THESIS

FADS2 EXPRESSION MODULATES EFFECT OF DIETARY POLYUNSATURATED FATTY ACIDS ON WESTERN DIET-INDUCED GLUCOSE INTOLERANCE

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ABSTRACT

FADS2 EXPRESSION MODULATES EFFECT OF DIETARY POLYUNSATURATED FATTY ACIDS ON WESTERN DIET-INDUCED GLUCOSE INTOLERANCE

Fatty Acid Desaturase 2 (*FADS2*) haplotypes associated with hyperactivity of its gene product, delta-6-desaturase (D6D), are associated with obesity and type-2 diabetes in humans. D6D regulates long-chain polyunsaturated fatty acid (PUFA) biosynthesis and is upregulated in several rodent models of obesity/insulin resistance, but its direct influence on diabetes is unclear. D6D activity might favor pathogenic effects of omega-6 FA linoleic acid (LA) by enhancing production of its product arachidonic acid (AA). Conversely, D6D may promote protective effects of omega-3 FA α-linolenic acid (ALA) by enhancing production of ALA to long-chain PUFAs that displace AA in cell membranes. It is hypothesized that abundant LA found in the modern western diet will be converted to AA promoting an inflammatory phenotype. The present study is to determine the interaction of heterozygous knockout (HET) or transgenic overexpression (TG) of *FADS2* in mice fed high fat diets (HFD), as well as the interaction of LA:ALA content in the HFD. Adult male mice with HET (low), wild type (WT; medium), and TG (high) expression of *FADS2* were fed HFD (45% w/w) containing 8% PUFA supplied by a balanced mix of LA and ALA (1:1), LA-rich (41:1), or ALA-rich (1:4) for 16 weeks. Glucose intolerance developed in WT mice, with no difference between diets. In HET mice, glucose intolerance was attenuated but this protection was removed by ALA rich diet. TG mice developed more glucose intolerance than WT. TG mice fed high LA diets were more glucose tolerant than high ALA and mixed diets. In conclusion, *FADS2* expression modulates metabolic responses to high fat feeding. HET provides some protection against glucose intolerance, except when given an ALA rich diet. Transgenic overexpression increases glucose intolerance while a high LA diet attenuates this effect. This is inconsistent with current hypotheses that AA production from LA increases metabolic risk.

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INTRODUCTION

Compared to the ancestral human diet, the modern western diet consists of much higher proportions of sucrose, saturated fat, and omega-6 fatty acids. This change in diet is thought to contribute to a monumental increase in obesity and type 2 diabetes (T2D) over the past few decades [1]. Worldwide, almost 360 million people have T2D [2]. Pre-diabetes affects a further 316 million people, which is defined as an intermediary blood glucose level above normal, but below diabetic levels.

The majority of glucose uptake from the blood to peripheral tissues is mediated by insulin, which primarily targets adipose tissue, muscle, and liver. In adipose tissue, insulin promotes the uptake and storage of fatty acids and inhibits lipolysis of stored triglycerides [3]. In insulin resistance, these actions are compromised, which leads to hyperglycemia, elevated free fatty acids, and pancreatic beta cell dysfunction [4].

Obesity and the associated metabolic disorders including insulin resistance, type 2 diabetes and non-alcoholic fatty liver disease are evidenced by inflammation linked to excess energy intake, as well as the quality of fats in the diet [5,6,7]. Chronic low grade inflammation in obesity disrupts cellular metabolism and impairs insulin signaling. However, the mechanisms for this have not been completely elucidated [8]. Tissue macrophages have an important role in promoting inflammatory signaling and injury that can impair normal insulin signaling [9,10]. Compared to other organs, the liver has the largest resident population of macrophages. Studies have established the involvement of hepatic macrophages in the pathogenesis of insulin resistance [11,12].

There is an immense interest in omega-3 polyunsaturated fatty acids (n3 PUFA) in regards to health benefits. These n3 PUFAs, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are derived from the essential n3 PUFA alpha-linolenic acid (ALA) or are consumed directly in the diet, primarily from fish oils. n3 PUFAs are thought to have an anti-inflammatory effect and also act to

lower triglycerides [13]. However, much controversy exists on their effects on glucose metabolism and insulin resistance. n6 PUFAs, mainly arachidonic acid(AA) produced from linoleic acid(LA) are commonly reported as pro-inflammatory, due to the fact that AA is a substrate for eicosanoids [14]. Dietary n3 PUFAs are believed to counteract the inflammatory actions of n6 AA by competing for common rate limiting desaturase enzymes in their biosynthesis, displacing AA in membrane phospholipids, or promoting the production of anti-inflammatory eicosanoids. However, despite widespread acknowledgement that inflammation contributes to diabetes and cardiovascular disease, studies that examined the effect of dietary n6 and n3 PUFAs on inflammation and contribution to these disorders have produced conflicting results [15].

