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Metabolic Benefits of Plant-based N-3 Polyunsaturated Fatty Acids on Brown Thermogenesis

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METABOLIC BENEFITS OF PLANT-BASED N-3 POLYUNSATURATED FATTY ACIDS ON BROWN THERMOGENESIS

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There are two essential fatty acids for humans, α-linolenic acid (ALA) and linoleic acid (LA), which should be taken from foods to maintain health. Once incorporated into cells, ALA and LA, which are omega-3 poly unsaturated fatty acid (n-3 PUFA) and n-6 PUFA respectively, undergo elongation and desaturation to generate longer and more unsaturated fatty acids influencing inflammation and immunological responses. Numerous studies showed a dietary reduction of n-6/n-3 PUFA ratio improves cardiovascular health, inflammation, and insulin resistance. Fish oil, the main resource for n-3 PUFA, is shown to increase these health benefits. In our lab, we investigated the efficacy of ALA-enriched butter (n3Bu) as an alternative to fish oil for n-3 PUFA. n3Bu-fed mice showed increased bioconversion of ALA to long-chain n-3 PUFA (LC n-3 PUFA) and attenuated high fat (HF) diet-induced insulin resistance and inflammation. Besides, these health benefits, n-3 PUFA is shown to improve obesity and its related diseases by regulating lipid metabolism in both white adipose tissue (WAT) and brown adipose tissue (BAT). Fish oil abundant with n-3 PUFA promotes BAT formation and increases its thermogenic activity in cold acclimation. However, the impact of ALA-enriched agricultural products on the BAT function is unknown. In this study, we investigated the effect of ALA-biofortified butter (n3Bu) on lipid metabolism and

thermogenic functions in BAT. Intake of n3Bu significantly reduced the whitening of BAT and increased the thermogenesis in response to acute-cold treatment. Consumption of n3Bu promoted bioconversion of LC n-3 PUFA, fatty acid elongation and desaturation, and mitochondrial biogenesis. Taken together, our results support that ALA-biofortified butter is a novel source of n-3 PUFA that potentiates the BAT thermogenic function.

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1.1 BAT thermogenesis contributes to attenuating obesity and metabolic syndrome

1.1.1 BAT thermogenesis, energy expenditure, and obesity prevention

Brown adipose tissue (BAT) is an energy-dissipating fat tissue that controls body temperature and whole-body energy expenditure. BAT is physiologically different from white adipose tissue (WAT) that stores energy in Triglyceride (TG) form. BAT is thought to be a therapeutic target of obesity and its associated metabolic diseases due to its thermogenic activity [1-3]. Obesity occurs due to energy imbalance caused by excess calorie intake and decreased energy expenditure [4], while increasing BAT thermogenesis, which controls body energy expenditure, is considered to alleviate obesity [5]. BAT is a significant contributor to body temperature homeostasis in mammals [3, 6, 7]. In humans, BAT has been considered to exist only in newborns and lost rapidly after birth. Recently, human studies have revealed that adult humans possess a significant amount of BAT in the supraclavicular and paraspinal regions, and the thermogenic activity remarkedly increases in response to cold temperature [1-3, 8-11]. In these studies, the BAT amount negatively correlated with age, and obesity-related parameters such as BMI and body fat mass [1, 2, 11]. Some reviews discussed the therapeutic potential of BAT-mediated energy expenditure in adult humans as pharmacological agents [12, 13]. A few studies showed an increase of BAT thermogenesis in human adults during cold exposure [9, 14-16], which is mediated by the sympathetic nervous system (SNS) that activates β3-

adrenergic receptor (AR) [17]. Based on this pathway, oral administration of β3-AR agonist, mirabegron, stimulated BAT thermogenesis in healthy human subjects with detectable BAT and increased energy expenditure [18]. In rodent studies, BAT thermogenic activity significantly increases during cold exposure [19, 20], and directly stimulating β3-AR using β3-AR agonist, increased BAT thermogenesis much more than subjects exposed to cold [21]. These implicate the effect of cold on BAT thermogenesis requires BAT innervation by SNS-activated β3-AR pathway [22]. Transplanting BAT to other animals increases the recipient's BAT thermogenesis with improved insulin sensitivity and ameliorated obesity [23-26]. However, the ablation in BAT reduced energy expenditure and induced obesity in transgenic mice [27]. In humans, obese subjects with early-onset type of obesity with positive family history showed reduced cold-induced thermogenesis compared to lean subjects [28]. Both the core temperature measured at the sternum and the temperature measured at 4^{th} intercostal space between the spine and scapula where the BAT is hypothesized to be located dropped faster and more markedly in obese subjects compared to lean subjects [28]. This result is consistent with the studies showing that both the presence of BAT and its thermogenic activity are negatively correlated to obesity [1, 2, 11] and the absence of BAT showed more dramatic drop of body temperature during the cold exposure [3]. Overall, these findings implicate BAT thermogenesis is a critical factor that controls body energy expenditure and contributes to preventing obesity.

1.1.2 BAT thermogenesis and UCP1-mediated pathway

When the macronutrients undergo fuel metabolism, it produces NADH and FADH2. During oxidative phosphorylation, NADH and FADH2 donates electrons to electron transport chain (ETC), which in turn pumps protons out from the mitochondrial matrix to intermembrane space forming an electrochemical gradient. ATP synthase utilizes this proton gradient to convert ADP to ATP, generating energy in the form of ATP. However, when the protons re-enter the mitochondrial matrix via uncoupling protein 1 (UCP1) bypassing the ATP synthase, the energy stored in the proton gradient is converted to heat instead of ATP [5, 29-31]. The UCP1 expression is very high in brown adipose tissue (BAT), but it is very low in white adipose tissue (WAT) [32]. BAT thermogenesis is stimulated by both cold and diets [33-36]. In cold conditions, the sympathetic nervous system (SNS) activates β-adrenergic receptors (βARs) to promote BAT thermogenic activity [34]. In the adult human, the development of BAT and intensified proton leak via boosted oxidative phosphorylation were observed with the daily exposure of cold for 4 weeks [37]. BAT thermogenesis is also induced by a high-calorie diet via the βARs-mediated pathway [5, 38, 39]. High fat diet-fed mice enhanced diet-induced thermogenesis (DIT), having protective effects against diet-induced obesity. However UCP1-ablated mice showed no UCP1-dependent DIT and developed more obesity [36]. High fat diet or cafeteria diet-fed mice had more UCP1 protein compared to chow-fed mice implicating DIT is UCP1-dependent thermogenesis [36, 40]. Knocking out UCP1 ablated DIT and

induced obesity even in obese resistant 129S mice [40]. These suggest diet-induced thermogenesis (DIT) is UCP1-dependent thermogenesis [36, 40]. UCP1-ablated mice showing obesogenic phenotypes supports increased energy expenditure by BAT thermogenesis is UCP1-dependent [41].

1.2 Metabolic benefits of n-3 PUFA on obesity and thermogenesis

n-3 poly unsaturated fatty acids (PUFA) have a double bond on the third carbon from the methyl end of the fatty acid chain. Long-chain (LC) n-3 PUFAs, especially EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) abundant in fish oil are well-known to stimulate BAT development, thermogenic activity, and thus increasing energy expenditure and ameliorating obesity [42-44]. According to the USDA, people in the US intake proteins heavily from meats such as beef, pork, and poultry (73%) and very little from seafood (5.2%) [45]. The fatty acid profile of chicken showed a very minimal amount of LC n-3 PUFAs, and the content further decreased after being processed to burgers and chicken nuggets [46]. Meats from beef, lamb, and pork showed high saturated FAs contents and a high n-6/n-3 PUFA ratio with high linoleic acid (LA) content [47]. The n-3 PUFA proportions were varying among livestock depending on n-3 PUFA abundance in their feedstock, for example, grass-fed livestock showed significantly increased n-3 PUFA contents, majorly alpha-linolenic acid (ALA), in their muscle and adipose tissues [48, 49]. Typical western diets heavily dependent on red meat, processed meat, and fries from LA-rich vegetable oil, but with limited consumption of both green leafy vegetables and seafood characterize

high n-6 PUFA and shortage of n-3 PUFA [50-53]. This imbalance in the n-6/n-3 PUFA ratio causes chronic inflammation, which can lead to obesity-induced metabolic symptoms such as insulin resistance (IR) and non-alcoholic fatty liver disease (NAFLD) [54-56]. Arachidonic acid (ARA) upregulates CCAAT/enhancer-binding protein (C/EBP) b/d-involved pathway and peroxisome proliferator-activated receptor (PPAR) b/d-involved pathway both of which promote PPARγ expression to produce PPARγ ligand for adipogenic gene activation [57-60]. In contrast, LC n-3 PUFA such as EPA and DHA, have anti-adipogenic characteristics by inhibiting ARA-mediated PPARγ activation [57]. During the postnatal period when the proliferation of adipose tissue is high, lowering n-6/n-3 PUFA ratio lowered adipogenic markers, including PPARγ, reduced size of cells in adipose tissue, and raised circulatory adiponectin level effective for increasing insulin sensitivity in offspring [57, 61]. In addition, these changed adipose morphology gave resistance against diet-induced obesity and insulin impairment in later life [61]. Intake of LC n-3 PUFAs is also effective at increasing BAT thermogenesis which contributes to higher energy expenditure and obesity prevention. Mice fed with EPA and DHA showed increased mRNA and protein expression of BAT thermogenic markers such as PPARα, PGC1α, and UCP1 in both BAT and WAT, suggesting increased thermogenic activity in BAT and browning of WAT [43, 62, 63]. Dietary EPA and DHA diminished lipogenesis, lipid accumulation, hypertrophy in BAT but still increased BAT thermogenic markers in mice [43, 62]. In addition, fish oil-fed rats showed increased BAT thermogenic activity thorough increased mitochondrial contents

and hyperplasia [42, 62]. These implicates the LC n-3 PUFAs in fish oil decrease the size of lipid droplets in BAT but increase the BAT thermogenic activity by increasing the number of cells in BAT. EPA and DHA also increased mitochondrial markers such as Pgc1α (PPARγ coactivator 1 alpha), Pgc1β, Nrf1 (Nuclear respiratory factor 1), Cox4 (Cytochrome c oxidase subunit 4), and Tfam (Mitochondrial transcription factor A) and raised the contents of UCP1, the mitochondrial inner membrane protein [43, 62, 64, 65]. Additionally, EPA treatment on HIB 1B cells, commonly used brown adipose cell line, increased both mitochondrial contents and mRNA expression of BAT thermogenic markers [65]. These suggest LC n-3 PUFAs in fish oil increase the UCP1-mediated BAT thermogenic activity by increasing BAT mitochondrial contents. Fibroblast growth factor 21 (FGF21) is closely associated with cold-induced BAT thermogenesis [66-68]. FGF21 treatment increased mRNA expression of BAT thermogenic markers such as PGC-1α and UCP1 in mice adipocytes [66, 69]. Dietary intake of EPA increased both FGF21 and its receptor, UCP1, in mice adipose tissue [62, 63, 65]. These implicate EPA acts as a ligand for FGF21 to induce UCP1-mediated thermogenic pathway. EPA and DHA also increased AMP-activated protein kinase (AMPK) and Carnitine palmitoyl transferase 1 (CPT1) thermogenic markers implicating LC n-3 PUFAs induce SNS-mediated BAT thermogenesis [62, 64]. As discussed in chapter 1, BAT thermogenesis is regulated by SNS via noradrenaline binding to β3AR. Kim et al. showed fish oil upregulated BAT thermogenic markers, β3AR and UCP1, in wild type mice, but this was not observed in transient receptor potential vanilloid 1 (TRPV1) KO

mice [70]. TRPV1 is known to induce SNS-β3AR-mediated thermogenesis and TRPV1 agonists such as capsaicin also increases BAT thermogenesis [71, 72]. Therefore, no BAT thermogenesis in TRPV1 KO mice under fish oil treatment suggests n-3 PUFAs act as ligands for TRPV1 to stimulate SNS-mediated BAT thermogenesis [70, 71]. AMP-activated protein kinase (AMPK) in the hypothalamus is also a major regulator of SNS-mediated BAT thermogenesis [71, 73, 74]. Fish oil abundant of n-3 PUFAs was effective at increasing AMPK activity in rats [75]. Rodríguez-Rodríguez et al. showed Carnitine palmitoyl transferase 1C (CPT1C) abundant in ventromedial nucleus of the hypothalamus (VMH) have a critical role in VMH AMPK-SNS-BAT pathway, and CPT1C KO mice exhibited impaired BAT thermogenesis [73]. In Bargut et al., fish oil increased BAT thermogenic markers including β3AR, CPT1, and UCP1 [76]. These implicates n-3 PUFAs target CPT1 for BAT thermogenesis which is involved in AMPK-SNS-BAT axis.

