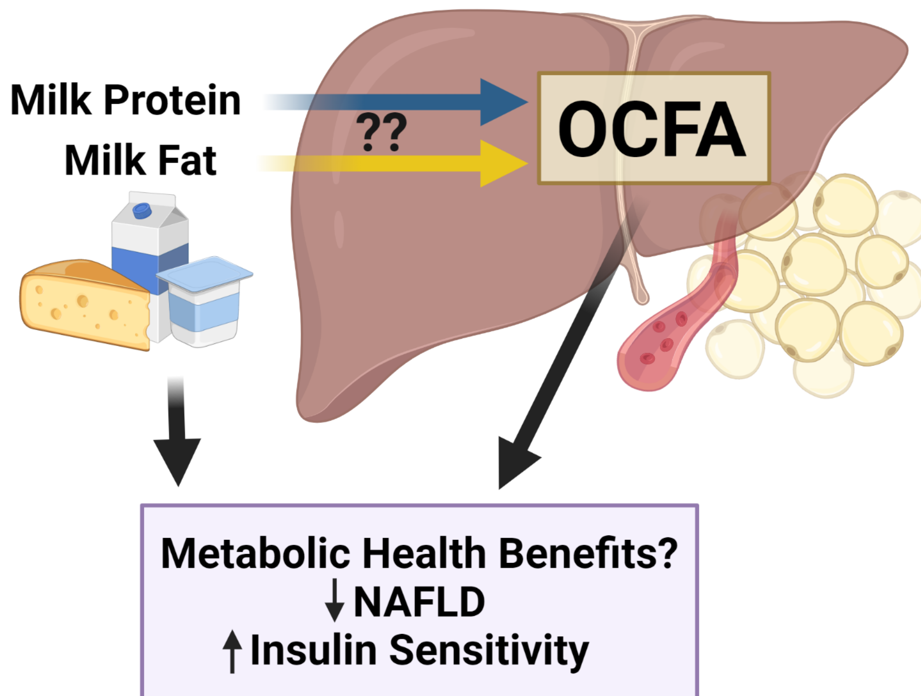


Figure 3. Aims of the study. Short- and long-term feeding studies will be utilized to address the impact of milk fat and milk protein supplementation on HF-diet induced metabolic perturbations and odd-chain fatty acid (OCFA) levels. This also includes the characterization of heptadecanoic acid (C17:0) supplementation to delineate OCFA specific roles. Figure created with BioRender.com. NAFLD, nonalcoholic fatty liver disease.

3 Methods

3.1 Animal maintenance

C57BL/6JRj male mice were purchased from Janvier Labs (Janvier, France), maintained on 12 hr light/dark cycle, and group-housed (2-3 mice per cage) at 23°C. At 12 weeks of age mice were given experimental diets for 1, 4 or 20 weeks (Study I, III, and II, respectively) (Section 2.1.1 – Study Design) with *ad libitum* access to food and water. Control low- and high-fat diets were used along with experimental high-fat diets with indicated supplementations (**Table 1**), with 40% energy from fat.

Table 1. Composition of experimental high-fat diets.

Composition of semisynthetic experimental low-fat (LF) and 40 Energy% high-fat (HF) diets supplemented with milk fat (HFMF), C17:0 (HFC17), milk protein (HFMP), leucine (HFL) or valine (HFV) produced by ssniff Spezialdiäten GmbH. [*] Calcium Caseinate 380 was provided by NZMP. [†]Anhydrous milk fat was provided by Uelzena. [#]Measured by bomb calorimetry.

Components	LF (g/kg)	HF (g/kg)	HFMF (g/kg)	HFC17 (g/kg)	HFMP (g/kg)	HFL (g/kg)	HFV (g/kg)
Casein[*]	140	140	140	140	280	140	140
Wheat starch	466.5	299.5	299.5	299.5	159.5	249.5	249.5
Maltodextrin	100	100	100	100	100	100	100
Dextrose	50	50	50	50	50	50	50
Sucrose	100	100	100	100	100	100	100
Lipids	43	210	70	160	210	210	210
Cellulose	50	50	50	50	50	50	50
Milk Fat[†]	-	-	140	-	-	-	-
C17:0	-	-	-	50	-	-	-
L-Leucine	-	-	-	-	-	50	-
L-Valine	-	-	-	-	-	-	50
Mineral mixture	35	35	35	35	35	35	35
Vitamin mixture	10	10	10	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5
L-Cysteine	3	3	3	3	3	3	3
Energy content (kJ/g)[#]	16.5	19.4	19.9	19.5	19.9	20.0	19.8

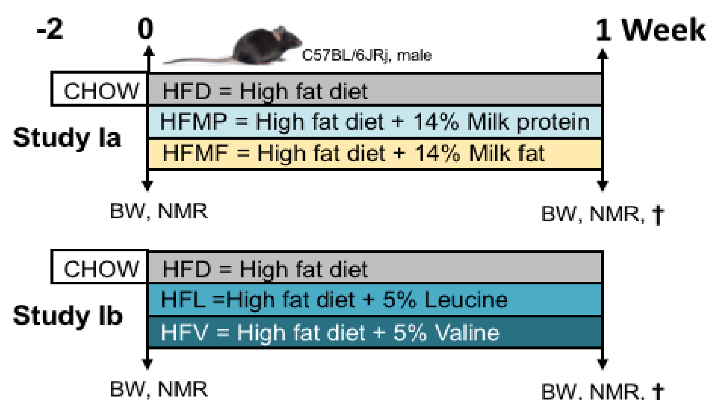
3.1.1 Study design

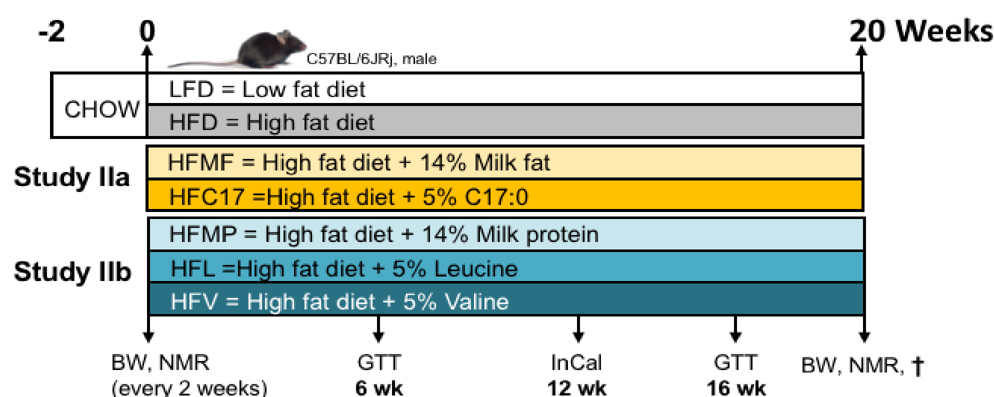
Graphical descriptions of the studies are shown in **Figure 4**, while all diets used are described in **Table 1**. At the end of the interventions, 2 hr fasted animals were euthanized by cervical dislocation at indicated times. Following blood collection, tissues were isolated, weighed and snap frozen in liquid nitrogen. For plasma collection, blood was transferred to heparin-coated tubes until centrifugation at 9000 xg for 10 min. All experiments were approved by the ethics committee of the Ministry for Environment, Health, and Consumer Protection of Brandenburg, Germany (approval no. 2347-17-2018).

As the liver is thought to be the main tissue for endogenous OCFA synthesis, Study Ia and Ib were performed first to determine which supplementations induce these FA, either milk fat or milk protein or individually supplemented BCAA. Mice were fed for 1 week with body weight and composition measurements performed at the beginning and end of the intervention (see Section 2.1.2 Metabolic Characterization).

Study IIa and IIb were performed to metabolically characterize the long-term health effects of these various supplementations. Mice were fed for 20 weeks with detailed phenotyping including indirect calorimetry measurements (Section 2.1.2 – performed at week 12) and assessment of glucose / insulin tolerance (see Section 2.1.3 Tolerance Tests – performed at week 6 / 16)—body weight and composition were taken bi-weekly throughout the intervention time.

Study III was performed to directly address insulin sensitivity of tissues after milk protein or individual BCAA supplementations, based on the findings of Study IIb. Following 4 weeks of intervention, mice were fasted for 2 hr and subsequently received an *i.p.* injection of insulin (0.75U/kg body weight). After 30 min, mice were euthanized by cervical dislocation and tissues collected as described above.





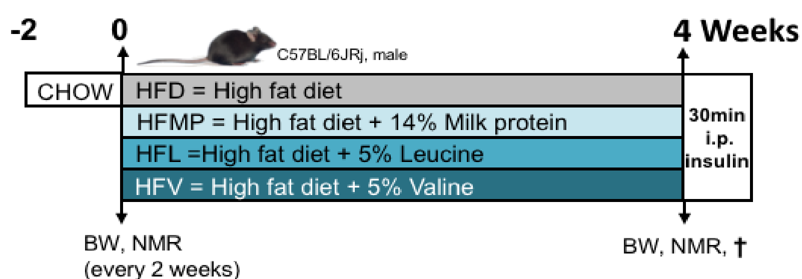


Figure 4. Study Designs. Male C57BL/7JRj mice were fed for various time points with indicated diets with subsequent phenotypic and molecular characterizations, as described in text. BW, body weight; GTT, glucose tolerance test; InCal, indirect calorimetry; NMR, nuclear magnetic resonance.

3.1.2 Metabolic characterization

Body weight and composition were taken bi-weekly, with the exception of Study Ia and Ib. Fat mass was measured using nuclear magnetic resonance spectrometer EchoMRI^{TM}-Analyzer (Echo Medical Systems, USA) and lean mass calculated as difference between fat mass and body weight (measured on a scale).

Indirect calorimetry measurements were measured in week 12 of Study II using the PhenoMaster System (TSE Systems GmbH, Germany). Mice were acclimatized to individual housing and the cages of the climate-controlled cabinet for 3 days prior to 24 hr indirect calorimetry, locomotor activity and energy expenditure measurements. Energy intake was calculated by multiplying food intake with the energy content of diets determined from bomb calorimetry, as previously described (Weitkunat, Schumann et al. 2015).

3.1.3 Tolerance tests

Oral glucose tolerance tests (oGTT) were performed in week 6 and 16 of Study II with a 20% glucose solution (2 g/kg body weight) after a 16 hr fast. Blood glucose and plasma insulin were measured using a Contour XT blood glucose meter (Bayer AG, Germany) and mouse ultrasensitive insulin ELISA (80-INSMSU-E01, ALPCO) at indicated time points, respectively. i.p. insulin tolerance test (ITT) was performed after 2 hr fasting at week 16 (0.75 U/kg body weight) with blood glucose measurements throughout.

3.2 Cell culture

Cells were maintained at 37°C and 5% CO_2 with respective cell culture media. Treatments used for *in vitro* studies are listed in **Table 2**.

Table 2. Cell Culture Reagents.

Reagents	Company	Ref. Number
(±)-Sodium β-hydroxyisobutyrate	Sigma	36105
BAY 876	R&D	6199
Bovine Serum Albumin (Fatty acid free)	Sigma	A6003
GW6471	Cayman Chemicals	11697
Heptadecanoic Acid (C17:0)	Sigma	H3500
Lipofectamine 2000 Transfection Reagent	ThermoFischer	11668027
L-Leucine	Sigma	L8912
L-Valine	Sigma	V0500
Palmitic Acid	Sigma	P0500
siGENOME Hacl1 siRNA	Dharmacon	D-001210-04-05
siGENOME NTC siRNA	Dharmacon	M-060754-00-0005
Wy-14643	Cayman Chemicals	70730

3.2.1 Primary hepatocyte culture

Primary hepatocytes were derived from livers of 10-14 weeks old C57BL/6JRj, male mice following collagenase digestion. Mice were first euthanized with isoflurane, and cannula was inserted into the *vena cava.* Perfusion buffer I (1X PBS (pH 7.4), 25 mM Glucose, 10 mM HEPES, 0.2 mM EDTA, 0.05% (w/v) KCl, 0.001% (v/v) Phenol red) was perfused for 5 min at 5 ml/min rate, before 5 min perfusion with perfusion buffer II (1X PBS (pH 7.4), 25 mM Glucose, 30 mM HEPES, 1 mM $CaCl_2$, 0.05% (w/v) KCl, 0.001% (v/v) Phenol red, 0.5 mg/mL Collagenase Type IV (C5138, Sigma)). Liver was then extracted and dissociated in perfusion buffer II, with subsequent filtration through a cell sieve (70 μm) (352350, Corning) and centrifugation at 50 xg for 3 min. This was repeated until supernatant appeared visually clear, resuspension of cell pellet was performed with DMEM after each centrifugation step. After the final centrifugation, tissue pellet was resuspended in DMEM (P04-03596, PAN Biotech) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (P30-3306, PAN Biotech), 50 mg/mL penicillin, 100 mg/ml streptomycin (P06-07100, PAN Biotech), 0.5 nM insulin, and 100 nM dexamethasone for plating. Hepatocytes were plated in 6-well plates pre-coated with rat tail collagen I (356400, Corning), at a density of 200,000 cells/mL. After 4 hr, media was changed to serum starvation media-DMEM containing 4.5 g/L glucose, 50 mg/mL penicillin, 100 mg/ml streptomycin, 0.5 nM insulin, 1 μM vitamin B12 and 100 nM dexamethasone until additional treatments were performed 16 hr later as further

described in figure legends. Insulin stimulation following specified treatments was performed 2 hr after media was changed, and insulin was pipetted into wells to a final concentration of 100 nM for 15 min.

3.2.2 C2C12 Myotube Culture

C2C12 cells were cultured in DMEM (P04-03596, PAN Biotech) with 10% FBS and 1% penicillin-streptomycin as described (Ost, Igual Gil et al. 2020). At 90% confluency, differentiation of cells was induced for 7 days with DMEM containing 3% horse serum (P30-0700, PAN Biotech). Cells were treated for indicated times for protein or gene expression analysis—insulin stimulation was performed as above. Glucose uptake was measured using the Glucose Uptake-Glo Assay kit (J1341, Promega), according to manufacturers' protocol.

3.3 Analytic procedures

3.3.1 LCFA measurements

Long-chain fatty acid (LCFA) composition was determined in liver and plasma phospholipid (PL) fractions by gas chromatography (GC) in collaboration with Andreas Wernitz (Department Molecular Epidemiology, German Institute of Human Nutrition, Germany) using 25 μl of plasma or 20 mg of tissue which was weighed and transferred to Teflon-coated vials, as previously described in detail (Weitkunat, Schumann et al. 2015). Total lipid extraction was performed with methyl tert-butyl ether (MTBE)/ methanol before subsequent solid-phase separation, hydrolysis and methylation with trimethyl sulfonium hydroxide. Samples were then measured with GC and analyzed. All PL LCFA data are represented as area percent of single fatty acids to total area of detected fatty acids, of which there were a total of 22 different fatty acids ranging from C14:0 to C22:0.

Analysis of PL fractions from primary hepatocytes were performed after 48 hr of treatment. Briefly, 600,000 cells were scraped in 1X PBS, centrifuged (85 xg at 4°C) for 5 min and supernatant was removed. Cells were then resuspended in 1 mL of deionized water and transferred to 16 mm × 100 mm Teflon-coated vials. Before storage, cells were overlaid with N_2 gas.

3.3.2 Plasma measurements

Circulating BCAA concentrations were determined in collaboration with Thorsten Henning (Department Molecular Toxicology, German Institute of Human Nutrition, Germany) with LC-MS/MS as described with some modifications (Prinsen, Schiebergen-

Bronkhorst et al. 2016). Briefly, 10 µL plasma samples were mixed with 40 µL of an internal standard solution (62.5 µM of isoleucine-$^{13}C_6$,^{15}N, leucine-$^{13}C_6$,^{15}N and valine-$^{13}C_5$ in 90% acetonitrile). Protein was precipitated by storing samples at -20°C for 10 min and subsequent centrifugation for 10 min at 17000 xg, 4°C. 2 µl of sample supernatant was then injected into the LC-MS/MS system.

Plasma levels of 3-HIB, beta-hydroxybutyrate (βHB) and acetoacetate (AcAc) were measured by GC-MS/MS after methylchloroformate derivatization at Bevital AS (Bergen, Norway; http://www.bevital.no) as described (Midttun, McCann et al. 2016). Total cholesterol levels were measured using the Cholesterol liquicolor kit (10017, Human Diagnostics).

3.3.3 Tissue measurements

3.3.3.1 Triglycerides

Measurements of tissue triglycerides (TG) levels were performed using the Triglyceride Determination Kit (TR0100, Sigma Aldrich). Samples were homogenized with 10 mM sodium hydrogen phosphate buffer (pH 7.4, 1 mM EDTA, 1% (v/v) polyoxyetylene-10-tridecyl ether) for 3 min at 50 Hz with the TissueLyser LT (Qiagen GmbH, Germany) (40 mg tissue: 800 µL buffer). After homogenization, samples were centrifuged (4°C, 23100 xg, 30 min) and supernatant was incubated at 70°C for 5 min in a thermal shaker. Heated samples were then stored on ice for 5 min before an additional centrifugation step (4°C, 23100 xg, 30 min). Supernatants (5 µL) were pipetted into a 96-well transparent plate in triplicates and mixed with 100 µL Reagent A, with subsequent incubation at 37°C for 5 min. Absorbance was measured at 540 nm with the Eon Plate Reader (Biotek Instruments, USA) to determine free glycerol. Then, 25 µL of Reagent B was added to each well and incubated at 37°C for 15 min, before an additional absorbance measurement at 540 nm to determine TAG content. TAG concentrations were determined by subtracting the absorbance of free glycerol values from the absorbance of the second reading, before determining concentrations from a glycerol standard curve (0.075-2.5 µg/µL).

3.3.3.2 Diacylglycerides

Diacylglyceride (DAG) measurements were performed using the Diacylglycerol Assay kit (ab242293, Abcam). Approximately 50 mg *M. quadriceps* were suspended in 1 mL of cold PBS before being sonicated for 5 min on ice. Subsequently, 1.5 mL methanol, 2.25 mL 1 M NaCl and 2.5 mL chloroform were all added to the sonicated sample and

vortexed. To separate the phases, samples were centrifuged at 1500 xg for 10 min (4°C). The upper aqueous phase was removed and discarded. The lower chloroform phase was washed 2 times with pre-equilibrated upper phase (PEU) (1:1:0.9 of chloroform: methanol: 1 M NaCl), where the phases were separated after each wash by centrifugation (1500 xg, 10 min, 4°C) and upper aqueous phase was discarded each time. The remaining lower phase was then dried in a Speedvac (RT , 0.7 mbar; ThermoFisher, Germany) and resuspended with 50 μL of the 1X Assay Buffer.

DAG standards were prepared according to the kit ranging from 0-2 mM DAG standard. For samples, standards and blanks, 20 μL were pipetted into a 96-well plate, and 20 μL of Kinase Mixture added to standards and one half of the paired samples (+Kin)—20 μL of 1X Assay Buffer were added to the other half of the paired samples (-Kin). The plate was then incubated for 2 hr at 37°C before 20 μL from each well was pipetted into a 96-well fluorescence black plate. Following the transfer, 40 μL of Lipase Solution was added to each well and then incubated at 37°C for 30 min. Finally, 50 μL of Detection Enzyme Mixture was added to each well, incubated at RT for 10 min and read with a fluorescence microplate reader for excitation 530-560 nm and emission 585-595 nm range. Average relative fluorescence units were determined by subtracting the -Kin values from paired +Kin values, and concentration determined from the standard curve.

3.3.3.3 Glycogen

Tissue glycogen concentrations were determined using the Starch Kit (10207748035, R-Biopharm AG). Tissues were first homogenized (skeletal muscle, 50 mg) for 90 sec at 50 Hz in 1 mL of 1 N NaOH. Homogenates were then incubated at 70°C for 45 min in a thermalshaker, then chilled on ice for 5 min before subsequent centrifugation (4°C, 12400 xg, 45 min). Supernatants were centrifuged again (4°C, 12400 xg, 30 min) and process was repeated until supernatants were clear.

For glycogen measurement, glycogen-derived glucose (A+) and free glucose (B-) were measured. Mixtures were made where 50 μL of sample was mixed with 1 μL acetic acid and either 100 μL amyloglucosidase (A+) or 100 μL DI water (B-). Mixtures were mixed and incubated at 60°C for 15 min, prior to centrifugation at RT (23100 xg, 10 min). In triplicates, 30 μL samples were mixed in 96-well plates with 100 μL DI water / 100 μL NADP/ATP and shaken at RT for 3 min. Absorbance was measured then at 340 nm, before 12 μL Hexokinase/Glucose-6-phosphate-dehydrogenase was added to each well and shaken at RT for 15 min. Subsequently, absorbance was measured again at 340

nm. The initial absorbance was subtracted from the second absorbance, and the absorbance of B- was subtracted from A+ to obtain values from the glycogen standard curve (0.01-3 μg/μL).

3.3.3.4 Histology

Histological analysis was performed on liver tissues that were fixed immediately in 4% paraformaldehyde after dissection. Liver sections were then embedded in paraffin and cut into 2 μM slices and subsequently stained with hematoxylin-eosin (H&E). Tissue slices were imaged with the Eclipse E800 microscope (Nikon GmbH, Germany) and relative fat content was calculated in cooperation with Prof. Dr. Janin Henkel (Department of Nutritional Biochemistry, University of Bayreuth). Random microphotographs of the H&E-stained liver sections were chosen containing no blood vessels (central veins or portal fields) and lipid content was determined using ImageJ software as described. The sum of histogram data in the range of 152-255 were defined for lipid droplet areas, which were then calculated relative to the overall density per field to determine relative lipid content.

3.3.4 Molecular biology / Biochemical methods

3.3.4.1 RNA isolation

Isolation of RNA was performed using the peqGOLD TriFast (30-2010, VWR) reagent, where 20 mg of tissue were homogenized (3 min, 50 Hz) with 1 mL TriFast reagent and incubated at RT for 5 min. Isolation of RNA from cells were performed in 6-well plates where 1 mL TriFast reagent was pipetted into the well after washes with 1X PBS, and mixture was pipetted up and down 8 times before transferring to an RNase-free reaction tube. Homogenates were then given 250 μL chloroform and mixture was incubated for 10 min at RT, with periodic mixing throughout, before centrifugation (4°C,18400 xg, 20 min). Aqueous upper phase (RNA-containing) was transferred to an RNase-free reaction tube containing 500 μL isopropanol. After inversion of reaction tube, samples were centrifuged again (4°C,18400 xg, 60 min). For cell culture samples, aqueous upper phase was incubated with isopropanol overnight at 4°C, and subsequently centrifuged as stated above. Precipitated RNA was then washed once with 75% (v/v) ethanol and again with 100% ethanol, before being air dried at RT. Samples were resuspended in 40 μL Diethyl pyrocarbonate (DEPC) water (MP Biomedicals LLC, USA) and incubated at 60°C for 5 min to improve solubility. RNA concentration was then measured with Take3 plate with the optical density (OD) at 260nm (BIOTEK, Gene5 2.05, USA). Purity of RNA

was determined using the ratio of the OD_{260nm}:OD_{280nm}, where values between 1.8-2.0 indicated a low protein contamination.

3.3.4.2 DNase digestion

Isolated RNA samples were subsequently treated with DNase to remove trace amounts of genomic DNA. Approximately 8 μg of RNA was combined with DNase I master mix (EN0521, Fisher Scientific) containing 2 U DNase I, 1X Reaction buffer (with $MgCl_2$), 0.75 U RiboLock (RNase-inhibitor), and DEPC water to a total volume of 30 μL. Samples were incubated at 37°C for 30 min for the DNase digestion. Following this incubation, samples were given 1 μL of 50 mM EDTA and incubated at 65°C for 10 min to inhibit the function of the DNase I and stop the reaction.

RNA integrity was then assessed using a 1% (w/v) RNA denaturing agarose gel (1X N-morpholino propane sulfonic acid (MOPS) buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA, pH 6.0) and formaldehyde). Aliquots of samples were mixed with equal volume of 2X RNA loading dye (R0641, Fermentas Life Science) and incubated at 70°C for 10 min. After brief incubation on ice, samples were pipetted into the gel and electrophoresis was performed at 120 V for 25 min. Integrity of 18S and 28S rRNA was visualized with the UV transilluminator gel documentation system (Biometra, Germany).

3.3.4.3 cDNA synthesis

Reverse transcription (RT) of DNase-treated RNA into complementary DNA (cDNA) was performed using the LunaScript RT SuperMix Kit (E3010, New England Biolabs). After quantification of RNA concentration after DNase treatment, 1 μg of RNA was combined with 1X LunaScript RT SuperMix (containing random hexamer primers, oligo dT primers, RNase inhibitor, and Luna Reverse Transcriptase) and DEPC water to a volume of 20 μL. RT-PCR was performed in a thermal cycler (25°C, 2 min; 55°C, 10 min; 95°C, 1 min) and subsequently diluted with DEPC water to a concentration of 5 ng/μL.

3.3.4.4 Quantitative real-time PCR (qPCR)

Gene expression analysis was performed with quantitative real-time PCR (qPCR) using the LunaUniversal qPCR Master Mix (M3003, New England Biolabs). Briefly, 1 μL of cDNA was combined with 4 μL of reaction mixture, containing 2.5 μL LunaUniversal Master Mix, 0.5 μL of gene-specific forward and reverse primers (2.5 μM stock) each, and 0.5 μL DEPC water, in a 384-well plate. Relative fluorescence was measured with the ViiA 7 Software v1.2 (Applied Biosystems, USA). Primers used are listed in **Table 3**.

PCR thermal cycling is listed in **Table 4**. Melting curves were analyzed to determine specificity of primer pairs and relative gene expression was calculated using the ddCT method, where genes of interest were normalized to the expression of reference genes (housekeeping genes), as noted below, and control groups set to a value of 1.

Table 3. Primer Sequence List.