The modern western diet, in addition to being high-fat and high-sugar, is also highly enriched with n6 PUFA linoleic acid(LA) compared to ancestral diets. This is due mainly because of an increased consumption of LA-rich oils from vegetables such as corn. This imbalance of n6:n3 deviates from the 1:1 ratio of our ancestral diet, and has been hypothesized to promote low grade inflammation that contributes to metabolic and cardiovascular disease. However, studies examining the effect of dietary n6 PUFAs on cardiovascular and metabolic risk have produced conflicting results, supporting impairment [15], improvement [16], or no effect [17]. Studies examining n3 PUFAs have similarly produced conflicting results [18,19,20]. Accumulating evidence suggests that the key modulating factor in a modern LA-rich diet may be an individual's propensity to convert essential PUFAs (n6-LA and n3-ALA) into their long-chain derivatives(n6-AA and n3-DHA). This pathway is modulated by the rate limiting enzyme delta-6-desaturase (D6D), which is encoded by the *FADS2* gene [21]. Single nucleotide polymorphisms in the *FADS2* gene that confer higher D6D enzyme activity have been associated with inflammation, type 2 diabetes, and metabolic syndrome in Western populations [22,23,24], suggesting an important role of FADS2 genotype on metabolic risk in the context of the modern diet. It is postulated that the effects of n3 PUFAs may be similarly affected by *FADS2* genotype, perhaps

augmenting the anti-inflammatory potential of n3-ALA [25]. Accordingly, we hypothesized that in the context of a high-fat/high-sucrose "Western" diet, *FADS2* genotype is an important modulator of dietary n6:n3 PUFA balance on metabolic risk.

The aim of this study was to determine the main and interaction effects of *Fads2* genotype and dietary n6:n3 PUFA ratio on glucose intolerance induced by a high fat diet (HFD) in mice. To clarify these effects, we fed a HFD with varying n6:n3 PUFA content to mice with transgenic overexpression or heterozygous knockdown of *Fads2* to model variations in *FADS2* genotype common in human populations. We hypothesized that dietary n6-PUFA would augment glucose intolerance, while n3-PUFA would be protective, and that higher *Fads2* expression would amplify these effects.

MATERIALS AND METHODS

Animal Subjects

Two strains of mice were used in this project. *Fads*2 heterozygous (*Fads2*$^{+/-}$; HET) mice were generated on a C57Bl/6 background from founder mice provided by Dr. Nakamura at the University of Illinois [26]. *Fads2*-HET mice have ~50% reduction of hepatic delta-6-desaturase expression (Supplemental Fig. 1) and were compared to C57BL/6J WT mice in the present study to represent human *FADS2* haplotypes associated with low D6D activity. *Fads*2 Transgenic (TG) mice were generated in our laboratory on a FVB background using the *Fads*2 cDNA sequence provided by Dr. Nakamura [27]. This sequence was inserted into a vector and placed downstream of a CMV promoter and upstream of a neomycin resistant gene, then injected into pronuclei of fertilized eggs. TG founder mice were identified by southern blot and PCR, and were backcrossed for 10+ generations to produce the *Fads2*-TG mice used in the present study to represent human *FADS2* haplotypes associated with higher D6D activity. *Fads2*-TG mice exhibit significant elevation in *Fads*2 mRNA in heart and liver, and increased D6D protein expression in liver (Supplemental Fig.1).

Thirty five C57Bl/6J(HET and WT) and thirty one FVB(TG and WT) adult male mice were randomly divided into three experimental groups and fed *ad libitum* custom high-fat "western" diets with 3 variable n6:n3 PUFA ratios as explained below (Harlan/Envigo). The nutrient composition of the experimental diets is provided in Table 1 of supplemental material. In general, the diet contains sucrose as a primary source of carbohydrate (26.7% total kcal), and cocoa butter (54% saturated fat) as the primary source of fat (27.7% total kcal), with 13.9% kcal coming from a mix of flax or corn oil providing n3 and n6 PUFAs. Importantly, all diets are free of any long-chain highly unsaturated fatty acids (e.g., AA or DHA), so all such PUFA are obtained only by endogenous synthesis from LA and ALA provided in the diets. The experimental diets provided PUFAs as corn oil (LA-rich) and flaxseed oil (ALA-rich) in three

proportions: 41:1 n6:n3 (LA), 1:4 n6:n3 (ALA) and 1:1 n6:n3 (Mix). Monounsaturated and saturated fats were equal in each diet, and were supplied by the oils and cocoa butter. The mice were group housed at the animal care facility at Colorado State University on a 12:12-h light-dark cycle, under controlled temperature (23C) and humidity (35%). During the treatment period, body weight and food intake were monitored semiweekly, and glucose tolerance was measured monthly for 4 months. All procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85–23, revised 2011), and were approved by the Colorado State University Institutional Animal Care and Use Committee.

Tissue collection

After 16 weeks of treatment on the HFD, mice were anesthetized with 5% isoflurane and killed by exsanguination following thoracotomy and removal of the heart. Tissues collected include: serum, heart, liver, brain, and gastrocnemius. Tissues were quickly frozen in liquid nitrogen or fixed in 10% neutral buffered formalin.

Intraperitoneal glucose tolerance test (GTT)

All animals were fasted for 8 hours prior to GTT. To perform the GTT, all mice received a glucose intraperitoneal injection at 2mg/g body weight. Blood glucose concentrations were measured prior to injection, and at 30, 60, 90 and 120 minutes after glucose administration with AlphaTRAK 2 test strips and AlphaTRAK2(Zoetis) device according to manufacturer's instructions. Data is expressed as mean with standard error bars.

Serum triglyceride analyses

Blood was collected by cardiac puncture under isoflurane anesthesia. Serum was separated from the clotted blood by centrifugation (1000g, 10 min, 4C). Serum triglycerides were measured using a Triglyceride Quantification Colorimetric/Fluorometric Kit from Biovision following the manufacturer's instructions.