1.3 Lipid remodeling during thermogenesis: BAT thermogenesis and FA composition.

1.3.1 FA desaturation, Stearoyl-CoA desaturase (SCD)

There is emerging evidence that intake of dietary LC n-3 PUFAs such as EPA and DHA increase brown adipose tissue development and its thermogenic activities [42, 65, 71, 77, 78]. FAs should undergo a series of elongations and desaturations to synthesize long-chain PUFAs [79]. Stearoyl-CoA desaturase-1

(SCD1), also called Δ9-desaturase, is a desaturase enzyme involved in the first step of PUFA synthesis [79-81]. SCD1 converts saturated fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0) into mono-unsaturated fatty acids (MUFAs) such as palmitoleic acid (C16:1) and Oleic acid (C18:1) respectively [79, 80]. These MUFAs can be further elongated and desaturated to produce PUFAs, including LC n-3 PUFAs such as EPA and DHA [79]. In Chp1, we discussed that the cold-induced BAT thermogenesis is a β3AR-mediated pathway. Either cold stimulation or β3AR activation upregulated protein expression of SCD1 in BAT, suggesting its enzymatic role in β3AR-mediated BAT thermogenesis during cold exposure [82, 83]. $SCD1^{-/-}$ mice showed impaired thermogenesis in cold exposure suggesting SCD1 is required for cold-induced thermogenesis [84]. In mammals, Δ5 and Δ6 desaturases encoded by Fads1 and Fads2 respectively are required for synthesizing LC-PUFAs from 18 carbon precursors [79, 85, 86]. Mammals also lack Δ-12 and Δ-15 desaturases, thus cannot synthesize n-3 and n-6 PUFAs. Therefore essential fatty acids such as ALA and LA must be consumed through diets [79, 87-89]. These desaturases which are required for PUFA synthesis have important roles in human health by affecting PUFA availability to tissue [90]. Desaturase polymorphism is known to exist among populations, and some of these alleles are more vulnerable to metabolic diseases and cardiovascular diseases [90-93]. In young children, lower mRNA-expressions of FADS2 were associated with a higher risk of atopic eczema due to impaired metabolism of synthesizing LC PUFAs [94]. Investigation of polymorphism in FADS1 and FADS2, which participate in LC

PUFAs synthesis such as arachidonic acid (ARA) and DHA showed that allele variation in FADS2 is closely related to Autism spectrum disorders (ASD) [95]. This is supported by the dietary DHA is required for brain and optical developments in children, and also effective at improving their visual and cognitive functions [96-99]. FADS1 gene encodes Δ5 desaturase, which is a rate-limiting enzyme in the metabolism of n-3 and n-6 PUFAs, and minor alleles of FADS1 locus polymorphisms are associated with reduced FADS1 expression and intra-hepatic fat accumulation [100]. Transgenic expression of n-3 PUFA synthesis can increase n-3 PUFA contents in animals. Fat-1 transgenic mice carry the fat-1 gene from *Caenorhabditis elegans* (*C. elegans*) which encodes n-3 FA desaturase enzyme that converts n-6 fatty acids to n-3 fatty acids [89, 101]. The tissue and organs of these fat-1 mice was abundant of n-3 FAs without taking dietary n-3 FAs [89]. Studies for potential transgenic livestock showed fat-1 transgenic pig and cow produced meats and milk abundant of n-3 PUFAs which can be alternatives of fish oil for sustainable n-3 PUFA products from land [102, 103]. In populations where red meat and milk is habitually consumed, these n-3 PUFA enriched meats and milk would provide health benefits from low n-6/n-3 ratio as discussed in the previous chapter. Overexpression of Δ6 and Δ15 FA desaturases activity showed increased ALA accumulation and decreased LA accumulation in blue-green algae, *Synechocystis* sp. PCC6803 [104].

1.3.2 FA elongation and BAT thermogenesis

Cold-induced BAT thermogenesis requires lipid remodeling, such as increased FA elongation activity and n-3 PUFA synthesis [105-107]. Fatty acid elongases are required for synthesizing the three types of n-3 PUFA involved in human physiology, ALA (18:3, n-3), EPA (20:5, n-3), and DHA (22:6, n-3) [108]. For example, elongation of very-long-chain fatty acids 6 (ELOVL6) catalyzes the first and rate-limiting step of fatty acid elongation [108, 109]. ELOVL6 converts C16 fatty acids to C18 fatty acids, then ELOVL3 converts C18 precursors to C20, C22, and C24 fatty acids [105, 106, 109]. Ablation of either Elovl3 or Elovl6 causes defects in BAT thermogenic activity due to reduced capacity to elongate saturated fatty acyl-CoAs into very-long-chain fatty acids [105, 106]. Xu et al. reported the increase of ELOVL3 activation and the accumulation of glycerophospholipid species (GLPs) of DHA, EPA, and ARA during the cold-induced browning of WAT in mice indicating the increased FA elongation activity for cold-induced BAT thermogenesis [107]. The study also suggests the increased FA elongation is involved in the increased synthesis of triglyceride species with higher carbon numbers and double-bond content, which is beneficial to type 2 diabetes (T2D) [107].

1.3.3 Increased cardiolipin synthesis and BAT thermogenesis

Cardiolipin is phospholipid exclusively found in the inner mitochondrial membrane and closely related to mitochondrial energy metabolism [110]. It is an

integral part of the electron transfer chain (ETC) complexes, which forms electrochemical proton gradient [111]. Mass spectrometry (MS) on lipid metabolism of adipose tissue during cold showed that the most increased lipid species were cardiolipin and phosphatidylglycerol, the precursor of cardiolipin, in all of the brown, beige, and white adipose tissues [112, 113]. Since cardiolipin is specific to the inner mitochondrial membrane, increased cardiolipin species level measured by MS can represent the increased mitochondrial contents in the adipose tissue [111, 112, 114]. Cardiolipin constitutes up to 20% of inner mitochondrial phospholipids and essential at stabilizing the mitochondrial morphology and its energy production by interacting with enzyme complex I to IV (electron carriers) of electron transfer chain (ETC) and ADP/ATP carrier of oxidative phosphorylation process [115, 116]. The absence of cardiolipin in crd1 null yeast showed reduced mitochondrial content and disabled ETC function with severely decreased oxidative phosphorylation [111]. Cardiolipin not only serves as essential enzymes for ATP synthesis by oxidative phosphorylation process, but also has a significant role for heat generation by the uncoupling process mediated by UCP1. Lee et al. showed that one UCP1 protein is tightly bound to three molecules of cardiolipin conferring stability [117]. Overexpression of cardiolipin synthase 1 (Crls1) in BAT increased Ucp1 mRNA level, while knocking out Crls1 decreased Ucp1 mRNA expression and significantly reduced uncoupled respiration in the BAT [113]. Therefore, cardiolipin has an essential role in stabilizing both coupling reaction of mitochondrial respiration and the uncoupling reaction of UCP1-mediated thermogenesis. Dietary DHA can reduce LA content

and accumulate DHA in the mitochondrial membrane and cardiolipin [118, 119]. Reviews discuss n-3 PUFAs may influence the protein-scaffold of cardiolipin in the mitochondrial membrane, which may be critical for mitochondrial structure and function [120, 121]. These suggest n-3 PUFAs can target cardiolipin, which has a critical role in uncoupling process of UCP1 [113]. This is further supported by the increase of cardiolipin species in BAT and beige cells during cold, which are the sites of thermogenic activity [112], and the increase of BAT thermogenesis by intake of n-3 PUFAs as discussed in Chapter 2.

1.4 Potential role of ALA on obesity, lipid remodeling and BAT thermogenesis

Alpha-linolenic acid (ALA) is one of two essential fatty acids (EFAs) along with linoleic acid (LA) in humans [122-124]. These essential fatty acids can be used for synthesizing longer chain fatty acids in the body, but the conversion from n-6 to n-3 fatty acids or vice versa doesn't occur in mammals including humans due to lack of FAT-1 protein, an n-3 fatty acyl desaturase [124, 125]. ALA is a precursor of LC n-3 PUFAs, such as EPA and DHA [124-127], whose beneficial function to health is supported by numerous studies. Studies show the conversion of dietary ALA to EPA is about 6 to 21%, and to the DHA is about 0 to 9% [125, 127-130]. Especially in the brain, ALA to DHA conversion was 0.2 to 1% and a significant amount of ALA undergo β-oxidation [124]. However, ALA-fed mice showed as much brain DHA content as DHA-fed mice compared to control mice suggesting dietary ALA can support sufficient DHA level in the

brain [131]. The conversion rate is low in the body because of the rate-limiting step of the bioconversion, the addition of a fourth double bond by Δ 6 desaturase [127, 128, 132] and most dietary ALA undergoes β-oxidation to produce energy or stored as fat [133]. As shown in figure1, ALA and LA compete for the same enzyme; therefore excess intake of n-6 PUFAs lowers the conversion rate of ALA to LC n-3 PUFAs [125, 134, 135]. Western diets with too much n-6 PUFAs and short of n-3 PUFAs can cause an imbalance of n-6/n-3 PUFAs ratio in our body causing chronic inflammation from pro-inflammatory cytokines derived from arachidonic acid (ARA), which can lead to CVD, obesity, and other metabolic diseases such as IR and NAFLD [54, 134, 136]. Studies show bioconversion of ALA to EPA and DHA is higher in women than in men [130, 137, 138], and the genetic variability affects an individual's ability to generate these LC n-3 PUFAs [139]. The conversion rate is also dependent on tissue types, and the intake of ALA is critical for maintaining LC n-3 PUFAs in vital organs such as the liver and brain. [124, 140]. Some studies showed ALA intake was sufficient to generate enough DHA for the brain in adults [131, 133]. However, maternal ALA deficiency significantly drops the concentration of brain DHA in new-born rats, which may affect neural development in the embryo [141]. These suggests dietary ALA is important for maintaining proper DHA levels in neural system. In liver, increasing even small amount of dietary ALA could boost the amount of DHA [142]. Therefore, dietary ALA is necessary for providing sufficient DHA in vital organs even with the low bioconversion rate of ALA to DHA in these tissues. In addition, there are numerous studies on the beneficial functions of ALA on CVD

and other metabolic diseases, which are considered independent of LC n-3 PUFAs such as EPA and DHA [143-145].

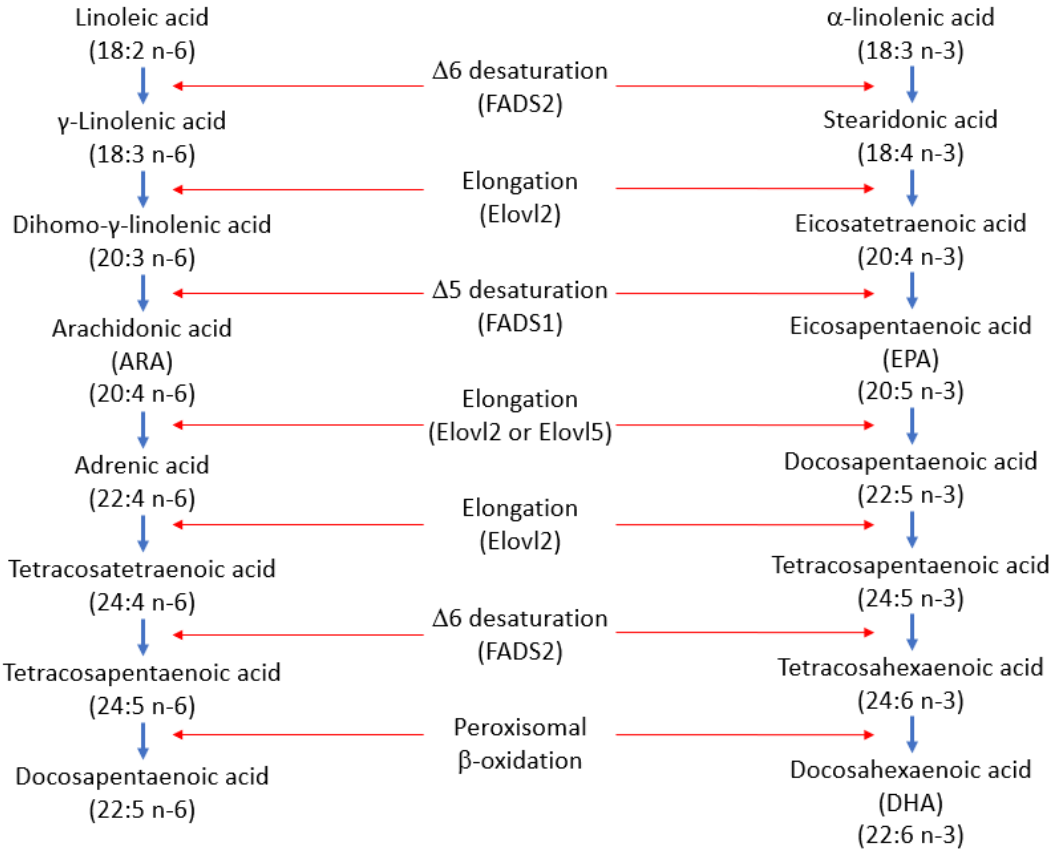

Figure 1. Synthesis of LC n-6 and n-3 PUFAs from essential fatty acids, LA and ALA, in human. LA and ALA share the common enzymes for synthesizing longer chain fatty acids via series of desaturation and elongation.

1.4.1 ALA attenuates CVD, obesity and associated metabolic diseases

ALA is mostly found in seed oils and green leafy vegetables, while EPA and DHA are predominantly present in fish oil [123, 124, 126, 128, 146]. The beneficial effects of marine LC n-3 PUFAs such as EPA and DHA on CVD, obesity, and obesity-induced metabolic symptoms are very well known, while the efficacy of ALA on these diseases is not studied as much as these LC n-3 PUFAs [126, 147, 148]. According to reviews, the dietary intake of ALA increased its content in plasma and tissue, but the effectiveness of ALA on CVD is still controversial [148-153]. Some studies discussed ALA intake was not associated with coronary heart diseases (CHD), but it could be effective at reducing stroke [152-157]. Modern western diets with increased n-6 PUFAs intake and decreased n-3 PUFAs contribute to diet-induced obesity and inflammation via the ARA-mediated pathway [158-160]. The obesity induced by imbalance of an n-6/n-3 PUFAs ratio can be alleviated by increasing intake of n-3 PUFAs such as ALA, which can both inhibit the ARA-mediated adipogenesis and promote lipid oxidation [159-161]. In obese or overweight subjects, consuming ALA-enriched diacylglycerol (DAG) showed anti-obesity characteristics increasing energy expenditure with a significantly reduced visceral fat area (VFA), decreased BMI, and increased dietary fat oxidation [162-164]. In school-age children, the low serum concentration of ALA was significantly related to greater adiposity gains indicating ALA is possibly effective in preventing pediatric obesity [165]. Obesity contributes to chronic systemic inflammation, which induces metabolic diseases such as IR and NAFLD [166-171]. Dietary ALA showed anti-obesity,

and anti-inflammation characteristics hence attenuated these metabolic symptoms [172-174]. Maternal intake of ALA was also effective at attenuating risk factors for these symptoms in adult offspring mice [175]. In Japanese elderly people, ALA intake was much more effective at improving insulin resistance compared to EPA and DHA [176]. ALA intake in obese Zucker rats decreased hepatic lipid accumulation and showed synergetic effects if combined with exercise indicating the beneficial effect of ALA on fatty liver disease [177].