Gene ID	Gene name	Primer sequence (5'→3')	
Acaca	Acetyl-CoA Carboxylase A	f	TTTCACTGTGGCTTCTCCAG
		r	TGCATTTCACTGCTGCAATA
Acot1	Acyl-CoA Thioesterase 1	f	GACAAGAAGAGCTTCATTCCCGTG
		r	CATCAGCATAGAACTCGCTCTTCC
Acox1	Acyl-CoA Oxidase 1	f	CACCATTGCCATTCGATACA
		r	TGCGTCTGAAAATCCAAAATC
Acss2	Acyl-CoA Synthetase Short Chain Family Member 2	f	TGTGTGATGGCCATACCTTC
		r	GTAGTCTGGTGTGGCAATGG
Atf4	Activating Transcription Factor 4	f	GGAATGGCCGGCTATGG
		r	TCCCGGAAAAGGCATCCT
B2m	Beta-2-Microglobulin	f	CCCCACTGAGACTGATACATACGC
		r	AGAAACTGGATTTGTAATTAAGCAGGTTC
Bcat2	Branched Chain Amino Acid Transaminase 2	f	CGCCTCTGCCTGCCAGACTT
		r	GCCGCACGTAGAGGCTCGTT
Bckdhb	Branched Chain Keto Acid Dehydrogenase E1 Subunit Beta	f	AGCTATTGCGGAAATCCAGTTT
		r	ACAGTTGAAAAGATCACCTGAGC
Bckdk	Branched Chain Keto Acid Dehydrogenase Kinase	f	GCCCGGTTCCCCTTCAT
		r	TGTGGCTCTCATGGCATTCTT
Ccl2	C-C Motfi Chemokine Ligand 2	f	CACTCACCTGCTGCTACTCA
		r	GCTTGGTGACAAAAACTACAGC
Cd36	Cluster of Differentiation 36	f	CCAAGCTATTGCGACATGAT
		r	ACAGCGTAGATAGACCTGCAAA
Cd68	Cluster of Differentiation 68	f	GGACTACATGGCGGTGGAATAC
		r	GAGAGCAGGTCAAGGTGAACAG
Chop	C/EBP Homologous Protein	f	AGAGTGGTCAGTGCGCAGC
		r	CTCATTCTCCTGCTCCTTCTCC
Col1a1	Collagen Type I Alpha 1 Chain	f	GTGCTCCTGGTATTGCTGGT
		r	GGCTCCTCGTTTTCCTTCTT
Cpt1a	Carnitine Palmitoyltransferase 1A	f	CCAAACCCACCAGGCTACA
		r	GCACTGCTTAGGGATGTCTCTATG

Gene ID	Gene Name		Primer sequence (5'→3')
Elovl6	Elongation of Very Long Chain Fatty Acids Protein 6	f	TGCAGGAAAACTGGAAGAAGTCT
		r	AGCGGCTTCCGAAGTTCAA
Fads1	Fatty Acid Desaturase 1	f	CTCGTGATCGACCGGAAGGT
		r	CCACAAAAGGATCCGTGGCAT
Fasn	Fatty Acid Synthase	f	TTGATGATTCAGGGAGTGGA
		r	TTACACCTTGCTCCTTGCTG
Fatp3	Fatty Acid Transport Protein 3	f	AGGACAAGCTGCTGAAGGAT
		r	TGTCTCCAGTACGATCGTGG
Fatp4	Fatty Acid Transport Protein 4	f	CTCAGCAGGAAACATCGTGG
		r	ACAGTCATGCCGTGGAGTAA
Glut1	Glucose Transporter 1	f	CGGGTATCAATGCTGTGTTC
		r	GTCCAGCTCGCTCTACAACA
Glut4	Glucose Transporter 4	f	CTATGCTGGCCAACAATGTC
		r	CCCTGATGTTAGCCCTGAGT
Hacl1	2-Hydroxyacyl-CoA Lyase 1	f	ACAGGCTTGATGCAGGTTCT
		r	CCACTTCCATGCCAGAAAAT
Hadha	Hydroxyacyl-CoA Dehydrogenase Subunt Alpha	f	TGCATTTGCCGCAGCTTTAC
		r	GTTGGCCCAGATTTCGTTCA
Hibadh	3-Hydroxyisobutyrate Dehydrogenase	f	GCAGCGGTGTGTTCTAGGTC
		r	ACACGTCATAGAGGATGAGTGG
Hibch	3-Hydroxyisobutyryl-CoA Hydrolase	f	GTGGAGGCGTCATAACGCTA
		r	AGGAATGTGTCAGGGTCTTGT
Hprt	Hypoxanthine Phosphoribosyltransferase 1	f	CAGTCCCAGCGTCGTGATTA
		r	AGCAAGTCTTTCAGTCCTGTC
Mut1	Methylmalonyl-CoA Mutase	f	GCACACTGCCAGACATCG
		r	TGCAGTGCGGACAATGTTAT
Pccb	Propionyl-CoA Carboxylase Subunit Beta	f	GAGAAGTTCGCCAACCCTTT
		r	TCCGAGCACGAGTAGAGGAT
Pgc1a	Peroxisome Proliferator-activated Receptor Gamma Coactivator 1-Alpha	f	AGCCGTGACCACTGACAACGAG
		r	GCTGCATGGTTCTGAGTGCTAAG
Phyh	Phytanoyl-CoA 2-Hydroxylase	f	ACTGCCTTCTCCCCGAGATT
		r	GTCCAGTGAAACACTCCA
Ppara	Peroxisome Proliferator-activated Receptor Alpha	f	ATTCGGCTGAAGCTGGTGTA
		r	AAGCGAATTGCATTGTGTGA
Pparg	Peroxisome Proliferator-activated Receptor Gamma	f	TGCCAAAAATATCCCTGGTT
		r	GGCGGTCTCCACTGAGAATA

Gene ID	Gene Name		Primer sequence (5'→3')
Ppm1k	Protein Phosphatase, Mg2+/Mn2+ Dependent 1K	f	ATGTTATCAGCGGCCTTCATTAC
		r	GTGGAGAAGTAGCAGGCAGG
Scd1	Stearoyl-CoA Desaturase 1	f	AGAGAGAGAGGTAGCCATATC
		r	TCAAATCTCACTAATCTCTGG
Scd2	Stearoyl-CoA Desaturase 2	f	GTTTGAAAGCTTTGGGTAGGG
		r	AAGGCCCTAAAGCCTCTCTCT
Slc3a2	Solute Carrier Family 3 Member 2	f	CAAAGTGCCAAGAAAAAGAGC
		r	CTGAGCAGGGAGGAACCAC
Srebf1	Sterol Regulatory Element Binding Transcription Factor 1	f	GAGGATAGCCAGGTCAAAGC
		r	AGGATTGCAGGTCAGACACA
Tnfa	Tumer Necrosis Factor Alpha	f	CCACCACGCTCTTCTGTCT
		r	GCTCCTCCACTTGGTGGTTT
Trib3	Tribbles 3	f	TCAAGCTGCGTCTTGTC
		r	AGCTGAGTATCTCTGGTCCCACGTA

Table 4. qRT-PCR Thermal Cycling Program.

Cycle Step	Temperature	Time	Cycles
Intial Denaturation	95°C	60 s	1
Denaturation	95°C	15 s	40
Extension	60°C	30 s (+plate read)	
Melt Curve	60-95°C	various	1

3.3.4.5 RIPA protein extraction

Ground tissues were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% (w/v) Na-Deoxycholate, 1% (v/v) Triton X-100 and 1X Halt Protease & Phosphatase Inhibitor Cocktail (#10085973, ThermoScientific)) at a ratio of 50 mg tissue to 1000 μL lysis buffer for 3 min at 50 Hz in a TissueLyser. Homogenates were incubated on ice for 30 min and subsequently centrifuged for 30 min (4°C, 21000 xg). Supernatants were centrifuged until lysates were clear at the same speed and duration, before storage at -20°C until further use.

Protein isolates from cell culture studies were isolated by adding 150 μL RIPA buffer to each well of a 6-well plate, and subsequent scraping with a cell scraper before transfer

into a reaction tube. Samples were then incubated on ice for 20 min, with periodic vortexing, and centrifuged for 30 min (4°C, 16000 xg).

3.3.4.6 Plasma Membrane Isolation

Isolation of plasma membrane fractions were performed according to a published protocol (Nishiumi and Ashida 2007). 50 mg of M. quadriceps was homogenized in 200 μL of Buffer A (50 mM Tris (pH 8.0), 0.5 mM Dithiothreitol (DTT), 0.1% (v/v) Triton X-100) for 3 min at 50Hz. Homogenates were then sheared by passaging through a 23-gauge needle. Samples were centrifuged (4°C, 1000 xg, 10 min) and precipitate was resuspended in 150 μL Buffer B (50 mM Tris (pH 8.0), 0.5 mM DTT) and incubated for 10 min on ice. The supernatant was stored for further use. After incubation, resuspended precipitates were centrifuged again (4°C, 1000 xg, 10 min). The supernatant was combined with the first supernatant. Precipitates were resuspended again with Buffer B and centrifuged. After centrifugation, supernatants were removed and this wash step was repeated twice, where the final precipitate was then resuspended in 100 μL of Buffer C (50 mM Tris (pH 8.0), 0.5 mM DTT, 1% (v/v) Triton X-100) and incubated on ice for 1 hr. This resuspended precipitate (plasma membrane fraction) was then centrifuged, along with the supernatants (post-plasma membrane fraction) from the initial steps (4°C, 16000 xg, 20 min).

3.3.4.7 Protein determination

Protein concentrations were determined using the Bio-Rad DC Protein Assay (#500-0116, BioRad) and performed according to manufacturer’s protocol. Protein samples were diluted accordingly (1:10 for muscle and cell culture studies; 1:20 for liver samples) and 15 μL were mixed with a 25 μL mixture of Reagent A and S in 96-well plates, done in triplicates. After brief mixing, 200 μL of Reagent B was added to each well and incubated for 15 min, RT. Absorbance was measured at 750 nm and protein concentration was determined using the bovine serum albumin (BSA) standard curve (0.1 – 1 μg/μL).

3.3.4.8 Western blot analysis

For western blot analysis, aliquoted protein samples were first diluted with 4X Laemmli buffer (0.25 M Tris-HCl, 8% (w/v) SDS, 40% (v/v) Glycerol, 0.04% (w/v) Bromophenol blue, pH 6.8) with 10% (w/v) 1 M DTT—3 volumes of protein to 1 volume of 4X Laemmli. Samples were then heated to 95°C for 5 min in a thermal shaker.

Gels containing a lower separation (running) gel and the upper stacking gel were prepared as described in **Table 5**. Gels were loaded appropriately with 20 µg sample and a ProSieve QuadColor Protein Marker (830537, Biozym), after filling running chambers with 1X TGS buffer (diluted from 10X TGS buffer (0.25 M Tris, 1.92 M Glycine, 1% (w/v) SDS (pH 8.3)). Gel electrophoresis was run first for 30min at 60V through the stacking gel and further run at 120 V for an additionally 90 min, minimum.

Table 5. Composition of SDS-PAGE gel.

Reagents	Running Gel (10%)	Stacking Gel (4%)
DI water	2.94 mL	1.6mL
10% (w/v) SDS	60µL	25µL
1.5M Tris-Base (pH 8.8)	1.5mL	-
0.5M Tris-Base (pH 6.8)	-	0.625mL
Acrylamide/Bis 30	1.5mL	0.25mL
APS 10% (w/v)	40µL	15µL
TEMED	6µL	2.5µL

Semi-dry western blotting transfer was then performed, where 12 filter papers (6 per side) and one PVDF membrane activated for 1 min in 100% methanol were equilibrated in transfer buffer (48 mM Tris, 1.3 mM SDS, 20% (v/v) Methanol, pH 9.2), before assembly of the sandwich. Transfer of proteins was performed using the TransBlot Turbo system (BioRad, USA) for 30 min at 2.5 A and 25 V. Efficiency of transfer was tested by staining the membrane with Ponceau-S (0.4% (w/v) Ponceau-S, 40% (v/v) Ethanol, 15% (v/v) Acetic acid). Membranes were then washed 3 times with 1X TBST (1X TBS Buffer (10X TBS Buffer: 200 mM Tris, 1.37 M NaCl), 0.1% (v/v) Tween20) before 1 hr blocking of membrane with 5% (w/v) milk powder or 5% (w/v) BSA, for total protein antibodies or phospho-protein antibodies, respectively. Following blocking, membranes were washed again 3 times with 1X TBST before overnight incubation with primary antibodies (**Table 6**) at 4°C. The following day, membranes were washed 3 times with 1X TBST and incubated with the appropriate horseradish peroxidase coupled secondary antibody (**Table 6**) diluted 1:5000 in 5% (w/v) milk powder for 1 hr at RT. After washing again, proteins were detected using the Western Bright Sirius chemiluminescent HRP substrate (K-12043, Biozym) and imaged using the Fusion SL Vilber Lourmat (Peqlab, Germany). GAPDH, ATUB and ponceau staining were used as loading controls for confirmation of equal loading. Western blot quantifications were performed with ImageJ, where intensity values were normalized to the control group after equalizing to the loading controls.

Table 6. Western Blot Antibodies.

Primary Antibody	Dilution	Company	Ref. Number
ATUB	1:1000 - 5% Milk	Sigma	T6074
AKT	1:1000 - 5% Milk	Cell Signaling	9272
pAKT Ser 473	1:1000 - 5% BSA	Cell Signaling	9271S
BCKDHA	1:1000 - 5% Milk	Santa Cruz	sc-67200
pBCKDHA Ser293	1:1000 - 5% BSA	Abcam	ab200577
CD36	1:1000 - 5% Milk	R&D	MAB2519
CDH2	1:1000 - 5% Milk	Cell Signaling	4061
CPT1	1:1000 - 5% Milk	Alpha Diagnostic	CPT1M11-A
GAPDH	1:1000 - 5% BSA	Ambion	AM4300
HACL1	1:1000 - 5% Milk	Sigma	HPA035496
mTOR	1:1000 - 5% BSA	Cell Signaling	4517
pMTOR Ser2448	1:1000 - 5% BSA	Cell Signaling	5536
PKCθ	1:1000 - 5% BSA	Cell Signaling	13643
Secondary Antibody	**Dilution**	**Company**	**Ref. Number**
Anti Rabbit	1:1000 - 5% Milk	Cell Signaling	7074
Anti Mouse	1:1000 - 5% Milk	Cell Signaling	7076
Anti Rat	1:1000 - 5% Milk	Santa Cruz	sc-2032

3.3.4.9 Fatty acid synthase activity assay

Activity of Fatty Acid Synthase was measured using a modification of published protocols (Nepokroeff, Lakshmanan et al. 1975). Liver tissue (50 mg) was homogenized (50 Hz, 3 min) in Phosphate-Bicarbonate Buffer, pH 8.0 (70 mM Potassium Carbonate, 85 mM Dipotassium Phosphate, 9 mM Monopotassium Phosphate and 1 mM DTT). Samples were cleared by centrifugation (23000 xg, 10 min, 4°C) and subsequently used in the activity assay. In a 96-well plate, 20 μL of supernatant, 150 μL of reaction buffer (100 mM Potassium Phosphate (pH 6.5), 1 mM DTT, 25 μM Acetyl- or Propionyl-CoA, and 150 μM NADPH) and 30 μL of 500 μM Malonyl-CoA were assayed for 30 min at 37°C — where absorbance was measured at 340 nm.

3.4 Statistical analysis

Statistical calculations were determined using GraphPad Prism 8 (GraphPad Software Inc., USA). All data are presented as mean ± SEM, except for plasma values which are represented as interleaved box and whisker (min to max) plots. Comparisons for normality were determined prior to further analysis. Normally distributed data were analyzed with an ordinary one-way ANOVA with Bonferroni's post hoc tests to relative control group. Non-normally distributed data were analyzed with Kruskal-Wallis tests. To determine outliers, Grubbs outlier tests were performed and subsequently removed from analysis. Pearson correlation coefficient R^2 were used to describe correlative data. Data were defined as statistically significant with $p<0.05$.

4 Results

4.1 Dairy intake

4.1.1 Short-term high-fat dairy fat or dairy protein feeding elevates hepatic OCFA

To address the relation of dairy intake and odd-chain fatty acid (OCFA) levels, Study Ia (see Section 2.1.1) sought to compare the short-term (1 week) supplementation of dairy fat, in the form of clarified butter, or dairy protein, specifically casein, in high-fat diets. As the ratio of circulating OCFA in humans differs in their ratio of C15:0 to C17:0 (1:2) when compared to the typical 2:1 ratio found in dairy fat, we hypothesized that additional components of dairy could also influence OCFA levels. The initial aim was thus to confirm that both dairy fat and dairy protein could successfully lead to an increase in hepatic OCFA. To clarify this, C57BL/6JRj mice were fed for 1 week with a control high-fat (HF) diet or HF diets supplemented with 14% milk fat (HFMF) or 14% milk protein (HFMP). After one week of feeding, there were no apparent changes in body weight or fat mass (**Figure 5A, B**), as well as no effect on liver weight or triglycerides (**Figure 5C, D**). While there was no effect of these supplements on phenotypic characteristics, HFMF feeding led to an increased food intake (**Figure 5E**). Importantly, both supplementations resulted in elevated hepatic levels of pentadecanoic acid (C15:0), and heptadecanoic acid (C17:0) levels (**Figure 5F**), demonstrating that dairy fat and protein intake both influence hepatic OCFA.

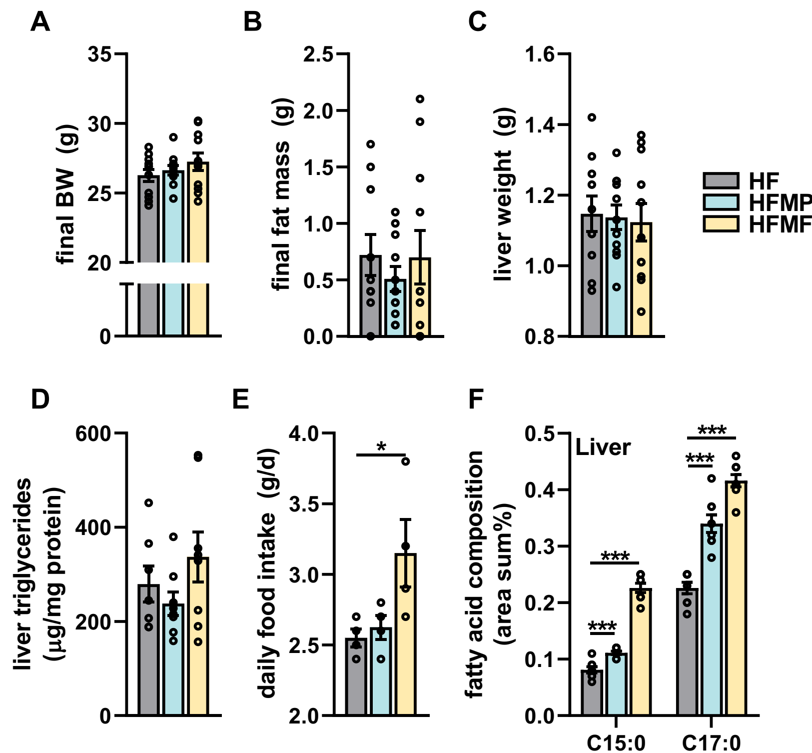


Figure 5. Dairy protein & dairy fat intake increases liver phospholipid odd-chain fatty-acids. (A) Final body weight in male C57BL/6JRj mice fed high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), or milk fat (HFMF) for 1 week (n = 8). **(B)** Final fat mass determined with NMR after 1 week (n = 8). **(C)** Final liver tissue weight (n = 8). **(D)** Liver triglycerides (n = 8). **(E)** Average food intake per day over 24 hr (n = 4). **(F)** Long-chain fatty acid composition of OCFA in liver phospholipid fraction after 1 week (n = 8). Results are expressed as area percentage of individual fatty acids to total area of detected fatty acids. Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. BW, body weight; C15:0, pentadecanoic acid; C17:0, heptadecanoic acid.

4.2 Dairy fat & OCFA supplementation

4.2.1 OCFA-rich diets increase circulating and tissue C15:0 & C17:0 levels

Following the confirmation that both supplementation of dairy fat (HFMF) and dairy protein (HFMP) resulted in increased OCFA levels, a long-term feeding study (Study IIa – Section 2.1.1) was performed in order to better understand the direct role of an increased OCFA intake on metabolic health with regard to diet-induced obesity models. To determine the long-term effect of milk fat or C17:0 supplementation on fatty acid composition, liver, eWAT and plasma phospholipid fractions were analyzed. As expected, C15:0 levels in liver and plasma were increased after HFMF feeding, and, notably, HFC17 feeding also induced C15:0 levels in liver and plasma (**Figure 6A, C, E**). Meanwhile, only HFC17 feeding led to a significant increase in C17:0 levels in all tissues analyzed (**Figure 6A, C, E**). Interestingly, HFC17-fed mice also had an induction of C15:0 and C17:0 levels in eWAT (**Figure 6C**)—though it has been described that the

liver is the main organ for endogenous OCFA synthesis. These increases in OCFA were accompanied with decreases in C16:0 and C18:0 levels after HFC17 feeding, in liver and plasma. HFMF feeding, on the other hand, led to significant increases in C16:0 levels in all fractions analyzed (**Figure 6B, D, E**).

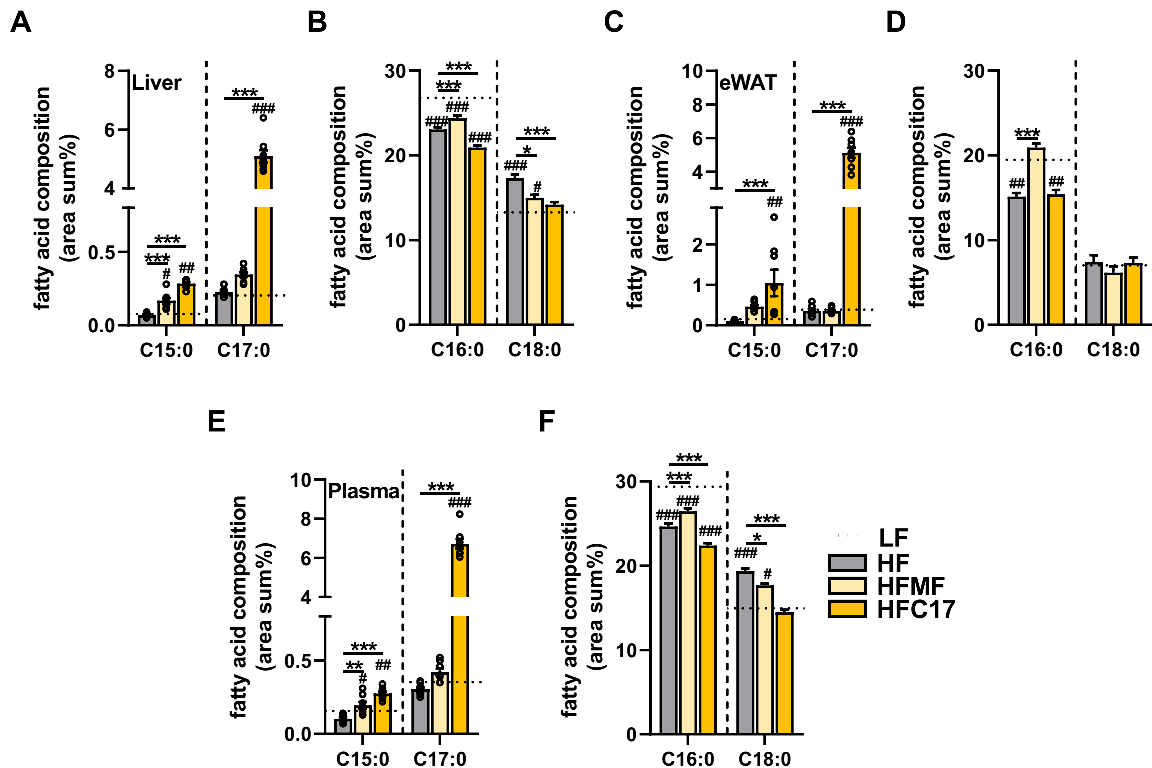


Figure 6. Long-term feeding of OCFA-rich diets increases liver, eWAT and plasma phospholipid C15:0 and C17:0. (A, C, E) Long-chain fatty acid composition of OCFA in liver, epididymal white adipose tissue (eWAT) and plasma phospholipid (PL) fractions in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF), or C17:0 (HFC17) for 20 weeks (n = 8). **(B, D, F)** Long-chain fatty acid composition of ECFA in liver, eWAT and plasma PL fractions (n = 8). Data are shown as mean ± SEM. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. OCFA, odd-chain fatty acid; C17:0, heptadecanoic acid; C15:0, pentadecanoic acid; C16:0, palmitic acid; C18:0, stearic acid.

4.2.2 OCFA-rich diets do not protect from HF-diet induced weight gain.

As the secondary aim in this long-term feeding study was to determine the metabolic implications of OCFA-rich diets in HF-feeding, C57BL/6JRj mice were fed a HF diet for 20 weeks with increased milk fat (HFMF) as a natural source of OCFA or heptadecanoic acid (HFC17) to determine direct actions of this FA. While all HF groups (HF, HFMF, and HFC17) resulted in increased body weight compared to LF, there were no differences between HF groups (**Figure 7A, B**) with additionally no difference in final lean body mass, fat mass or tissue weights (**Figure 7C-E**), suggesting that these supplementations had no impact on HF diet induced weight gain and fat accumulation.

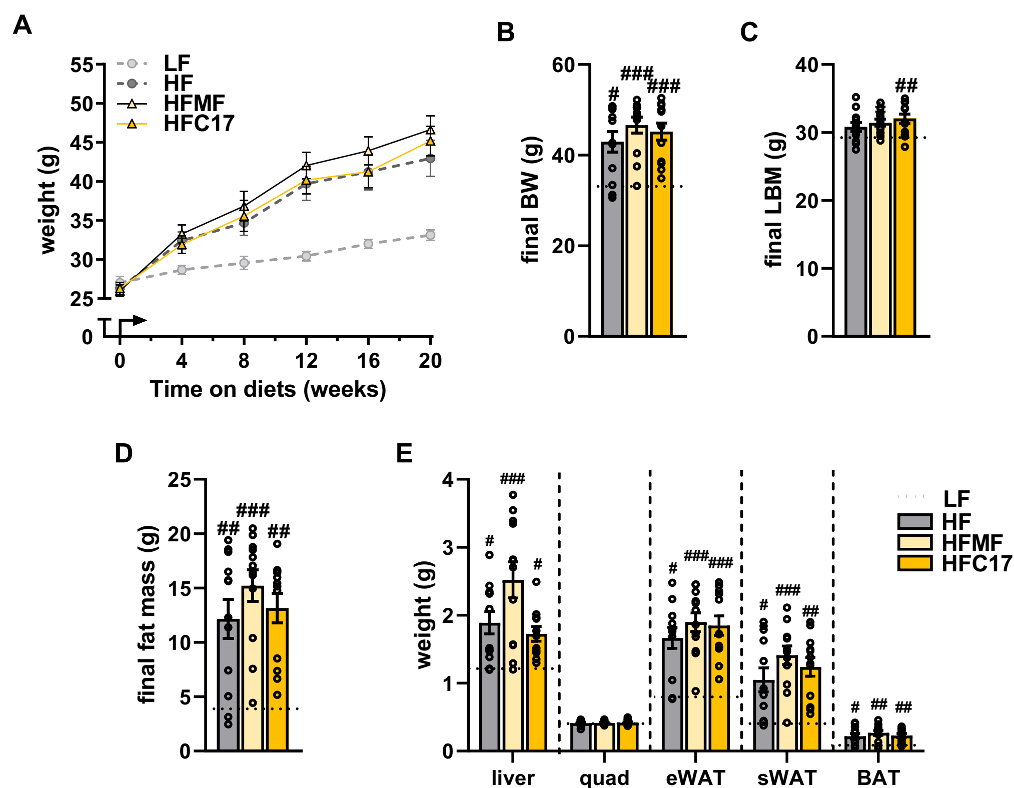


Figure 7. OCFA-rich diets do not protect from HF-diet induced weight gain & adiposity. (A) Body weight development in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF) or C17:0 (HFC17) for 20 weeks (n = 12). **(B)** Final body weight at 20 weeks (n=12). **(C)** Final calculated lean body mass (n = 12). **(D)** Final fat mass determined with NMR (n = 12). **(E)** Final tissue weights for liver, muscle and adipose tissue depots (n = 12). Data are mean ± SEM, LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. BW, body weight; LBM, lean body mass; eWAT, epididymal white adipose tissue; sWAT, subcutaneous white adipose tissue; BAT, brown adipose tissue; quad, quadriceps muscle.

These data are supported by the fact that none of the HF diets appeared to have any effect on daily food or energy intake (**Figure 8A, B**). Further metabolic characterization with indirect calorimetry performed at week 12 of the intervention revealed no strong differential effect on locomotor activity or energy expenditure (EE), but all HF-diets had reduced respiratory exchange ratios (RER) as compared with LF fed mice (**Figure 8C-E**)— which is expected with the increased fat content of the diets. Essentially, both experimental diets (HFMF and HFC17) had no impact on alterations in metabolic phenotype.

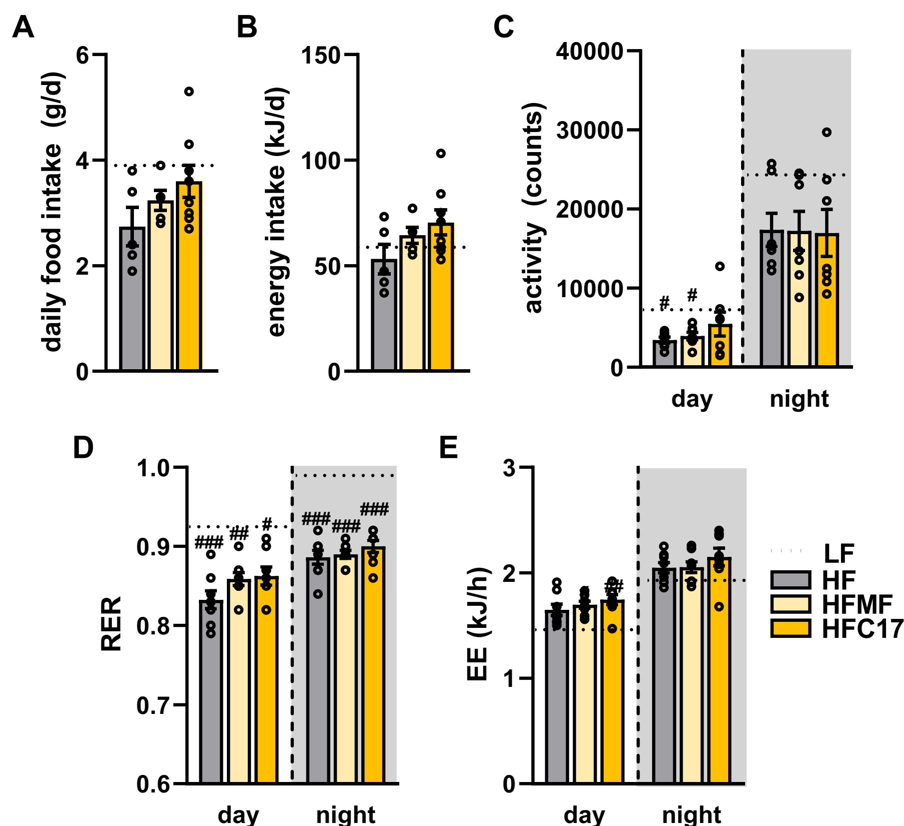


Figure 8. OCFA-rich diets have no impact on HF-diet induced metabolic phenotype. (A) Average food intake per day over 24 hours in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF) or C17:0 (HFC17) at week 12 of the intervention (n = 5-7). **(B)** Calculated energy intake per day from average food intake and energy content of diets. (n = 5-7). **(C)** Average locomotor activity (beam breaks) x1000 per 12 hr period (day and night phase) (n = 7). **(D)** Respiratory exchange ratio (RER; n = 8). **(E)** Average energy expenditure (EE) per hour per 12 hr period (day and night phase) (n = 8). Data are mean ± SEM, LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF.