Isolation of hepatocytes and hepatic macrophages

After removing sections of the liver for histology, approximately ½ of the liver remained and was used to isolate hepatocytes and hepatic macrophages. The remaining liver was homogenized and placed into 8ml of digestion buffer (0.02 mg collagenase type II, 94ul of 1M CaCl2, ~8ml DPBS) at 37C for 1 hour. After 1 hour, 80ul of 0.5M EDTA was added to stop reaction. The digestion was filtered through a 100 micron strainer and centrifuged at 50g for 3 min and 4C. The pellet contained the hepatocytes, and was resuspended in 0.5ml PBS to be frozen. The supernatant was collected and centrifuged at 580g for 5 min. The resulting supernatant was aspirated to 4ml, and 8ml of PBS was added and then centrifuged at 580g for 10min. Supernatant was aspirated to 2 ml and 4 ml PBS added. This 6 ml solution was deposited on a 14% HistoDenz solution and centrifuged at 800g for 25min. Macrophages were found at the interface of the gradient.

Fatty acid phospholipid extraction

Phospholipids were extracted from hepatocytes and macrophages by homogenization in 600uL of methanol. The non-phospholipid portion was pelleted by centrifugation at 900g for 5min. The supernatant containing the phospholipids was added to 25ul of sodium methoxide and incubated at room temperature to generate fatty acid methyl esters. 75ul of methanolic HCl was added to stop the reaction after three minutes. The fatty acid methyl esters were extracted by adding 700ul of hexane. This upper hexane layer containing the fatty acid methyl esters was transferred to gas chromatography vials and dried under a stream of nitrogen. Hepatocyte phospholipids were suspended in 50ul of hexane and macrophage phospholipids were suspended in 20ul of hexane. Gas chromatography analysis was performed using an Agilent DB-225 30m x 0.250 mm x .25um column (J&W scientific model 122-2232) on an Agilent 6890 Series Gas Chromatographer with a flame ionization detector. Using a flow rate of 1.7ml/min and split ratio of 14.1:1 in 25 minutes, the initial temperature of the oven was 120C with an

initial ramp temperature of 10C/min for 8 minutes, then 2.5C/min for 4 minutes and held at 210C for the remainder of the run. Fatty acid data are represented as % total fatty acids.

Separation and quantification of macrophage and hepatocyte phospholipids

Lipids from hepatocytes and hepatic macrophages were extracted using Folch extraction solution with 25 mg/L BHT added. After adding the extraction solution, vortex sample and add 1ml H20 and centrifuge at 2500rpm for 10 min. Aspirate the top layer, as the lower layer contains lipids. The suspended lipids were dried under nitrogen stream and resuspended in 60ul of hexane. Separation of phospholipids was achieved by normal phase High pressure liquid chromatography (Agilent Zorbax Rx-Sil column, 4.6x250mm, 5um). Two mobile phases consisting of hexane:isopropanol:0.3mM potassium acetate:acetic acid(424:566:10:0.1, mobile phase a) and hexane:isopropanol:5.0mM potassium acetate:acetic acid(385:515:100:1, mobile phase b) were used. The run began with 100% mobile phase a and went to 100% mobile phase b in 6 minutes. 100% Mobile phase b was maintained for 5 minutes, and then returned to 100% mobile phase a in 1 minute. Fractions were collected based on known standard elution times, dried under nitrogen stream, and methylated using sodium methoxide and methanolic HCl with the protocol seen above.

Histology and immunohistochemistry

Histology was performed in 10um sections of paraffin-embedded liver samples using hematoxylin and eosin stain and F4/80 antibody for quantitative assessment of liver lipid droplet area, and macrophage number, respectively. Quantitation was performed using Nikon's NIC Elements Advanced Research Microscope Imaging Software. Data was averaged from two separate sections of each liver, and is represented as % of total area.

Statistical analysis

Given the complexity of experimental design, analyses of results were clarified as sub-studies. Study 1 evaluated the effect of dietary PUFA composition in wild-type (control) mice. Study 2 examined

the influence of *Fads*2 expression when dietary intake of n6:n3 PUFA was balanced, and study 3 investigated the interaction of *Fads*2 expression with dietary PUFA composition. Values were reported as mean ± S.E.M., and significant differences between mean values were determined by t-tests, one-way and two-way analysis of variance with post-hoc comparisons by Fisher's least significant difference as indicated in the text and figure legends. $P < 0.05$ was considered statistically significant for all analyses.

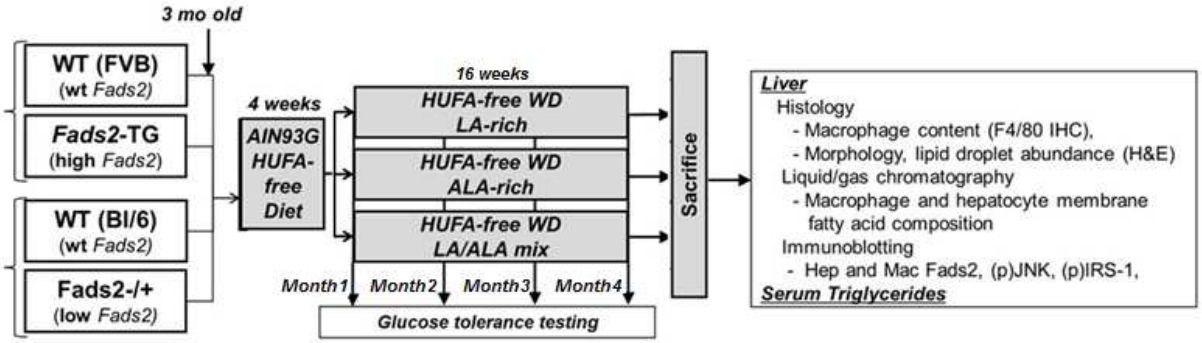

Chart 1. Experimental Design

RESULTS AND DISCUSSION

Study 1: Effect of dietary PUFA on HFD-fed WT mice

n6-PUFA augments, while n3 may attenuate glucose intolerance in wild type HFD-fed mice