1.4.2 ALA and mitochondrial biogenesis

In chapter 1, we discussed cold-induced BAT thermogenesis is the Ucp1-mediated pathway, which is in the inner mitochondrial membrane [178]. Marine LC n-3 PUFAs such as EPA and DHA showed increased thermogenic activity in BAT by increasing mitochondrial contents, thus raising Ucp1 protein levels [43, 179, 180]. Similar to the beneficial effects of EPA and DHA on mitochondrial contents, dietary ALA can promote mitochondrial biogenesis [181, 182]. Mitochondria is the primary site of β-oxidation of fatty acids which contributes to energy expenditure. ALA from plant sterol ester enhanced mitochondrial biogenesis and mitochondrial β-oxidation, increasing mitochondrial biogenesis markers, Pgc1α, Nrf1, and Tfam, and mitochondrial β-oxidation markers, Pparα and Cpt1a [182]. In the study of Zhou et al., dietary ALA increased mitochondrial contents and its lipid oxidation in WT mice but not in AMPK-ablated mice suggesting the promoting effect of ALA on mitochondrial biogenesis involves

AMPK-mediated pathway [181]. This is supported by the studies that Pgc1α, a key regulator of mitochondrial biogenesis, is mediated by AMPK and Sirtuin 1 (SIRT1) and it is upregulated in BAT by cold [183-187]. ALA also enhanced the positive effect of exercise on increasing mitochondrial contents of obese Zucker rats [177]. The upregulation of both AMPK and SIRT1 by exercise is known to participate in PGC1α-mediated mitochondrial biogenesis [188]. Besides the AMPK-SIRT1-PGC1α axis, mitochondrial biogenesis can be stimulated by PGC1α binding to the complex of PPARα, PPARγ and retinoid x receptor (RXR) [188-191]. Plant seed oils rich in ALA also increases mitochondrial biogenesis. Chia seed oil rich in ALA increased PGC1α in skeletal muscle, implicating the positive effect of ALA on increasing mitochondrial biogenesis [192]. Linseed oil upregulated SIRT1 and PGC1α in hepatic mitochondria and restored mitochondrial biogenesis alleviating insulin resistance and fatty liver disease [193]. This confirms the role of SIRT1-PGC1α in mitochondrial biogenesis. Perilla seed oil as plant-based n-3 PUFA source stabilized mitochondrial membrane potential which is the driving force of oxidative phosphorylation and protected mitochondrial dysfunction from reactive nitrogen species stress suggesting its role in protecting mitochondria and maintaining mitochondrial contents [194].

1.4.3 Effects of ALA on lipid profile

As discussed in chapter3, lipid remodeling such as increased FA desaturation and elongation, and increased cardiolipin contents were observed during the thermogenesis in BAT [105-107, 195]. Considering dietary ALA is effective at attenuating obesity and inflammation as discussed in chapter 4.1, it is assumed that dietary ALA may induce these lipid metabolism modifications. However, there are not extensive research efforts that show the direct effect of ALA on these lipid metabolism changes. SCD1, FADS1 and FADS2 are FA desaturases that participate in FA metabolism in human body, and they are widely used as the desaturation indices in human FA composition [87, 196-199]. SCD1 is an enzyme that converts saturated fatty acids (SFAs) such as stearic acid (C18:0) and palmitic acid (C16:0) into monounsaturated fatty acids (MUFAs) such as oleic acid (C18:1 cis-9) and palmitoleic acid (C16:1 n-7), respectively, both of which are the most abundant MUFAs in human adipose tissue [199-203]. Compared to these long-chain SFAs, dietary MUFAs including oleic acid abundant in olive oil have an increased fatty acid β-oxidation rate and energy expenditure, therefore less likely to induce obesity, chronic inflammation and insulin resistance [203-206].

However, some studies show increased SCD1 activity is closely associated to these symptoms. In rodent experiments, increased SCD1 activity was positively correlated to obesity, insulin resistance, and hepatic steatosis [196, 207, 208], and reducing SCD1 activity using SCD1 inhibitors such as GSK993 and sterculic oil reversed these symptoms and improved metabolic

phenotypes with reduced LDL-C and triglyceride (TG) levels in the liver and the plasma [209-211]. In addition, inhibiting SCD1 in mice using SCD1-specific antisense oligonucleotide inhibitors (ASOs) increased fatty acid oxidization, and reduced fatty acid synthesis and secretion in the liver [212]. In overfeeding conditions, SCD1-null mice (ab^J/ab^J; ob/ob, SCD1 -/-) had higher fatty acid oxidation, oxygen consumption, and energy expenditure which attenuated obesity, insulin resistance and hepatic steatosis compared to their littermate controls (ob/ob) [207, 213-215]. Abnormal hepatic lipid metabolism such as high VLDL-TG level is closely related to obesity, T2D, and steatohepatitis [216-222]. SCD1 products, palmitoleate, and oleate are essential to cholesterol synthesis in the liver [208, 215, 223-226]. Palmitoleate and oleate of liver cholesteryl esters and triglycerides are major components of very-low-density lipoprotein (VLDL) which transports fatty acids to muscle and adipose tissues [207, 208, 215, 227], and the VLDL-TG levels in liver and blood plasma were significantly reduced in SCD1-deficient mice which are protected from obesity and hepatic steatosis [207-209, 215, 224]. In obese humans, SCD activity was positively correlated to the plasma TG levels contributing to obesity [224]. In morbidly obese patients, SCD1 protein levels in both visceral and subcutaneous adipose tissues were significantly correlated to BMI, and insulin resistance [228]. In obese children, SCD indices were positively correlated to BMI but inversely related to DHA content in plasma phospholipids, which was not affected by ALA intake [199].

In some studies, dietary ALA decreased SCD1 activity and improved these symptoms [175, 181, 199, 229, 230]. Dietary ALA as well as SCD1 siRNA

significantly reduced SCD1 activity via farnesoid-X-receptor (FXR) which suppresses sterol regulatory element-binding protein 1c (SREBP1C) which is one of the transcription factors for SCD1 expression [230, 231]. This reduced SCD1 activity by ALA in cholesterol-transporting macrophage-derived foam cells (MDFCs) increased cholesterol efflux and decreased cholesterol accumulation in foam cells implicating the protective effects of ALA against atherosclerosis [230, 231]. Dietary ALA in high fat-fed mice decreased the expression of genes involved in lipogenesis including SCD1 reducing adiposity which are dependent of AMP-activated protein kinase (AMPK) [181]. Maternal ALA intake attenuated adiposity, obesity-induced insulin resistance, and hepatic steatosis, and showed lower SCD1 activity in adult mice offspring compared to those fed maternal SFA [175]. According to Shomonov-Wagner et al. (2015), maternal ALA intake reduced the HOMA index as well as EPA and DHA, but ALA was significantly more effective at reducing SFA-induced liver fat accumulation compared to EPA and DHA [229]. Interestingly, both ALA and DHA showed significantly lower SCD1 activity compared to SFA, while EPA showed high SCD1 activity comparable to SFA in this study [229].

Even though increased SCD1 activity seems to be closely associated to obesity, insulin resistance, and hepatic steatosis, it is not determined if the increased SCD1 activity directly contributes to these metabolic diseases or it is the marker for these symptoms [196, 228]. Deleting Scd1 from both adipocytes and liver reduced Scd1 products but it didn’t protect mice from either genetically-induced or diet-induced obesity and insulin resistance [232]. In leptin-deficient

obese mice, SCD1 deficiency severely aggravated diabetes with reduced insulin secretion because β-cells are impaired by its greater exposure to SFA, triglycerides, free fatty acid, and free cholesterol whose elevated levels are associated with β-cell impairment [214]. In human adipocytes treated with exogenous palmitate (C16:0), palmitate-intolerant adipocytes as well as SCD1-knockdown adipocytes showed low SCD1 activity and high SFA: MUFA composition in phospholipids compared to palmitate-tolerant adipocytes, in addition, SCD1-kncokdown adipocytes had reduced cell membrane fluidity and impaired insulin sensitivity implicating the critical role of SCD1 in phospholipid FA composition and insulin regulation in human adipocytes [233]. Hepatic SCD1 activity was correlated positively with insulin sensitivity and negatively with liver fat in obese participants, but not in lean participants, implicating the protective role of SCD1 in obesity-induced insulin resistance and liver fat accumulation [234]. These suggest SCD1 have a protective role against insulin resistance and hepatic steatosis in obesity, and this could be the reason for the increased SCD1 activity in obese subjects.

FADS1 and FADS2 encode Δ5 and Δ6 desaturases, respectively [235-237]. Genetic variations of FADS1 and FADS2 affect bioconversion of 18C PUFAs such as LA and ALA into longer chain PUFA synthesis [199, 235-237]. This endogenous LC PUFA synthesis from 18C PUFAs as well as dietary LC PUFAs influence body n-6/n-3 LC PUFA ratio and their derivative productions which affect obesity and its associated symptoms [124, 200, 237-241]. Genetic variations on the FADS gene affect the LC PUFA synthesis in humans [237-239,

242]. GG allele at rs174537 of FADS1 on chromosome 11q12.2- q13.1 is more frequent in African ancestry populations compared to European and Asian ancestry populations [238, 239, 242]. This GG allele at rs174537 is more efficient at converting dihomo-γ-linolenic acid (DGLA) to ARA compared to TT or TG alleles, therefore African ancestry populations are more likely to have a greater amount of ARA and its oxidative products with a higher risk of developing chronic-inflammation and its associated disorders with traditional Western diets abundant in LA [238, 239, 242]. Interestingly, this study points out the FADS1 rather than FADS2 is the rate-limiting step of the LC-PUFAs synthesis from 18C PUFAs based on the allelic effect of FADS1 on DGLA to ARA ratio [242]. Women with minor allele homozygotes in FADS1 and FADS2 had low ARA, EPA, and DHA levels in their plasma and breastmilk compared to major allele carriers [243, 244]. These researches implicate the importance of FADS on LC PUFA synthesis in the human body. In a human study with 3-week dietary intervention, saturated fat-rich diet increased Δ6 desaturase, and SCD1 activity and decreased Δ5 desaturase activity, while rapeseed oil-diet rich in MUFA and PUFA showed opposite effects [245]. Mice fed with ALA-enriched diet increased hepatic mRNA expression of both Δ5 and Δ6 desaturases which can partly explain the increased EPA synthesis in both liver and adipocytes in these mice [246]. This makes sense because both Δ5 and Δ6 desaturases are essential enzymes for converting ALA to LC n-3 PUFAs. In the study investigating the effect of either ALA- or LA-enriched diet on rat LC PUFA metabolism, both diets increased hepatic Δ6 desaturase activity, but only ALA-enriched diet increased hepatic Δ5-

desaturase activity, therefore dietary ALA activated both Δ5 and Δ6 desaturases in liver [247]. In addition, these two rodent studies showed ALA-enriched diets increased LC n-3 PUFAs but decreased LC n-6 PUFAs components in liver, adipocytes, and brain [246, 247]. However, neither LA- nor ALA-enriched diets affected Δ5 and Δ6 desaturases activities in the brain [247, 248]. In diet-induced obese rats, ALA-enriched diet increased EPA, and DHA and decreased ARA concentrations in the liver, but the ALA-enriched diet didn't significantly increase the mRNA expression of Fads-1 and Fads-2 [200]. It seems the desaturase activities of FADS1 and FADS2 do not necessarily match the bioconversion of ALA to LC n-3 PUFAs and it is tissue specific.

ELOVL2 and ELOVL5 genes encode FA elongase-2 and FA elongase-5, respectively, that add two carbons to two essential FAs, LA and ALA, during the LC PUFA synthesis in humans [249-252]. ELOVL5 is involved in elongating 18 and 20 carbon PUFAs and ELOVL2 elongate specific for 20 and 22 carbon PUFAs [252]. Genetic variations in ELOVL2 and ELVOL5 affected LC n-3 PUFA metabolism implicating the importance of ELOVLs on endogenous LC n-3 PUFA synthesis [249, 253-255]. Asian sea bass (*Lates calcarifer*) fed with ALA diets significantly increased hepatic mRNA expression of ELOVL5/2, which elongates 18- and 20- carbon PUFAs to 20- and 22-carbon LC PUFAs in this fish, compared to the fish fed with commercial diet [256]. In a mice study, dietary ALA increased the bioconversion of ALA to EPA and the associated mRNA expressions, Fads1, Fads2, and ELOVL5 in the liver, but too much ALA intake (> 5%) decreased these gene expressions [257]. Even though there are not many

studies which measured the effect of ALA on ELOVLs activity, considering that ELOVLs participate in the bioconversion of ALA to LC n-3 PUFAs and dietary ALA increased the endogenous EPA and DHA synthesis, it can be assumed ALA intake activates the ELOVLs [241, 252, 258-261].