4.2.3 C17:0 supplementation elicits minor effects on glucose-stimulated insulin secretion

As OCFA have been described to have an inverse association with T2DM and insulin sensitivity, we next addressed the possible direct impact of OCFA on insulin and glucose sensitivity, in relation to a modified fat composition intake. In order to probe for an impact of these diets, oral glucose tolerance tests (oGTT) and an insulin tolerance test (ITT) were performed. Fasting blood glucose levels after 6 and 16 weeks of feeding showed no significant differences between HF groups (**Figure 9A**), while HFMF had elevated fasting insulin levels compared to HF at week 6, these differences were not apparent after 16 weeks of feeding (**Figure 9B**). oGTTs were performed at both time points; however, neither 6 nor 16 weeks of feeding resulted in altered glucose clearance in any of the HF diets as seen in the incremental area under the curve (iAUC) (**Figure 9C, D**). Glucose-stimulated insulin secretion was unaffected after 6 weeks of feeding; however, at week 16, HFC17 insulin levels were significantly reduced, specifically at 15 min post gavage, compared to HF levels (**Figure 9E, F).** Additionally, an insulin tolerance test (ITT) performed at 16 weeks showed no significant difference between HF groups, but significant differences of all groups compared to LF (**Figure 9G, H**). Taken together, these data provide evidence that C17:0 supplementation has only a minor effect on glucose-stimulated insulin homeostasis under HF conditions.

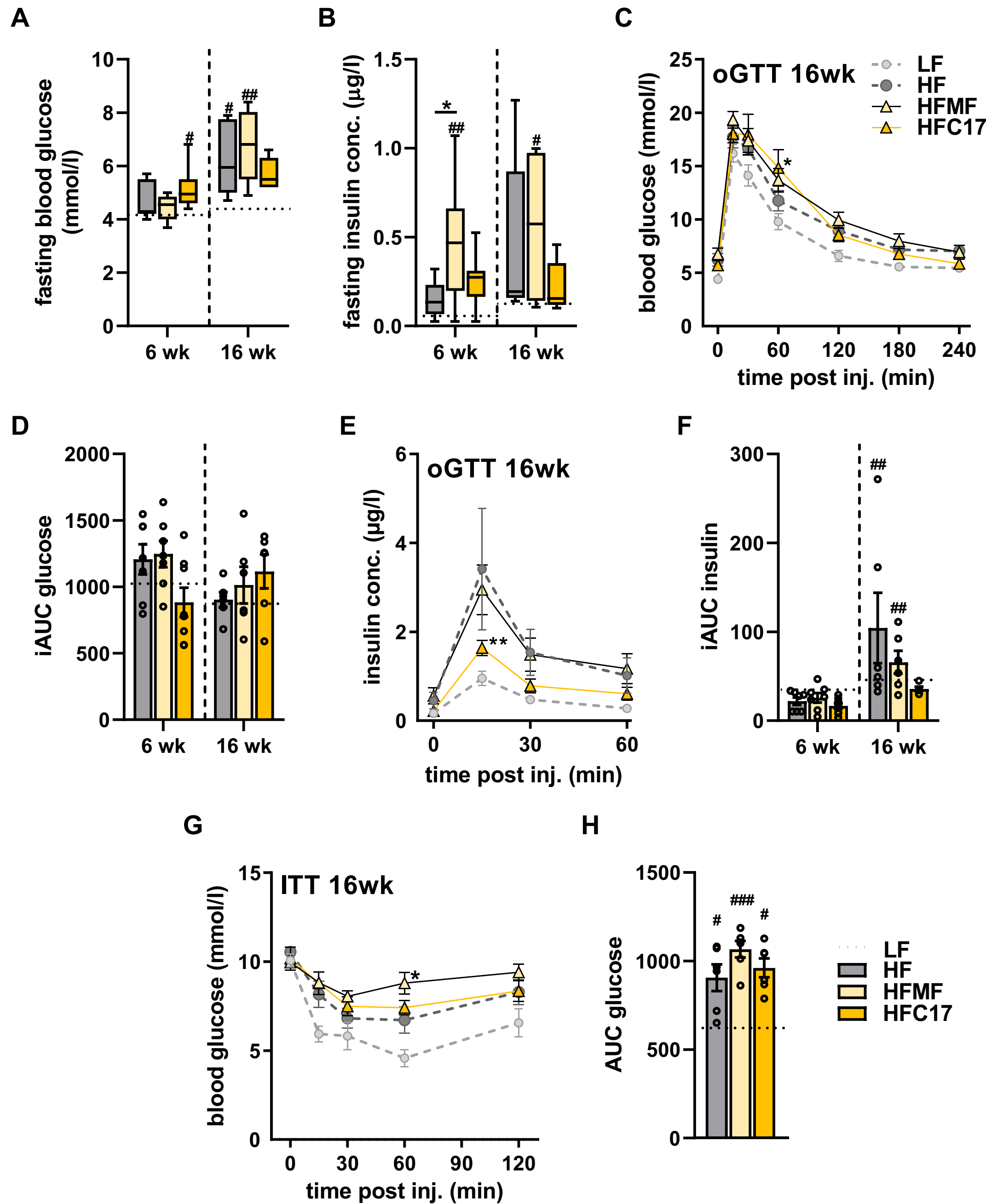


Figure 9. OCFA-rich diets elicit negligible effect on glucose & insulin homeostasis. (A) 16hr fasting blood glucose in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF), or C17:0 (HFC17) for 6 or 16 weeks (n = 6-8). **(B)** 16hr fasting insulin at 6 or 16 weeks (n= 6-8). **(C)** Glucose measurements during an oral glucose tolerance test (oGTT) at 16 weeks of feeding (n = 6-8). **(D)** Incremental area under the curve (iAUC) of glucose levels during oGTT at week 6 or 16 of experiment (n = 6-8). **(E)** Insulin measurements during oGTT after 16 weeks of feeding (n = 6). **(F)** iAUC of insulin levels during oGTT at week 6 or 16 of experiment (n = 6-8). **(G)** Glucose measurements during an i.p. ITT at 16 weeks (n = 6). **(H)** Area under the curve (AUC) of glucose levels during an i.p. ITT (n = 6). Plasma data (A,B) are expressed as interleaved box and whiskers (min to max) plots. All other data are shown as mean ± SEM. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. C17:0, heptadecanoic acid

4.2.4 Long-term dairy fat feeding worsens the HF-induced liver phenotype, independently of C17:0

Surprisingly, despite the increase in OCFA after supplementation of both diets, intrahepatic lipid content was significantly increased in HFMF feeding compared to HF, but was unaffected after HFC17 feeding (**Figure 10A**). The HFMF-induced increase in lipid content was more pronounced in hepatic triglycerides (**Figure 10B**). Cholesterol, a component of butter that is a major lipotoxic molecule critical in the development of NASH, was significantly elevated in the circulation of HFMF fed mice (**Figure 10C**). In line with this, certain inflammation and fibrotic markers were significantly elevated in HFMF-fed mice (**Figure 10D**), which is reflective of the hepatic fat content. HFMF feeding induced gene expression of *Pparg* and its target, *Cd36,* a fatty acid translocase (**Figure 10E**). While not significant on gene expression, protein expression of the fatty acid transporter, CD36, was highest in HFMF (**Figure 10F**). HFC17 feeding, however, exhibited no apparent effect on the HF-induced hepatic phenotype.

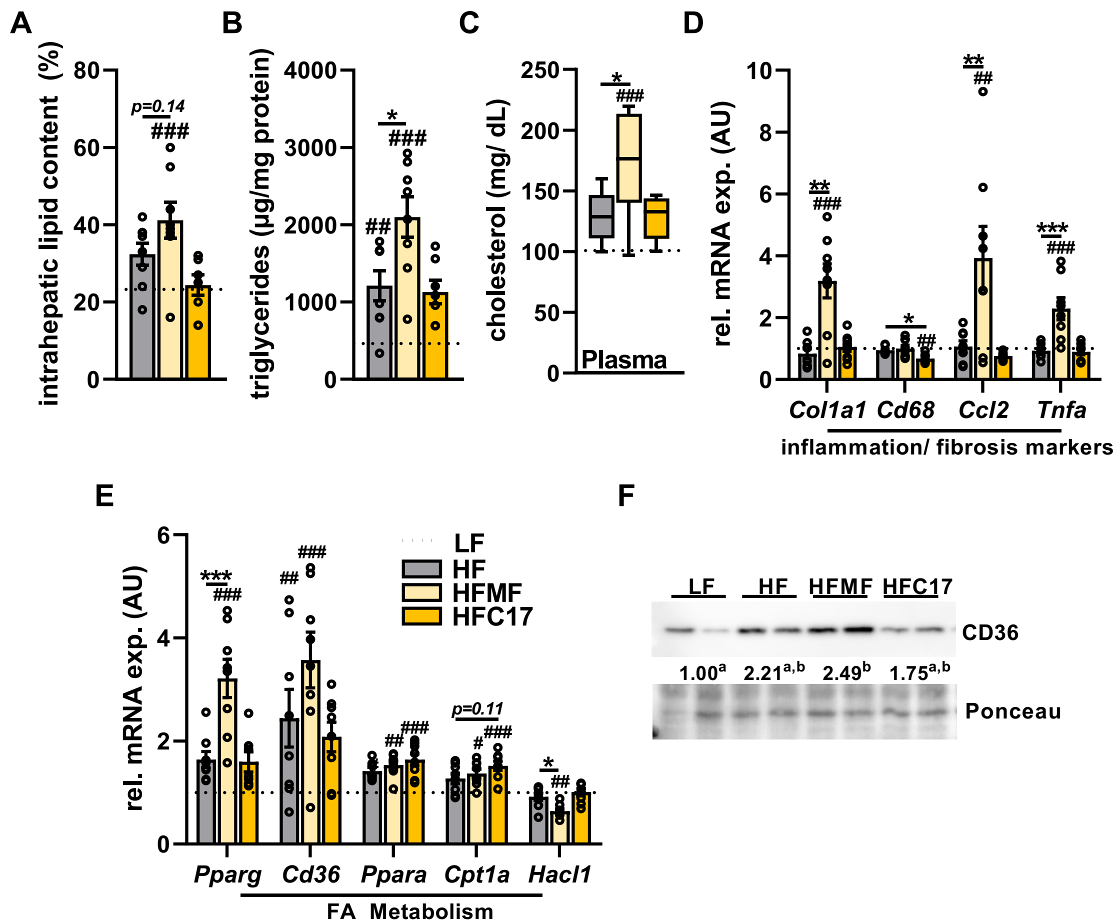


Figure 10. Milk fat intake worsens hepatic lipid accumulation. (A) Relative liver fat content as determined by H&E in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF), or C17:0 (HFC17) for 20 weeks (n = 8). **(B)** Liver triacylglycerides (n= 8). **(C)** 2 hr fasting plasma total cholesterol after 20 weeks (n = 8). **(D)** qPCR of key genes in inflammation / fibrosis normalized to LF in livers at 20 weeks with *B2m* as reference gene (n = 8). **(E)** qPCR of key genes in FA uptake / β-oxidation normalized to LF in livers at 20 weeks with *B2m* as reference gene (n = 8). **(F)** Representative western blot of CD36 in liver normalized to LF (n = 6). Plasma data (C) are expressed as interleaved box and whiskers (min to max) plots. Data are shown as mean ± SEM. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. Different letters represent significant difference between groups in western blots. H&E, hematoxylin and eosin; C17:0, heptadecanoic acid.

4.3 Dairy protein & BCAA supplementation

4.3.1 Short-term high-fat valine supplementation elevates hepatic OCFA

As it was possible to confirm that milk protein leads to an induction of hepatic OCFA in Study Ia, it was important to determine specific amino acids that could influence OCFA production *in vivo* under obesogenic conditions (Study Ib – Section 2.1.1). The BCAA, which compromise 15-25% of total protein intake, were the focus of these studies as, biochemically, Val catabolism contributes to propionyl-CoA (Pr-CoA) formation, a possible primer for *de novo* OCFA synthesis, while Leu contributes to acetyl-CoA (Ac-CoA) alone (**Figure 2B**). Ile, however, can contribute to both Pr-CoA and Ac-CoA pools. In a short-term (1 week) dietary intervention, male C57BL/6JRj mice were fed a control HF diet, along with experimental HF-diets supplemented with either 5% Leu (HFL) or Val (HFV) to uncover acute differential effects in hepatic lipid metabolism. Data presented for study Ib are modified from previously published data (**Publication 3 – see Publication List**).

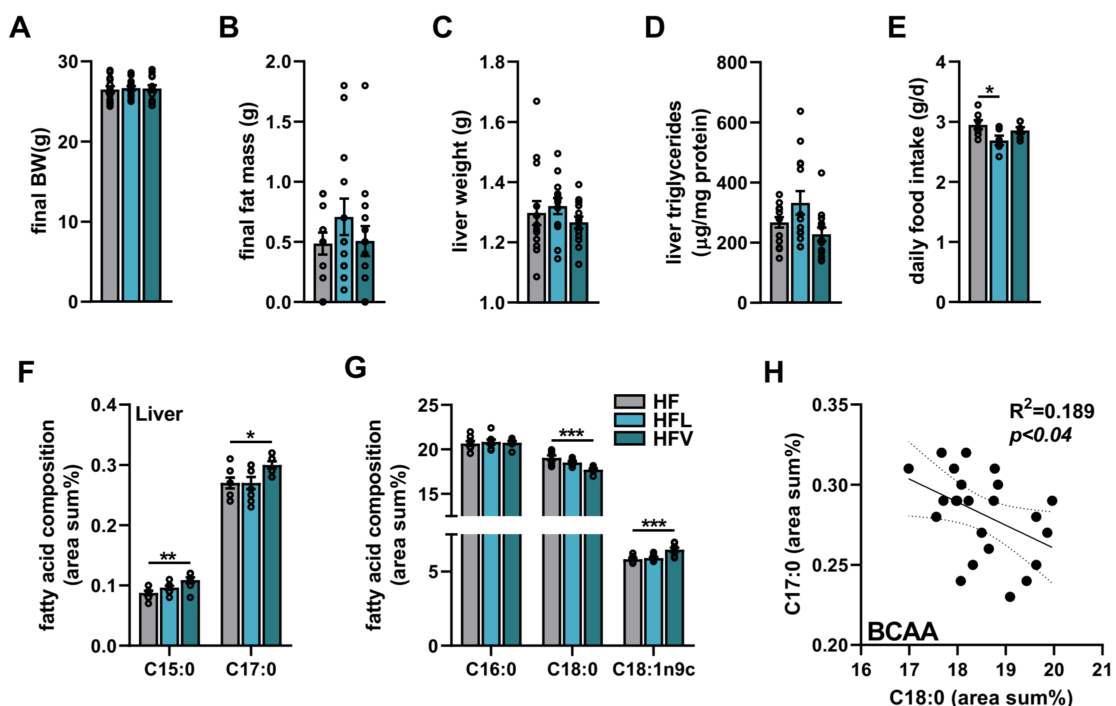


Figure 11. Dietary supplementation of valine, not leucine, induces hepatic odd-chain fatty acid levels. (A) Final body weight in male C57BL/6JRj mice fed high-fat diet (HF) or experimental HF diets supplemented with leucine (HFL), or valine (HFV) for 1 week (n = 14). **(B)** Final fat mass determined with NMR after 1 week (n = 14). **(C)** Final tissue weights for liver (n = 14). **(D)** Liver triglycerides (n = 8). **(E)** Average food intake per day over 24 hr (n = 7-8). **(F)** Long-chain fatty acid (LCFA) composition of OCFA in liver phospholipid (PL) fraction (n = 8). **(G)** Long-chain fatty acid composition of even-chain fatty acids in liver PL fraction (n = 8). **(H)** Pearson correlation of stearic acid (C18:0) with heptadecanoic acid (C17:0) in liver. Results of LCFA are expressed as area percentage of individual fatty acids to total area of detected fatty acids. Data are mean ±

SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF. BW, body weight; C15:0, pentadecanoic acid; C16:0, palmitic acid; C18:1n9, oleic acid; OCFA, odd-chain fatty acid.

After 1 week of feeding, neither of the experimental diets (HFL or HFV) had an effect on final body weight, fat mass, liver weight or hepatic triglycerides (**Figure 11A-D**); however, HFL feeding did result in a lower daily food intake (**Figure 11E**). While there were no significant effects of Val or Leu on the fat accumulation, changes in the hepatic phospholipid LCFA profile were present after Val feeding. Most notably, C15:0 and C17:0 levels were significantly increased, which was accompanied by a decrease in stearic acid (C18:0) and an increase in the monounsaturated fatty acid oleic acid (C18:1n9) (**Figure 11F, G**). The decrease in C18:0 correlated negatively with C17:0 levels (**Figure 11H**).

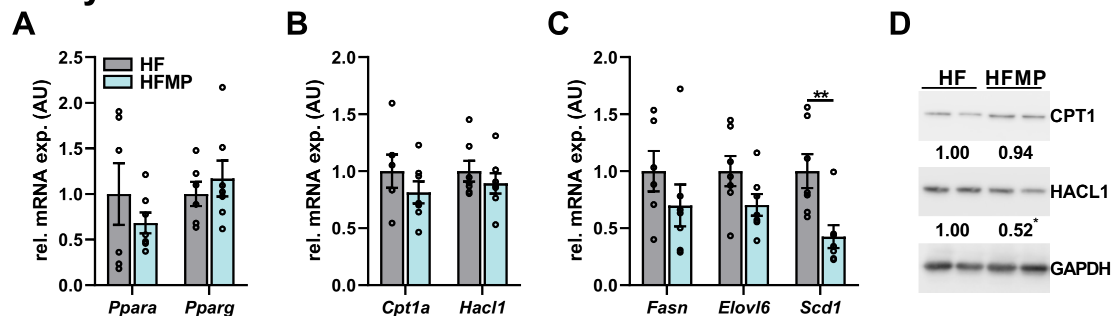
4.3.2 Short-term valine supplementation induces PPARα-target genes

As Pr-CoA has multiple fates within the cell, including fatty acid synthesis, anaplerosis to the tricarboxylic acid (TCA) cycle, and function as a post-translational modification (Bhagavan 2002), we examined the impact of these dietary interventions with protein or BCAA supplementations on expression of key mediators of these pathways. Gene and protein expression data from Study Ia indicated that there was no impact of a high-fat milk protein (HFMP) feeding on gene expression of key lipid mediators (*Ppara* and *Pparg)* as well as key α- and β-oxidation genes (*Hacl1* and *Cpt1a*, respectively); however, HFMP tended towards a lower expression of *de novo* lipogenesis genes (**Figure 12A-D**).

After Val supplementation, however, there was a significant increase in *Ppara* expression, but no other lipid metabolism regulator (**Figure 12E**). In line with this, there was a significant increase in gene expression of PPARα target genes, specifically *Acot1* and *Hacl1* in HFV-fed mice (**Figure 12F**). While there were no effects on CPT1, protein expression of HACL1 tended (*p = 0.1)* towards an increase in the HFV group (**Figure 12G**). Furthermore, there were no differences in gene expression of most lipogenic genes; however, *Scd2* was upregulated in Val-fed mice (**Figure 12H**)—likely a driver for the increased oleic acid (C18:1n9) in this group (see **Figure 11G**). As it has been previously suggested that propionate can act as a primer for fatty acid synthesis of OCFA, it was important to test the ability of Ac-CoA or Pr-CoA to act as primers for fatty acid synthesis in this study. Using an *ex vivo* Fatty Acid Synthetase (FASN) Activity assay, it was possible to confirm that both metabolites exhibit equal capacity to serve as

primers for FASN activity (**Figure 12I**); however, no significant differences were observed between the experimental groups and control HF diet.

Study Ia



Study Ib

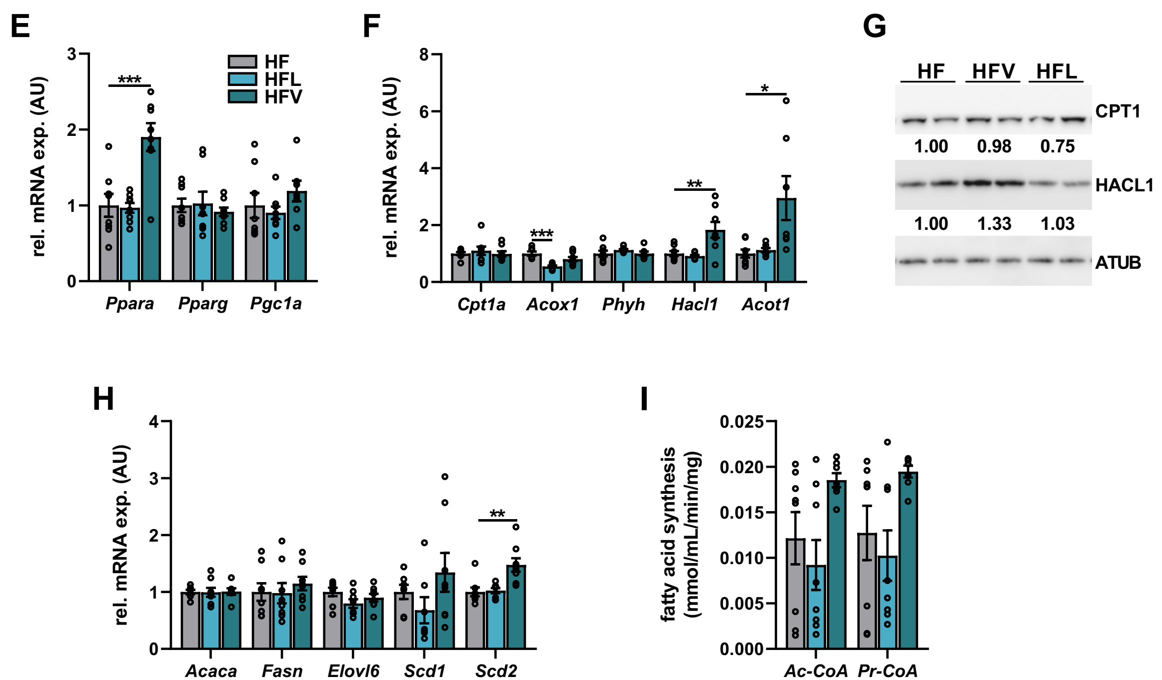


Figure 12. Short-term HF valine feeding induces key enzymes in α-oxidation. (A) qPCR of transcriptional regulators in livers of male C57BL/6JRj mice fed high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP) for 1 week (n = 6). **(B)** qPCR of key FA oxidation genes in liver (n = 8). **(C)** qPCR of key FA synthesis genes in liver (n = 8). **(D)** Representative western blot for key FA oxidation enzymes in liver (n = 3). **(E)** qPCR of transcriptional regulators in livers of male C57BL/6JRj mice fed high-fat (HF) or experimental HF diets supplemented with leucine (HFL), or valine (HFV) for 1 week (n = 8). **(F)** qPCR of key FA oxidation genes in liver (n = 8). **(G)** Representative western blot for key FA oxidation enzymes in liver (n = 6). **(H)** qPCR of key FA synthesis genes in liver (n = 8). **(I)** Fatty acid synthase activity assay (n = 7-8). Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared to respective control. Ac-CoA, acetyl-CoA; Pr-CoA, propionyl-CoA.

4.3.3 PPARα-agonism induces α-oxidation and OCFA formation *in vitro*

It has been suggested before that α-oxidation, the single carbon removal from a fatty acid, may play an important part in endogenous OCFA synthesis (Jenkins, de Schryver et al. 2017). To address this in connection with the short-term Val-induced OCFA accumulation, it was necessary to further elucidate the direct roles of PPARα, a key regulator of FA oxidation, as well as a key protein in α-oxidation, 2-Hydroxyacyl-CoA Lyase 1 (HACL1). First, the role of PPARα in OCFA formation was tested using primary mouse hepatocytes treated with 50 µM PPARα agonist Wy-14643 (Wy) or vehicle (DMSO) control. Confirming the action of this potent PPARα agonist, key targets (*Cpt1, Acox1, Acot1*) were significantly elevated on both gene and protein expression after 24 hr stimulation (**Figure 13A, B**). Meanwhile, 48 hr stimulation of hepatocytes with Wy led to significant changes in the phospholipid LCFA profile. Specifically, C17:0 levels were increased by this treatment, with concomitant decreases in C18:0 levels (**Figure 10C, D**). Wy treatment had no effects on C15:0 levels, suggesting that PPARα agonism plays no role in C15:0 formation (**Figure 13C**). While this data clearly provides evidence for the importance of PPARα in OCFA formation, it was necessary to further confirm that this was dependent on the enzyme HACL1. Again, using primary mouse hepatocytes, cells were treated with 50 nM siRNA targeted against *Hacl1* or non-targeting control and phospholipid LCFA profiles were determined. After confirming that siRNA treatment led to a significant reduction of *Hacl1* mRNA (**Figure 13E**), it was apparent that knockdown of this enzyme led to a significant reduction of C17:0 levels alone, while there was no effect on C15:0 (**Figure 13F**). In line with this decrease of C17:0 after the knockdown, there was additionally an increase in C18:0 levels (**Figure 13G**). Data presented here have been partially published (**Publication 3 – Publication List**).

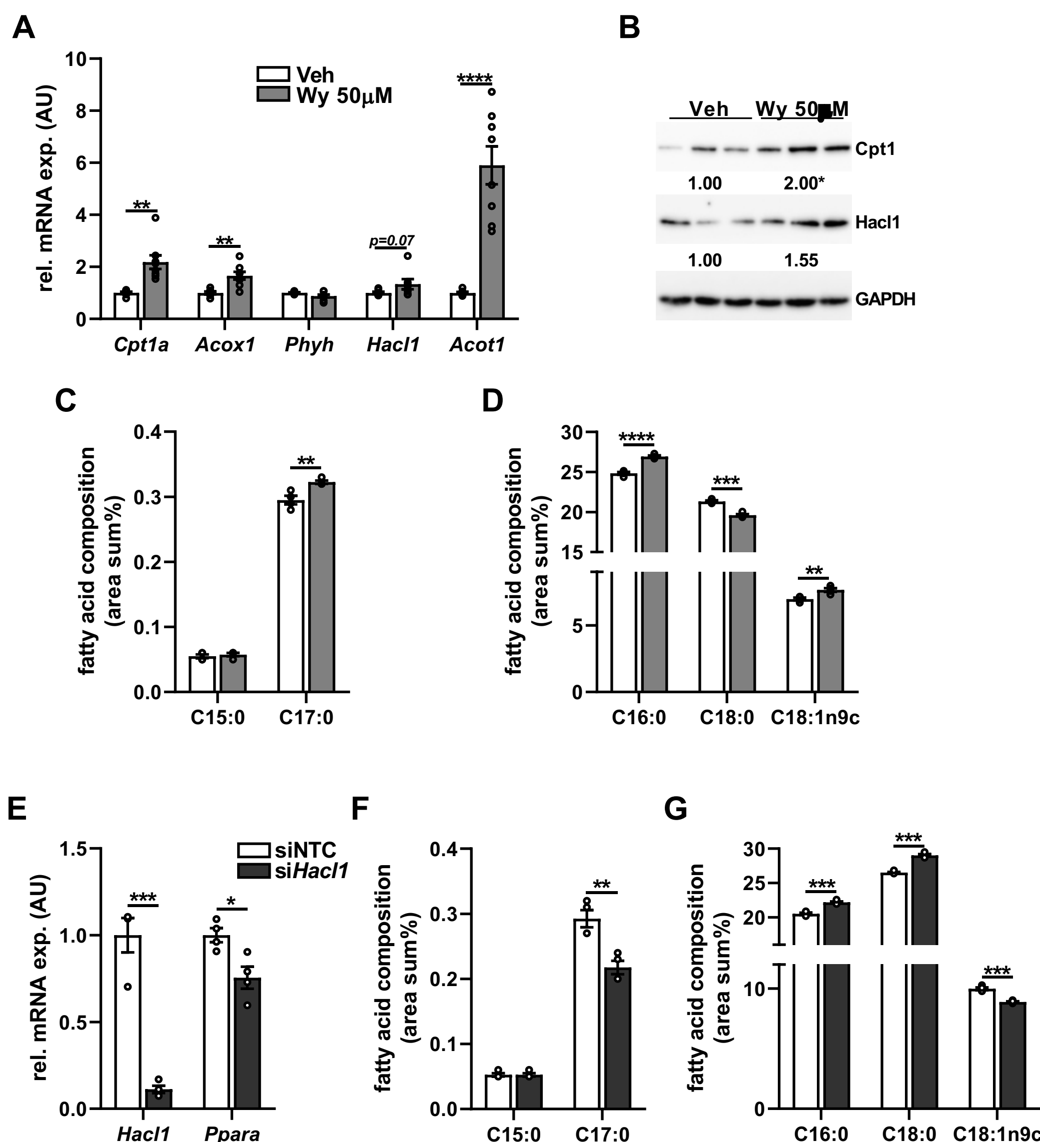


Figure 13. PPARα agonism increases hepatic odd-chain fatty acids through Hacl1 induction. (A) qPCR of PPARα-target genes in primary hepatocytes treated with 50 µM PPARa-agonist (Wy-14643) for 24 hr (n = 4). **(B)** Representative western blot for key FA oxidation enzymes after 24 hr WY treatment (n = 3). **(C, D)** Long-chain fatty acid (LCFA) composition of phospholipid (PL) fraction after 48 hr Wy treatment (n = 4). **(E)** qPCR of *Hacl1* and *Ppara* in primary hepatocytes treated with 50 nM siRNA targeting *Hacl1* for 24 hr (n = 4). **(F, G)** LCFA composition of PL fraction in primary hepatocytes treated with 50 nM siRNA targeting *Hacl1* for 48 hr (n = 4). LCFA results are expressed as area percentage of individual fatty acids to total area of detected fatty acids. Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared to respective control. C15:0, pentadecanoic acid; C17:0, heptadecanoic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:1n9c, oleic acid.