To determine the effects of dietary PUFA composition in the absence of genetic engineering of *Fads2* expression, we fed our FVB and C57BL/6J wild-type mice the high fat diets with varying proportions of n3:n6 PUFAs: 1:1(Mix), 4:1(ALA), and 1:41(LA). Effects of the diets were similar in C57BL/6J and FVB mouse cohorts, with LA-diet fed mice being more glucose intolerant than ALA-diet fed mice(p=0.0264 and p=0.0140, respectively)(Fig. 1A). After normalizing our two strains of mice to the Mix diet, 1-way ANOVA showed a significant difference (p=0.0131) between the means in total area under the curve (AUC) for the GTT (Fig. 1B). Glucose tolerance was reduced in WT LA diet compared to ALA diet (p=0.004). While not statistically significant, WT LA diet GTT AUC was trended higher than the Mix-fed mice (p=0.057) and lower than mix (p=.18). Thirty minutes following glucose injection during the GTT, blood glucose in mice fed the LA-diet was significantly higher than ALA-diet in both strains of mice (C57BL/6J p=0.0304, FVB p=0.0354), while ALA-diet tended to be lower than Mix diet in C67BL/6J (p=0.08)(Fig. 1C). Very similar results were seen at the 60 min time point of the GTT, showing LA-diet being more glucose intolerant than ALA-diet (C57BL/6J p=0.0049, FVB p=0.0133) (Fig. 1D).
These results are similar to what Mustad et. al found when they fed differing n3 PUFAs(ALA, EPA, DHA) to diabetic ob/ob mice[28]. Enriching the diet with ALA improved insulin sensitivity and glycemic responses, while Mustad showed supplementation with EPA and DHA had no improvement. Our data further support that ALA rich diets may be more effective in controlling glucose intolerance that its long-chain derivatives (EPA and DHA) in diabetes. Another study reported that ALA in plasma phospholipids was inversely associated with type-2 diabetes in humans, but EPA and DHA were not [29].

While ALA may attenuate glucose intolerance in WT Mice, we found that high n6 PUFA in a high fat diet augmented glucose intolerance. This is consistent with studies that have suggested that n6 and n3 PUFAs elicit opposing effects on systemic inflammation markers, including glucose intolerance [16,21]. Studies have also shown that the n6:n3 ratio plays an important role in obesity due to the inflammatory effects of AA eicosanoid production and anti-inflammatory effects of DHA lipid species [30]. Our findings agree that increasing consumption of n3 and decreasing n6 PUFA in a high fat diet in mice with "normal" activity of D6D will improve glucose tolerance.