As discussed in Chapter3, cardiolipin (CL) is a mitochondrial phospholipid that is essential for stabilizing electron transport chain complexes which generates an electrochemical proton gradient across the mitochondrial inner membrane for ATP synthesis via oxidative phosphorylation [262, 263]. Besides this, CL has a critical role for heat generation by the UCP1-mediated uncoupling process in BAT [112, 113, 117, 264]. During the cold exposure, CL and phosphatidylglycerol, a cardiolipin precursor, were the most increased lipid species in BAT and WAT implicating the importance of CL in cold-induced BAT thermogenesis [112]. Actually, CL binds to and stabilizes UCP1 protein which is essential for BAT thermogenesis [117]. In mice, adipocytes with transiently overexpressed cardiolipin synthase1 (Crls1), increased Ucp1 mRNA expression was observed in BAT [113]. In addition, adipose-specific Crls1 knockout (AdCKO) mice showed depletion of all cardiolipin species and disrupted mitochondrial cristae in iBAT [113]. AdCKO mice had reduced cold-tolerance and Ucp1 transcription was not induced by cold in iBAT and scWAT [113]. These imply CL synthesis is indispensable for cold-induced thermogenesis in BAT and beige fat. N-3 LC PUFAs such as EPA and DHA are known to increase CL contents in mitochondria and decrease the components of n-6 PUFAs such as ARA and LA in CL [118, 119, 265, 266]. ALA, the precursor of these LC n-3

PUFAs, are also expected to increase CL synthesis during cold-induced BAT thermogenesis. Isolated rat cardiomyocytes treated with ALA had significantly increased 18:3 containing CL species and it had protective effects against cardiomyocytes ischemia suggesting ALA changed CL composition and contributed to survival of cardiomyocytes [150]. In the study of incorporation of 18-carbon unsaturated FAs into cardiolipin (CL), oleic acid (18:1) was the most efficient at being incorporated into CL, while LA and ALA were further desaturated and elongated to long-chain fatty acids to be incorporated into CL [267]. This implicates LC PUFAs including EPA and DHA are more efficient at CL synthesis than ALA. Even though there is not much study directly measuring the effect of dietary ALA on CL synthesis, the anti-obesity characteristic of ALA is expected to contribute to BAT thermogenesis via CL remodeling and its enhanced synthesis as LC n-3 PUFAs do. Therefore, the research on the effect of ALA on CL remodeling in BAT during cold would further reveal the role of ALA as an activator of CL synthesis on UCP-1 mediated BAT thermogenesis.

CHAPTER 2. EXPERIMENTS AND RESULTS

2.1 Introduction

Obesity has become a major public health challenge whose prevalence doubled since 1980s in the world [268, 269]. Increased obesity rate burdens the health care system by increasing the expense for treating obesity-related comorbidities and rises the indirect social costs for the loss of productivity from being absent in work for illness [268-270]. In 2017 to 2018, over 40% of Americans were obese from aged 20 to 60 and over [271]. Obesity is also prevalent in children and adolescents, and about one third of them are obese or overweight [268, 272, 273]. Childhood obesity is closely associated with developing insulin resistance, fatty liver, atherosclerosis, and dyslipidemia which were previously considered as ‘adult’ diseases, in addition, children with obesity are more vulnerable to severe obesity, T2D, hepatic steatosis, and coronary heart disease in later life [272, 274-277].

BAT is a unique type of adipocyte that specialized in producing heat energy involved in body energy expenditure. Therefore, the thermogenic activity of BAT is considered as a therapeutic target of obesity [278, 279]. EPA and DHA abundant in fish oil have shown its beneficial effects on alleviating obesity via promoting BAT development and its thermogenic activity [65, 70]. These marine n-3 PUFAs were effective at increasing mitochondrial uncoupling protein UCP1 which is essential for the thermogenic ability of BAT [65, 70, 278, 280]. Long-chain PUFAs serve as activators of the UCP1 uncoupling process, which carries a proton from inter-membrane space to matrix space generating heat [280, 281].

Dietary intake of ALA, the metabolic precursor of EPA and DHA, are effective at reducing visceral fat adiposity, preventing obesity and its comorbidities such as insulin resistance, hepatic steatosis, and dyslipidemia [162, 282-284]. Therefore, ALA is expected to be effective at promoting BAT thermogenic activity, as it showed anti-obesity characteristics and improved metabolic syndromes in human and animal studies [162, 284, 285].

Despite of the beneficial effects of fish-derived n-3 PUFAs on obesity-induced metabolic symptoms, seafood consumption in the US is far below the USDA recommendations [286, 287]. People in the US aged 19 and above are consuming seafood average 3 oz per week, which is less than 8 oz per week as recommended by the U.S. Dietary Guidelines for Americans [287-290], thus not intaking enough EPA and DHA to reduce the risk of CVD as recommended by American Heart Association, 1g per day [287, 289-293]. According to the USDA survey, beef, pork, and poultry are much more consumed as protein sources compared to seafood in the US [45]. Typical Western diet characterized by heavy meat consumption with little seafood intake causes high SFA ingestion and n-6/n-3 PUFA imbalance inducing chronic inflammatory diseases such as obesity, CVD, and fatty liver diseases [52, 54, 294]. On the other hand, using plant oil rich in ALA as n-3 PUFA source significantly lowered the n-6/n-3 PUFA ratio in blood serum, improved lipid metabolism, reduced inflammation, and oxidative stresses [295, 296]. Flaxseed oil rich in ALA also attenuated obesity, improved lipid metabolisms in both plasma and liver, and reduced liver fat accumulation in HFD-fed rats [284]. Additionally, maternal ALA was effective at preventing

SFA-induced insulin resistance and hepatic lipid accumulation in mice offspring [229]. There are many n-3 PUFA-enriched dairy products such as EPA- and DHA-fortified milk, eggs, and cheese, but there are only a few ALA-enriched dairy products and most of them are infused with flaxseed oil [297-300]. Some EPA- and DHA-fortified food products are infused with fish oils, which is not attractive to vegetarians and have some fish flavors unacceptable to some consumers [301, 302]. Eggs from fish oil source had unpleasant fish odor, while eggs produced from flaxseed diet had similar odor compared to regular eggs [303]. Compared to seafoods, land-based n-3 PUFA is more sustainable way to produce foods [304, 305], because industrialized fishing is depleting many fish communities in the world disrupting marine ecosystems [305, 306]. Overfishing is considered to precede all of the other factors such as anthropogenic climate change and water pollution for disturbing marine ecosystems [306]. In addition, seafood consumption is the major route of human exposure to neurotoxic mercury in the organic form of methyl mercury highly accumulated in high trophic fishes [307-310]. Children have lower body weight compared to adults and are more vulnerable to environmental threats due to their specific developmental periods, making them more vulnerable to being poisoned by mercury through fish consumption which is commonly recommended as DHA source essential for their brain development during the childhood [98, 308, 311-313]. In these reasons, ALA-enriched dairy products could be the alternative source of marine n-3 PUFAs. Plant seed oils such as flaxseed, canola, walnut, rapeseed and soybean oils are rich in ALA, they can replace vegetable oils high in pro-inflammatory n-6

PUFAs such as grapeseed, safflower, hempseed, sesame, and sunflower oils, and unhealthy palm oil rich in SFA [314-316]. Numerous studies showed ALA is as effective as EPA and DHA for lowering n-6/n-3 PUFA ratio and alleviating metabolic symptoms associated with high n-6 PUFA consumption. In broiler chickens, ALA from linseed oil was effective as much as fish oil at reducing n-6/n-3 PUFA ratio in meat, enhancing growth and behavioral performances, and improving lipid metabolism [317]. In rat fed with western diets characterized by high fat and high fructose, ALA was effective at alleviating obesity and improving lipid metabolism in liver and blood plasma [136]. And plant oils rich in ALA lowered n-6/n-3 PUFA ratios in rats by increasing hepatic contents of ALA and its metabolically derived LC n-3 PUFAs along with reduced LA and ARA contents [318]. Therefore, consuming ALA-enriched dairy products, and plant seed oils would increase the n-3 PUFA ingestion and alleviate metabolic symptoms caused by n-6/n-3 PUFA imbalance. Considering little seafood consumption in typical American diet, unsustainable conventional agricultural practices in seafood production, the risk of mercury poisoning, and unattractive fish flavors, the land-based n-3 PUFA, ALA, would be the alternative source of marine n-3 PUFAs, EPA and DHA. However, there are not many dairy products fortified with ALA nor many researches on health benefits of these products. Furthermore, most ALA-enriched dairy products used infusion of flaxseed oil powder into the products, however this requires extra steps of mixing microencapsulated flaxseed oil powder to milk before pasteurization [298]. Milk naturally containing high ALA-contents would save cost and time for this process.

Therefore, further researches on ALA-enriched dairy products would be remarkable and compelling. ALA-enriched butter used in this study is made from ALA-enriched milk from bio-fortified cow fed with fermented feedstock rich in ALA. In this research, the ALA-enriched dairy product, ALA-enriched butter (n3Bu), was investigated for its effect on lipid metabolism and BAT thermogenic activity compared to conventional butter rich in SFA, and Margarine with high n-6 PUFA contents.

2.2 Central Hypothesis, purpose, and Specific Aims

A. Purpose of the Study

The purpose of this study is to investigate the impact of the biofortified butter with α-linolenic acid (ALA), the precursor of LC n-3 PUFAs, on the thermogenic BAT function and whole-body energy metabolism.

B. Central Hypothesis

Chronic consumption with ALA-biofortified butter promotes thermogenic activity of BAT by modifying FA metabolism and increasing its thermogenic capacity.

C. Specific Aims

Specific Aim 1: Assess the effects of ALA-biofortified butter on remodeling FA metabolism in BAT such as n-6/n-3 PUFA ratio, FA desaturation, and FA elongation.

Specific Aim 2: Determine the impact of ALA-biofortified butter on mitochondrial biogenesis in BAT during cold-induced adaptive thermogenesis.

2.3 Material and Methods

2.3.1 Animals and diet preparation

All animals were maintained in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska-Lincoln. C57BL/6J mice were purchased at 6 weeks of age from Jackson Laboratory. The mice were randomly assigned into four groups (n=8/group), chow group (chow) or isocaloric high fat diet groups (45% total calories from fat) from conventional butter (Bu, Highland Dairy Foods), n-3 enriched butter (n3Bu, Sunseo Omega Inc), or margarine (Ma, Land O'Lakes), and fed for 10 weeks *ad libitum*. Diets were formulated based on the AIN-93M purified rodent formula (diet composition in **Table S1**). Bodyweight and food intake were measured weekly.

2.3.2 Acute cold treatment and rectal temperature

To measure thermogenic potential, mice were exposed to cold temperature (4°C) acutely (3 hours). Core body temperature was detected using a rectal probe for mice (RET-3, Kent Scientific Corp). Infrared (IR) camera (A655sc, FLIR Systems) was used to detect thermal release and capture images of the surface body temperature. FLIR Research IR program software was used to display surface heat release via color palette representing temperatures between 10 and

34°C. To determine the fatty acid profile change during cold stress, mice were housed either at room temperature (Rm, 22°C) or cold temperature (Cold, 4°C) for 48 hours. At the time of necropsy, brown adipose tissue (BAT) were collected, snap-frozen in liquid nitrogen, and kept at −80°C until analysis.

2.3.3 Fatty acid profile of diet and BAT

To determine FA profile in the BAT, total lipids were extracted as we previously described [319]. Briefly, approximately ~100 mg of BAT was minced, and the extracted total lipids were converted into fatty acid methyl ester. Gas chromatography was performed on Agilent Technologies using a capillary HP-88 column (100 m x 0.25 mm x 0.2 μm film thickness). The identity of lipid species was determined by comparing its relative retention times with the commercial mixed-FA standard (NU-CHEK PREP). The area percentages for all resolved peaks were analyzed using the ChemStation Software (Agilent Technologies). To calculate the C18: C16 and SCD ratio, we used the formulation below.

Elongation (C18:C16) ratio = [C18:0+C18:1n7+C18:1n9/C16:0+C16:1n7]

SCD ratio = [C18:1n7+C18:1n9+C16:1n7/C16:0+C18:0]

2.3.4 qPCR and quantification of mtDNA/gDNA ratio

Total RNA was extracted from ~50 mg of BAT using TRIzol™ Reagent and treated with DNA-free™ DNA removal kit (ThermoFisher Scientific). Then the RNA was reverse-transcribed for cDNA synthesis (iScript, BioRad). Real-time

PCR was carried out on a QuantStudio 6 Flex (Applied Biosystems) using SYBR Green (Fisher scientific). Equal amounts of cDNAs prepared from the individual animal were pooled (n=4 per group) and qPCR reactions were performed in triplicate. The relative gene expression was calculated based on the $2^{-\Delta\Delta CT}$ method with normalization of the raw Ct values to Hprt (hypoxanthine-guanine phosphoribosyltransferase) (Figure S1, S2). Primer sequences are available in Table S2. To determine the mitochondrial DNA to genomic DNA ratio, total DNA was isolated using DNAzol (Life Technologies), as we described previously [320].

2.3.5 Western blot analysis

Protein was extracted from BAT using RIPA buffer containing protease and phosphatase inhibitors (Sigma). Proteins were fractionated using 10% SDS-PAGE, transferred to PVDF membranes, and incubated with antibodies agonist uncoupling protein 1 (UCP1), PR-domain containing 16 (PRDM16), CD11c, F4/80, stearoyl-Coenzyme A desaturase 1 (SCD-1), elongation of long-chain fatty acid-like family member 6 (Elovl6), voltage-dependent anion channel 1 (VDAC1), pyruvate dehydrogenase (PDH), respiratory oxidative phosphorylation protein (OxPhos), and β-actin. Chemiluminescence from ECL solution (Western Lightning) was detected using an ODYSSEY FC Imaging System (LI-COR). Details of antibody information are available in Table S3.

2.3.6 H&E staining

The BAT samples were fixed in 10 % buffered formalin, embedded in paraffin, and cut to 5 μm sections. After deparaffinization, BAT sections were stained with hematoxylin and eosin (H&E) staining as previously described [319].