4.3.4 *In vitro* valine treatment increases OCFA formation in a PPARα-dependent manner

To determine the extent to which Val drives expression and action of PPARα, and to investigate whether it directly increases OCFA formation, primary mouse hepatocytes were treated with Val or Leu. After a 48 hr treatment with 0.5 mM of Val or Leu, increases

in C15:0 and C17:0 levels were observed in the Val group (**Figure 14A**). These increases were not, however, complimented with a decrease in C18:0 levels (**Figure 14B**), and no correlation was present between these two FA (**Figure 14C**). While 0.5 mM Val treatment did not lead to an increase in *Ppara* or *Hacl1* expression, it was apparent that increasing concentrations of Val led to a dose-dependent increase in *Ppara* expression (**Figure 14D**) while *Hacl1* gene expression was significantly elevated after treatment with 2 mM of Val (**Figure 14F**). Similar to the short-term *in vivo* study with BCAA (Study Ib), there were no effects of Val or Leu on *Cpt1* expression even at higher concentrations (**Figure 11E**).

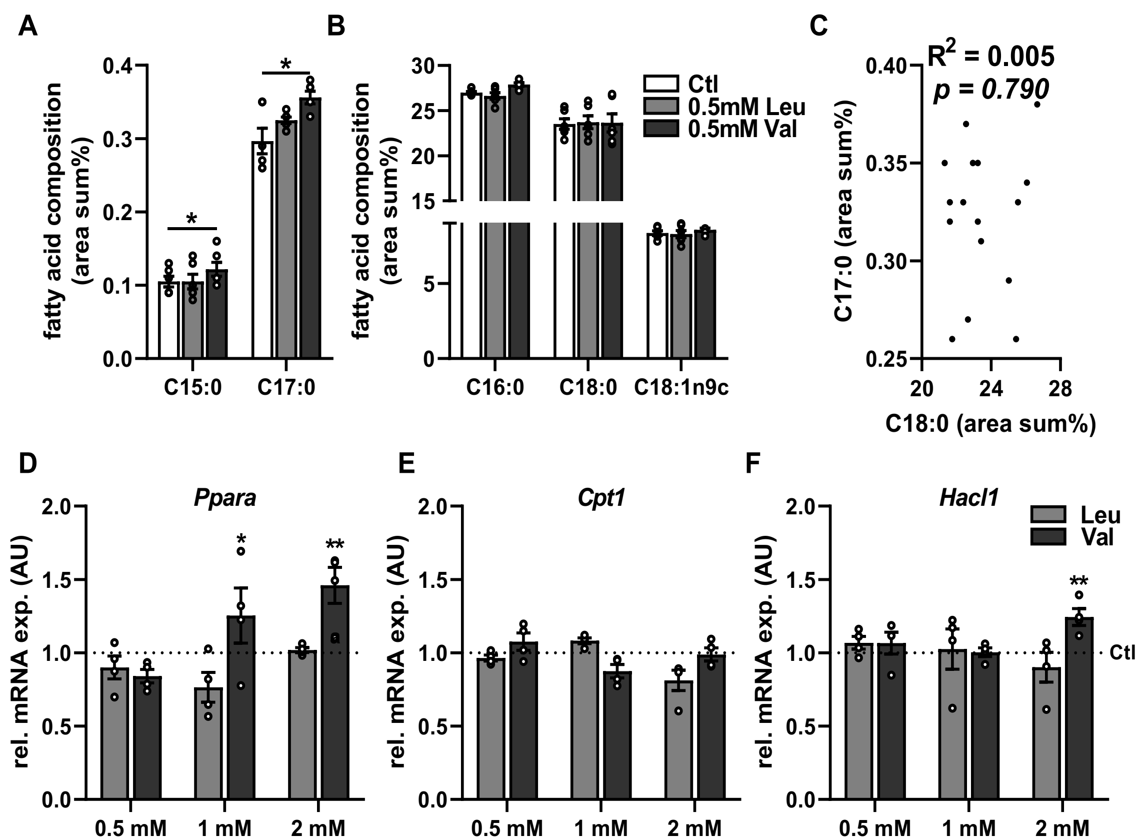


Figure 14. Valine treatment in primary hepatocytes increases OCFA and *Ppara* expression. (A,B) Long-chain fatty acid composition of phospholipid fraction after 48 hr Val or Leu (0.5 mM) treatment in primary hepatocytes (n = 4). **(C)** Pearson correlations of stearic acid (C18:0) with heptadecanoic acid (C17:0). **(D-F)** qPCR of *Ppara, Cpt1* and *Hacl1* in primary hepatocytes after 24 hr Val or Leu dose treatment (n = 4). Results are expressed as area percentage of individual fatty acids to total area of detected fatty acids. Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared to respective control. Ctl, control; C15:0, pentadecanoic acid; C16:0, palmitic acid; C18:1n9c, oleic acid.

In order to further confirm the role of PPARα in valine-induced OCFA formation, primary hepatocytes were pre-treated with the PPARα antagonist, GW6471, before subsequent Val treatment. LCFA composition of the phospholipid fraction showed that treatment with the antagonist prevented any increase in C15:0 or C17:0 levels after Val treatment, with no additional effects on C18:0 levels (**Figure 15A-C**). In line with this, *Ppara* and *Hacl1* levels were significantly decreased in all groups treated with the antagonist (**Figure 15D, E**), further underlining the role of PPARα in Val-mediated increases of OCFA.

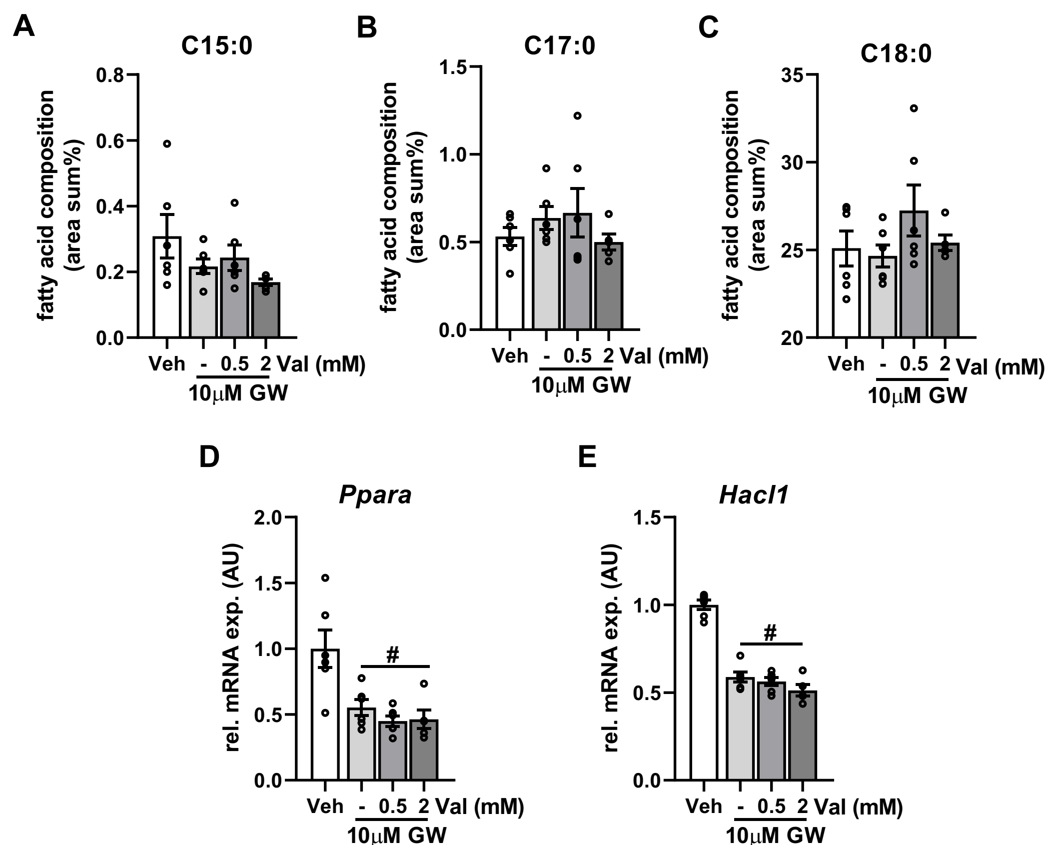


Figure 15. PPARα antagonist inhibits OCFA formation after valine treatment. (A-C) Long-chain fatty acid composition of phospholipid fraction in primary hepatocytes pre-treated with 10 μM PPARα antagonist (GW; GW6471) with subsequent 0.5-2 mM valine treatment for 48 hr (n = 6). **(D, E)** qPCR of *Ppara* (D) and *Hacl1*(E) after treatments for 24 hr (n = 6). Data are mean ± SEM and normalized to vehicle (Veh) treated cells. *p < 0.05; **p < 0.01; ***p < 0.001. # indicates significantly different from Vehicle (Veh) treatment. C15:0, pentadecanoic acid; C17:0, heptadecanoic acid; C18:0, stearic acid.

4.3.5 Connection of long-term milk protein & BCAA feeding in OCFA formation.

To further clarify the connection of dairy protein intake and the beneficial associations of OCFA, a 20-week feeding study was performed (Study IIb – Section 2.1.1). The study aims were two-fold here: (1) to further elucidate the long-term effects of a higher milk protein intake, as well as the roles of the individual BCAA (Leu and Val), in metabolic health and (2) to determine whether these supplementations impact OCFA formation on the long-term. Using the same diets as in the short-term, mice were fed experimental diets (milk protein – HFMP; Leu – HFL; Val – HFV) and control LF and HF diets for 20 weeks with metabolic characterization performed throughout. LCFA analysis of the phospholipid fractions of the liver, eWAT and plasma revealed that HFMP feeding was able to induce both C15:0 and C17:0 levels in the long term only in liver and plasma fractions (**Figure 16A, E**) and were rather decreased in eWAT (**Figure 16C**). These increases were independent of C18:0 levels (**Figure 16B, D, F**), suggesting these increases were likely driven more by FA synthesis using Pr-CoA as a primer for *de novo* lipogenesis in the liver, as opposed to being derived from α-oxidation. Interestingly, HFL fed mice also had increased circulating C17:0 levels compared to HF, whereas, HFV feeding had no impact on either liver or plasma OCFA levels (**Figure 16A, E**).

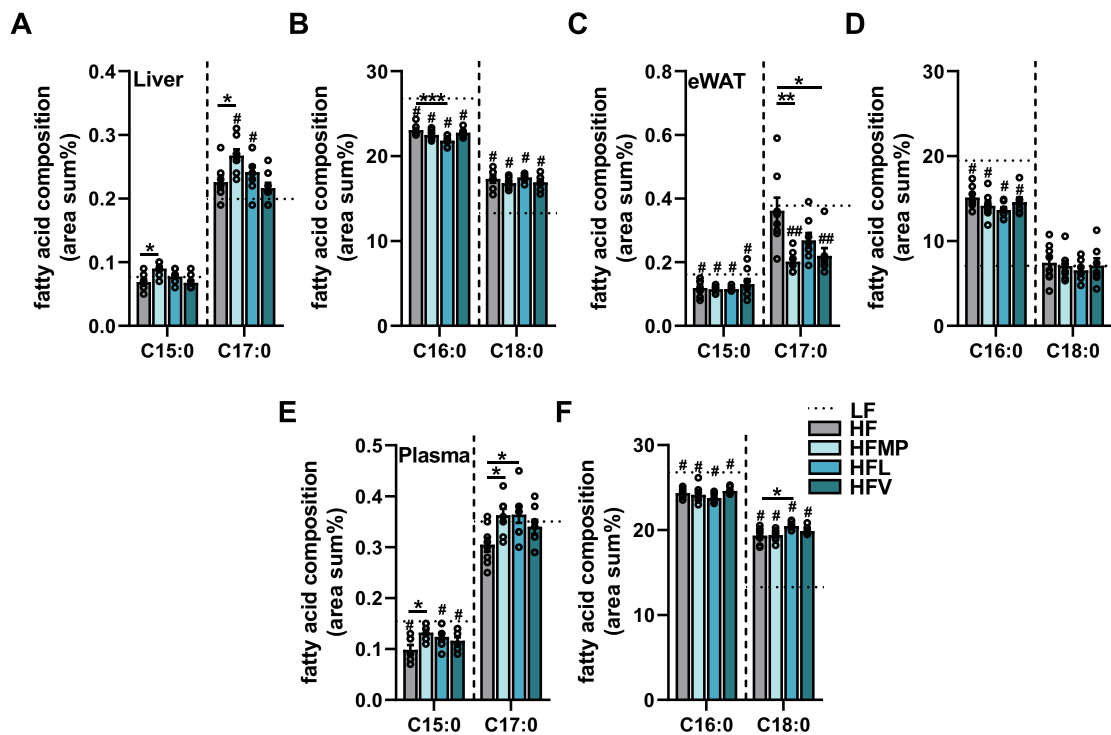


Figure 16. Long-term dairy protein feeding induces liver and plasma OCFA levels. (A, B) Long-chain fatty acid (LCFA) composition of odd-chain fatty acids (OCFA, C15:0 and C17:0) or even-chain fatty acids (ECFA, C16:0 and C18:0) in liver phospholipid (PL) fraction (n = 8). **(C, D)** LCFA composition of OCFA (C15:0 and C17:0) or ECFA (C16:0 and C18:0) in epididymal white adipose tissue (eWAT) PL fraction (n = 8). **(E, F)** LCFA composition of OCFA (C15:0 and C17:0) or ECFA (C16:0 and C18:0) in plasma PL fraction (n = 8). Results are expressed as area percentage of individual fatty acids to total area of detected fatty acids. Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. C15:0, pentadecanoic acid; C17:0, heptadecanoic acid; C16:0, palmitic acid; C18:0, stearic acid.

4.3.6 Long-term high-fat milk protein or individual BCAA supplementation elicit differential metabolic health outcomes

After 20 weeks, HF feeding, as expected, resulted in increased body weight, specifically due to an accumulation of fat mass, with no effect on lean body mass—HFMP and HFL feeding protected mice from this HF-induced weight gain (**Figure 17A-D**). On the contrary, while HFV did not significantly differ from HF-fed mice in body weight, there was a significant expansion of fat depots, more specifically of subcutaneous white adipose tissue (sWAT) (**Figure 17E**). At week 12 of the feeding intervention, mice were metabolically characterized using metabolic cages with indirect calorimetry. Here, data indicated that the differences in body weight gain were independent of food or energy intake, as there were no significant differences between the groups (**Figure 17F, G**). Locomotor activity, as well as respiratory exchange ratio (RER), were also not different among HF fed groups (**Figure 17H, I**). However, HFL feeding, apparently, led to a

significant increase in energy expenditure (EE) compared to HF in both the day and night phases (**Figure 17J**). Together, these data highlight the protective effects of a Leu supplementation or higher milk protein intake against the deleterious effects of HF diet-induced fat accumulation, which is not apparent after HFV feeding.

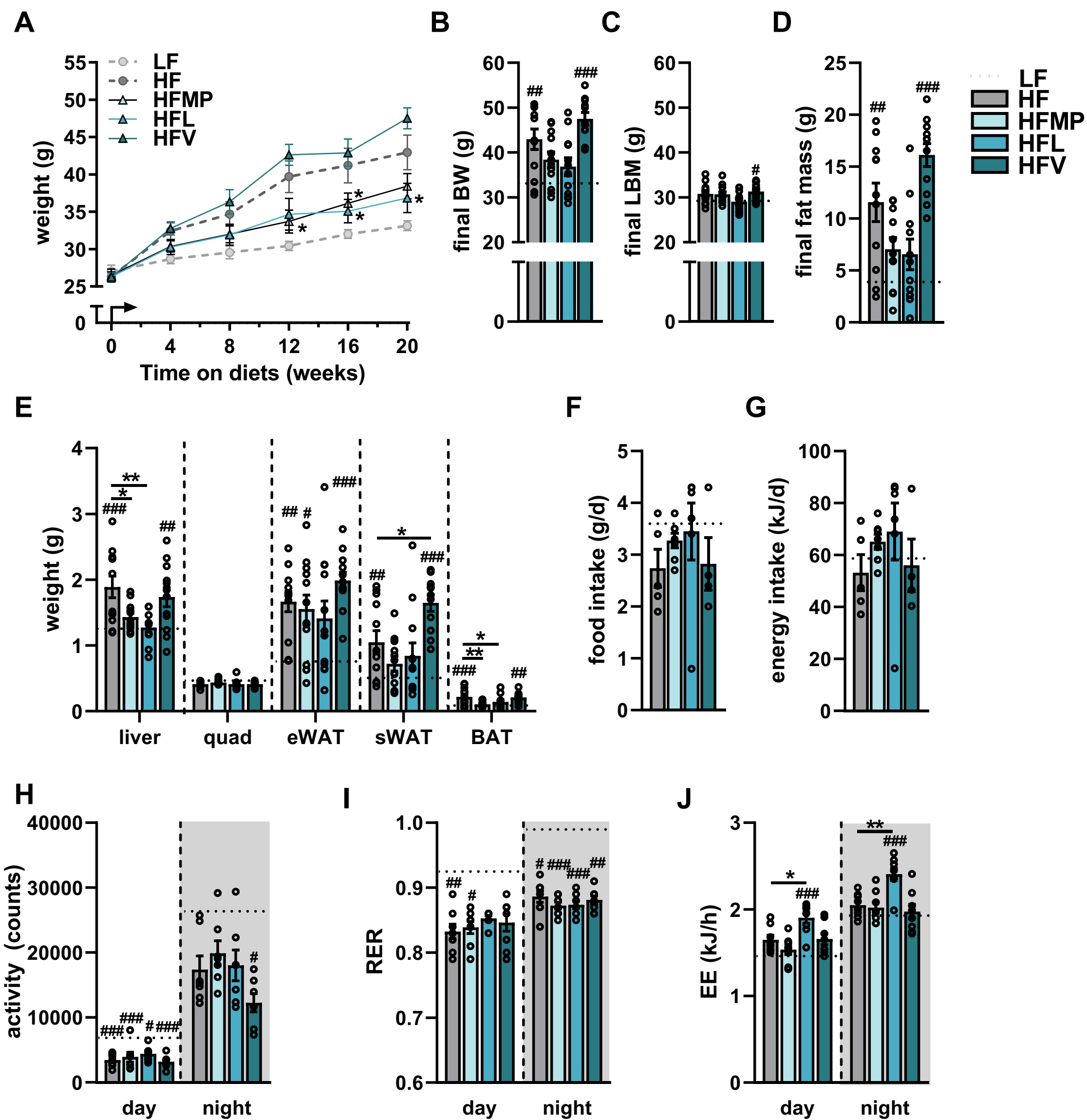


Figure 17. Differential long-term effects of milk protein or BCAA supplementation under high-fat diet conditions. (A) Body weight development in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks (n = 12). **(B)** Final body weight at 20 weeks (n=12). **(C)** Final calculated lean body mass (n = 12). **(D)** Final fat mass determined with NMR (n = 12). **(E)** Final tissue weights for liver, muscle and adipose tissue depots (n = 12). **(F)** Average food intake per day over 24 hr (n = 5-7). **(G)** Calculated energy intake per day from average food intake and energy content of diets. (n = 5-7). **(H)** Average locomotor activity (beam breaks) x1000 per 12 hr period (day and night phase) (n = 7). **(I)** Respiratory exchange ratio (RER; n = 8). **(J)** Average energy expenditure (EE) per hour per 12 hr period (day and night phase) (n = 8). Data are mean ± SEM, LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. BW, body weight; LBM, lean body mass; eWAT, epididymal white adipose tissue; sWAT, subcutaneous white adipose tissue; BAT, brown adipose tissue; quad, quadriceps muscle.

4.3.7 Respective protective & deleterious effects of Leu & Val on glucose tolerance.

While the role of Leu with regards to insulin sensitivity and glucose tolerance has been previously explored, little is known about the specific effects of Val. For that reason, oral glucose tolerances tests (oGTT) were performed at week 6 and 16, as a means to better understand their roles. While no differences were detected at week 6 of the intervention, HFL fed mice tended towards lower fasting insulin and glucose levels at week 16—no differences were observed for HFMP and HFV fed mice (**Figure 18A, B**). Glucose clearance was strongly delayed in the HFV group, which was apparent already after 6 weeks of intervention, but was exacerbated after 16 weeks, as seen in the incremental area under the curve (iAUC) (**Figure 18C, D**). These mice, however, exhibited comparable levels of insulin during the oGTT to the HF-fed mice, suggesting peripheral insulin resistance (IR) in the HFV mice (**Figure 18E, F**). Meanwhile, HFL feeding had no significant impact on glucose clearance compared to HF, but displayed insulin values similar to LF fed mice (**Figure 18C-F**) at week 16, indicating an improved insulin sensitivity. To further confirm the effect on insulin sensitivity, an intraperitoneal insulin tolerance test (i.p.ITT) was performed at week 16. HFL mice showed an insulin response similar to that of LF fed mice (**Figure 18G, H**). These experiments revealed no significant differences between HF and HFMP, suggesting no strong contribution of dairy protein intake alone on insulin sensitivity—possibly due to counteracting roles of Val and Leu on insulin sensitivity. However, through these interventions, it was possible to confirm the suggested protective effects of Leu, while highlighting a causal role for Val in glucose intolerance.

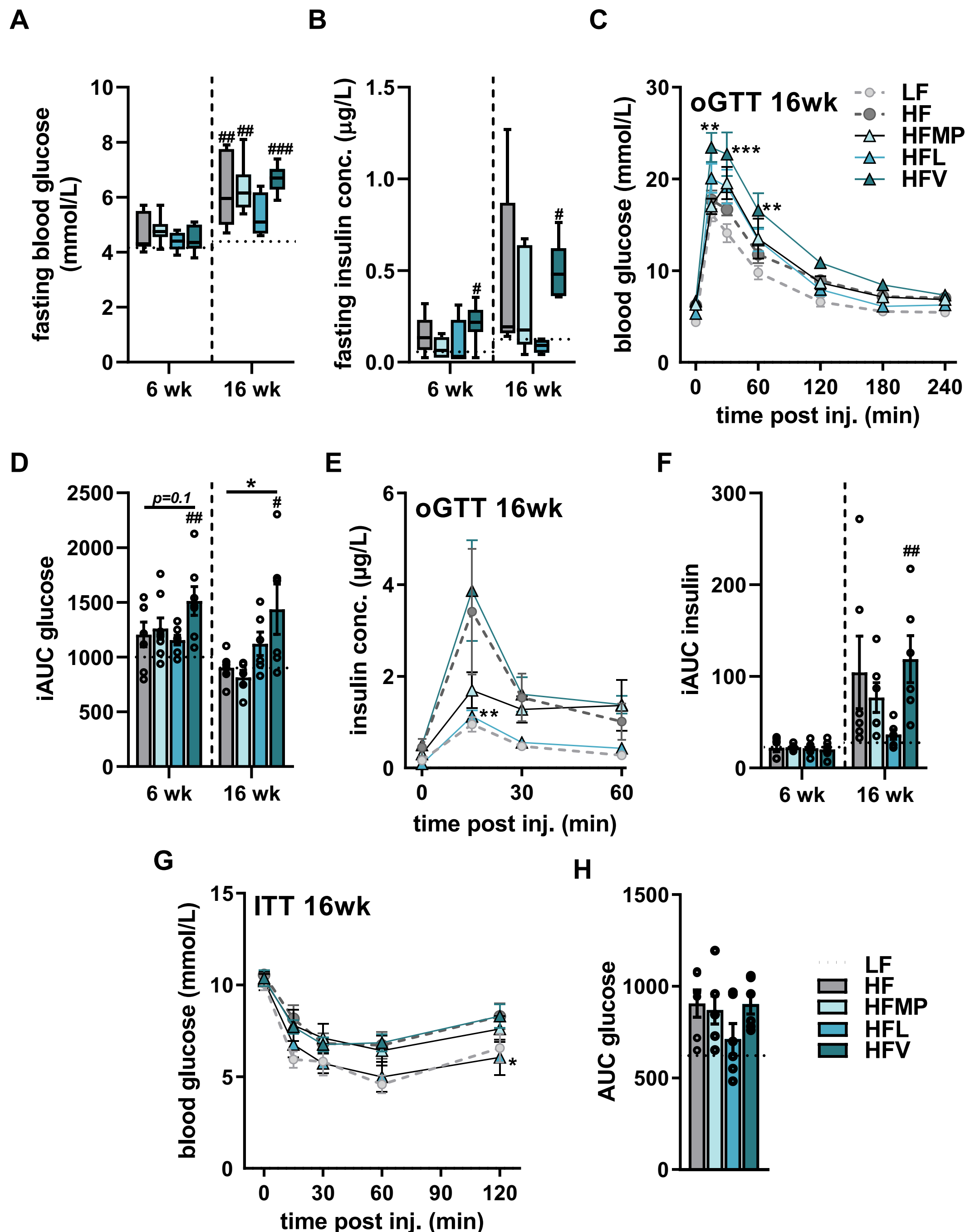


Figure 18. Glucose clearance is impaired after long-term valine feeding, independent of glucose stimulated-insulin secretion. (A) 16 hr fasting blood glucose in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 6 or 16 weeks (n = 6-8). **(B)** 16 hr fasting insulin at 6 or 16 weeks (n= 6-8). **(C)** Glucose measurements during an oGTT at 16 weeks of feeding (n = 6-8). **(D)** Incremental area under the curve (iAUC) of glucose levels during oGTT at week 6 or 16 of experiment (n = 6-8). **(E)** Insulin measurements during oGTT after 16 weeks of feeding (n = 6). **(F)** iAUC of insulin levels during oGTT at week 6 or 16 of experiment (n = 6-8). **(G)** Glucose measurements during an i.p. ITT at 16 weeks (n = 6). **(H)** Area under the curve (AUC) of glucose levels during an i.p. ITT (n = 6). Plasma data (A,B) are expressed as interleaved box and whiskers (min to max) plots. All other data are shown as mean ± SEM. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF.

4.3.8 Medium-term milk protein or individual BCAA supplementation in HF diets mirrors the long-term effects of diets

In an effort to further understand the effects of these supplementations on insulin sensitivity, a medium-term, 4-week feeding study was performed with these diets, with an acute injection of insulin at the end-point of the intervention (Study III – Section 2.1.1). There were no apparent differences between the groups in body weight development throughout this intervention; however, HFV-fed mice displayed a significantly increased fat mass accumulation (**Figure 19A-D**). Furthermore, while tending towards an increase in HFV-fed mice, there were no significant differences in the tissue weights between the HF groups, including liver weight, quadriceps and various adipose tissue depots (epididymal-, subcutaneous-, brown adipose tissue) (**Figure 19E**).

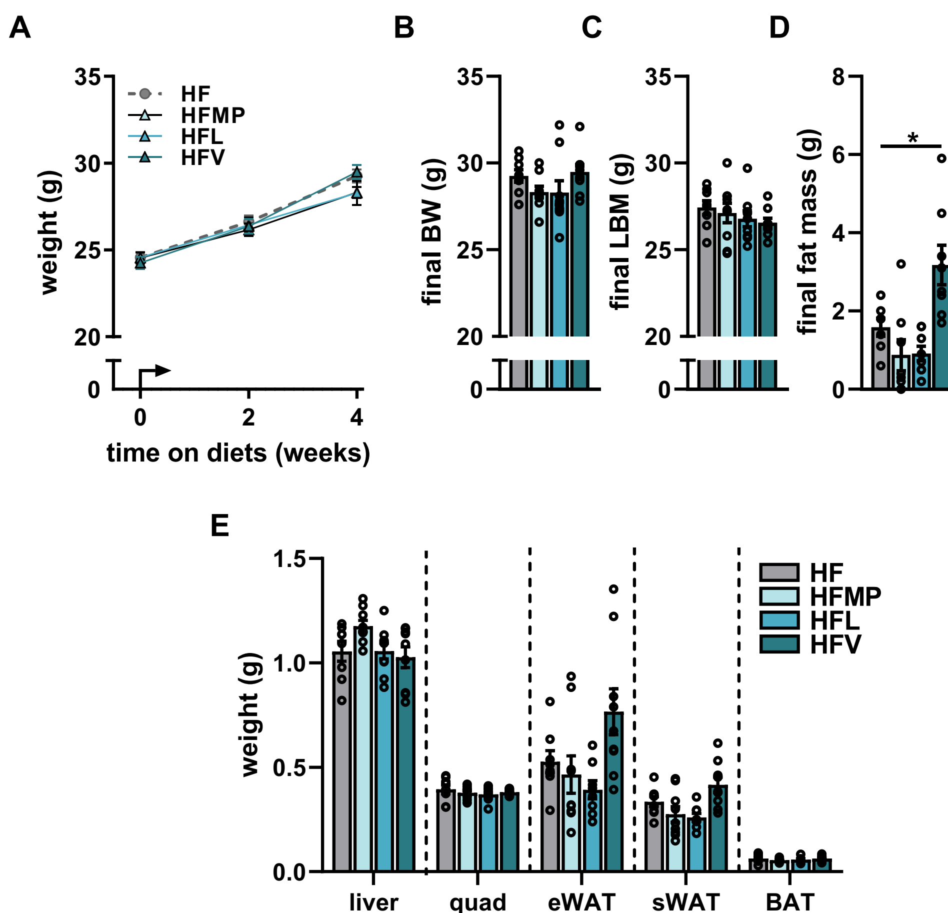


Figure 19 Medium-term supplementation of valine is sufficient for adipose tissue expansion. (A) Body weight development in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 4 weeks (n = 9-10). **(B)** Final body weight at 4 weeks (n = 9-10). **(C)** Final calculated lean body mass (n = 9-10). **(D)** Final fat mass determined with NMR (n = 9-10). **(E)** Final tissue weights for liver, muscle and adipose tissue depots (n = 9-10). Data are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF. BW, body weight; LBM, lean body mass; quad, quadriceps muscle; eWAT, epididymal white adipose tissue; sWAT, subcutaneous white adipose tissue; BAT, brown adipose tissue.