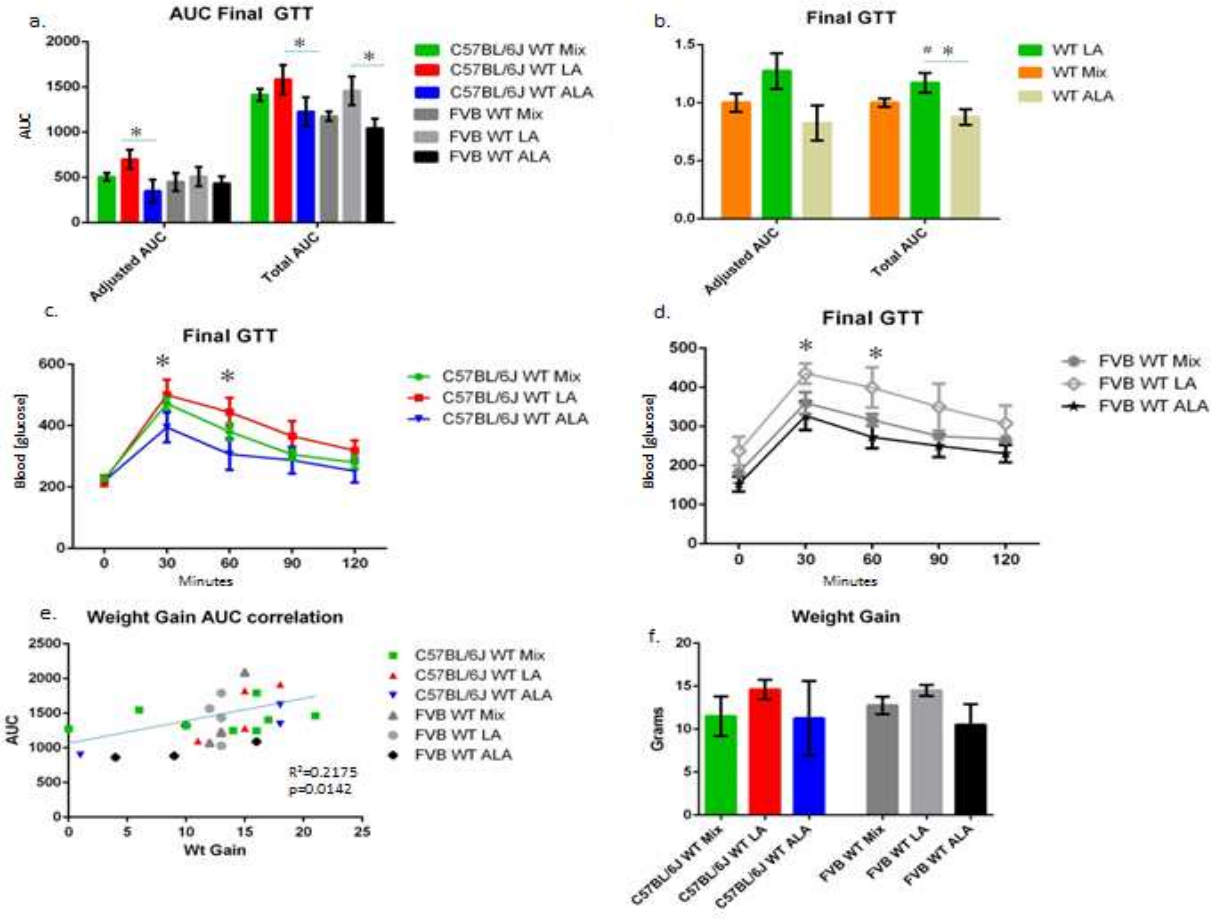

Figure 1. Diet affects glucose tolerance and weight gain. C57Bl/6J WT mice fed LA diet were more glucose intolerant than ALA diet (adjusted AUC p=0.0276 total AUC p=0.0264). FVB WT mice fed LA diet were also more glucose intolerant than ALA diet (total AUC p=0.014)(a). After normalizing genotypes to the Mix diet and combining them, glucose intolerance was augmented in WT LA diet compared to ALA diet(p=0.0038). WT LA diet was also trending higher than Mix diet (p=0.057)(b). In C57BL/6J WT mice, at thirty minutes of GTT, LA diet was higher than ALA diet (p-0.0304) and also at sixty minutes (p=0.0049)(c). In FVB WT mice, LA diet was higher than ALA diet at thirty and sixty minutes (p=0.0354 and p=0.0133 respectively)(d). Weight gain correlates with AUC in WT mice. Correlation R^2=0.2175 and p=0.0142 (e). No significant differences between weight gained were noted(f).
*p<.05 #p<.1

Weight gain predicts glucose intolerance in WT mice, and diet may affect weight gain in parallel with glucose tolerance in WT mice

The significant correlation of weight gain and GTT AUC established that weight gain was reliable in predicting glucose intolerance in WT mice with all 3 diets (p=0.0142) (Fig. 1E). Despite this correlation, there was no significant difference in weight gain between any of the diets, and no significant change in food intake or its correlation with weight gain (Supplemental Fig. 2). While not statistically significant, weight gain was altered across n6 and n3 and Mix diets, and paralleled changes in glucose tolerance AUC. While not being significant, these results are consistent with previous studies suggesting opposing effects of n6 and n3 PUFAs on weight gain which may be through altered adipogenesis [31], lipolysis [32], and/or inflammation [33]. Further investigation is required.

n6 PUFA promotes hepatic macrophage accumulation in WT mice

Studies have established the involvement of hepatic macrophages and inflammation in the pathogenesis of insulin resistance [12,13]. Therefore, the effect of dietary PUFAs on hepatic macrophage accumulation was evaluated in WT mice fed a HFD by probing liver tissue sections for F4/80-positive cells by immunohistochemistry. While there were no significant differences between groups, hepatic macrophage accumulation in the LA-diet was trending higher than other groups in FVB mice (p=.09) and when both WT strains were combined (p=0.01) (Fig. 2A).

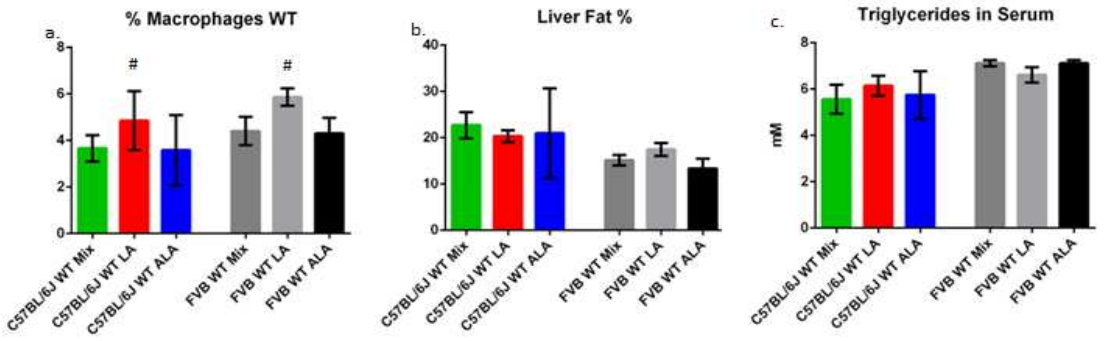

Figure 2. Diet affects macrophage accumulation. Macrophage accumulation in LA diet is not significant but is trending higher in C57BL/6J mice(p=0.0858) and FVB mice(p=.0994)(a).
No differences in liver fat was seen(b). No differences in triglyceride concentration in serum was seen(c). #p<.1
Representative IHC images of macrophages and liver fat provided in Supplemental Figure 3.