2.3.7 Statistics

All data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests or Student's t-test, *P<0.05 and ***P<0.001. All analyses were performed using Graph Pad Prism (Version 6.02).

2.4 Results

2.4.1 Supplementation with ALA-biofortified butter promoted thermogenic potential in the BAT

Fatty acid (FA) analysis by GC/MS revealed that butter made out of biofortified milk (referred bio-fortified butter) contained approximately ~4% of ALA (C18:3) similar to margarine, while conventional butter was nearly absent of ALA. Except for ALA content, FA composition was identical between conventional butter and n-3 PUFA fortified butter. Made out of vegetable oil (~80%), margarine contained less amount of saturated FA (both C16:0 and C18:0) and palmitoleic acid (C16:1n7), but possessed a 10-fold higher amount of linoleic acid (LA, C18:2) than conventional butter or ALA-biofortified butter. In all

samples, other PUFA levels, such as arachidonic acid (20:4, ARA), DHA, and EPA, were negligible (Figure 2A).

C57BL/6 mice were fed for ten weeks with one of the isocaloric high fat diets prepared from conventional butter (Bu), ALA-biofortified butter (n3Bu), and margarine (Ma). HF feeding with Bu or Ma, but not n3Bu, significantly increased the BAT weight compared to chow (Figure 2B, C). H&E staining of BAT section revealed that feeding with Bu or Ma remarkably induced white adipocyte-like morphological changes in the BAT, but a significantly lesser degree with n3Bu feeding (Figure 2B). Reflecting the dietary LA content, Ma diet induced ~2-fold increase in LA levels in the BAT. Intriguingly, 10 weeks of n3Bu diet significantly reduced ARA content, while promoted EPA content in the BAT compared with Bu or Ma diet (Figure 2D). Consequently, n3Bu feeding decreased intracellular n-6/n-3 FA ratio in the BAT by ~4-fold compared to Bu or Ma (Figure 2E). These results suggest that bioconversion ALA to n-3 LC PUFA is facilitated in n3Bu-fed BAT compared to Ma-fed BAT.

Next, we asked whether the dietary reduction of n-6/n-3 FA ratio in ALA-biofortified butter alters BAT thermogenic activity. When mice were placed to cold temperature (4°C) acutely (3 hours), n3Bu-fed mice were able to maintain a higher core-body temperature than Bu or Ma-fed mice, as comparable to chow-fed mice (Figure 3A). Heat release captured by IR camera was higher in n3Bu-fed mice than Bu or Ma-fed mice (Figure 3B). The qPCR analysis of brown-specific gene expressions revealed that Ma feeding substantially decreased the brown signature gene expressions of Ucp1, Prdm16, Pgc1α, Cidea, and Dio2, while

n3Bu showed a tendency to increase these gene expressions compared to Bu control (Figure S1). Consistently, protein expression levels of uncoupling protein 1 (UCP1) were reduced in Ma-fed mice compared to Bu or n3Bu. The expression levels of PRDM16, a key transcription factor for brown adipogenesis, were higher in n3Bu-fed BAT than Bu or Ma-fed BAT. Importantly, BAT from n3Bu feeding showed a remarkable decrease in F4/80 and CD11c expression levels, implicating that the n3Bu suppresses macrophage infiltration and proinflammatory M1 polarization compared to the Bu- or Ma-fed group (Figure 3C).

2.4.2 Supplementation with ALA-biofortified butter altered FA composition in the BAT

Emerging evidence suggests that thermogenic activation of BAT is associated with FA remodeling including augmented n-3 LC PUFA synthesis and FA elongation [321]. Based on this literature suggestion, we next investigated the impact of n3Bu on lipid metabolism in the BAT during thermogenic activation. The GC/MS analysis revealed that cold treatment (CT, 4°C) for 48 hours resulted in a rapid increase of EPA and DHA in the BAT in response to n3Bu feeding compared to mice kept at ambient temperature (RT, 22°C) (Figure 4A). However, these changes in n-3 LC PUFA were less prominent in the BAT with Bu feeding and almost completely absent with Ma feeding (Figure 4A). We also examined the differential impact of Bu, n3Bu, and Ma feeding on FA elongation and desaturation. Cold treatment decreased the content of C16 FA (palmitic and palmitoleic acid) but increased the C18 FA (stearic acid) in n3Bu-fed BAT and, to

a lesser degree, Bu-fed BAT. However, Ma-fed BAT failed to alter FA elongation in response to cold temperature (Figure 4B). Therefore, the FA elongation ratio (see the formula in the method section) in the BAT was most evident with n3Bu feeding (Figure 4C). Also, the degree of FA desaturation was higher in n3Bu-fed BAT than in Bu- or Ma-fed BAT upon cold treatment (Figure 4D). The reductions in SCD-1 and ELOVL6 expression in Ma-fed BAT were significant (Figure 4E), although the difference between n3Bu- and Bu-fed BAT did not reach statistical significance. Consistently, there was an increase in transcription levels of Scd-1, Elovl6, and Elvol3 in the n3Bu-fed BAT compared to Bu, but a substantial decrease in these genes in Ma-fed BAT (Figure S2). However, the changes in delta-5 and delta-6 desaturase levels were similar between the groups (Figure S2). Collectively, these results suggest that ALA-biofortified butter facilitates cold-mediated n-3 LC PUFA synthesis and FA elongation/desaturation.

2.4.3 Supplementation with ALA-biofortified butter facilitated mitochondrial biogenesis

Molecular events for thermogenic activation include mitochondrial biogenesis in the BAT [322]. Next, we investigated whether the improved thermogenic function by n3Bu feeding is linked with mitochondrial biogenesis. There was a substantial increase of mitochondrial proteins in the n3Bu-fed BAT compared to Bu- or Ma-fed BAT, including 1) voltage-dependent anion channel 1 (VDAC1) located in mitochondrial outer membrane, 2) pyruvate dehydrogenase (PDH) located in mitochondrial matrix, and 3) oxidative phosphorylation proteins

(OxPhos) located in mitochondrial inner membranes (Figure 5A). Conforming to the increased mitochondrial mass, the mitochondrial DNA to genomic DNA ratio (mtDNA/gDNA) was significantly higher in n3Bu-fed BAT compared to Bu- or Ma-fed BAT (Figure 5B).

Emerging evidence also suggest that sirtuin 3(SIRT3), a NAD+-dependent deacetylase in mitochondria, is a key modulator for brown thermogenesis [323]. Consistent with this study, the transcriptional levels of Sirt3 and SIRT3 protein expression levels were higher in n3Bu-fed BAT than Bu or Ma-fed BAT (Figure 5C, D). Collectively, these data support that ALA-biofortified butter effectively promotes thermogenesis, partly through the facilitation of mitochondrial biogenesis in the BAT.

2.5 Discussion

Previously, we have reported that ALA-enriched butter was effective in attenuating HF diet-induced insulin resistance compared to the other isocaloric diets prepared from conventional butter or margarine [324]. The metabolic improvement by ALA-biofortified butter was associated with augmented bioconversion into n-3 LC PUFA, reduced inflammation in the metabolic tissues (i.e., liver and WAT), and systemic production of anti-inflammatory oxylipins [324]. Here we investigated the impact of ALA-biofortified butter in regulating the BAT thermogenesis. The present work demonstrated that ALA-biofortified butter 1) attenuated HF diet-mediated BAT whitening and inflammation (Figure

2), and 2) increased the brown fat specific gene and protein markers and thermogenic activity (Figure 3). In terms of mechanism, intake of ALA-biofortified butter facilitates cold-mediated lipid remodeling by promoting n-3 LC PUFA conversion, by increasing ELOVL6 and SCD activities and by stimulating mitochondrial biogenesis (proposed working model in Figure 6). Taken together, our work suggests that ALA-biofortified butter could be an alternative of fish oil in activating brown thermogenesis.

Accumulating evidence suggest that FA are critical modulators of non-shivering adaptive thermogenesis [325]. At least three different modes of FA regulation have been reported to promote thermogenic regulation in the BAT including, 1) increased n-3 LC PUFA levels [326, 327], 2) increased FA elongation and desaturation [321], and 3) increased cardiolipin (CL) synthesis [328, 329]. Here, we discussed our results based on these FA regulations for thermogenic activation.

The endogenous synthesis of n-3 LC PUFA requires a series of reactions by elongases and desaturases. In general, LC n-6 PUFA synthesis is the favored pathway over n-3 PUFA formation due to overabundance of LA to ALA [330, 331]. It is important to note that BAT possesses a unique feature of increasing the n-3 LC PUFA content in response to thermogenic stimuli [327]. Our previous results showed that fish oil supplementation elevated the n-3 LC PUFA content upon cold treatment [13]. Similarly, the supplementation with ALA-biofortified butter induced an increase of n-3 LC PUFA levels in basal as well as cold-stimulated conditions (Figure 4A). The increased bioconversion from ALA to n-3

LC PUFA (i.e., DHA and EPA) is dependent on the reduced dietary n-6/n-3 PUFA ratio. Despite the same ALA content, margarine supplementation was unable to increase n-3 LC PUFA content presumably due to high LA content (Figure 4A). The precise mechanism by which cold treatment promotes bioconversion of n-3 LC PUFA in the BAT is yet to be determined. One mechanism could be the induction of GRP120, a well-known membrane receptor for n-3 PUFA, in the BAT during cold exposure [332]. It is possible that n-3 PUFA released from the WAT are redistributed into the BAT through GRP120. This scenario could increase the n-3 PUFA levels in the BAT independent of transcriptional modulation of delta-5 or delta-6 desaturase, the crucial enzymes for PUFA synthesis. Our system may fit in this scenario as ALA-biofortified butter consumption promoted cold-induced n-3 PUFA content without modification of mRNA levels of Fads1 and Fads2 (Figure S2). Nonetheless, our results strongly support that increased availability of n-3 PUFA in the BAT upon intake of ALA-biofortified butter stimulates thermogenic activation.

In addition to an increase of n-3 LC PFUA content, BAT should undergo other compositional changes in FA in order to activate BAT, including the modulation of FA elongation and desaturation [327]. The enzyme ELOVL6 has been demonstrated to regulate thermogenic capacity; Tan et al. revealed that cold treatment significantly promotes the C18:C16 ratio in the BAT due to ELOVL6 activity [321]. In parallel, thermogenic activation of BAT is associated with elevated desaturase index and SCD-1 activity. Conversely, genetic ablation of Elovl6 was unable to induce a full thermogenic recruitment of BAT. More

interestingly, the activation of ELOVL6 activities in the BAT is linked with mitochondrial function [333], suggesting that ELOVL6 activity is required for the remodeling of mitochondria for enhancing thermogenic potential. Consistent with this literature support, our results showed that indices of cold-induced FA elongation and desaturation were higher with ALA-biofortified butter than conventional butter or margarine (Figure 4C). These FA compositional changes by ALA-biofortified butter consumption were correlated with enhanced mitochondrial biogenesis (Figure 5), implicating that FA remodeling is required for mitochondria in the BAT. Recently, Sebaa et al. demonstrated that an increase of mitochondrial deactylation by SIRT3 plays a key role for UCP1 regulation for thermogenic activation. ALA-biofortified butter increased the SIRT3 protein and gene expression in the mitochondria fraction in BAT, suggesting that ALA-biofortified butter may upregulate the deacetylation. We are currently under investigation regarding the mechanism by which increased n-3 PUFA levels promote SIRT3 activation and mitochondrial deacetylation.

Cardiolipins (CL) are unique phospholipids that are predominantly found in the mitochondrial inner membrane. CL plays critical functions, including formation of the respiratory supercomplex [334, 335]. Lipidomic analysis revealed that cold adaptation induces de novo CL synthesis as well as remodeling of CL with the longer and less saturated acyl chains in the BAT [329]. These results are consistent with our observation that ALA-biofortified butter increased ELOVL6 and SCD-1 activities in the BAT. Unfortunately, we were unable to measure the CL lipid content due to a shortage of BAT samples. Also, no

significant differences were found in mRNA expression levels of CL synthase (Crls1) among the isocaloric dietary groups (Figure S1). Nonetheless, we speculate that the CL species would be different in ALA-biofortified butter fed BAT based on an enhanced FA elongation and desaturation. It is of interest to determine the impact of differential dietary fat intake on mitochondrial CL species regulation and its association with mitochondrial thermogenic capacity as a future research.

Besides, there is growing evidence showing that conjugated linoleic acid (CLA), either trans-10, cis-12 isomer alone, or the mixture with cis-9, trans-11 isomer, increases the thermogenic activity in the adipose tissue [336-338]. The thermogenic action by CLA is most evident in the WAT with a minor impact on the BAT [338]. It is quite distinctive from the thermogenic activity exerted by ALA enrichment in this work, since n3Bu supplementation promotes thermogenesis in the BAT, while causes a minimum impact on WAT. It would be of interest to investigate whether CLA supplementation also induces similar FA remodeling, including augmentation of desaturation, elongation and LC n-3 PUFA synthesis.