At the end of the intervention, mice were *i.p.* injected with insulin and tissues were subsequently harvested after 30 min, in order to determine the effects on insulin sensitivity in various insulin-responsive tissues. After 30 min, the change in blood glucose levels was not significantly different between the groups; however, HFV feeding did elicit the smallest change in glucose levels (**Figure 20A**). Upon investigation of the insulin signaling cascade, by means of western blotting for one of its key mediators protein kinase B (AKT), it was apparent that there were no differences in hepatic insulin signaling of these mice (**Figure 20B**). In the quadriceps muscle, however, glycogen synthesis appeared to be impaired as glycogen levels in HFV fed mice were lower when compared to HF (**Figure 20C**). Along these lines, AKT phosphorylation in quadriceps was significantly decreased after HFV feeding (**Figure 16D**). While both HFMP and HFL showed no significant differential effects, these disturbances in muscle insulin sensitivity of HFV-fed mice could partially explain the impaired glucose tolerance of these mice.

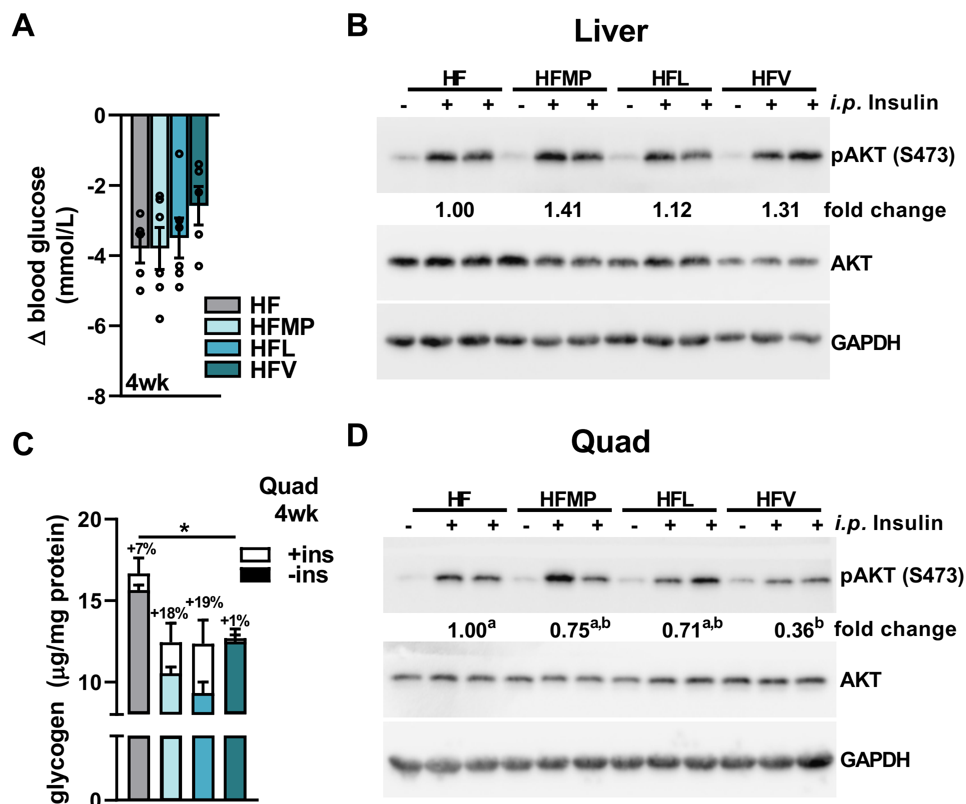


Figure 20. Medium-term valine feeding drives impairments in skeletal muscle insulin signaling. (A) Delta blood glucose after 30 min insulin stimulation from male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 4 weeks (n = 6). **(B)** Representative western blot of insulin stimulated (30min) AKT phosphorylation in liver of mice fed for 4 weeks (n = 6). **(C)** Quadriceps (Quad) glycogen levels after 30 min insulin stimulation from 4 week fed mice (n = 6). **(D)** Representative western blot of insulin stimulated (30 min) AKT phosphorylation in Quad of mice fed for 4 weeks (n = 6). Data are shown as mean ± SEM except for western blot quantification where only mean fold change is indicated below representative blots, normalized to HF. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF. Different letters represent significant difference between groups in Western blots. Quad, quadriceps. ins, insulin.

4.3.9 Differential effects of long-term dairy protein or BCAA supplementation on BCAA catabolism

As the functionality of the BCAA catabolic pathway could impact OCFA synthesis via Pr-CoA formation, it was important to characterize the molecular effects of these supplementations. Using both the diet composition and food intake data from the metabolic cage, it was calculated that the intake of Leu and Val was 2-fold higher in HFMP-fed mice compared to the HF group (**Figure 21A, B**). The calculated daily Leu and Val intake showed an approximately 6-fold and 4-fold increased intake by HFL and HFV feeding, respectively (**Figure 21A, B**). After 20 weeks of feeding, but not yet apparent after 4 weeks, HFMP feeding led to significantly higher fasting plasma concentrations of Leu and Ile, compared to HF, while HFL feeding induced only a mild (trending, *p=0.12*) increase in circulating Leu levels (**Figure 21C, D**). Strikingly, HFV

mice had a 4-fold increase in circulating Val levels, already evident after 4 weeks of feeding, with no effects on the levels of Leu or Ile (**Figure 21C, D**). Interestingly, the circulating Val concentrations at 20 weeks also correlated with final fat mass (R^2=0.371; *p<0.02*), sWAT weight at 20 weeks (R^2=0.417; *p<0.008*) and the iAUC of blood glucose levels from the oGTT at week 16 (R^2=0.535; *p<0.05*). Along with the increased Val levels, HFV feeding also led to an increase in circulating levels of the Val-derived metabolite 3-hydroxyisobutyrate (3-HIB) (**Figure 21E**).

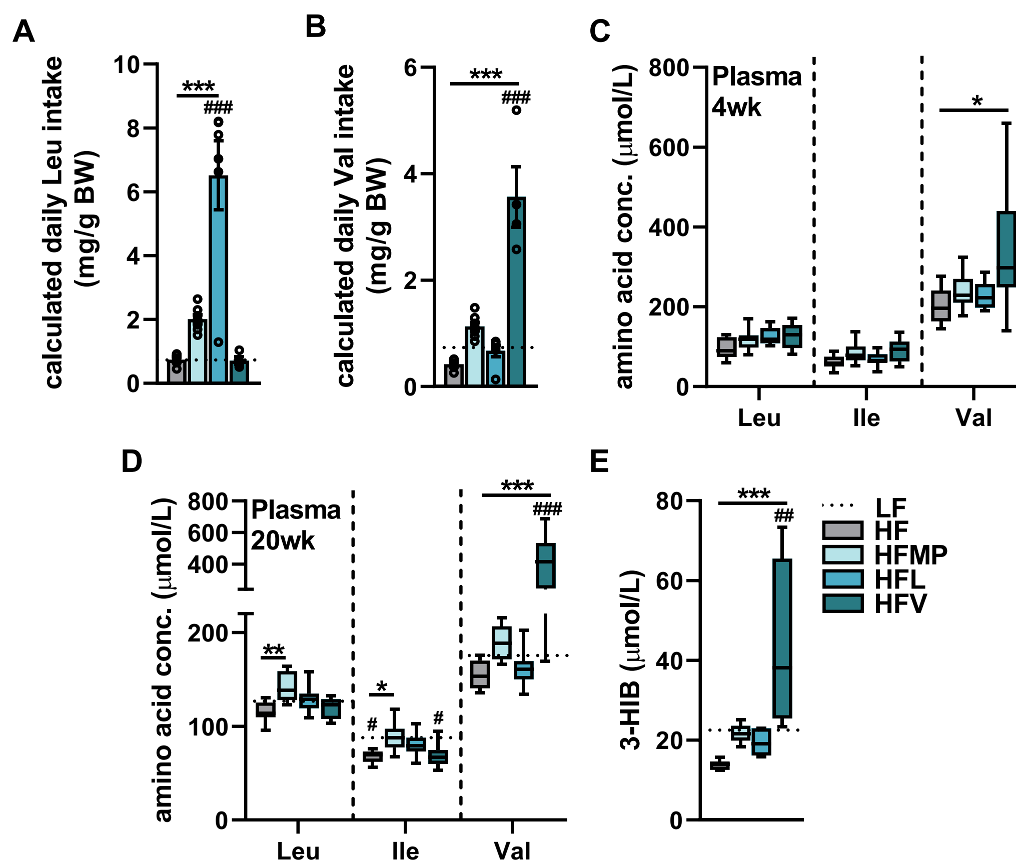


Figure 21. Accumulation of valine and its metabolite 3-hydroxyisobutyrate after valine supplementation. (A) Calculated daily leucine (Leu) intake for male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks (n = 8). **(B)** Calculated daily valine (Val) intake (n = 8). **(C)** 2 hr fasting plasma BCAA levels after 4 weeks (n = 8). **(D)** 2 hr fasting plasma BCAA levels after 20 weeks (n = 8). **(E)** 2 hr fasting plasma 3-hydroxyisobutyrate (3-HIB) levels at 20 weeks (n = 6). Plasma data (C-E) are expressed as interleaved box and whiskers (min to max) plots. All other data are shown as mean ± SEM. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. BW, body weight; Ile, isoleucine.

Gene expression analysis of the BCAA catabolic pathway revealed that HFMP and HFV feeding had no significant effects on expression of key genes in the liver; however, HFL feeding increased the expression of branched-chain amino transaminase (*Bcat2*) and protein phosphatase 1K (*Ppm1k*) (**Figure 22A**), the enzyme responsible for activating

the branched-chain keto-acid dehydrogenase (BCKDHA) via dephosphorylation. HF diet feeding led to an increase in this inhibitory phosphorylation of BCKDHA in the liver, which was reversed with HFMP and HFL feeding, but not with HFV feeding (**Figure 22B**)—suggesting that hepatic BCAA catabolism in both HFMP- and HFL-fed mice is protected from HF-induced disturbances. After HFV feeding, BCAA catabolism appears to be shunted towards the skeletal muscle—these mice exhibited an increased muscle gene expression of one of the BCAA transporters (*Slc3a2*) and certain key catabolic genes (*Bcat2, Bckdh, Ppm1k, Hadha*) (**Figure 22C**). To this point, the phosphorylation of BCKDHA was also unaffected (**Figure 22D**).

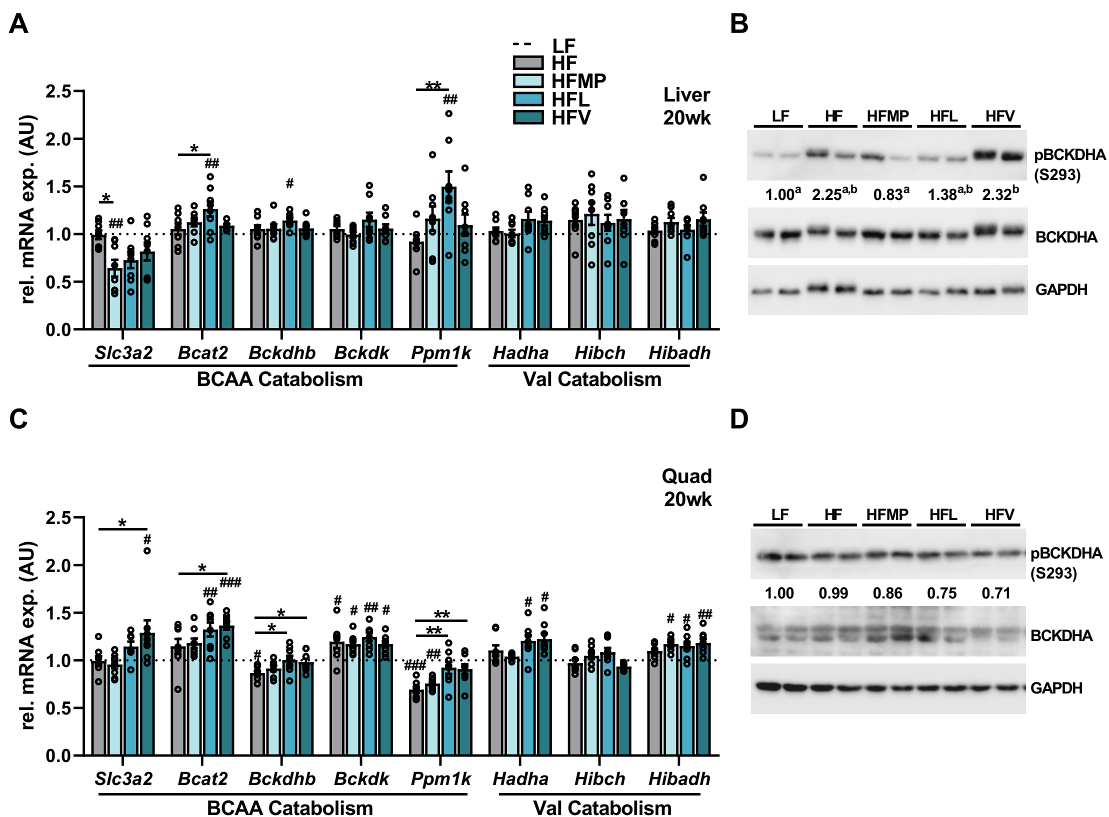


Figure 22. Valine supplementation increases BCAA catabolic gene expression in skeletal muscle. (A) qPCR of key genes in BCAA oxidation of male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks, normalized to LF in liver with *B2m* as reference gene (n = 8). **(B)** Representative western blot of BCKDHA phosphorylation in liver normalized to LF (n = 6). **(C)** qPCR of key genes in BCAA oxidation normalized to LF in quadriceps (Quad) muscle at 20 weeks with *B2m* as reference gene (n = 8). **(D)** Representative western blot of BCKDHA phosphorylation in Quad normalized to LF (n = 6). Data are shown as mean ± SEM except for western blot quantification where only mean is indicated below representative blots. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. Different letters represent significant difference between groups in Western blots.

4.3.10 Long-term hepato-protective effects of dairy protein & Leu supplementations in HF diets

The liver is a key metabolic hub important for the intersecting of fatty acid processing / synthesis and amino acid catabolism. While HF feeding, as well as HFV, appeared to have an impaired hepatic BCAA catabolism, both HFMP and HFL feeding protected from the HF-induced hepatic deteriorations. To further clarify the protective aspects of these diets with regards to hepatic lipid metabolism, gene and protein analysis were performed. As expected, HF increased the ectopic hepatic fat accumulation, which was prevented by both HFMP and HFL, but not by HFV feeding (**Figure 23A**). These findings were supported by the decreased triglyceride concentrations (**Figure 23B**). The effects of long-term HFMP and HFL feeding also protected from HF-induced upregulation of several genes of inflammation and fibrotic markers in the liver (**Figure 23C).** The hepato-protective effects were, at least partially, mediated by the downregulation of gene and protein expression of a key FA transporter, CD36, after milk protein or Leu supplementation (**Figure 23D, E**). HFV feeding, on the other hand, appeared to induce additional FA transport gene expression (*Fatp3, Fatp4*) and protein levels of CD36 (**Figure 23D, E**)—however, this had no overall effect on hepatic lipid content. Unlike the short-term BCAA feeding study (Study Ib), long-term HFV feeding had no apparent effect on *Ppara* or *Hacl1* gene expression (**Figure 23F**). Altogether, these findings highlight the beneficial effects of HFMP feeding, which may be mediated at least in part by the actions of Leu.

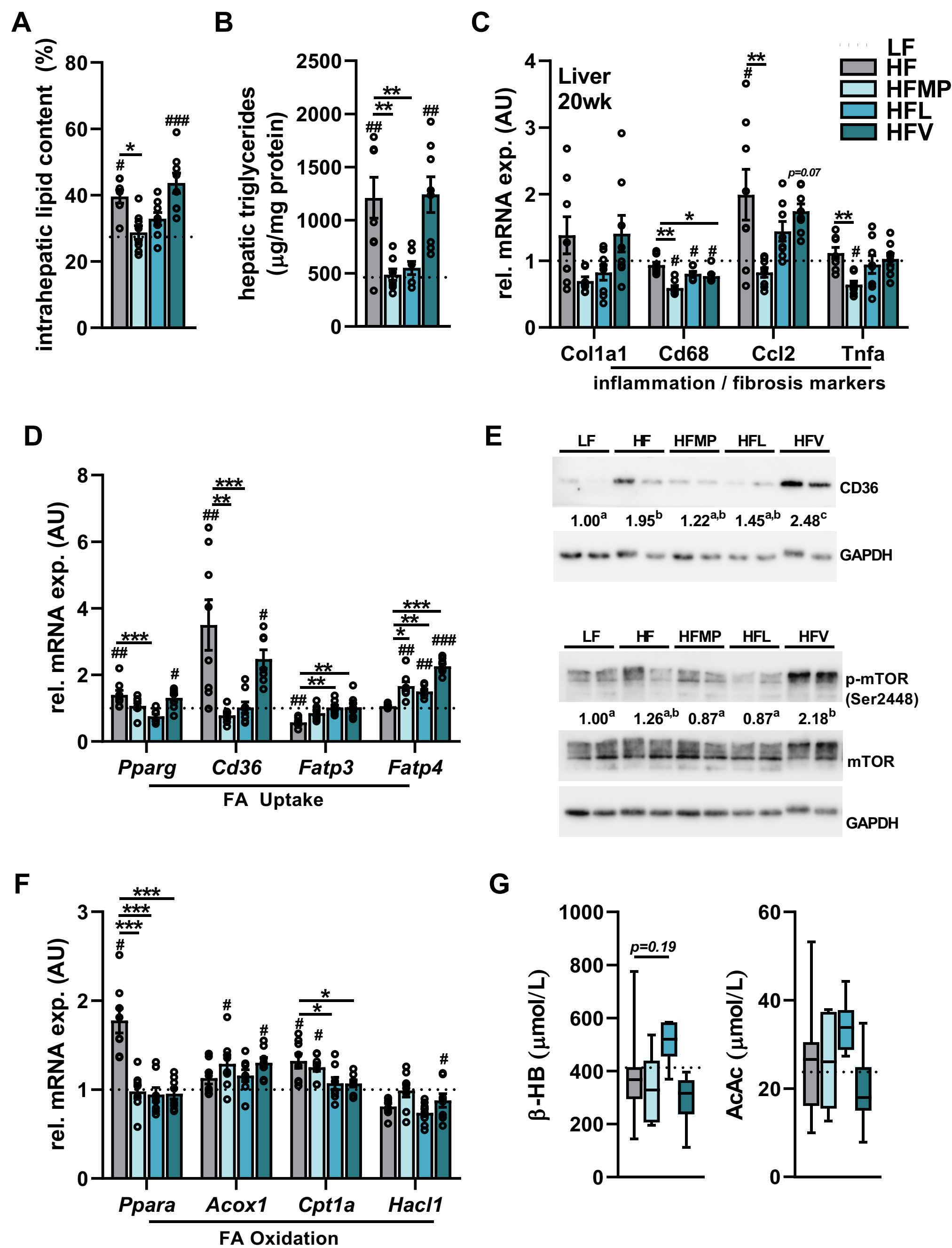


Figure 23. Milk Protein & leucine supplementation protect from long-term HF-induced hepatic fat accumulation and inflammation. (A) Relative liver fat content as determined by H&E staining in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks (n = 8). **(B)** Liver triglycerides (n = 8). **(C)** qPCR of key genes in inflammation / fibrosis normalized to LF in livers at 20 weeks with *B2m* as reference gene (n = 8). **(D)** qPCR of key genes in FA uptake normalized to LF in livers at 20 weeks with *B2m* as reference gene (n = 8). **(E)** Representative western blot of FA transport protein CD36 and mTOR phosphorylation in liver at 20 weeks normalized to LF (n = 6). **(F)** qPCR of key genes in FA oxidation normalized to LF in livers at 20 weeks with *B2m* as reference gene (n = 8). **(G)** 2 hr fasting plasma ketone bodies levels at 20 weeks (n = 6). Plasma data (G) are expressed as interleaved box and whiskers (min to max) plots. All other data are shown as mean ± SEM except for western blot quantification where only mean is indicated below representative blots. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 within groups and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. Different letters represent significant difference between groups in Western blots. β-HB, β-hydroxybutyrate; AcAc, acetoacetate.

4.3.11 Valine metabolite 3-HIB driven glucotoxicity impairs insulin signaling

Given the compromised insulin signaling in skeletal muscle of HFV-fed mice, further investigation was carried out to unravel the underlying mechanism for this impairment. After 20 weeks of feeding, HFV mice had increased basal glycogen levels, and tended towards an increased circulating lactate (**Figure 24A, B**). In line with this, gene expression of the basal glucose transporter, *Glut1*, was significantly upregulated after HFV feeding, while *Glut4* expression was downregulated in all HF groups compared to LF (**Figure 24C**). Indicative of increased intracellular glucose-6-phosphate (G6P) levels, HFV-fed mice also had the highest levels of diacylglycerides (DAG), with no difference in TG levels compared to HF (**Figure 24D, E**). While membrane localization of protein kinase C-θ (PKC θ), a negative regulator of insulin receptor substrate 1 (IRS1) increases with increasing levels of DAGs (Timmers, Schrauwen et al. 2008), there were no differences between HF- and HFV-fed mice (**Figure 24F, G**)—rather it was reflective of TG content. Interestingly, HFV feeding did, however, lead to an upregulation of glucotoxicity stress marker Tribbles 3 (*Trib3*), a regulator of AKT (Zhang, Wu et al. 2016) (**Figure 24H**).

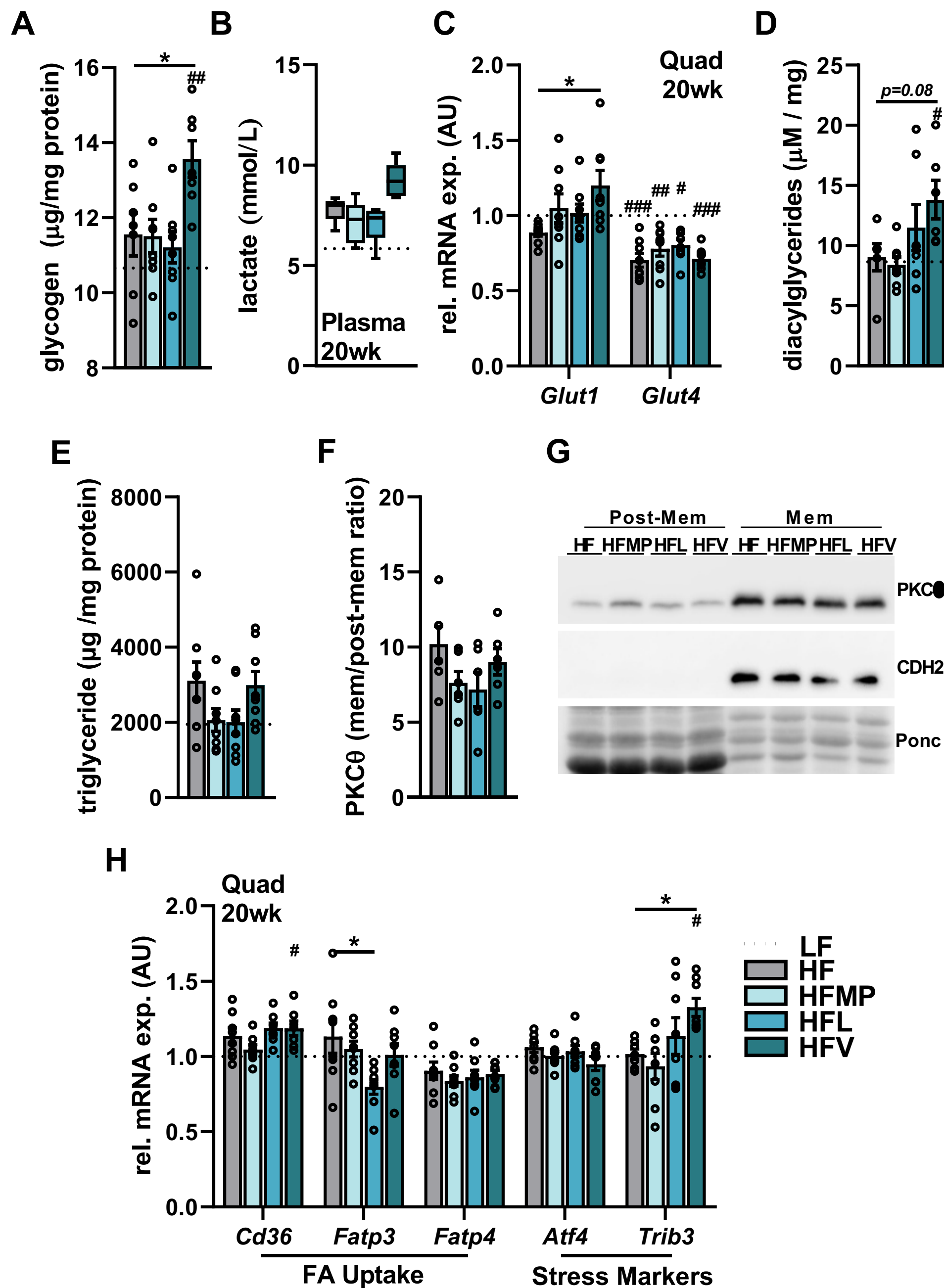


Figure 24. Valine feeding induces glucotoxicity in skeletal muscle. (A) Quadriceps (quad) glycogen levels in male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks (n = 8). **(B)** 2 hr fasting plasma lactate levels at 20 weeks (n = 6). **(C)** qPCR of key genes in glucose transport normalized to LF in quad at 20 weeks with *Hprt* as reference gene (n = 8). **(D)** Quad diacylglyceride levels at 20 weeks (n = 6-7). **(E)** Quad triglyceride levels at 20 weeks (n = 8) **(F)** Ratio of PKCθ localization from membrane (mem) to post membrane (post-mem) fraction (n = 6) **(G)** Representative western blot of PKCθ membrane fractionation normalized to LF. **(H)** qPCR of key genes in FA uptake and ER stress normalized to LF with *Hprt* as reference gene (n = 8). Plasma data (B) are expressed as interleaved box and whiskers (min to max) plots. All other data are shown as mean ± SEM except for western blot quantification where only mean of signal or fold change is indicated below representative blots. LF is represented as dotted line. *p < 0.05; **p < 0.01; ***p < 0.001 compared to HF and #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LF. FA, fatty acids.

In order to test for a direct effect of Val or its metabolite 3-HIB in driving glucotoxicity, differentiated C2C12 mouse myotubes were used. After 24 hr treatment with 2 mM Val or 3-HIB, there was a significant upregulation of both *Glut1* and *Trib3* gene expression under lipid loaded conditions (i.e. in the presence of 250 μM palmitic acid (PA)) (**Figure 25A**). This upregulation of *Glut1* by both treatments also led to a significant upregulation of basal glucose uptake *in vitro* (**Figure 25B**). Both Val and 3-HIB treatments led to significant reductions in AKT phosphorylation after acute insulin stimulation (**Figure 25C**). To confirm whether these effects were dependent on the upregulated basal glucose uptake, a GLUT1 inhibitor (Bay-876) was used in combination with the treatments. While Val and 3-HIB still led to upregulated *Glut1* expression, Bay treatment was able to reverse the *Trib3* increases (**Figure 25D, E**). Treatment with this GLUT1 inhibitor was also able to rescue the 3-HIB-mediated decrease in AKT signaling (**Figure 25F**).