Chronic inflammation is a key mechanism that links obesity and T2D. Therefore, these trends for higher hepatic macrophage content in the LA group might partially account for the observed effects on glucose tolerance [34]. n3 PUFAs have shown anti-inflammatory effects in models with chronic inflammation [35,36], either through an inhibition of AA metabolism to pro-inflammatory eicosanoids or production of anti-inflammatory eicosanoids and resolvins from DHA [37]. Macrophage accumulation was similar in the ALA-rich and Mix diet, indicating that increasing dietary ALA above a 1:1 ratio with LA does not confer additional anti-inflammatory effects. Moreover, this indicates that the greater glucose tolerance seen in the ALA-diet group compared to Control was not due to reduced macrophages in hepatic tissue, suggesting that some other mechanism was responsible.

Macrophages can span a spectrum of phenotypes, from M1, very pro-inflammatory (classically activated) to M2, anti-inflammatory (alternatively activated) [38]. In obesity, macrophage polarization is more inclined to the M1 phenotype, which secretes cytokines such as TNFα and IL-1b that increase local inflammation as well as endocrine effects from being secreted into systemic circulation [39]. M2 macrophages primarily serve a role in tissue repair and growth as they have low proinflammatory cytokine expression, and high expression of anti-inflammatory cytokines such as IL-10 and IL-1[40]. Cytokine signaling from M1 macrophages can activate kinases such as c-jun end terminal kinase-1/2 (JNK1/2) to impair insulin signaling through serine phosphorylation of insulin receptor substrate-1 (IRS-1) [41]. Phenotypic changes to macrophages can be investigated by evaluating macrophage expression of inducible nitric oxide synthase (iNOS; M1) and arginase-1 (Arg-1; M2). Experiments are currently underway to evaluate effects of dietary PUFA composition on hepatic macrophage phenotype (iNOS and Arg-1 expression ratio) by immunoblotting cells isolated from livers of mice in each of the experimental groups.

No clear effect of dietary PUFA ratio on liver fat accumulation or serum triglyceride concentration in WT mice.

To examine the effects of PUFA ratio on hepatic steatosis (liver droplet accumulation), hematoxylin and eosin staining was performed on sections of liver and then imaged. Surprisingly, there was no effect of dietary PUFA ratio on liver fat accumulation (Fig. 2B). Similarly, PUFA ratio did not affect serum triglyceride concentration in WT mice (Fig. 2C), consistent with no effect of hepatic lipid storage or release. Serum triglyceride concentration can also be affected by lipoprotein lipase activity, however that was not measured.

As tissues become more insulin resistant adipose tissue lipolysis activity increases. This results in increased delivery of fatty acids to the liver, where they contribute to non-alcoholic fatty liver disease (NAFLD) [42]. Elevated glucose levels in insulin resistant individuals also suppress hepatic fatty acid oxidation to further increase hepatic fat accumulation. Hepatic lipid droplets were observed in all groups as expected with chronic high-fat feeding (Supplemental Fig.3). However, no differences in liver fat accumulation were seen between dietary PUFA groups, despite differing glucose tolerance levels. While some studies have shown that n3-PUFA supplementation increases insulin sensitivity and lowers hepatic triglyceride levels in rodent models [43,44], other studies have concluded that NAFLD and T2D progression are unrelated [45]. Based on the results of our studies, dietary PUFAs seem unlikely to modulate glucose tolerance through effects on hepatic lipid storage and release; at least in WT mice.

Macrophage and hepatocyte phospholipid AA and DHA levels follow diet

Membrane phospholipids are able to initiate and amplify inflammatory signaling through the action of phospholipase A2, which catalyzes the release of free AA for synthesis of inflammatory prostaglandins and other lipid species [46]. One study examining the effects of eicosanoids concluded that levels of inflammatory prostaglandins are increased by elevated glucose in macrophages [47]. Therefore, we evaluated the fatty acid composition of phospholipids extracted from hepatocytes and

macrophages isolated from liver tissue of mice in our experimental groups. In general, hepatocyte levels of n6-AA and n3-DHA paralleled the dietary PUFA content of their precursors n6-LA and n3-ALA, respectively. In WT mice hepatocytes, n6-AA was highest in LA diet and lowest in ALA diet, with Mix diet being in the middle. n3 DHA in hepatocytes was higher in the ALA and Mix diet than LA diet. Macrophage phospholipid composition paralleled hepatocytes. AA was highest in LA diet and lowest in ALA diet, with Mix diet being between the two. DHA in macrophages from ALA diet and Mix diet was higher than LA diet (Fig. 3A-D). These results are generally consistent with glucose intolerance levels, suggesting that dietary PUFA composition may influence hepatic glucose handling by altering the PUFA composition of hepatocyte or macrophage membranes. However, future studies are needed to confirm any causal relationship between cellular phospholipids and glucose intolerance.