Bio-fortification of n-3 PUFA is widely provided to farm animals, such as pigs, chicken, and cows, by supplementing the feed with a plant source of n-3 PUFA, i.e., flaxseed oil or algae [339]. However, endogenous conversion rate from ALA to n-3 PUFA is very low in ruminant animals. The most significant barrier is suppressing the bio-hydrogenation of ruminal microbes, which revert dietary unsaturated FA into SFA in the rumens. Without modulation of gut

microbes, the dietary unsaturated FA are unable to reach the small intestine for absorption and incorporation [340]. Numerous efforts were made to increase n-3 PUFA content in cattle in hopes of yielding n-3 PUFA-enriched dairy products such as milk, cheese, and butter [341]. For example, the generation of transgenic cattle was attempted by introducing the Fat-1 gene, the n-3 FA desaturase derived from C. elegans [342]. However, this approach is unfavorable to customers based on an unavoidable dispute regarding the health concerns related to genetically modified foods. Industrial incorporation of fish oil into the dairy products by enzymatic inter-esterification also has limitations due to difficulties in eliminating fish odor. New techniques obtained from the development of system biology have been applied to improve or manipulate the ruminal microbial community to modulate the bacterial population with bio-hydrogenation capacity [343]. Several studies have reported that inclusion of linseed oil in cattle feed improves the FA profiles (decrease of n-6/n-3 ratio) and accumulates the ALA content by modulating the bio-hydrogenation capacity [344]. In our study, milking cows were fed with partially fermented cattle feed that contains two different sources of ALA (giant kelp, and pomace of perilla seeds) resulting in a significant reduction in n-6/n-3 FA ratio in the systemic levels, including meats, fats and milk (data not shown). We cautiously speculate that our cattle feed may trigger bypassing of bio-hydrogenation or suppression of the bacterial population that have bio-hydrogenation capacity. Currently, it is under investigation whether our cattle feed induces a population shift of ruminal bacteria and suppresses bio-hydrogenation capacity in rumens.

2.6 Conclusion

This study provides a novel insight that ALA-biofortified agricultural-products could be an alternative source of n-3 PUFA other than fish. The ALA-enriched butter can recapitulate the enhanced thermogenic energy expenditure similar to fish oil supplementation. The thermogenic activation of BAT was associated with improved biosynthesis of n-3 LC PUFA, FA elongation/desaturation, and mitochondrial biogenesis. By thoroughly evaluating the effectiveness of ALA bio-fortified butter on thermogenesis in comparison with conventional butter and margarine, our study opens a new research avenue for designing health-promoting (or therapeutic) dairy products via manipulation of animal nutrition, metabolism, and presumably ruminal microbiome.

CHAPTER 3. DISCUSSION, LIMITATIONS, AND FUTURE STUDIES

3.1 Discussion

In this report, we investigated the effect of ALA-biofortified butter (n3Bu) on lipid metabolism and thermogenic potential in BAT compared to SFA-rich conventional butter (Bu) and margarine (Ma) with high LA contents. N3Bu lowered n-6/n-3 PUFA ratio reducing n-6 PUFA contents such as ARA in BAT, while increasing n-3 PUFA contents such as ALA and EPA (Figure 2D, E). Compared to Bu and Ma, n3Bu significantly lowered ARA contents in BAT, implicating high ALA contents in n3Bu was effective at suppressing the bioconversion of LA into its LC n-6 PUFA, ARA. This is consistent to the studies that plant oils rich in ALA was effective at lowering n-6/n-3 PUFA ratio in diverse tissues of animals [241, 318, 345]. LA and ALA share the common enzymatic pathway for biosynthesizing their LC PUFAs, therefore they compete each other for the corresponding enzymes [240, 247]. The dietary LA/ALA ratio significantly influences the bioconversion of LA and ALA to their longer chain homologous [240, 241]. Accordingly, it can be said the ALA in n3Bu was effective at suppressing the bioconversion of LA to ARA, but it promoted the biosynthesis of EPA from ALA in BAT. In addition to modifying n-6/n-3 PUFA ratio in BAT, n3Bu significantly attenuated WAT-like morphology in BAT and reduced BAT weight compared to Bu- and Ma-fed BAT (Figure 2B, C). Fish oil abundant of EPA and DHA is well known for reducing weight gain in WAT and attenuating lipid accumulation in diverse tissues via promoting lipid oxidation and inhibiting lipogenesis [70, 346-350]. Fish oil is also effective at upregulating

thermogenic markers such as UCP1, PGC1α, PRDM16, and β3-AR in both WAT and BAT implicating the efficacy of fish oil on browning of WAT and enhancing BAT thermogenic activity [70, 76, 346]. As well as fish oil, n3Bu in this study promoted cold-induced thermogenic activity which enhances body energy expenditure and upregulated BAT thermogenic markers such as UCP1 and PRDM16 (Figure 3A, B, C).

Intake of excessive n-6 PUFAs and SFAs in modern Western diet remarkably enhances pro-inflammatory cytokines secretion in adipocytes and causes low-grade chronic inflammation and inflammatory diseases such as depressive symptoms, CVD, cancer, obesity, insulin resistance, and obesity-induced fatty liver diseases [351-354], while increasing marine n-3 PUFAs intake attenuates these chronic inflammations via inhibition of nuclear factor-kappa B (NF-kB) and toll-like receptor 4 (TLR4), which stimulate pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and C-reactive protein (CRP) [352, 353, 355, 356]. Obesity increases the macrophage infiltration into obese WAT and these adipose tissue macrophages become pro-inflammatory M1 macrophages [351, 357, 358]. M1 macrophages release pro-inflammatory cytokines such as TNF-α, IL-6, and IL-β which induces further M1 macrophage differentiation from monocytes and causes inflammation that inhibits insulin action in adipocytes [351, 357-360]. On the other hand, healthy adipocytes in lean people is predominated by M2 macrophages that secrete anti-inflammatory cytokines [358, 361-363]. Adiponectin confers anti-inflammatory effects on multiple cell types and tissue by

reducing pro-inflammatory cytokine secretion and promoting monocyte-derived macrophage polarization into anti-inflammatory M2 phenotype [360, 363-365]. N-6 PUFA and SFA downregulated adiponectin and increased macrophage infiltration in adipose tissue, but n-3 PUFA restored these adiponectin gene expressions and prevented macrophage infiltration suggesting the protective action of n-3 PUFA on adipocytes from high-fat diet-induced inflammation [365]. In addition, n-3 PUFA intake downregulated inflammatory cytokines, increased anti-inflammatory cytokines, and reduced inflammatory macrophage in adipose tissue decreasing M1 macrophage markers such as CD11c, F4/80, MCP-1, CCL2 and IL-6 and increasing M2 macrophage markers such as IL-10, arginase 1, and MGL1, [365-369]. This suggests n-3 PUFAs shifted macrophage polarization from pro-inflammatory M1 to anti-inflammatory M2 phenotype. Consistent to these results, n3Bu-fed BAT in this study had low expression of pro-inflammatory M1 macrophage markers, CD11c and F4/80, compared to Bu- or Ma-fed BAT (Figure 3C). This suggests the n3Bu has anti-inflammatory effects as well as fish oil via attenuating high-fat diet-induced M1 macrophage polarization.

Fish oil rich in EPA and DHA is well known for promoting cold-induced BAT thermogenesis via increasing thermogenic markers expression and body energy expenditure in cold conditions [43, 71, 76]. Therefore, it is assumed increased LC n-3 PUFA synthesis is required for BAT thermogenic activity during cold acclimation. Even though there are not many studies that directly show endogenous LC n-3 PUFA synthesis is required for BAT thermogenic activity, the beneficial effects of dietary LC n-3 PUFAs on BAT thermogenesis in

cold suggests endogenous LC n-3 PUFA synthesis also contributes to BAT thermogenic activity. In this study, n3Bu was effective at promoting the biosynthesis of EPA and DHA in BAT during cold acclimation compared to Bu- and Ma-fed BAT (Figure 4A). This suggests n3Bu promoted bioconversion of ALA into EPA and DHA in cold implicating the critical role of endogenous LC n-3 PUFA synthesis on cold-induced BAT thermogenesis. Besides the endogenous LC n-3 PUFA synthesis, BAT thermogenic activity requires FA metabolism modifications such as increased desaturation and elongations. SCD1 is a desaturase enzyme that converts C16- and C18-saturated fatty acids into monounsaturated fatty acids such as palmitoleic acid (C16:1) and oleic acid (C18:1) which are major components of membrane phospholipids, triglycerides and cholesteryl esters and have a critical role at lipogenesis [207, 213, 370]. SCD1 is often considered as the therapeutic target of obesity, and its associated metabolic symptoms, because SCD1 is closely associated with obesity, diabetes, atherosclerosis, and cancer, while depleting SCD1 in mice prevented obesity via reducing adiposity and increasing energy expenditure [213, 371, 372]. $SCD1^{-/-}$ mice had lean phenotype with increased lipolysis, decreased lipogenesis, and increased fatty acid β-oxidations and were resistant to obesity-related metabolic symptoms [84, 213, 370, 371]. Leptin is known to suppress lipogenesis of SCD1 activity, and leptin-deficient ob/ob mice become profoundly obese [207, 371, 373]. Consistently, the obese phenotype of leptin-deficient ob/ob mice were attenuated by SCD1 deficiency due to reduced adiposity and increased energy metabolism [207, 371, 373]. However, SCD1-deficient mice ($scd1^{-/-}$) showed

severe cold intolerance that failed them to maintain their body temperature in cold (4 C°) which led to death due to cold stress in several hours [84, 373]. $SCD1^{-/-}$ mice had increased basal thermogenesis in room temperature with upregulation of BAT thermogenic markers expression such as UCP1, β3-AR and PGC1α compared to $SCD1^{+/+}$ mice, however $SCD1^{-/-}$ mice developed hypothermia in cold temperature even with increased UCP1 expression [84]. This suggests the SCD1 has an essential role for adaptive thermogenesis in cold condition which is vital for mammals to survive in cold temperature. Consistent to this, n3Bu in this study was effective at increasing cold-induced BAT thermogenesis (Figure 3) and significantly increased SCD ratio in BAT during cold acclimation, while Bu- and Ma-fed BAT failed to show significant increase of SCD ratio (Figure 4D). In addition, SCD1 protein expression in Ma-fed BAT was significantly lower than Bu- and n3Bu-fed BAT (Figure 4E). This implicates n3Bu was effective at increasing SCD activity in cold which is considered to be essential FA metabolic modification for cold-induced BAT thermogenic activity, while high LA contents in Ma inhibited the remodeling of FA metabolism for the adaptive thermogenesis in BAT. In addition to the SCD1 activation, FA elongations are also required for BAT thermogenic activity. Both Elovl3 and Elovl6 genes belong to Elovl gene family that encodes enzymes for very long-chain fatty acid (VLCFA) elongation [106, 374, 375]. ELOVL3 elongates SFAs and MUFAs up to 24 carbons [105], and ELOVL6 is the major enzyme that elongates C16 FAs to C18 FAs [106, 375, 376]. Some studies showed activation of Elovl3 in BAT is closely associated with BAT thermogenic capacity during cold acclimation [105, 374], and Elovl3

expression is dramatically upregulated in BAT during the cold exposure [82, 374, 377-379]. Elovl6 is also essential for BAT thermogenic activity via regulating mitochondrial contents [106]. Deletion of Elovl6 ablated the cold-induced increase of elongating C16 to C18; and Elovl6 KO mice failed to increase the expression of BAT mitochondrial ETC proteins during cold [106]. These implicate Elovl6-mediated FA elongation is necessary for BAT thermogenic activity via regulating mitochondrial functions. N3Bu in this study also activated these FA elongations in BAT during cold (Figure 4B, C), indicating the modification of FA metabolism occurred in n3Bu-fed BAT during the adaptive thermogenic activity in cold. Protein expression of ELOVL6 was increased in n3Bu-fed BAT compared to Bu- and Ma-fed BAT (Figure 4E), implicating n3Bu was effective at inducing FA metabolism change for BAT thermogenic activity in cold via regulating mitochondrial contents. This is consistent to the increased mitochondrial proteins in n3Bu-fed BAT (Figure 5A). Therefore, n3Bu is effective at remodeling FA metabolism such as FA desaturation and elongation to promote BAT thermogenic activity in cold.

Mitochondria is important for UCP-1 mediated thermogenesis in BAT because BAT thermogenic activity is mediated by uncoupling process of UCP1 which is located on inner mitochondrial membrane in BAT. Fish oil abundant in EPA and DHA is well known for activating mitochondrial biogenesis. As well as fish oil, n3Bu in this study also increased mitochondrial protein contents in BAT compared to Bu- and Ma-fed BAT (Figure 5A). And this is further supported by the increased mitochondrial-DNA/genomic-DNA ratio in n3Bu-fed BAT (Figure

5B). Sirtuin is protein deacetylases that is involved in cellular energy homeostasis [380, 381]. Among mammalian Sirtuin family members that control metabolic processes, sirtuin3 (Sirt3) resides in mitochondria and involves in mitochondrial biogenesis [380, 382, 383]. Sirt3 and Ucp1 are upregulated in BAT during cold exposure; and constitutive expression of Sirt3 increases UCP1 and PGC1-α expressions suggesting Sirt3 has a critical role in cold-induced BAT thermogenesis [384]. N3Bu significantly increased mRNA and protein expressions of Sirt3 in BAT compared to Bu- and Ma-fed BAT (Figure 5 C and D). This implicates n3Bu is as effective as fish oil for modifying mitochondrial functions which is essential for BAT thermogenic activity in cold.

As shown in this research, ALA-biofortified butter had beneficial effects on inducing BAT thermogenesis in cold. N3Bu was effective at maintaining BAT thermogenic capacity attenuating WAT-like morphology induced by high-fat diets, and increased body energy expenditure during cold exposure along with increased BAT thermogenic markers. In addition, n3Bu modified FA metabolism and regulated mitochondrial biogenesis in BAT both of which are considered essential for cold-induced thermogenic activity in BAT. The positive effect of n3Bu on BAT thermogenic activity in this research suggests ALA-biofortified dairy products could be a reliable source of land-based n-3 PUFA that can replace marine n-3 PUFAs abundant in fish oil. Consumption of 18C PUFAs such as LA and ALA from land-based agricultural products such as vegetable oils and dairy products are much greater than the utilization of EPA and DHA abundant in fish oil. In this aspect, comparing the biochemical characteristics between n-6 and n-3

18C PUFAs on BAT metabolism contributes to identifying the differential effects of these essential FAs on BAT thermogenic activity, the therapeutic target of obesity.