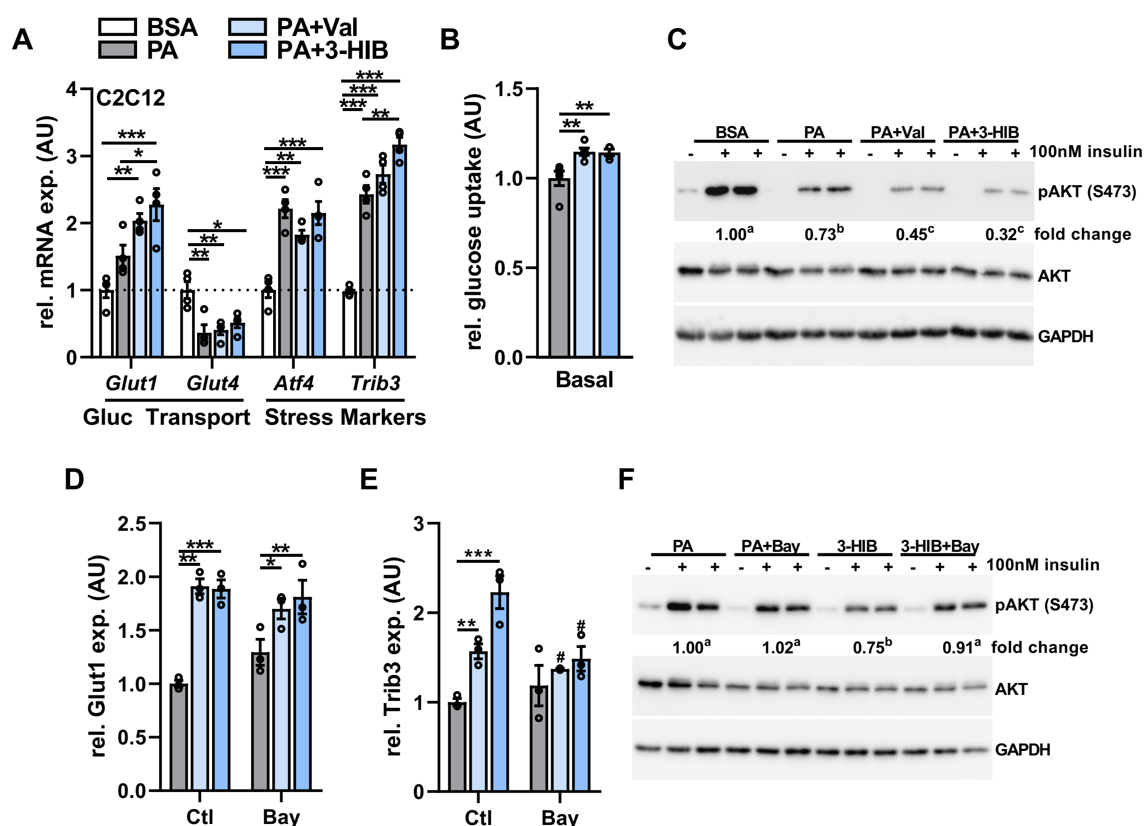


Figure 25. Valine-metabolite, 3-HIB, drives basal glucose uptake and glucotoxicity-mediated impairment of myotube insulin signaling. (A) qPCR of glucose transporters and stress markers in C2C12 mouse myotubes after treatment with 2 mM Val or 3-HIB in presence of 250 µM PA for 24 hr normalized to BSA control with *B2m* as reference gene (n = 4). **(B)** Relative basal glucose uptake in differentiated C2C12 mouse myotubes after treatment with 2 mM valine (Val) or 3-HIB for 48 hr normalized to PA (n = 8). **(C)** Representative western blot of insulin stimulated (15 min) AKT phosphorylation in differentiated C2C12 mouse myotubes after treatment with 2 mM Val or 3-HIB for 48 hr normalized to BSA control (n = 4). **(D-E)** qPCR of glucose transporter and stress markers in C2C12 mouse myotubes after treatment with 2 mM Val or 3-HIB in presence of 250 µM PA and 25 nM BAY876 (Bay) for 24 hr normalized to PA-Ctl with *B2m* as reference gene (n = 4). **(F)** Representative western blot of insulin stimulated (15 min) AKT phosphorylation in differentiated C2C12 mouse myotubes after treatment with 2 mM Val or 3-HIB in presence of 25 nM BAY876 normalized to PA control (n = 4). Fold change in Western blots (C, F) refers to insulin induced change normalized to BSA control (C) or PA control (F). Data are shown as mean ± SEM except for western blot quantification where only mean of fold change is indicated below representative blots.*p < 0.05; **p < 0.01; ***p < 0.001 compared to respective control and .#p < 0.05 comparing Ctl to Bay treated groups. Different letters represent significant difference between groups in Western blots. BSA, bovine serum albumin; PA, palmitic acid; Bay, BAY-876.

4.3.12 OCFA levels demonstrate inverse associations with metabolic health outcomes

Data from these long-term feeding studies confirmed that a higher dairy protein intake in an obesogenic diet can induce OCFA formation *in vivo* (see **Figure 16**). In line with previous findings, correlations between the circulating OCFA (C15:0 and C17:0) and certain metabolic health parameters were observed. Namely, C15:0 had a strong inverse association with final fat mass, fasting insulin levels, and liver fat accumulation (**Figure 26A**). On the other hand, C17:0 only exhibited a weaker correlation with final fat mass (**Figure 26B**). Furthermore, certain circulating amino acids, which biochemically can also contribute to the Pr-CoA pool—thus, likely contributing to OCFA formation— were also correlating with C15:0 levels. These included: isoleucine (R^2=0.159; $p<0.02$), methionine (R^2=0.144; $p<0.02$) and threonine (R^2=0.128; $p<0.03$), but not valine levels (R^2=0.053; $p=0.158$).

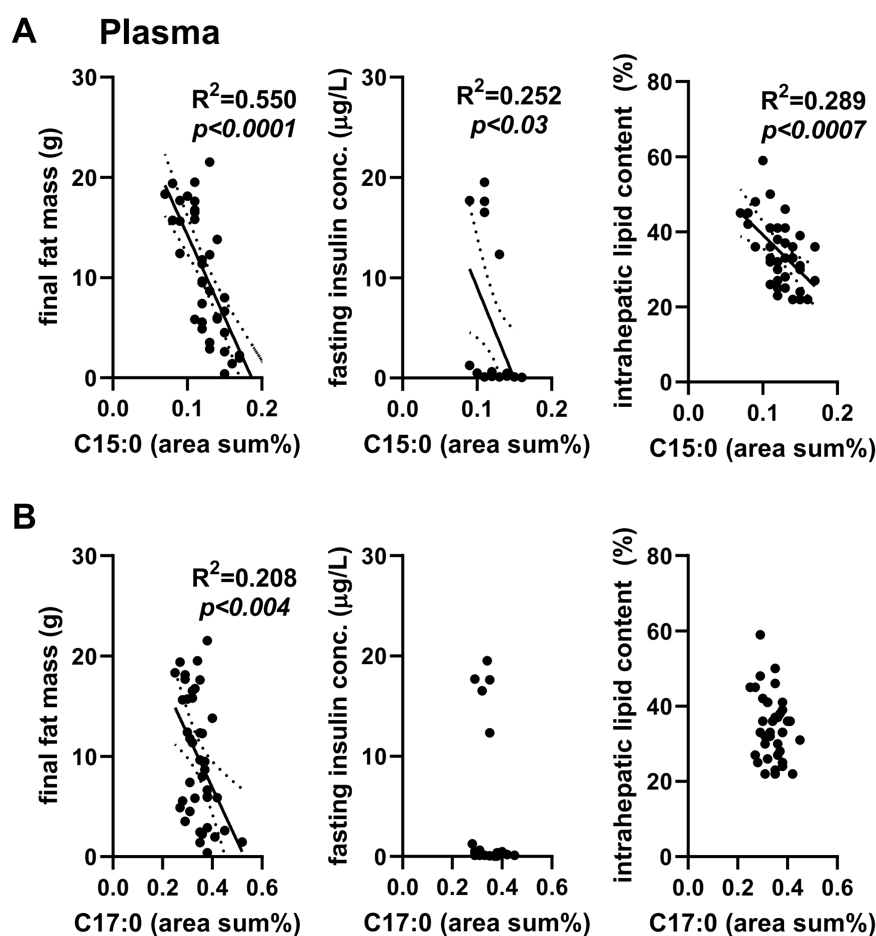


Figure 26. OCFA exhibit inverse associations with certain metabolic parameters. (A, B) Pearson correlations of pentadecanoic acid (C15:0) or heptadecanoic acid (C17:0) in plasma phospholipid fraction with final fat mass, fasting insulin concentration, and intrahepatic lipid content.

5 Discussion

In recent years, odd-chain fatty acids (OCFA), namely pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), and their role / involvement in metabolic health have gained mounting interest. Initially, OCFA had been suggested as biomarkers of dietary dairy fat intake; however, increasing numbers of studies have demonstrated the inverse association of these fatty acids with numerous metabolic disturbances including: adiposity (Aglago, Biessy et al. 2017), CVD (Khaw, Friesen et al. 2012), T2DM (Forouhi, Koulman et al. 2014) and NASH (Yoo, Gjuka et al. 2017). While these saturated fatty acids (SFA) (C15:0 and C17:0) are present in whole fat milk—they represent only 1% and 0.5%, respectively, of the total fatty acids—recent studies indicate that endogenous production from additional sources needs to be further investigated. Intake of dairy products is also generally described as having an inverse association with incidence of the metabolic syndrome and related disease (McGregor and Poppitt 2013, O'Connor, Lentjes et al. 2014, Jakobsen, Trolle et al. 2021); however, the biological mechanisms are generally unclear.

Thus, this PhD thesis aimed at determining sources of and addressing the direct role for OCFA in metabolic health, in connection with dairy intake—both dairy fat and dairy protein. Both short-term (1 week) and long-term (20 week) mouse feeding studies under obesogenic conditions (HF diets) were utilized to (1) confirm that both milk fat and milk protein induce OCFA levels *in vivo* and that (2) the increases in OCFA by milk protein supplementation could in part be explained by catabolism of the BCAA valine (Val) to propionyl-CoA (Pr-CoA) and PPARα-induced α-oxidation, but only on the short-term. Despite both milk protein and milk fat inducing hepatic and circulating OCFA levels, (3) only milk protein demonstrated beneficial long-term effects with regards to adiposity and hepatic fat accumulation, likely mediated in part by Leu. Finally, these data further (4) revealed no significant role for C17:0 in prevention of HF-induced health impairments, even with correlations of this FA with positive health outcomes.

5.1 Dietary sources of OCFA under short-term obesogenic conditions

Unraveling the dietary sources of OCFA and the action of these source metabolites is key in determining whether the beneficial associations attributed to these FA are a result of a direct causal action or rather simply a correlation. The discrepancy between the ratio of OCFA in milk fat (2 to 1) versus the ratio present in humans (1 to 2) calls into question the validity of the OCFA as a biomarker merely for dairy fat intake. Herein, this thesis

showed that supplementation of either dairy fat (HFMF) or dairy protein (HFMP) can induce both hepatic phospholipid C15:0 and C17:0 levels on the short-term in Study Ia (**Figure 5F**), highlighting that phospholipid OCFA levels can be influenced by additional sources apart from just dairy fat. This point has been recently an expanding topic of research, as studies have shown the induction of OCFA levels after dietary fiber supplementation, or its microbial fermentation product propionate—a short-chain fatty acid (SCFA), via elongation from Pr-CoA (Weitkunat, Schumann et al. 2017, Weitkunat, Stuhlmann et al. 2017). These studies highlighting the impact of dietary fiber and SCFA on OCFA levels further support the data demonstrating an association of OCFA with fiber-rich foods (Forouhi, Koulman et al. 2014). Furthermore, endogenous production of C17:0 through the single carbon removal process of α-oxidation, via the enzyme HACL1, was recently shown to be an additional modifier of C17:0 levels only (Jenkins, de Schryver et al. 2017).

The studies showing an induction of OCFA by dietary fiber and SCFA supplementation posited that these increases in OCFA were through Pr-CoA as a primer for fatty acid synthesis (Weitkunat, Schumann et al. 2017), similarly to OCFA synthesis described in ruminants (Massart-Leen, Roets et al. 1983). This hypothesis is supported by the fact that in two different inherited diseases of propionyl-CoA metabolism, propionic acidemia and methylmalonic acidemia, where the genes converting propionyl-CoA to enter the TCA cycle are absent, patients present with elevated levels of propionyl-CoA and OCFA (Wendel, Baumgartner et al. 1991, Sperl, Murr et al. 2000). Dietary management guidelines for these disorders are primarily a low protein intake, to limit exposure to Pr-CoA precursor amino acids including Val, Ile, Met, and Thr (Baumgartner, Horster et al. 2014)—further supporting the thought that increased protein intake, composed of roughly 20% branched-chain amino acids (Val, Ile, Leu) (Layman and Walker 2006), impacts hepatic OCFA levels.

In line with the increases after HFMP feeding, short-term Val supplementation in HF diet (HFV – Study Ib) was also able to induce hepatic phospholipid OCFA levels (both C15:0 and C17:0), with a concomitant decrease in stearic acid (C18:0) levels (**Figure 11F, G**). On one hand, these changes are likely via an increase in Pr-CoA for *de novo* lipogenesis—as discussed, Val catabolism contributes fully to the Pr-CoA pools while Leu just to acetyl-CoA (Ac-CoA) and Ile to both Pr-CoA and Ac-CoA pools (**Figure 2A**). Despite the fact that there were no differences between groups in Study Ib with regards to fatty acid synthase activity, this thesis could prove that Ac-CoA and Pr-CoA elicit the same activity of this enzyme when used as primers for fatty acid synthesis (**Figure 12I**).

As previously mentioned, the catabolism of other amino acids, as well as the nucleobase thymine, can contribute to the pools of Pr-CoA (**Figure 2B**)—thus, the increases in OCFA in the HFMP feeding could also be attributed to these amino acids, including Val.

While the involvement of HACL1 in endogenous production of C17:0 has been previously demonstrated *in vivo* (Jenkins, de Schryver et al. 2017), transcriptional regulation of this event had not been shown. As PPARα is a key mediator of FA oxidation (Guillou, Martin et al. 2008), it was possible to demonstrate that activation of PPARα activity with the potent agonist Wy-14643 could induce C17:0 levels in vitro, with no effect on C15:0 (**Figure 13C**). Along these lines, HFV feeding induced both *Ppara* and *Hacl1* expression, likely driving an induction of α-oxidation in these mice, as indicated by the inverse association between C17:0 and C18:0 levels (**Figure 12A, 11H**). Important to point out is the fact that HFV feeding did, however, still induce C15:0 levels, suggesting the importance of both (1) de novo OCFA synthesis via Pr-CoA and (2) α-oxidation in endogenous OCFA synthesis.

Additionally, what drives the Val-mediated *Ppara* induction remains unclear, as this could be mediated by multiple pathways. For example, *Acot1*, a thioesterase controlling the rate limiting step of CoA liberation from palmitoyl-CoA to yield palmitate has been implicated in controlling PPARα action by regulating availability of FA in fasting conditions (Franklin, Sathyanarayan et al. 2017), and is highly upregulated by HFV feeding (**Figure 12F**). It remains a possibility that certain FA produced by hepatocytes may be driving this increase in expression and action as well. So called “new fat”, endogenously synthesized, has been shown to elicit stronger effects on hepatic metabolism as opposed to “old fat”, i.e. FA derived from adipose tissue lipolysis (Chakravarthy, Pan et al. 2005, Sanderson, Degenhardt et al. 2009). In part, we could confirm that neither C17:0 nor oleic acid (C18:1n9c) treatment of primary hepatocytes elicited an induction of *Ppara* or *Hacl1* expression (data shown in: (Bishop, Schulze et al. 2020); however, treatment with Val in increasing concentrations led to a dose-dependent increase in both (**Figure 14D, F**). It is also possible that a molecule in the Val catabolic pathway elicits these responses, namely, 3-aminoisobutyrate (BAIBA) (**Figure 2B**) a metabolite that has been previously described as a strong activator of hepatic PPARα and driver of FA oxidation (Roberts, Bostrom et al. 2014).

While HFV feeding (Study Ib) did lead to an induction of the PPARα-HACL1 axis, likely driving the increases in C17:0, but not the driver of the C15:0 induction, HFMP feeding

(Study Ia) on the short term did not induce this axis, suggesting that the increases of OCFA observed in this feeding study are likely due to *de novo* lipogenesis from the increased Pr-CoA availability through the various amino acid degradation pathways. All together, these data highlight that OCFA content of the liver, the key site of endogenous OCFA synthesis, is influenced by milk fat, as shown in Study Ia, as well as milk protein, through multiple pathways involving several amino acids (**Figure 27**).

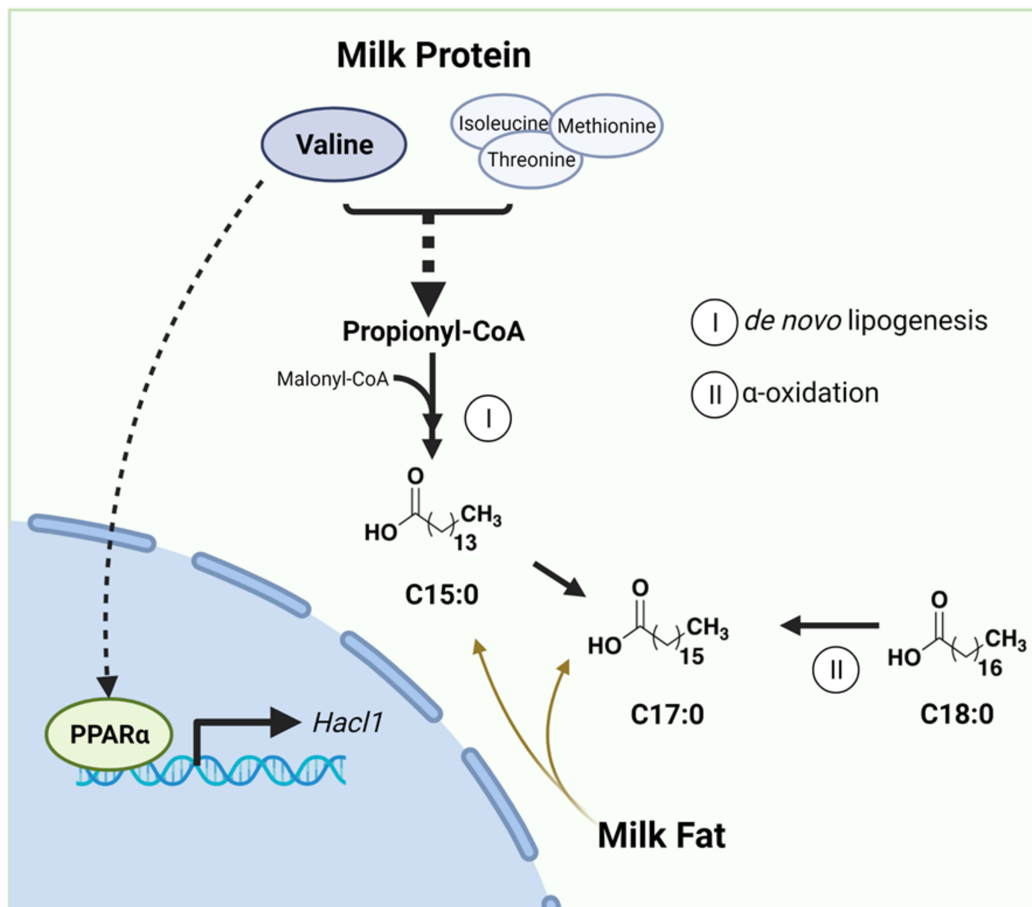


Figure 27. Pathways of hepatic OCFA Synthesis. OCFA formation occurs via two mechanisms, de novo lipogenesis from propionyl-CoA (Pr-CoA) or α-oxidation via a PPARα-HACL1 axis. Milk fat feeding directly increases OCFA levels due to the presence in milk fat, whereas milk protein metabolism leads to increases in Pr-CoA levels through the metabolism of multiple amino acids or activation of PPARα via valine. Figure created with BioRender.com.

5.2 Influences of long-term HF feeding on OCFA formation

Recently, it has been demonstrated that 4 week HF diet feeding leads to reductions in hepatic and circulating levels of both C15:0 and C17:0—dependent on multiple mechanisms induced by HF diet, including impaired liver lipid metabolism (Ampong, John Ikwuobe et al. 2022). This finding holds true in the long-term HF-feeding study (Study II) where HF fed mice have reduced levels of OCFA compared to LF; however, only in the plasma phospholipid fraction (**Figure 6E, 16E**). Similar to the short-term

feeding study (Study Ia), HFMF feeding led to increases in C15:0 and C17:0 levels in the hepatic and plasma fractions (**Figure 6A, E**), in both cases essentially in a 1:2 ratio, despite the different ratio found in the supplementation (see **Table 7**). The epididymal white adipose tissue (eWAT) fraction, on the other hand, clearly showed only increases in C15:0 levels (**Figure 6C**)—in line with the previous suggestion that adipose tissue OCFA may serve as the optimal biomarker for dairy fat intake (Wolk, Furuheim et al. 2001, Baylin, Kabagambe et al. 2002), with a ratio of 2:1 (C15:0 to C17:0). This follows with the fact that WAT is a major destination for dietary fats by free diffusion or via transport proteins. For example, post-prandial lipoprotein lipase (LPL), a lipase that hydrolyzes triglycerides into free fatty acids, is elevated in WAT (Teusink, Voshol et al. 2003), likely promoting the uptake and storage of essential dietary FA.

Table 7 Ratios of C17:0 to C15:0 in different phospholipid and diet fatty acid compositions.

Diets	C15:0 : C17:0 Ratio			
	Liver	eWAT	Plasma	Diet
LF	**2.63**	2.54	2.25	-
HF	3.34	3.62	3.04	-
HFMF	2.15	0.82	2.27	0.49
HFC17	18.03	9.31	24.75	788.2
HFMP	2.98	1.75	2.74	-
HFL	3.12	2.16	2.96	-
HFV	3.23	1.59	2.94	-

Milk fat composition, specifically bovine milk, is primarily triglycerides but contains other lipid types including di- and mono-acylglycerides, FFA, cholesterol and phospholipids (Lubary, Hofland et al. 2011). While present in most fractions, OCFA are enriched in the phospholipid and FFA fractions (Fievez, Colman et al. 2012) and likely incorporated into triglycerides within chylomicrons by intestinal enterocytes and released to the blood for uptake by adipose tissue (Carreiro and Buhman 2019). The plasma OCFA appear to reflect more directly the hepatic levels (**Figure 6A**) in HFMF fed mice, indicating that they are rather endogenously produced or modified further by the liver—possibly by chain length modifications or further utilization for very-long chain FA synthesis, which was not measured in these studies. In both the plasma and liver PL fractions of HFMF-fed mice, there was also a reduction of C18:0 levels, indicative of possible contribution of α-oxidation.

HFC17 feeding, however, increased C15:0 and C17:0 levels in all fractions (**Figure 6**), despite containing very little C15:0 (**Table 7**), suggesting that saturating levels of C17:0 can lead to increases of C15:0 by β-oxidation. OCFA have already been hypothesized to contribute to the Pr-CoA pool via β-oxidation (Sbaï, Narcy et al. 1994). Similar results were obtained in a previous study from our lab, where treatment of primary hepatocytes with C17:0 led to concomitant increases in C15:0 as well (Bishop, Schulze et al. 2020).

With regards to the milk protein or BCAA supplementations (Study IIb), it was possible to confirm that HFMP feeding did induce hepatic OCFA levels after long-term feeding, confirming the results from the short-term study (Study Ia). This was also reflected in the plasma OCFA levels, which were elevated similarly (**Figure 16A, E**). Meanwhile, α-oxidation was apparently not induced (no induction of *Hacl1* gene expression and no difference in C18:0 levels (**Figure 16B, 23F**)). While it is known that the basal, endogenous levels of hepatic C17:0 are regulated in part by HACL1 action (Jenkins, de Schryver et al. 2017), the synthesis of OCFA from milk protein supplementation seems to be limited to *de novo* lipogenesis via Pr-CoA. This is further supported by the correlations of C15:0 levels with Ile, Met and Thr—all amino acids catabolically contributing to the Pr-CoA pool.

Unlike the short-term BCAA supplementation (Study Ib), HFV supplementation had no effects on hepatic or circulating OCFA levels on the long-term (**Figure 23**). Accumulation of Val and its metabolite 3-HIB in the plasma after already 4 weeks of feeding indicate that catabolism of this amino acid was likely impaired (**Figure 21C-E**). Increases in circulating BCAA levels have been recapitulated in several genetic models of obesity and IR, including ob/ob mice and Zucker fatty rats, which has been attributed to reductions in BCAA catabolic genes (Lynch and Adams 2014). While HF feeding alone did not induce circulating BCAA levels, consistent with other DIO mouse models (Newgard, An et al. 2009), HFV feeding led to a large accumulation of Val in the plasma. This could, in part, be due to the HF-induced inhibitory hyper-phosphorylation of BCKDH, the flux controlling step in BCAA catabolism (Brosnan and Brosnan 2006). Both HF- and HFV-fed mice had the highest levels of BCKDH phosphorylation, likely due to the fact that both groups were not protected from ectopic lipid accumulation in the liver (**Figure 22B, 23A**). Hepatic lipotoxicity can have detrimental effects on BCAA oxidation, as FA and their metabolites inhibit BCKDH activity, a result of adverse effects on redox state or increased Ac-CoA concentrations (Lynch and Adams 2014), thus limiting hepatic BCAA oxidation. In fact, it has been demonstrated that obese and diabetic mice

redistribute their BCAA oxidation, with it being shunted towards skeletal muscle (Neinast, Jang et al. 2019)—similar to the HFV-fed mice. Clearly, the discrepancy between HFV on the short-term versus the long-term with regards to OCFA formation can, at least in part, be explained by impaired hepatic BCAA oxidation in these mice. HFMP- and HFL-fed mice, on the other hand, were both protected from HF-induced hepatic lipid accumulation (**Figure 23A, B**), likely via the reduced expression of FA transporters, confirming previous results (Freudenberg, Petzke et al. 2012). It seems very likely, that this protection from fat accumulation in the liver is the reason for the low levels of BCKDH phosphorylation, and, in the case of HFMP, the increased OCFA levels (**Figure 16A**).

Additionally, HFV did not affect *Ppara* or *Hacl1* expression and C18:0 levels (**Figure 16B, 23F**). Thus, there was no increase in OCFA after long-term Val supplementation, via either *de novo* lipogenesis or α-oxidation. These data are fitting, as a human study has previously shown that the BCAA and OCFA have a strong covariance, highest between C17:0 and Val/Ile, which is lost in certain disease states such as T2DM and morbid obesity (Al-Majdoub, Geidenstam et al. 2017).

Interestingly, HFMP- and HFV-fed mice exhibited reduced levels of OCFA in the eWAT PL fraction (**Figure 16C**). While it has previously been shown that *in vitro* BCAA supplementation leads to significant increases of OCFA in adipocytes via the catabolism of Val and Ile (Crown, Marze et al. 2015), these data further support the notion that, *in vivo*, adipose tissue OCFA levels are more representative of direct dietary FA intake, rather than endogenous production. Analysis of whole-body BCAA catabolism and oxidation utilizing isotope tracing also highlighted the fact that oxidation of BCAA or disposal into protein was lowest in WAT (Neinast, Jang et al. 2019).

These long-term feeding studies (Study II) shed light onto the relation of either milk fat or milk protein feeding on circulating and tissue (liver and eWAT) levels of OCFA, highlighting the importance of liver metabolism on endogenous OCFA synthesis and the role of eWAT on storing dietary derived fatty acids.

5.3 Long-term OCFA-rich diets elicit minimal effects on metabolic health outcomes

This study, in part, sought to further clarify the impact of an increased milk fat intake on the development of HF-induced obesity and related metabolic disturbances. Epidemiological studies have suggested an inverse association between dairy intake

and several components of the metabolic syndrome; however, this was very dependent on the types of dairy products and was not always the case (van Meijl, Vrolix et al. 2008). Thus, a major question arising from these studies is which components are driving these effects. As milk fat was previously suggested as the main source of OCFA found in humans, this was the obvious starting point to address the relation of dairy intake, OCFA and metabolic health.

Despite being a direct source of OCFA (Vlaeminck, Fievez et al. 2006), HF feeding with milk fat supplementation (HFMF) did not prevent HF-induced body weight gain and fat mass accumulation, nor did it have any impact on glucose and insulin homeostasis on the long term (**Figure 9**). Dietary recommendations have often suggested the avoidance of high-fat dairy products, i.e. butter, cheese and cream, as they are a major source of cholesterol increasing saturated FA (Weinberg, Berner et al. 2004, de Oliveira Otto, Mozaffarian et al. 2012). However, with regards to whole-body metabolism, plant-based fats (Hoevenaars, van Schothorst et al. 2012), as present in our control HF diet, and fats primarily sourced from butter, appear to have similar metabolic effects. The findings of this thesis are similar to those demonstrated in a mouse HF feeding study comparing the effects of butter and margarine as the fat sources, where the two supplementations had similar long-term metabolic effects with regards to body weight and fat mass, as well as glucose tolerance (Fan, Kim et al. 2020). Furthermore, it has been shown that intake of HF diets rich in saturated FA or poly-unsaturated FA, but similar omega-6/omega-3 ratios, exhibited no differences in adiposity or whole body energy balance (Duivenvoorde, van Schothorst et al. 2015), as is the case in the diets of this thesis as well. These findings further highlight that, when comparing fat sources, the ratio of omega-6/omega-3 likely have a stronger impact on energy metabolism.

Interestingly, HFC17 feeding appeared to have no impact on HF-induced body weight gain (**Figure 7, 8**), in spite of the fact that OCFA are inversely associated with adiposity (Aglago, Biessy et al. 2017). This suggests that C17:0 supplementation likely does not play a direct role in driving this association. This is in line with a recent publication, showing that 12 weeks of feeding had no impact on body weight or glucose and insulin levels in mice (Venn-Watson, Lumpkin et al. 2020). The results presented in this thesis suggest a mild effect of C17:0 on lowering insulin levels induced during an oGTT (**Figure 9F**), similarly to results from a cohort of dolphins fed a modified, fish-based diet rich in C17:0 (Venn-Watson, Parry et al. 2015). However, further confirmation of these findings with more direct causal studies is required.