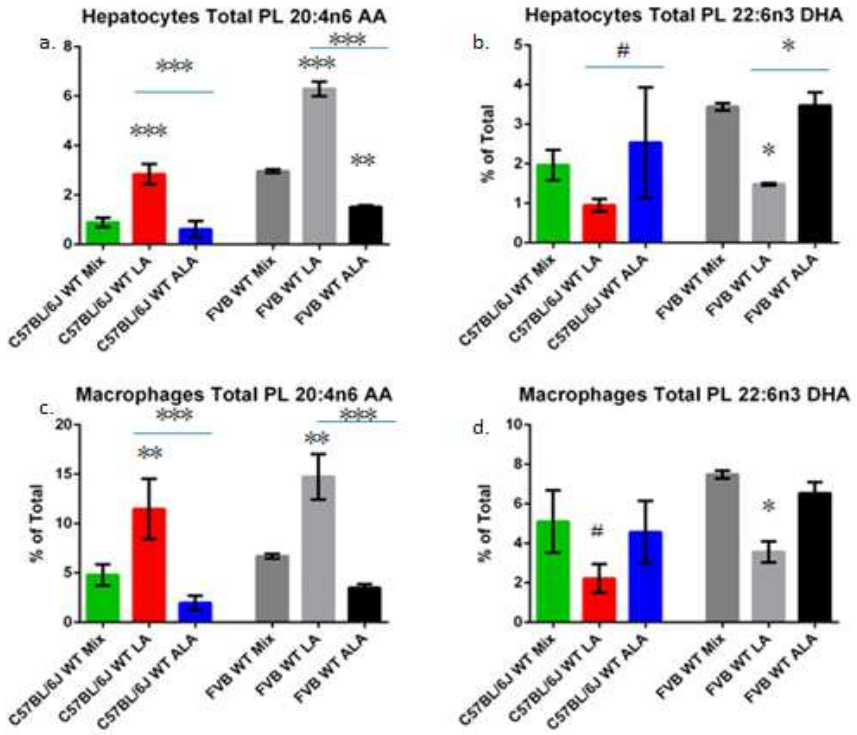

Figure 3. Phospholipid Content in Hepatocytes and Macrophages is affected by diet. In C57BL/6J hepatocytes, total AA was increased in LA(p=0.001) and LA diet was higher than ALA diet (p=0.001). In FVB Hepatocytes, total AA was increased in LA diet(p=0.001) and decreased in ALA diet(p=0.001)(a). In C57BL/6J hepatocytes, total DHA was trending lower in LA diet than ALA diet (p=0.0631) and in FVB mice LA diet was lower than Mix (p=0.0313) and ALA (p=0.0286)(b). In C57BL/6J macrophages, AA was higher in LA diet compared to Mix(p=0.0057) and ALA(p=0.001). Similar results in FVB mice with AA higher in LA diet than Mix(p=0.0059) and ALA(p=0.0003)(c). For DHA in macrophages, in C57BL/6J mice LA was trending lower than Mix(p=0.0749), and in FVB mice LA was lower than Mix(p=0.047)(d). ***p<.001 **p<.01 *p<.05 #p<.1

Study 2: Effect of *Fads2* genotype on HFD-induced metabolic syndrome

Fads2 genotype modulates glucose tolerance in n6:n3 balanced HFD

After examining the effects of dietary PUFA composition on HFD induced glucose intolerance in WT mice, we evaluated the effects of *Fads2* expression on the same parameters in mice fed a HFD with a balanced (1:1 n3:n6; Mix) PUFA ratio (Fig. 4). Consistent with *FADS2* haplotype associations with T2D in humans, glucose tolerance was inversely influenced by *Fads2* expression. *Fads2*-HET mice (lower hepatic *Fads2* expression) were more glucose tolerant than their corresponding C57Bl/6J WT, and *Fads2*-TG (higher hepatic *Fads2* expression) were less glucose tolerant than corresponding FVB WT mice. These differences were seen in total GTT AUC (HET vs WT p=0.0056 and TG vs WT p= 0.0259) (Fig. 4A) as well as at 30 (HET vs WT p=0.0002, TG vs WT p=0.035) and 60 minutes (HET vs WT p=0.0161, TG vs WT p=0.0123)(Fig. 4B-C) following bolus glucose injection.