3.2 Limitation and future studies

In this research, n3Bu significantly increased thermogenic potential in BAT with increased SCD1 and ELOVL6 protein expressions compared to Ma-fed BAT (Figure 3, 4E). However, these enzyme expressions are closely related to obesity, and its associated metabolic symptoms such as insulin resistance and hepatic steatosis, while suppressing these enzymes are known to attenuate these symptoms [196, 228, 385-389]. SFA is known to increase SCD1 activity; and SCD1 deficiency significantly reduced lipogenic gene expression and it was protective against metabolic disorders induced by excessive SFAs intake [390-393]. On the other hand, both n-3 and n-6 PUFAs suppressed SCD1 expression and attenuated obesity-induced metabolic symptoms [394-398]. ELOVL6 is also involved in obesity-induced insulin resistance and hepatic steatosis [375, 386, 387]. ELOVL6-deficeint ($ELVOL6^{-/-}$) mice were protected from obesity-induced insulin resistance via suppressing pro-inflammatory cytokines such as TNF-α, IL-1β, and inflammasome gene expressions including NLR family pyrin domain-containing 3 (NLRP3), even under the same hepatic steatosis and obesity conditions [375, 386-389]. Weiss-Hersh et al. showed fish oil significantly suppressed the hepatic gene expressions of both SCD1 and ELOVL6 [399]. In

these respects, the high SFA content in n3Bu might have increased expressions of SCD1 and ELOVL6, while margarine made from vegetable oil abundant of PUFAs might have been effective at reducing these enzyme expressions (Figure 2A, 4E). In regard to the effects of SFA, and PUFA on the lipid metabolism and thermogenic potential in BAT, SFA: PUFA ratio could be fixed in this study. Therefore, in the future, to better identify the effects of n-6/n-3 PUFA ratio on lipid metabolism, ALA-enriched margarine could be compared to conventional margarine rich in n-6 PUFAs.

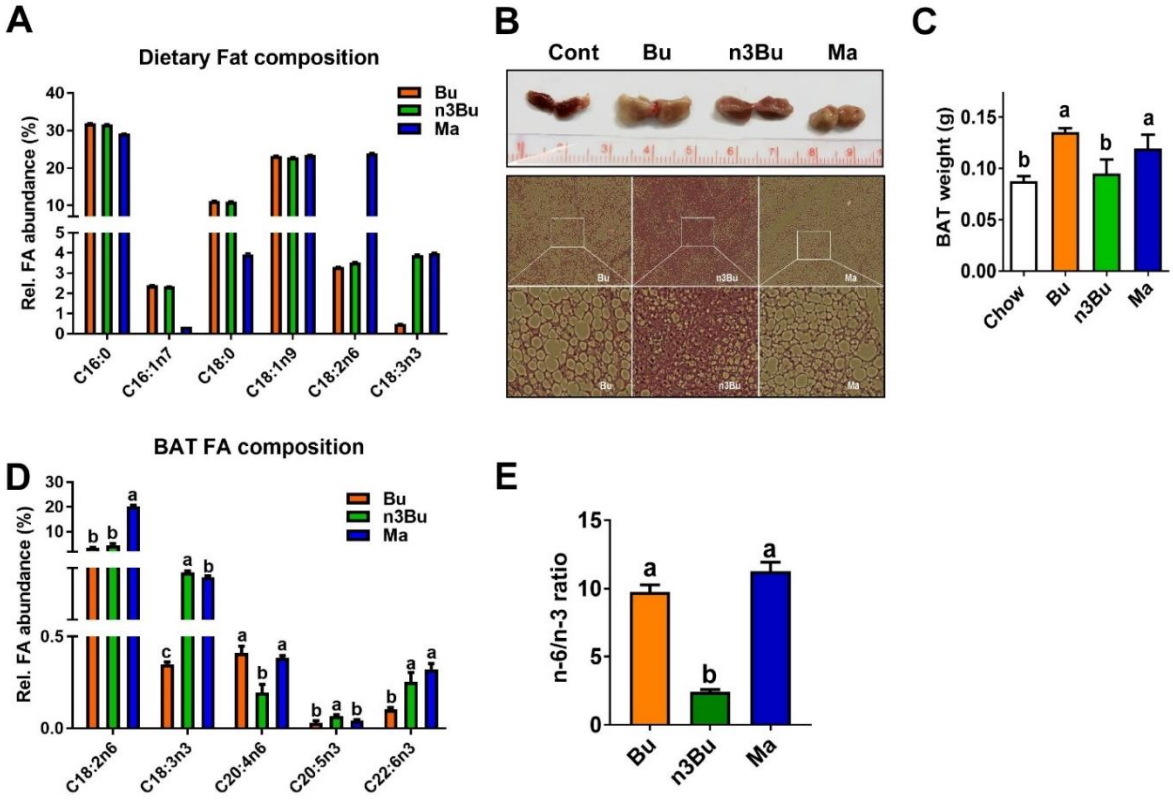

Figure 2. Supplementation with ALA-biofortified butter decreased triglyceride accumulation and n-6/n-3 PUFA ratio in the BAT. A. Fatty acid composition of the isocaloric HF diets made of conventional butter (Bu), ALA-biofortified butter (n3Bu), and margarine (Ma). B. Gross image (upper) and histology of BAT (lower) after 10 weeks of HF diet feeding (representative of n=8/group). C. BAT weight. D. Fatty acid profile in the BAT after supplementation. E. n-6/n-3 PUFA ratio in the BAT. All data represented as mean ± SEM. Treatments with different letters are significantly different from one another ($P<0.05$) by one-way ANOVA with Tukey's multiple comparison tests.

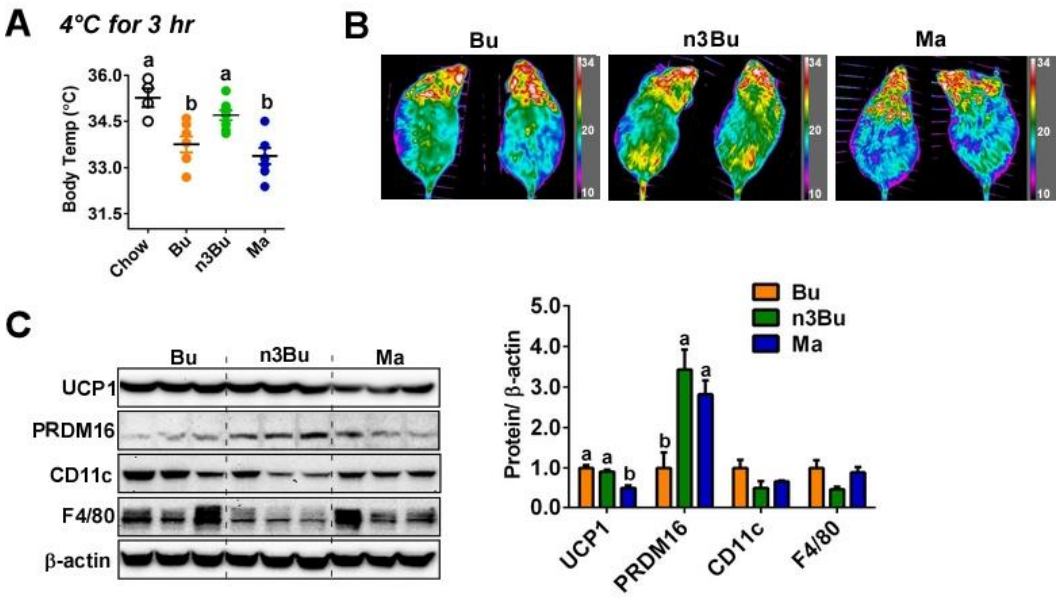

Figure 3. Supplementation with ALA-biofortified butter promoted thermogenesis and suppressed inflammation in the BAT. A. Core body temperature after exposing animals to 4°C acutely for 3 hours (n=4 for chow, n=8 for HF-fed animals). B. Heat release captured by IR camera at the end of a 3-hour cold exposure. C. Western blot analysis of UCP1, PRDM16, CD11c, and F4/80. β-actin used as a loading control for quantification. All data represented as mean ± SEM. Treatments with different letters are significantly different from one another ($P<0.05$) by one-way ANOVA with Tukey's multiple comparison tests.

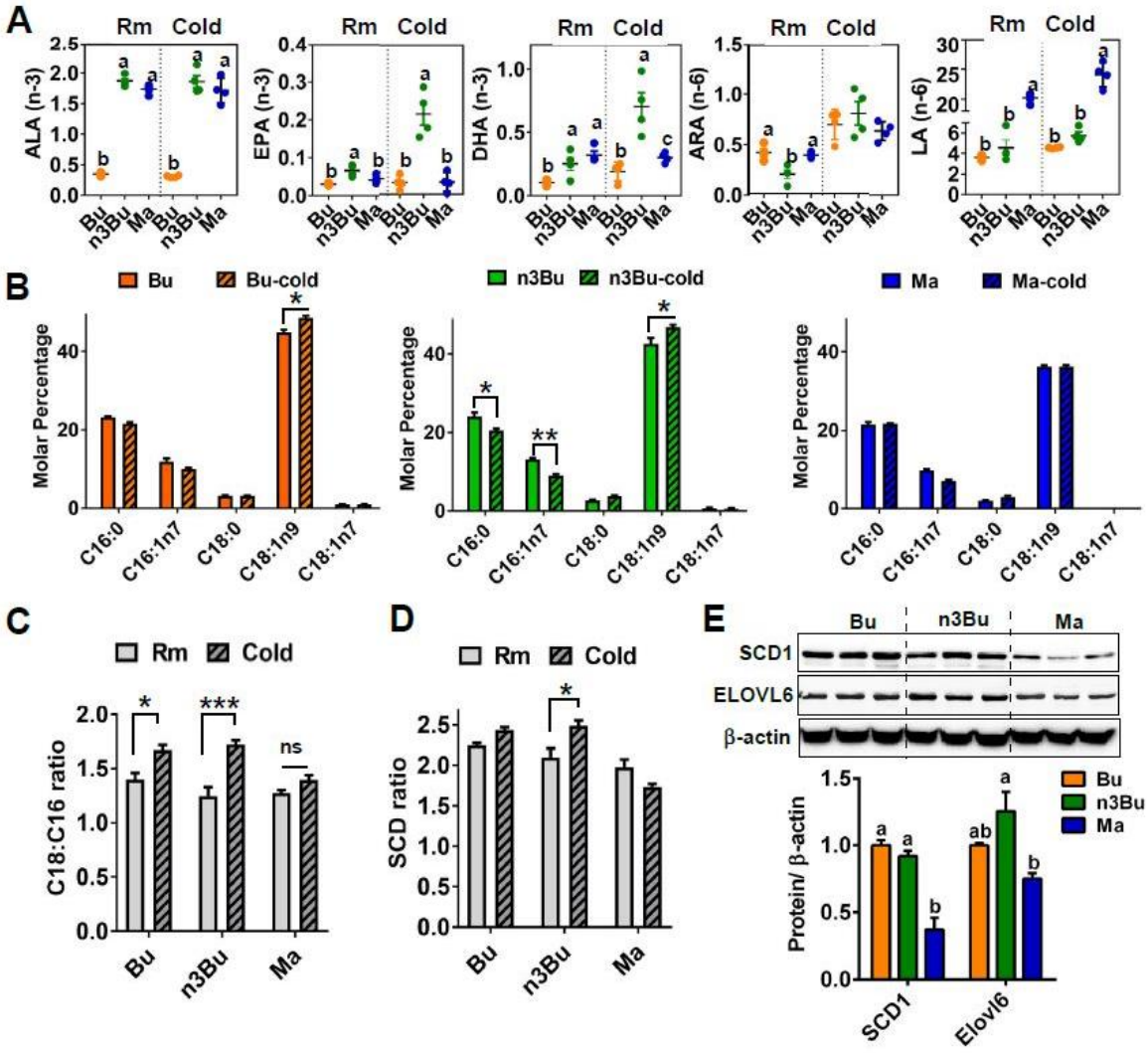

Figure 4. Supplementation with ALA-biofortified butter increased n-3 PUFA content and degree of desaturation and elongation of fatty acids in the BAT in response to cold exposure. A. Composition of n-3 (ALA, EPA, and DHA) and n-6 (ALA and LA) PUFA in the BAT at room temperature (Rm) or cold exposure (Cold) for 48 hours (n=4 per group). B. Fatty acid methyl ester analysis of BAT at the Rm and Cold. C. C18:C16 ratio. D. SCD ratio. E. Western blot analysis of stearoyl-CoA desaturase 1 (SCD-1), elongation of long chain fatty acid-like family member 6 (ELOVL6). Each lane represents individual animal (n=3) and β-actin used as a control for quantification (below). In A and E, treatments with different letters are significantly different from one another ($P<0.05$) by one-way ANOVA. All data represented as mean ± SEM. In B, C, and D, *, $P<0.05$, and ***, $P<0.001$ by Student's t-test.