While little impact on whole-body metabolism (compared to HF diet) was observed with the HFMF feeding, this diet did have a rather detrimental effect on the hepatic phenotype, with increased hepatic lipid accumulation, triglycerides, and expression of markers for inflammation, fibrosis and immune cell-recruiting chemokines (**Figure 10**). The milk fat diet, in contrast to the control HF diet, is composed of approximately 25% palmitic acid (C16:0), and additionally contains cholesterol, which is present in butter.

Whether the increase in circulating cholesterol is purely dependent on cholesterol intake alone or is also affected by the increase in SFA intake, which has been shown to drive cholesterol levels in humans (Sun, Neelakantan et al. 2015), needs further investigation. However, in both cases, both SFA and cholesterol are known for driving the development of hepatic steatosis, as described in part by the "multiple hit" theory of NAFLD progression (Buzzetti, Pinzani et al. 2016). Fat accumulates in the liver as triglycerides as a way to reduce the harmful effects of the increased FFA released from aberrant lipolysis of WAT or *de novo* lipogenesis in the liver. However, this is not necessarily hepatotoxic but rather is suggested to occur simultaneously with the increased levels of FFA, cholesterol and other metabolites. This increase in hepatic FA and related metabolites results in a lipotoxicity, inducing ER stress, mitochondrial dysfunction and increased inflammatory profiles; which, if left untreated, can lead to further progression to NASH (Buzzetti, Pinzani et al. 2016).

Palmitic acid, the most common circulating FFA, has been shown to be elevated in patients with NASH (de Almeida, Cortez-Pinto et al. 2002) and thus has garnered interest for its role in liver health. Studies *in vivo* have demonstrated that FFA accumulation via an inhibition of triglyceride synthesis results in hepatic injury and fibrosis (Yamaguchi, Yang et al. 2007) and thus palmitate's role has been considered crucial in the pathogenesis of NAFLD (Tilg and Moschen 2010, Ogawa, Imajo et al. 2018). The clarification of the mechanism by which palmitate drives lipid accumulation and injury has been explored in several models, one of which posits that palmitate directly upregulates PPARγ via PGC1α, key regulators of FA metabolism (Maruyama, Kiyono et al. 2016). From the data presented in this thesis, it is apparent that HFMF induces expression of PPARγ, as well as its downstream target CD36 (**Figure 10E**), a FA transporter which has been discussed as a key driver in lipotoxicity (Rada, González-Rodríguez et al. 2020).

The role of increased cholesterol intake, however, is also very important to consider when interpreting the results of this thesis. As discussed before, cholesterol intake is a

major risk factor for cardio-metabolic health and studies have consistently shown its function in the development in NASH in several animal models (Wouters, van Bilsen et al. 2010, Subramanian, Goodspeed et al. 2011, Van Rooyen, Larter et al. 2011, Henkel, Coleman et al. 2017). Human lipidomic analysis also revealed that cholesterol levels were increased in NASH with no differences in FFA (Puri, Baillie et al. 2007, Caballero, Fernández et al. 2009). It is likely that the increased cholesterol and C16:0 levels together are driving this overall detrimental hepatic phenotype—as discussed before, the synergistic action of cholesterol and fat drive the worsening of hepatic steatosis (Savard, Tartaglione et al. 2013).

C17:0 supplementation had no apparent effect on the HF-induced hepatic fat accumulation (**Figure 10A, B**), indicating that it also plays little to no role in this context. In HF feeding studies with C57BL/6J mice, only C15:0 supplementation, and not C17:0, could reduce body weight and improve the inflammatory profile (Venn-Watson, Lumpkin et al. 2020). The same study further clarified that C15:0 supplementation in a NASH-inducing diet fed to New Zealand white rabbits could attenuate the fibrosis score, with no effect on steatosis grading. The results presented here support the fact that feeding of OCFA, at least C17:0, do not elicit any significant beneficial metabolic health effects in HF feeding. While direct effects of C15:0 cannot be ruled out, it appears that the attributed beneficial aspects of dairy intake and OCFA are possibly mediated rather from intake of other components of dairy, i.e. milk protein, calcium, or other micronutrients.

5.4 Addressing the protein paradox: beneficial aspects of HF-milk protein supplementation can be attributed to Leu effects

Dietary protein intake with regards to metabolic health has to date rather been deemed the protein paradox: positive anabolic effects of high protein intake in intervention studies have been shown, but epidemiological studies demonstrate an association of improved health with low protein diets (Klaus, Pfeiffer et al. 2018). Furthermore, the BCAA have been highlighted as possible mediators for these conflicting effects. The long-term feeding of milk protein (HFMP), a high source of BCAA, or individual BCAA (Val, HFV; Leu, HFL) (Study IIb) was performed, in part, to address these conflicting results and better illustrate which components of milk intake drive these beneficial associations previously described (van Meijl, Vrolix et al. 2008).

Multiple components of milk, including whey protein and casein have been suggested as the drivers of the beneficial effects of dairy intake (Rice, Cifelli et al. 2011). Evidence in

humans has revealed the role of dietary protein in weight control and weight loss maintenance (Larsen, Dalskov et al. 2010) partially mediated by the effects on satiety (Paddon-Jones, Westman et al. 2008). Importantly, the data presented in this thesis confirm that HFMP and HFL feeding protect from the development of HF-induced adiposity (**Figure 17**), as has been previously demonstrated in various mouse studies (Halton and Hu 2004, Freudenberg, Petzke et al. 2012, Ma, Zhou et al. 2020). Despite the evidence suggesting a role of protein intake in increasing satiety, there were no significant differences between HF-fed groups, suggesting other mechanisms at play. The obesity resistance elicited by HFL feeding has been attributed to an increased energy expenditure (Binder, Bermudez-Silva et al. 2013), which has been replicated with HFL but was not present in the HFMP-fed group (**Figure 17J**). It is possible that the beneficial effects of HFMP feeding on adiposity are partially mediated by the increased Leu intake; however, further research is required to delineate the exact molecular mechanism in this case. For example, HF-diets supplemented with other amino acids, including Ile and Thr, mediate beneficial effects on adiposity as well (Ma, Zhou et al. 2020, Ma, Zhou et al. 2020).

With regards to glucose homeostasis, several studies have already demonstrated that either whey protein or Leu supplementation has varying effects; either no change or improved responses to glucose or insulin challenges have been described (Brunetta, de Camargo et al. 2018). While both HFMP and HFL had beneficial effects on adiposity, only HFL feeding demonstrated an improved glycemia and insulin sensitivity (**Figure 18**). Leu treatment has been reported to induce insulin secretion acutely; however, this effect is more likely not apparent in the long-term, due to adaptive responses that lead to improved insulin sensitivity (Zhang, Guo et al. 2007). Vital to point out are the results from previous mouse studies that robustly demonstrated that HF-BCAA supplementation protects mice from HF-induced obesity, but, rather paradoxically results in IR (Newgard, An et al. 2009, Zhang, Zhao et al. 2016), while others showed no difference on IR or adiposity (Lee, Vijayakumar et al. 2021). The beneficial effects of Leu or Ile already described are inconsistent with the data on BCAA supplementations, suggesting a possible compensatory negative effect of Val, that could be driving IR, and at least partially account for the inconsistent findings on protein supplementation as a whole on glucose homeostasis.

The role of Val in metabolic health and whole-body metabolism has been only recently addressed and rather indirectly. Initially, studies demonstrated the improvement of metabolic health with the restriction of BCAA (Fontana, Cummings et al. 2016), which

was further expounded upon to show that Val or Ile restriction alone was sufficient to restore metabolic health to DIO mice (Yu, Richardson et al. 2021). This thesis demonstrates that, while there is no effect on body weight, food intake or EE, HFV feeding drives an apparent impairment of glucose clearance during an oGTT, which was not due to different levels of insulin secretion throughout the intervention (**Figure 18D, F**). These data suggest a peripheral IR in the HFV mice, which was further addressed in the medium-term feeding study (Study III) with the aim to determine which insulin-sensitive tissue might display impaired insulin signaling after an acute (30 min) insulin injection. 4 weeks of feeding was sufficient to demonstrate that HFV feeding, but not HFMP or HFL, could lead to substantial reductions in skeletal muscle insulin sensitivity (**Figure 20D**).

Taken together, it is warranted to suggest that these apparent differential effects of Leu and Val on metabolic health under HF conditions are, at least in part, an explanation for the conflicting literature for BCAA and IR development. These counteracting roles demonstrate the importance of dietary protein quality when addressing glucose and insulin homeostasis. For example, when comparing whey and casein proteins in DIO rats, whey supplementation resulted in the strongest improvement of glucose tolerance (Pezeshki, Fahim et al. 2015). While whey protein contains a higher amount of total BCAA compared to casein, casein itself does have a higher proportion of Val (Hall, Millward et al. 2003). Further investigations are required to determine whether the ratio of the BCAA present in a diet may elicit varying effects on obesity and IR. In the case of this thesis, it is possible that HFMP feeding only exerted positive effects with regards to adiposity and not insulin sensitivity due to the counteracting roles of Val and Leu.

As described in Section 5.2, HFMP and HFL feeding protected mice from hepatic fat accumulation, mediated by the decreases in FA transport expression (**Figure 23D**), as has been demonstrated before (Zhang, Guo et al. 2007, Freudenberg, Petzke et al. 2012, Ma, Zhou et al. 2020). Interestingly, the interplay of BCAA with hepatic health are emerging to show an equally puzzling role—a NASH mouse model supplemented with BCAA demonstrated an alleviated hepatic steatosis and liver injury (Honda, Ishigami et al. 2017). In the human situation there is amassing evidence for the efficacy of BCAA supplementation for patients with NASH in improving quality of life (i.e. improved fatigue, sleep), glucose tolerance and serum albumin concentrations (Tajiri and Shimizu 2018). On the other hand, BCAA supplemented in a HF diet resulted in significant liver damage, due to an impaired hepatic autophagy mediated via mTOR signaling (Zhang, Zhao et al. 2016). HFV fed mice do not exhibit this protection from excess lipid accumulation;

however, the hepatic phenotype is not worsened by this diet when compared to HF diet alone (**Figure 23**). Though, there is a significant upregulation of mTOR activation and increased expression of FA transporters in HFV fed mice (**Figure 23E**), which could implicate Val as a driving force in development of NAFLD in an even longer intervention. An accumulation of circulating BCAA and a down-regulation of hepatic BCAA catabolism are hallmarks of NAFLD and NASH in humans (Brownlee 2001) which further suggest that the shift of BCAA catabolism in these mice to skeletal muscle likely drives the impaired insulin sensitivity.

While multiple mechanisms for BCAA-induced IR have been suggested (for review see (White and Newgard 2019)), the data in this thesis point towards the 3-HIB-mediated glucotoxicity in driving the apparent impairments in muscle insulin signaling. This study demonstrates that, while HFV feeding led to an accumulation of DAG in muscle, the concomitant increases in membrane-bound PKCθ were not present (**Figure 24D-F**), suggesting another mechanism than one previously proposed for 3-HIB action (Jang, Oh et al. 2016). Rather, 3-HIB appears to mediate the induction of *Glut1* expression and increased basal glucose uptake (**Figure 25A, B**). It has been previously shown that overexpression of GLUT1 in mice leads to the development of IR via increased intracellular glucose levels (Buse, Robinson et al. 1996). Humans with obesity or T2DM, as well as HF-fed mice, exhibit an increase in *Trib3* expression in skeletal muscle and TRIB3 knockout mice were shown to be protected from glucose-induced IR (Liu, Wu et al. 2010, Koh, Toyoda et al. 2013, Zhang, Liu et al. 2013). Inhibition of GLUT1 appeared to reverse the upregulation of *Trib3* by 3-HIB and rescue the impaired AKT signaling (**Figure 25D-F**), which is in line with studies showing TRIB3 action in inhibiting AKT (Kwon, Eom et al. 2018). Taken together, it is valid to posit that Val-mediated hyperglycemia due to an increased basal glucose uptake, by action of 3-HIB, may result in glucotoxicity and subsequent TRIB3-mediated inhibition of insulin signaling (**Figure 28**).

While further investigation is required into the effects of the BCAA and their interaction in metabolic health, it was possible to highlight the beneficial role of an increased milk protein intake in adiposity, at least partially via the action of Leu, contributing to the associated positive health outcomes attributed to dairy intake in epidemiological studies.

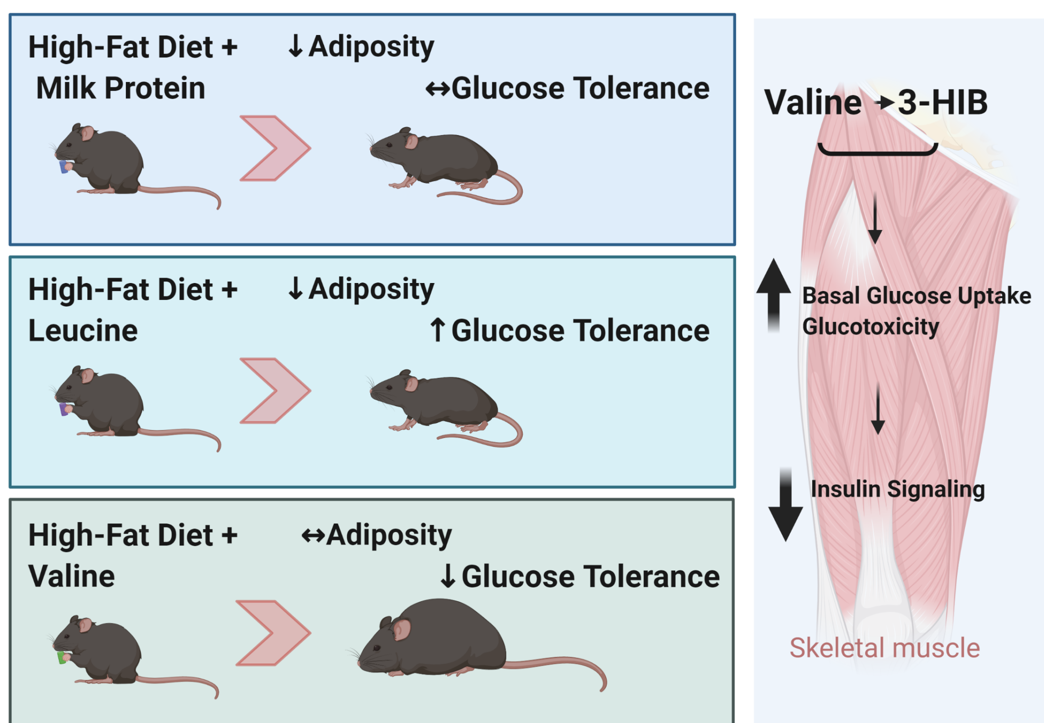


Figure 28. Graphical description of HF milk protein or individual BCAA supplementations. Both HF milk protein (HFMP) and leucine (HFL) -fed mice are protected from HF-induced adiposity; however, only HFL-fed mice resulted in an improved glucose tolerance. HF valine (HFV) feeding, on the other hand, had no effect on adiposity but results in a worsened glucose tolerance. HFV mice have an accumulation of valine and its metabolite 3-hydroxyisobutyrate (3-HIB), which drives basal glucose uptake in the skeletal muscle, with a resulting glucotoxicity-induced insulin resistance. Figure created with BioRender.com.

5.5 OCFA and dairy intake: correlation does not equal causation

The overall aim of this thesis was to further understand the connection of dairy intake with metabolic health, while simultaneously elucidating the involvement of OCFA. While milk protein supplementation induced OCFA levels and demonstrated varying inverse associations with specific metabolic parameters, these associations seemed to be more limited to C15:0 (**Figure 24**). Interestingly, however, is the fact that C17:0 supplementation had no strong effects on overall metabolic health, in line with the lack of associations seen in Study IIb. Furthermore, despite being able to induce OCFA levels, milk fat does not have an apparent beneficial outcome. These findings together highlight that C17:0 likely does not play a role in adiposity, insulin sensitivity or NAFLD development. Whether the OCFA have any direct signaling effects remains to be fully clarified, though initial data suggest a possible involvement of C15:0 in improved metabolic health (Venn-Watson, Lumpkin et al. 2020). However, both OCFA-rich diets in this thesis (Study IIa) indicate otherwise.

It is possible that OCFA can serve as a good neutral storage pool for excess Pr-CoA, removing excess from the plasma. This is a possible rescue mechanism to avoid the accumulation of Pr-CoA in the mitochondria, as it is known that excess Pr-CoA, in propionyl-CoA carboxylase or methyl-malonyl-CoA mutase deficient hepatocytes for example, lead to impaired oxidative phosphorylation and impaired mitochondrial function (Toyoshima, Watanabe et al. 1995, Schwab, Sauer et al. 2006). As Pr-CoA can serve as a metabolite for gluconeogenesis, OCFA are also a possible manner by which the cell can store excess Pr-CoA without directly increasing circulating blood glucose levels in a HF setting.

These correlations of OCFA with improved health outcomes have been demonstrated before in other mouse studies as well, where mice fed HF diets supplemented with dietary fibers or SCFA displayed increased OCFA (Weitkunat, Schumann et al. 2016, Weitkunat, Stuhlmann et al. 2017). The data from these studies, as well as this thesis, highlight the influence of dairy intake and dietary fibers in modifying OCFA levels. However, the data presented herein suggest that the correlations do not equal causation for the improved metabolic health. It is possible to suggest that the OCFA increases merely reflect an increase in metabolites that induce OCFA, but that the beneficial effects are mediated by the metabolites themselves and not the OCFA. For example, the positive effects on adiposity of milk protein intake are likely mediated by Leu, or other amino acids (Zhang, Guo et al. 2007, Ma, Zhou et al. 2020, Ma, Zhou et al. 2020). With regards to dietary fibers and SCFA, it has long been suggested that the G-protein coupled receptors (GPR) 41 and 43 mediate the improvements in energy homeostasis after an increased SCFA intake through multiple mechanisms (Zhang, Zhao et al. 2021). In both cases, it seems that the metabolites themselves, and not the OCFA, are the key mediators in improved adiposity, insulin sensitivity and overall health.

6 Summary & Outlook

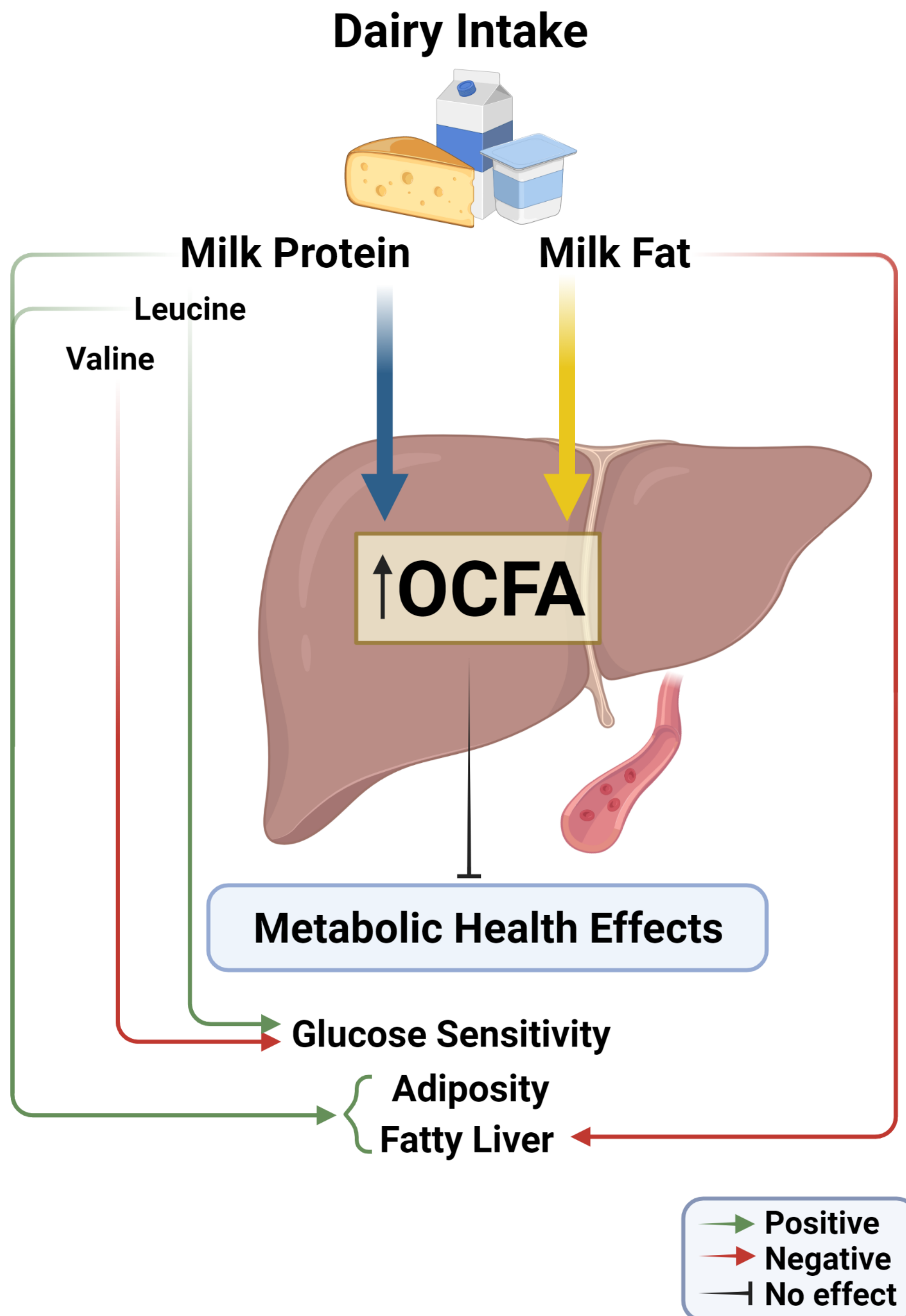
As of late, interest in the involvement of OCFA in metabolic health has grown significantly, though little is known about the direct sources and metabolic effects of these FA.

The studies presented in this thesis contribute to the improved comprehension of OCFA sources and function, or lack thereof, under HF diet settings. Using both short-term (Study I) and long-term feeding studies (Study II), it was possible to determine that both milk fat and milk protein supplementation contribute to OCFA levels. Milk fat is a direct source of OCFA, while milk protein contains certain amino acids that, when catabolized, contribute to the Pr-CoA pool for endogenous OCFA synthesis and in some cases induce a PPARα-HACL1 axis for increased α-oxidation. Importantly, the endogenous synthesis of OCFA primarily occurs in the liver, and the circulating PL levels of OCFA directly reflect the hepatic production, suggesting that circulating levels of OCFA are not a suitable biomarker for direct dairy intake.

Despite both fractions of milk leading to inductions of OCFA, in-depth metabolic phenotyping revealed that milk protein was the only one to elicit beneficial effects—suggesting the importance of dairy protein intake on driving the beneficial associations of total dairy intake. While milk protein was able to reduce adiposity, similar to Leu, there were no sensitizing effects on glucose homeostasis, which could be due to the detrimental contribution of Val to insulin sensitivity. While apparent inverse associations exist between OCFA and poor metabolic health, these data revealed little biological significance for C17:0 in the prevention of HF-induced health impairments (**Graphical Summary**).

While this study begins to shed more light into the physiologic relevance of OCFA, there are some limitations to this study, and future research is required. Recent studies have revealed the differential partitioning of OCFA, and the beneficial associations were dependent on the lipid type and sex (Prada, Wittenbecher et al. 2021). This thesis investigated the OCFA levels in the phospholipid fraction, similar to previous epidemiological studies (Forouhi, Koulman et al. 2014); however, further research into the influence of specific metabolites on OCFA levels in different lipid types is required. This recent study from Prada et al. also highlights that the inverse associations with T2DM risk were stronger in women, while the studies presented here were performed only in male mice. Finally, the direct role of C15:0 was not explored in this thesis, though

evidence is emerging that indicates its involvement in health outcomes (Venn-Watson, Lumpkin et al. 2020). Despite C17:0 demonstrating little contribution to improving energy homeostasis, a further understanding of sources of OCFA and their direct metabolic influence may aid in improving dietary recommendations for the future.



Graphical Summary. Both milk fat and milk protein increased hepatic and circulating odd-chain fatty acid (OCFA); however, only milk protein elicited protective effects on adiposity and hepatic fat accumulation—likely mediated by the anti-obesogenic effects of an increased Leu intake. Val and Leu feeding had divergent effects on glucose sensitivity, where Leu led to an improved insulin sensitivity and Val rather resulted in glucotoxicity-induced insulin resistance in skeletal muscle mediated by its metabolite 3-hydroxyisobutyrate (3-HIB). While OCFA levels correlated with improved health outcomes, C17:0 produced negligible effects in preventing HF-diet induced health impairments. Figure created in BioRender.com.

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8 Supplemental Materials

Supplemental Table 1. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study Ia.

Supplemental Table 2. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIa.

Supplemental Table 3. eWAT Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIa.

Supplemental Table 4. Plasma Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIa.

Supplemental Table 5. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study Ib.

Supplemental Table 6. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIb.

Supplemental Table 7. eWAT Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIb.

Supplemental Table 8. Plasma Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIb.

Supplemental Table 1. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study Ia. Hepatic LCFA spectra for male C57BL/6JRj mice fed high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), or milk fat (HFMF) for 1 week (n = 8). Different letters indicate differences between groups.

Liver PL	**HF**	**HFMP**	**HFMF**	**p-value**
C14:0	0.215 ± 0.015	0.230 ± 0.015	0.238 ± 0.012	n.s.
C15:0	0.081 ± 0.005[a]	0.111 ± 0.003[b]	0.226 ± 0.008[b]	0.004
C16:0	23.66 ± 0.36[a]	22.07 ± 0.24[b]	24.89 ± 0.36[c]	0.03
C16:1n7c	0.630 ± 0.063[a]	0.349 ± 0.034[b]	1.03 ± 0.090[c]	0.01
C17:0	0.226 ± 0.010[a]	0.340 ± 0.016[b]	0.416 ± 0.011[b]	0.0001
C18:0	15.95 ± 0.17[a]	16.66 ± 0.28[a]	15.06 ± 0.30[b]	0.04
C18:1n9c	6.55 ± 0.11[a]	6.78 ± 0.10[a]	8.13 ± 0.14[b]	0.0001
C18:1n7c	0.673 ± 0.039[a]	0.583 ± 0.027[a]	1.09 ± 0.111[b]	0.0007
C18:2n6t	0.153 ± 0.009[a]	0.233 ± 0.006[b]	0.181 ± 0.014[a]	0.0001
C18:2n6c	19.78 ± 0.31[a]	19.99 ± 0.33[a]	15.37 ± 0.57[b]	0.0001
C20:0	0.279 ± 0.021[a]	0.425 ± 0.036[b]	0.249 ± 0.017[a]	0.001
C18:3n6	0.303 ± 0.013[a]	0.389 ± 0.014[b]	0.373 ± 0.031[a]	0.01
C18:3n3	0.471 ± 0.018[a]	0.518 ± 0.019[a]	0.230 ± 0.018[b]	0.0001
C20:1n9	0.219 ± 0.012[a]	0.306 ± 0.017[b]	0.191 ± 0.006[a]	0.0002
C20:2n6	0.244 ± 0.019[a]	0.340 ± 0.019[b]	0.136 ± 0.009[c]	0.0009
C20:3n6	1.16 ± 0.07[a]	1.17 ± 0.07[a]	1.45 ± 0.10[b]	0.04
C20:4n6	15.82 ± 0.30	16.52 ± 0.24	16.65 ± 0.57	n.s.
C20:5n3	0.473 ± 0.014[a]	0.383 ± 0.011[b]	0.528 ± 0.035[a]	0.02
C22:4n6	0.208 ± 0.001[a]	0.195 ± 0.001[a]	0.238 ± 0.001[b]	0.002
C22:5n6	0.124 ± 0.005[a]	0.136 ± 0.007[a]	0.338 ± 0.022[b]	0.0001
C22:5n3	0.639 ± 0.022[a]	0.456 ± 0.001[b]	0.632 ± 0.035[a]	0.0001
C22:6n3	12.14 ± 0.13	11.82 ± 0.12	12.36 ± 0.22	n.s.

Supplemental Table 2. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIa. Hepatic LCFA spectra for male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF) or C17:0 (HFC17) for 20 weeks (n = 8). Different letters indicate differences between groups.