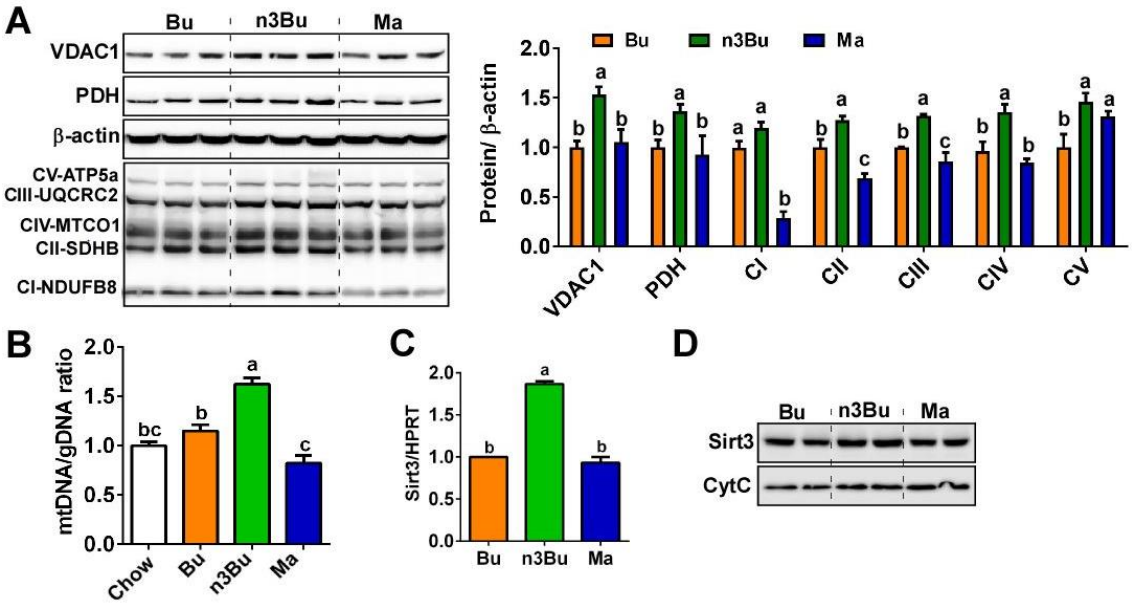

Figure 5. Supplementation with ALA-biofortified butter increased mitochondrial biogenesis in the BAT. A. Western blot analysis of mitochondrial proteins of VDAC1, PDH, and respiratory protein complexes I-V (left). β-actin was used as a control for quantification (right). **B.** mtDNA to gDNA ratio in BAT by qPCR (n=6 per group). **C.** *Sirt3* mRNA expression in BAT by qPCR (n=6 per group). **D.** Western blot analysis of Sirt3 in the mitochondrial fraction. Each lane represents individual animals in duplication. Cyt C was used as a control. All data represented as mean ± SEM. Treatments with different letters are significantly different from one another by one-way ANOVA ($P<0.05$).

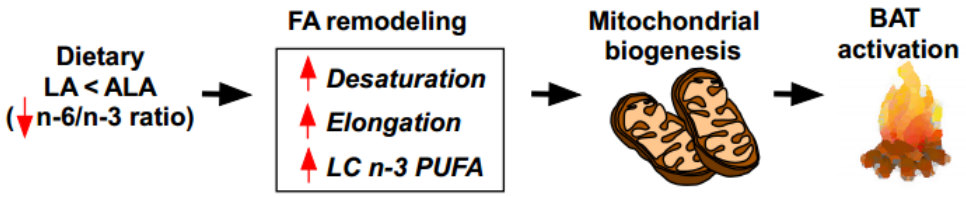

Figure 6. Working model of dietary ALA enrichment on thermogenic activation in the BAT. The reduced n-6/n-3 ratio by dietary supplementation with ALA enriched foods induces fatty acid remodeling, including the augmentation of desaturation, elongation, and long-chain (LC) n-3 PUFA formation. These FA profile changes facilitate mitochondrial biogenesis, leading to the thermogenic activation of the BAT.

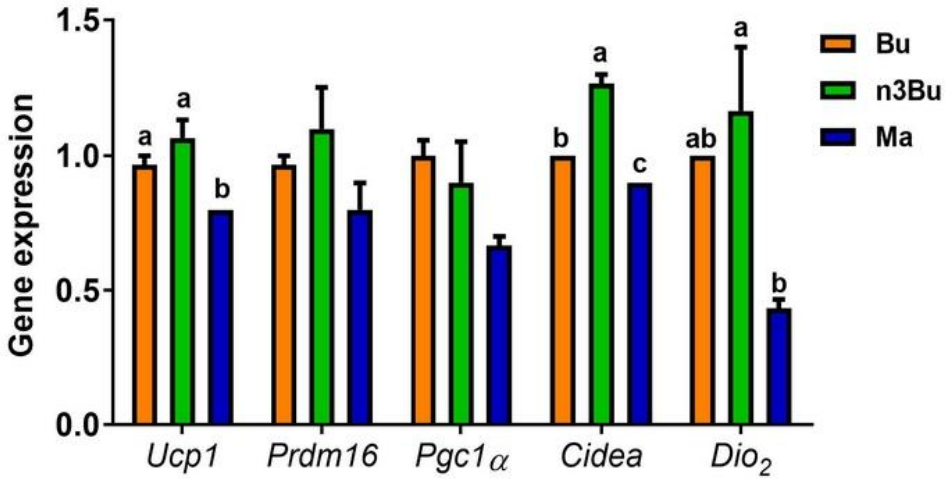

Figure S1. Supplementation with ALA-biofortified butter alters thermogenic genes expression in the BAT. mRNA expression levels of *Ucp1*, *Prdm16, Pgc1α*, *Cidea*, and *Dio2* from the pooled DNA (n=8 per group) with triplicated assay. All data represented as mean ± SEM. Treatments with different letters are significantly different from one another by one-way ANOVA ($P<0.05$).

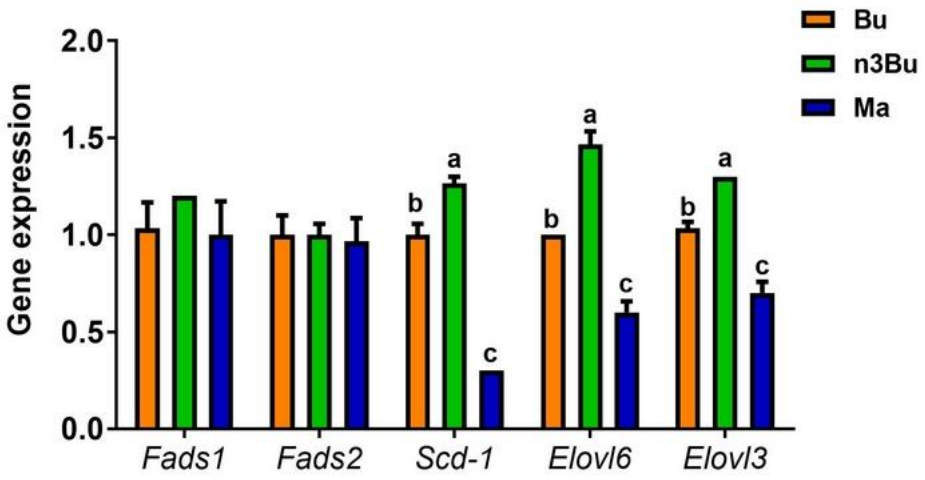

Figure S2. Supplementation with ALA-biofortified butter increased fatty acid denaturation and elongation related genes expression in the BAT. mRNA expression levels of *Fads1*, *Fads2*, *Scd-1*, *Elovl6*, and *Elovl3* from the pooled DNA (n=8 per group) with triplicated assay. All data represented as mean ± SEM. Treatments with different letters are significantly different from one another by one-way ANOVA ($P<0.05$).

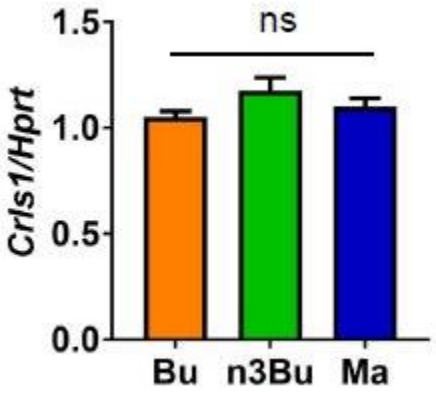

Figure S3. Supplementation with ALA-biofortified butter did not alter cardiolipin (CL) synthase (*Crls1*) expression in the BAT. mRNA expression *Crls1* in the BAT (n=4 of individual animals per group). All data represented as mean ± SEM. Treatments with different letters are significantly different from one another by one-way ANOVA ($P<0.05$).

Table S1. Diet composition of HF diet

Ingredients (g)	Diet		
	Bu	**n3Bu**	**Ma**
Casein	235	235	235
L-cystine	5	5	5
Corn starch	210	210	210
Maltodextrin	100	100	100
Sucrose	100	100	100
Cholesterol	2	2	2
Cellulose	50	50	50
Mineral mix	35	35	35
Calcium carbonate	30	30	30
Vitamin mix	10	10	10
Choline bitartrate	2.5	2.5	2.5
Solid Fat			
Hiland Butter	240	-	-
Sunseo Omega Butter		240	-
Land O Lake Margarine	-	-	240
% calories from Fat	45.4	45.4	45.4
% calories from CHO	34.4	34.4	34.4
% calories from Protein	20.2	20.2	20.2
Kcal/g diet	4.67	4.67	4.67

Table S2. Primer sequences for qPCR.

Primer	Primer sequence
Ucp1	F: 5'-AGGCTTCCAGTACCATTAGGT-3' R: 5'-CTGAGTGAGGCAAAGCTGATTT-3'
Prdm16	F: 5'-CAG CAC GGT GAA GCC ATT C-3' R: 5'-GCG TGC ATC CGC TTG TG-3'
Pgc1α	F: 5'-CCCTGCCATTGTTAAGACC-3' R: 5'-TGCTGCTGTTCCTGTTTTC-3'
Cidea	F: 5'-TGCTCTTCTGTATCGCCCAGT-3' R: 5'-GCCGTGTTAAGGAATCTGCTG-3'
Dio2	F: 5'-CAGTGTGGTGCACGTCTCCAATC-3' R: 5'-TGAACCAAAGTTGACCACCAG-3'
Fads1	F: 5'-TCAGTCTTTGGCACCTCGAC-3' R: 5'-TCCTTGCGGAAGCAGTTAGG-3'
Fads2	F: 5'-TCCTGTCCCACATCATCGTCATGG-3' R: 5'-GCTTGGGCCTGAGAGGTAGCGA-3'
Scd-1	F: 5'-GGGACAGATATGGTGTGAAACTATG-3' R: 5'-TTACAGACACTGCCCCTCAAC-3'
Elovl6	F: 5'-CGTAGCGACTCCGAAGATCAGCC-3' R: 5'-AGCGTACAGCGCAGAAAACAGGA-3'
Elovl3	F: 5'-CTTCGAGACGTTTCAGGACTTAAG-3' R: 5'-TCTGGCCAACAACGATGAG-3'
16S rRNA	F: 5'-CCGCAAGGGAAAGATGAAAGAC-3' R: 5'-TCGTTTGGTTTCGGGGTTTC-3'
Hexokinase	F: 5'-GCCAGCCTCTCCTGATTTTAGTGT-3' R: 5'-GGGAACACAAAAGACCTCTTCTGG-3'

Table S3. List of primary antibodies

Antibody	Host	Dilution	Company	Catalog no.
UCP1	Rabbit	1:1000	Abcam	Ab155117
PRDM16	Mouse	1:1000	Santa Cruz	Sc130243
CD11c	Rabbit	1:1000	Cell Signaling	97585
F4/80	Rabbit	1:1000	Abcam	Ab6640
SCD1	Rabbit	1:1000	Cell Signaling	2794
Elovl6	Rabbit	1:1000	Abcam	Ab69857
VDAC1	Rabbit	1:1000	Cell Signaling	4661
PDH	Rabbit	1:1000	Cell Signaling	3205
OxPhos	Mouse	1:250	Abcam	ab110413
SIRT3	Rabbit	1:1000	Cell Signaling	5490
β-actin	Rabbit	1:1000	Cell Signaling	4967

APPENDIX:

Abbreviations

AdCKO—Adipose-specific Crls1 knockout

ALA—Alpha linolenic acid

AMPK—AMP-activated protein kinase

ASO—Antisense oligonucleotide inhibitors

BAT—Brown adipose tissue

BMI—Body mass index

Bu—Conventional butter

CCL2—Chemokine (C-C motif) ligand 2

CL—Cardiolipin

CPT1—Carnitine palmitoyl transferase 1

Crls1—Cardiolipin synthase 1

CRP—C-reactive protein

DAG—Diacylglycerol

DGLA— Dihomo-γ-linolenic acid

DHA—Docosahexaenoic acid

ELOVL—Elongation of very long-chain fatty acids protein

EPA—Eicosapentaenoic acid

ETC—Electron transport chain

FGF21—Fibroblast growth factor 21

FXR—Farnesoid-X-receptor

IL-1β—Interleukin-1β

IL-6—Interleukin-6

IL-10—Interleukin-10

IR—Insulin resistance

LA—Linoleic acid

Ma—Margarine

MCP-1—Monocyte chemoattractant protein-1

MDFC—Macrophage-derived foam cells

MGL1— Macrophage galactose-type C lectin 1

NAFLD—Non-alcoholic fatty liver disease

NF-kB—Nuclear factor-kappa B

N3Bu—ALA-biofortified butter

OxPhos—Oxidative phosphorylation protein

PDH—Pyruvate dehydrogenase

PRDM16—PR-domain containing 16

PUFA—Polyunsaturated fatty acid

RXR—Retinoid x receptor

SCD—Stearoyl-CoA desaturase protein

SFA—Saturated fatty acid

SIRT1—Sirtuin 1

SIRT3—Sirtuin 3

SNS—Sympathetic nervous system

TG—Triglyceride

TLR4—Toll-like receptor 4

TNF-α—Tumor necrosis factor α

TRPV1—Transient receptor potential vanilloid 1

T2D—Type 2 diabetes

UCP1—Uncoupling protein 1

VDAC1—Voltage-dependent anion channel 1

VFA—Visceral fat area

VMH—Ventromedial nucleus of the hypothalamus

WAT—White adipose tissue

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