Liver PL	**LF**	**HF**	**HFMF**	**HFC17**	**p-value**
C14:0	0.171 ± 0.009[a]	0.186 ± 0.018[a]	0.299 ± 0.011[b]	0.169 ± 0.010[a]	0.0001
C15:0	0.076 ± 0.003[a]	0.069 ± 0.005[a]	0.168 ± 0.019[b]	0.284 ± 0.010[c]	0.0001
C16:0	26.82 ± 0.26[a]	23.04 ± 0.25[b]	24.38 ± 0.32[c]	20.92 ± 0.26[d]	0.01
C16:1n7c	1.44 ± 0.10[a]	0.50 ± 0.02[b]	1.42 ± 0.04[a]	0.57 ± 0.03[b]	0.0001
C17:0	0.201 ± 0.012[a]	0.226 ± 0.011[a]	0.345 ± 0.016[a]	5.10 ± 0.21[b]	0.0001
C18:0	13.20 ± 0.39[a]	17.30 ± 0.44[b]	15.01 ± 0.35[c]	14.18 ± 0.31[a,c]	0.001
C18:1n9c	8.55 ± 0.28[a]	6.09 ± 0.12[b]	10.43 ± 0.61[c]	5.74 ± 0.15[b]	0.005
C18:1n7c	1.91 ± 0.18[a]	0.67 ± 0.06[b]	2.23 ± 0.26[a]	0.79 ± 0.04[b]	0.003
C18:2n6t	0.105 ± 0.006[a]	0.136 ± 0.010[a]	0.119 ± 0.012[a]	0.984 ± 0.026[b]	0.0001
C18:2n6c	12.72 ± 0.80[a]	18.01 ± 0.87[b]	12.22 ± 0.78[a]	16.20 ± 0.43[b]	0.01
C20:0	0.185 ± 0.018[a]	0.293 ± 0.029[b]	0.181 ± 0.025[a]	0.229 ± 0.008[a,b]	0.006
C18:3n6	0.258 ± 0.017[a]	0.234 ± 0.017[a,b]	0.169 ± 0.020[b]	0.225 ± 0.010[a,b]	0.003
C18:3n3	0.144 ± 0.018[a]	0.356 ± 0.030[b]	0.169 ± 0.017[a]	0.254 ± 0.015[c]	0.03
C20:1n9	0.225 ± 0.009[a,b]	0.223 ± 0.006[a,b]	0.271 ± 0.022[a]	0.190 ± 0.005[b]	0.0007
C20:2n6	0.171 ± 0.007[a]	0.260 ± 0.007[b]	0.185 ± 0.012[a]	0.226 ± 0.005[c]	0.04
C20:3n6	1.92 ± 0.16[a]	2.05 ± 0.20[a]	2.98 ± 0.22[b]	2.23 ± 0.16[a]	0.04
C20:4n6	16.67 ± 0.42[a,b]	15.69 ± 0.59[a,b]	15.20 ± 0.44[a]	17.02 ± 0.32[b]	0.04
C20:5n3	0.420 ± 0.005[a]	0.599 ± 0.036[a,b]	0.693 ± 0.063[b]	0.539 ± 0.023[a,b]	0.001
C22:4n6	0.284 ± 0.027[a]	0.164 ± 0.009[b]	0.231 ± 0.017[a,b]	0.209 ± 0.004[b]	0.02
C22:5n6	0.475 ± 0.155[a]	0.064 ± 0.006[b]	0.171 ± 0.026[a,b]	0.133 ± 0.006[b]	0.03
C22:5n3	0.639 ± 0.015	0.634 ± 0.022	0.699 ± 0.061	0.679 ± 0.016	n.s.
C22:6n3	13.42 ± 0.30	13.20 ± 0.23	12.43 ± 0.37	13.15 ± 0.22	n.s.

Supplemental Table 3. eWAT Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIa. Epididymal white adipose tissue (eWAT) LCFA spectra for male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF) or C17:0 (HFC17) for 20 weeks (n = 8). Different letters indicate differences between groups.

eWAT PL	**LF**	**HF**	**HFMF**	**HFC17**	**p-value**
C14:0	1.99 ± 0.10^{a}	1.85 ± 0.14^{a}	3.59 ± 0.33^{b}	2.02 ± 0.10^{a}	0.0001
C15:0	0.164 ± 0.007^{a}	0.136 ± 0.019^{a}	$0.459 \pm 0.039^{a,b}$	1.05 ± 0.325^{b}	0.004
C16:0	19.47 ± 0.34^{a}	15.12 ± 0.44^{b}	20.91 ± 0.48^{a}	15.42 ± 0.50^{b}	0.0001
C16:1n7c	6.75 ± 0.41^{a}	2.48 ± 0.19^{b}	6.51 ± 0.36^{a}	2.61 ± 0.13^{b}	0.0001
C17:0	0.380 ± 0.040^{a}	0.361 ± 0.042^{a}	0.353 ± 0.025^{a}	5.12 ± 0.30^{b}	0.0001
C18:0	7.06 ± 0.74	7.43 ± 0.78	6.17 ± 0.74	7.32 ± 0.62	n.s.
C18:1n9c	31.10 ± 0.48^{a}	28.65 ± 1.07^{b}	35.97 ± 1.07^{c}	25.72 ± 1.00^{b}	0.002
C18:1n7c	3.25 ± 0.173^{a}	1.62 ± 0.096^{b}	2.51 ± 0.184^{c}	1.55 ± 0.047^{b}	0.004
C18:2n6t	0.073 ± 0.005^{a}	0.055 ± 0.006^{a}	0.059 ± 0.002^{a}	0.136 ± 0.006^{b}	0.0001
C18:2n6c	23.84 ± 0.34^{a}	36.57 ± 0.58^{b}	18.91 ± 0.22^{c}	32.64 ± 0.66^{d}	0.0001
C20:0	0.134 ± 0.010^{a}	0.094 ± 0.007^{b}	0.099 ± 0.004^{b}	0.101 ± 0.007^{b}	0.02
C18:3n6	0.061 ± 0.005^{a}	$0.049 \pm 0.003^{a,b}$	0.038 ± 0.003^{b}	$0.049 \pm 0.002^{a,b}$	0.0003
C18:3n3	1.29 ± 0.09^{a}	2.34 ± 0.18^{b}	1.17 ± 0.10^{a}	1.84 ± 0.09^{c}	0.04
C20:1n9	0.640 ± 0.036^{a}	0.344 ± 0.011^{b}	0.308 ± 0.013^{b}	0.343 ± 0.012^{b}	0.0001
C20:2n6	0.113 ± 0.008^{a}	0.116 ± 0.004^{a}	0.066 ± 0.004^{b}	0.110 ± 0.005^{a}	0.0001
C20:3n6	0.309 ± 0.025	0.248 ± 0.014	0.246 ± 0.026	0.259 ± 0.016	n.s.
C20:4n6	1.99 ± 0.30	1.34 ± 0.22	1.64 ± 0.28	2.06 ± 0.31	n.s.
C20:5n3	0.065 ± 0.005	0.088 ± 0.008	0.070 ± 0.009	0.086 ± 0.006	n.s.
C22:4n6	0.151 ± 0.020	0.104 ± 0.011	0.123 ± 0.016	0.154 ± 0.011	n.s.
C22:5n6	0.064 ± 0.007^{a}	0.029 ± 0.003^{b}	0.025 ± 0.002^{b}	0.065 ± 0.012^{a}	0.004
C22:5n3	0.190 ± 0.023	0.231 ± 0.027	0.209 ± 0.034	0.244 ± 0.027	n.s.
C22:6n3	0.918 ± 0.105^{a}	0.746 ± 0.118^{a}	0.576 ± 0.067^{a}	1.11 ± 0.186^{b}	0.03

Supplemental Table 4. Plasma Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIa. Plasma LCFA spectra for male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk fat (HFMF) or C17:0 (HFC17) for 20 weeks (n = 8). Different letters indicate differences between groups.

Plasma PL	**LF**	**HF**	**HFMF**	**HFC17**	**p-value**
C14:0	0.284 ± 0.032	0.283 ± 0.033	0.231 ± 0.027	0.295 ± 0.048	n.s.
C15:0	0.154 ± 0.011[a]	0.104 ± 0.009[a]	0.195 ± 0.023[b]	0.275 ± 0.014[c]	0.004
C16:0	29.28 ± 0.10[a]	24.66 ± 0.36[b]	26.45 ± 0.38[c]	22.40 ± 0.26[d]	0.001
C16:1n7c	0.838 ± 0.055[a]	0.271 ± 0.012[b]	0.518 ± 0.030[c]	0.279 ± 0.016[b]	0.01
C17:0	0.349 ± 0.030[a]	0.305 ± 0.014[a]	0.421 ± 0.023[a]	6.72 ± 0.25[b]	0.0001
C18:0	15.95 ± 0.17[a]	15.95 ± 0.17[b]	16.66 ± 0.28[c]	15.06 ± 0.30[a]	0.009
C18:1n9c	8.43 ± 0.26[a]	4.65 ± 0.11[b]	7.54 ± 0.15[c]	4.60 ± 0.12[b]	0.006
C18:1n7c	1.82 ± 0.15[a]	0.721 ± 0.040[b]	1.61 ± 0.147[a]	0.700 ± 0.018[b]	0.0001
C18:2n6t	0.103 ± 0.002[a]	0.103 ± 0.003[a]	0.099 ± 0.006[a]	0.565 ± 0.012[b]	0.0001
C18:2n6c	22.18 ± 0.66[a]	27.85 ± 1.18[b]	22.78 ± 0.83[a]	27.54 ± 0.71[b]	0.003
C20:0	0.214 ± 0.012[a,b]	0.273 ± 0.027[a]	0.170 ± 0.024[b]	0.213 ± 0.006[a,b]	0.001
C18:3n6	0.169 ± 0.009[a]	0.126 ± 0.008[b]	0.108 ± 0.011[b]	0.133 ± 0.006[b]	0.03
C18:3n3	0.168 ± 0.012[a,b]	0.223 ± 0.021[a]	0.124 ± 0.013[b]	0.205 ± 0.015[b]	0.006
C20:1n9	0.234 ± 0.006[a]	0.206 ± 0.007[b]	0.154 ± 0.007[c]	0.164 ± 0.004[c]	0.02
C20:2n6	0.176 ± 0.007[a]	0.229 ± 0.009[a]	0.195 ± 0.008[c]	0.209 ± 0.003[b,c]	0.001
C20:3n6	2.06 ± 0.17[a]	2.12 ± 0.22[a]	3.38 ± 0.26[b]	2.23 ± 0.17[a]	0.004
C20:4n6	10.26 ± 0.29	9.99 ± 0.87	9.96 ± 0.70	10.36 ± 0.43	n.s.
C20:5n3	0.306 ± 0.026[a]	0.363 ± 0.022[a]	0.484 ± 0.026[b]	0.325 ± 0.017[a]	0.005
C22:4n6	0.173 ± 0.012[a]	0.103 ± 0.005[b]	0.126 ± 0.009[b]	0.120 ± 0.003[b]	0.002
C22:5n6	0.274 ± 0.062[a]	0.051 ± 0.005[b]	0.136 ± 0.007[b]	0.338 ± 0.022[b]	0.0001
C22:5n3	0.458 ± 0.017	0.466 ± 0.011	0.500 ± 0.037	0.501 ± 0.012	n.s.
C22:6n3	7.13 ± 0.36	7.57 ± 0.22	7.19 ± 0.23	7.60 ± 0.27	n.s.

Supplemental Table 5. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study Ib. Plasma LCFA spectra for male C57BL/6JRj mice fed high-fat (HF) or experimental HF diets supplemented with leucine (HFL), or valine (HFV) for 1 week (n = 8). Different letters indicate differences between groups.

Liver PL	**HF**	**HFL**	**HFV**	**p-value**
C14:0	0.163 ± 0.006	0.179 ± 0.009	0.169 ± 0.005	n.s.
C15:0	0.088 ± 0.004[a]	0.096 ± 0.003[a,b]	0.109 ± 0.005[b]	0.003
C16:0	20.67 ± 0.27	20.75 ± 0.17	20.83 ± 0.28	n.s.
C16:1n7c	0.322 ± 0.030	0.336 ± 0.047	0.382 ± 0.041	n.s.
C17:0	0.270 ± 0.009	0.270 ± 0.010	0.300 ± 0.006	0.064
C18:0	19.06 ± 0.27[a]	18.53 ± 0.16[a]	17.72 ± 0.15[b]	0.0005
C18:1n9c	5.83 ± 0.09[a]	5.91 ± 0.07[a]	6.48 ± 0.14[b]	0.003
C18:1n7c	0.651 ± 0.034	0.670 ± 0.055	0.814 ± 0.122	n.s.
C18:2n6t	0.188 ± 0.014	0.185 ± 0.014	0.178 ± 0.010	n.s.
C18:2n6c	18.94 ± 0.32	18.02 ± 0.76	18.62 ± 0.23	n.s.
C20:0	0.416 ± 0.024	0.476 ± 0.035	0.420 ± 0.036	n.s.
C18:3n6	0.399 ± 0.010	0.410 ± 0.016	0.398 ± 0.021	n.s.
C18:3n3	0.325 ± 0.016	0.323 ± 0.029	0.344 ± 0.013	n.s.
C20:1n9	0.283 ± 0.015	0.299 ± 0.012	0.312 ± 0.011	n.s.
C20:2n6	0.338 ± 0.017	0.343 ± 0.018	0.373 ± 0.023	n.s.
C20:3n6	1.38 ± 0.046	1.32 ± 0.046	1.34 ± 0.052	n.s.
C20:4n6	17.78 ± 0.31[a]	19.34 ± 0.64[b]	18.38 ± 0.20[a]	0.046
C20:5n3	0.440 ± 0.024	0.385 ± 0.010	0.429 ± 0.028	n.s.
C22:4n6	0.225 ± 0.007	0.224 ± 0.005	0.236 ± 0.006	n.s.
C22:5n6	0.149 ± 0.005[a]	0.178 ± 0.004[b]	0.181 ± 0.009[b]	0.016
C22:5n3	0.454 ± 0.023	0.400 ± 0.009	0.426 ± 0.012	n.s.
C22:6n3	11.65 ± 0.21	11.95 ± 0.08	11.41 ± 0.21	n.s.

Supplemental Table 6. Liver Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIb. Hepatic LCFA spectra for male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks (n = 8). Different letters indicate differences between groups.

Liver PL	**LF**	**HF**	**HFMP**	**HFL**	**HFV**	**p-value**
C14:0	0.171 ± 0.009	0.186 ± 0.018	0.203 ± 0.011	0.208 ± 0.009	0.189 ± 0.015	n.s.
C15:0	0.076 ± 0.003[a,b]	0.069 ± 0.005[a]	0.090 ± 0.004[b]	0.078 ± 0.004[a,b]	0.068 ± 0.004[a]	0.002
C16:0	26.82 ± 0.26[a]	23.04 ± 0.25[b]	22.50 ± 0.19[b,c]	21.81 ± 0.20[c]	22.74 ± 0.16[b]	0.03
C16:1n7c	1.44 ± 0.10[a]	0.50 ± 0.02[b]	0.53 ± 0.02[b]	0.54 ± 0.02[b]	0.55 ± 0.03[b]	0.0001
C17:0	0.201 ± 0.012[a]	0.226 ± 0.011[a]	0.268 ± 0.010[b]	0.241 ± 0.011[a,b]	0.216 ± 0.008[a]	0.0001
C18:0	13.20 ± 0.39[a]	17.30 ± 0.44[b]	16.81 ± 0.23[b]	17.49 ± 0.18[b]	16.91 ± 0.29[b]	0.0001
C18:1n9c	8.55 ± 0.28[a]	6.09 ± 0.12[b]	6.09 ± 0.12[b]	6.21 ± 0.25[b]	6.56 ± 0.22[b]	0.0001
C18:1n7c	1.91 ± 0.18[a]	0.67 ± 0.06[b]	0.72 ± 0.04[b]	0.61 ± 0.03[b]	0.82 ± 0.04[b]	0.0001
C18:2n6t	0.105 ± 0.006[a]	0.136 ± 0.010[a,b]	0.180 ± 0.012[b,c]	0.148 ± 0.009[b]	0.114 ± 0.008[a,b]	0.03
C18:2n6c	12.72 ± 0.80[a]	18.01 ± 0.87[b]	16.94 ± 0.44[b]	17.37 ± 0.39[b]	16.80 ± 0.40[b]	0.0003
C20:0	0.185 ± 0.018[a]	0.293 ± 0.029[b]	0.315 ± 0.014[b]	0.303 ± 0.012[b]	0.259 ± 0.016[a,b]	0.002
C18:3n6	0.258 ± 0.017[a,b]	0.234 ± 0.017[a]	0.284 ± 0.019[a,b]	0.315 ± 0.022[b]	0.215 ± 0.012[a]	0.03
C18:3n3	0.144 ± 0.018[a]	0.356 ± 0.030[b]	0.331 ± 0.017[b]	0.323 ± 0.013[b]	0.330 ± 0.017[b]	0.0001
C20:1n9	0.225 ± 0.009[a]	0.223 ± 0.006[a]	0.266 ± 0.006[b]	0.229 ± 0.006[a]	0.241 ± 0.012[a,b]	0.02
C20:2n6	0.171 ± 0.007[a]	0.260 ± 0.007[b]	0.281 ± 0.005[b]	0.271 ± 0.011[b]	0.273 ± 0.013[b]	0.0001
C20:3n6	1.92 ± 0.16[a,b]	2.05 ± 0.20[a,b]	1.86 ± 0.11[a,b]	1.50 ± 0.08[a]	2.30 ± 0.14[b]	0.003
C20:4n6	16.27 ± 0.42[a]	15.69 ± 0.59[a]	18.30 ± 0.11[b]	17.67 ± 0.37[b]	16.81 ± 0.26[a]	0.04
C20:5n3	0.420 ± 0.005[a]	0.599 ± 0.036[b]	0.510 ± 0.018[a,b]	0.375 ± 0.023[c]	0.528 ± 0.024[a,b]	0.01
C22:4n6	0.284 ± 0.027[a]	0.164 ± 0.009[b]	0.183 ± 0.004[b,c]	0.213 ± 0.004[d]	0.190 ± 0.005[c]	0.04
C22:5n6	0.475 ± 0.155[a]	0.064 ± 0.006[b]	0.098 ± 0.008[b,c]	0.135 ± 0.016[c]	0.083 ± 0.005[b,c]	0.006
C22:5n3	0.639 ± 0.015[a]	0.634 ± 0.022[a]	0.481 ± 0.013[b]	0.501 ± 0.019[b]	0.605 ± 0.010[a]	0.006
C22:6n3	13.42 ± 0.30	13.20 ± 0.23	12.95 ± 0.25	13.71 ± 0.18	13.21 ± 0.23	n.s.

Supplemental Table 7. eWAT Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIb. Epididymal white adipose tissue (eWAT) LCFA spectra for male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks (n = 8). Different letters indicate differences between groups.

eWAT PL	**LF**	**HF**	**HFMP**	**HFL**	**HFV**	**p-value**
C14:0	1.99 ± 0.10[a,b]	1.85 ± 0.14[a]	2.16 ± 0.05[a,b]	2.40 ± 0.19[b]	1.80 ± 0.08[a]	0.03
C15:0	0.164 ± 0.007[a]	0.119 ± 0.010[b]	0.115 ± 0.004[b]	0.116 ± 0.003[b]	0.131 ± 0.015[a]	0.01
C16:0	19.47 ± 0.34[a]	15.12 ± 0.44[b]	14.13 ± 0.51[b]	13.62 ± 0.36[b]	14.58 ± 0.54[b]	0.0001
C16:1n7c	6.75 ± 0.41[a]	2.48 ± 0.19[b,c]	2.58 ± 0.07[b,c]	3.23 ± 0.21[c]	2.25 ± 0.15[b]	0.0001
C17:0	0.380 ± 0.040[a]	0.361 ± 0.042[a]	0.201 ± 0.012[b]	0.269 ± 0.023[a,b]	0.220 ± 0.024[b]	0.03
C18:0	7.06 ± 0.74	7.43 ± 0.78	7.04 ± 0.58	6.54 ± 0.47	7.10 ± 0.87	n.s.
C18:1n9c	31.10 ± 0.48	28.65 ± 1.07	29.25 ± 0.31	28.03 ± 0.38	27.99 ± 1.41	n.s.
C18:1n7c	3.25 ± 0.173[a]	1.62 ± 0.096[b]	1.56 ± 0.05[b]	1.51 ± 0.04[b]	1.77 ± 0.05[b]	0.0001
C18:2n6t	0.073 ± 0.005	0.055 ± 0.006	0.043 ± 0.005	0.050 ± 0.007	0.050 ± 0.014	n.s.
C18:2n6c	23.84 ± 0.34[a]	36.57 ± 0.58[b]	37.38 ± 0.59[b]	38.38 ± 0.44[b]	37.17 ± 0.81[b]	0.0001
C20:0	0.134 ± 0.010[a]	0.094 ± 0.007[b]	0.098 ± 0.005[b]	0.093 ± 0.006[b]	0.084 ± 0.006[b]	0.002
C18:3n6	0.061 ± 0.005[a,b]	0.049 ± 0.003[a,b]	0.064 ± 0.004[a,b]	0.060 ± 0.004[a]	0.044 ± 0.003[b]	0.02
C18:3n3	1.29 ± 0.09[a]	2.34 ± 0.18[b,c]	2.68 ± 0.05[b,c]	2.20 ± 0.13[c]	2.09 ± 0.14[b]	0.02
C20:1n9	0.640 ± 0.036[a]	0.344 ± 0.011[b,c]	0.403 ± 0.013[b]	0.311 ± 0.008c	0.320 ± 0.020[b,c]	0.04
C20:2n6	0.113 ± 0.008	0.116 ± 0.004	0.125 ± 0.004	0.126 ± 0.006	0.119 ± 0.007	n.s.
C20:3n6	0.309 ± 0.025	0.248 ± 0.014	0.291 ± 0.009	0.251 ± 0.007	0.254 ± 0.014	n.s.
C20:4n6	1.99 ± 0.30	1.34 ± 0.22	1.18 ± 0.05	1.64 ± 0.13	1.48 ± 0.23	n.s.
C20:5n3	0.065 ± 0.005[a,c]	0.088 ± 0.007[b]	0.074 ± 0.004[a,b,c]	0.068 ± 0.003[a,b,c]	0.065 ± 0.006[c]	0.04
C22:4n6	0.151 ± 0.020	0.104 ± 0.011	0.105 ± 0.004	0.114 ± 0.003	0.116 ± 0.009	n.s.
C22:5n6	0.064 ± 0.007[a]	0.029 ± 0.003[b]	0.033 ± 0.003[b,c]	0.041 ± 0.004[c]	0.025 ± 0.002[b]	0.04
C22:5n3	0.190 ± 0.023	0.231 ± 0.027	0.170 ± 0.002	0.181 ± 0.012	0.220 ± 0.031	n.s.
C22:6n3	0.820 ± 0.044	0.746 ± 0.118	0.711 ± 0.034	0.828 ± 0.043	0.629 ± 0.071	n.s.

Supplemental Table 8. Plasma Phospholipid (PL) Long-Chain Fatty Acid (LCFA) Spectra Study IIb. Plasma LCFA spectra for male C57BL/6JRj mice fed low-fat (LF), high-fat (HF) or experimental HF diets supplemented with milk protein (HFMP), leucine (HFL) or valine (HFV) for 20 weeks (n = 8). Different letters indicate differences between groups.

Plasma PL	**LF**	**HF**	**HFMP**	**HFL**	**HFV**	**p-value**
C14:0	0.284 ± 0.032	0.283 ± 0.033	0.348 ± 0.033	0.401 ± 0.066	0.295 ± 0.030	n.s.
C15:0	0.154 ± 0.011[a]	0.098 ± 0.009[b]	0.133 ± 0.005[a,b]	0.124 ± 0.007[a,b]	0.116 ± 0.007[b]	0.01
C16:0	29.28 ± 0.10[a]	24.66 ± 0.36[b]	24.14 ± 0.34[b]	23.77 ± 0.16[b]	24.59 ± 0.16[b]	0.0001
C16:1n7c	0.838 ± 0.055[a]	0.271 ± 0.012[b]	0.280 ± 0.016[b]	0.290 ± 0.015[b]	0.266 ± 0.008[b]	0.0001
C17:0	0.349 ± 0.030[a,b]	0.305 ± 0.014[a]	0.363 ± 0.013[b]	0.364 ± 0.016[a,b]	0.340 ± 0.014[a,b]	0.0001
C18:0	14.96 ± 0.46[a]	19.35 ± 0.33[b]	19.38 ± 0.23[b]	20.46 ± 0.17[b]	19.87 ± 0.16[b]	0.0001
C18:1n9c	8.43 ± 0.26[a]	4.65 ± 0.11[b]	4.83 ± 0.10[b]	4.72 ± 0.07[b]	4.76 ± 0.11[b]	0.0001
C18:1n7c	1.82 ± 0.15[a]	0.721 ± 0.040[b]	0.720 ± 0.027[b]	0.659 ± 0.026[b]	0.769 ± 0.028[b]	0.0001
C18:2n6t	0.103 ± 0.002[a]	0.103 ± 0.003[a]	0.139 ± 0.005[b]	0.121 ± 0.005[b]	0.118 ± 0.004[a]	0.01
C18:2n6c	22.18 ± 0.66[a]	27.85 ± 1.18[b]	27.57 ± 0.48[b]	27.47 ± 0.50[b]	27.57 ± 0.80[b]	0.0002
C20:0	0.214 ± 0.012[a]	0.273 ± 0.027[a,b]	0.319 ± 0.009[b]	0.323 ± 0.012[b]	0.283 ± 0.009[b]	0.02
C18:3n6	0.169 ± 0.009[a]	0.126 ± 0.008[b]	0.148 ± 0.007[a]	0.154 ± 0.004[a]	0.115 ± 0.005[b]	0.02
C18:3n3	0.168 ± 0.012[a]	0.223 ± 0.021[a,b]	0.254 ± 0.014[b]	0.210 ± 0.011[a,b]	0.218 ± 0.014[a,b]	0.002
C20:1n9	0.234 ± 0.006[a,b]	0.206 ± 0.007[a]	0.251 ± 0.008[b]	0.239 ± 0.011[a,b]	0.221 ± 0.014[a,b]	0.02
C20:2n6	0.176 ± 0.007[a]	0.230 ± 0.000[b]	0.244 ± 0.006[b]	0.246 ± 0.006[b]	0.236 ± 0.004[b]	0.0001
C20:3n6	2.06 ± 0.17[a,b]	2.12 ± 0.22[a,b]	1.75 ± 0.10[a,b]	1.52 ± 0.07[a]	2.25 ± 0.19[b]	0.02
C20:4n6	10.26 ± 0.29	9.99 ± 0.87	11.12 ± 0.21	10.62 ± 0.40	9.93 ± 0.41	n.s.
C20:5n3	0.306 ± 0.026[a,b]	0.363 ± 0.022[a]	0.283 ± 0.011[b]	0.205 ± 0.013[b]	0.279 ± 0.017[b]	0.01
C22:4n6	0.173 ± 0.012[a]	0.103 ± 0.005[b]	0.121 ± 0.004[b,c]	0.136 ± 0.004[c]	0.113 ± 0.005[b,c]	0.007
C22:5n6	0.274 ± 0.062[a]	0.051 ± 0.005[b]	0.070 ± 0.004[b,c]	0.091 ± 0.005[c]	0.059 ± 0.002[b]	0.01
C22:5n3	0.458 ± 0.017[a]	0.466 ± 0.011[a]	0.371 ± 0.011[b]	0.389 ± 0.018[b]	0.447 ± 0.007[a]	0.01
C22:6n3	7.13 ± 0.36	7.57 ± 0.22	7.17 ± 0.16	7.37 ± 0.26	7.15 ± 0.28	n.s.

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Curriculum Vitae

*Page 124 (Curriculum vitae) contains personal data, and is therefore not part of the publication

List of Publications

1. **Bishop, CA,** Machate, T, Henning, T, Henkel, J, Pueschel, G, Weber, D, Grune, T, Klaus, S, Weitkunat, K (2022). Detrimental effects of branched-chain amino acids in glucose tolerance can be attributed to valine induced glucotoxicity in skeletal muscle. Nutrition & Diabetes *in review.*

2. Weitkunat, K, **Bishop, CA**, Wittmüss, M, Machate, T, Schifelbein, T, Schulze, MB, & Klaus, S (2021). Effect of Microbial Status on Hepatic Odd-Chain Fatty Acids Is Diet-Dependent. Nutrients, 13(5), 1546.

3. **Bishop, CA**, Schulze, MB, Klaus, S, & Weitkunat, K (2020). The branched-chain amino acids valine and leucine have differential effects on hepatic lipid metabolism. FASEB journal, *34*(7), 9727–9739.

4. Yan, X*, Wang, Z*, **Bishop, CA***, Weitkunat, K, Feng, X, Tarbier, M, Luo, J, Friedländer, MR, Burkhardt, R, Klaus, S, Willnow, TE, & Poy, MN (2018). Control of hepatic gluconeogenesis by Argonaute2. Molecular metabolism, *18*, 15–24.
   *These authors contributed equally to this work

5. Seidl, SE, Pessolano, LG, Jr, **Bishop, CA**, Best, M, Rich, CB, Stone, PJ, & Schreiber, BM. (2017). Toll-like receptor 2 activation and serum amyloid A regulate smooth muscle cell extracellular matrix. PloS one, *12*(3), e0171711.

Conference Contributions

“High-fat valine feeding drives adiposity & glucose intolerance” **CA. Bishop**, T. Henning, T. Machate, S. Klaus & K. Weitkunat; Metabolism Month; 2021; *online*

“Role of branched-chain amino acids in hepatic lipid metabolism” **CA. Bishop**, K. Weitkunat, & S. Klaus; Metabolism in Action; 2019; Copenhagen (Denmark)

“Role of dietary derived propionyl-CoA and odd-chain fatty acids in hepatic lipid metabolism” K. Weitkunat, **CA Bishop**, M. Uhlemann, & S. Klaus; NuGO week 2020; 2019; Bern (Switzerland)

Declaration of Academic Honesty

I hereby declare that the entirety of this PhD thesis entitled "Influences of Dairy Intake on Odd-Chain Fatty Acids & Energy Metabolism" is my original work, apart from the denoted sources. I confirm that this thesis has not been submitted elsewhere for an academic degree.

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Christopher Allen Bishop