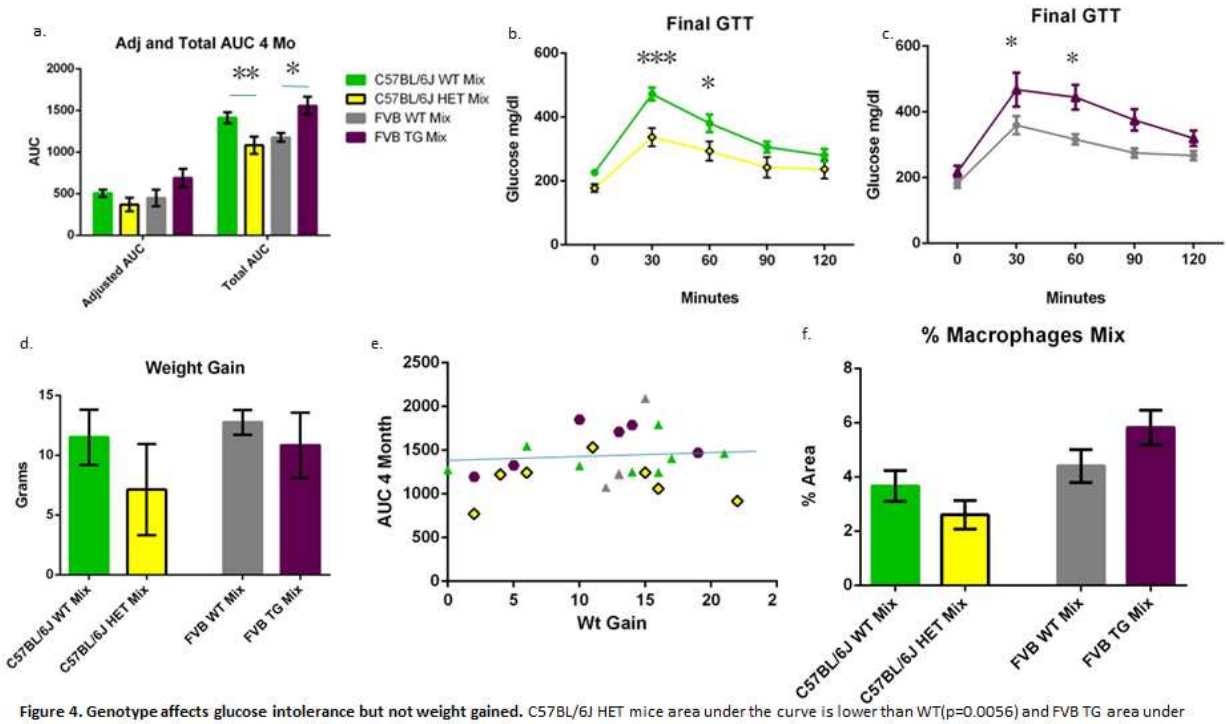

Figure 4. Genotype affects glucose intolerance but not weight gained. C57BL/6J HET mice area under the curve is lower than WT(p=0.0056) and FVB TG area under the curve was higher than WT(p=0.0259)(a). Glucose levels at 30 and 60 minutes were lower in HET than WT(p=0.0002 and p=0.0161)(b) and higher in TG than WT(p=0.035 and p=0.0123)(c). Weight gain was not changed in either genotype (d) and did not correlate to AUC(e). There was a difference in means in hepatic macrophage accumulation(ANOVA p=0.0073) however post-hoc analysis differences were not significant(f). ***p<.001 **p<.01 *p<.05

Weight gain does not predict glucose tolerance in Fads2 genotypes, and D6D expression does not affect weight gain

Contrary to the effect of altering dietary PUFA in WT mice, effects of *Fads2* genotype on GTT AUC were not associated with parallel effects on weight gain (Fig. 4D-E). This is contrary to studies that have linked *Fads2* TG mice to obesity, glucose intolerance, insulin resistance and hyperlipidemia compared to WT mice on a chow diet in our laboratory [48]. We can therefore postulate that in a HFD background, the influence of *Fads2* genotype on glucose tolerance is not dependent on weight gain, but is caused by other mechanisms.

Fads2 genotype predicts hepatic macrophage accumulation in PUFA balanced HFD

To determine if *Fads2* genotype alters glucose tolerance by affecting hepatic macrophage accumulation, number of F4/80-positive cells was evaluated in liver sections from the TG, HET and associated WT mice fed a MIX diet by immunohistochemistry (Supplemental Fig. 4A). Results showed expected trends and a significant difference across groups (ANOVA p-value=0.0073) (Fig. 4F).; however, trends for lower macrophage accumulation in HET vs. WT(p=0.1833) and higher in TG than WT were not statistically significant in post-hoc analyses (p= 0.18 and 0.16, respectively).

This suggests that the effects of *Fads2* genotype on glucose tolerance may be due, at least in part, to changes in hepatic macrophage accumulation in hepatic tissue. Hepatic insulin resistance in obesity is linked to increased inflammatory signals through the production of inflammatory cytokines from resident or infiltrated M1 macrophages [49]. We speculate that the small changes observed in hepatic macrophage number, or perhaps phenotype (M1 vs. M2), could have potent effects on inflammatory signaling that at least partially explain the observed effects of Fads2 genotype on glucose tolerance herein. To further investigate this hypothesis, hepatic macrophage phenotype and inflammatory cytokines profile (IL-1b, TNFa, IL-6) should be measured and related to changes in GTT AUC.

No clear effect of Fads2 on liver fat accumulation or serum triglyceride concentration

Similar to results obtained from WT mice fed the different dietary PUFA ratios, *Fads*2 genotype had no clear effect on hepatic steatosis in mice fed a MIX diet, as well as no effect on serum triglyceride concentration with a HFD background (Supplemental Fig. 4). Therefore, we conclude that the effects of high-fat feeding either overwhelm or negate effects of *Fads2* genotype on these parameters, and that neither is responsible for the observed effects on glucose tolerance.

No clear effect of Fads2 on phospholipid content of hepatocytes and macrophages

In Mix diet fed mice, *Fads2* genotype had unexpected effect on hepatocyte and macrophage AA and DHA levels that are difficult to interpret (Fig. 5). In hepatocytes, Fads2 genotype did not significantly affect phospholipid AA (Fig. 5A) or DHA (Fig. 5B) levels compared to respective WT mice. In hepatic macrophages, both HET and TG mice had lower AA than their respective WT controls (Fig. 5C, D). These results are difficult to interpret and suggest a more complex handling of cellular PUFA than is reflected by changes in phospholipid fatty acid composition. Importantly, LA and ALA are acted upon by D6D and downstream elongase and desaturase enzymes as CoA-esters following hydrolysis from membrane phospholipids by phospholipases, and are subsequently re-esterified to membrane phospholipids by selective acyltranferase enzymes [50]. Therefore, it is plausible that changes in the levels of "free" PUFA CoA-esters or their derivatives (e.g., eicosanoids) might better reflect *Fads2* genotype and its interaction with diet. Regardless, these results indicate that changes in liver phospholipid PUFA composition do not predict the effects of Fads2 genotype on glucose tolerance, at least in mice fed a HFD with balanced n3:n6 PUFA composition.

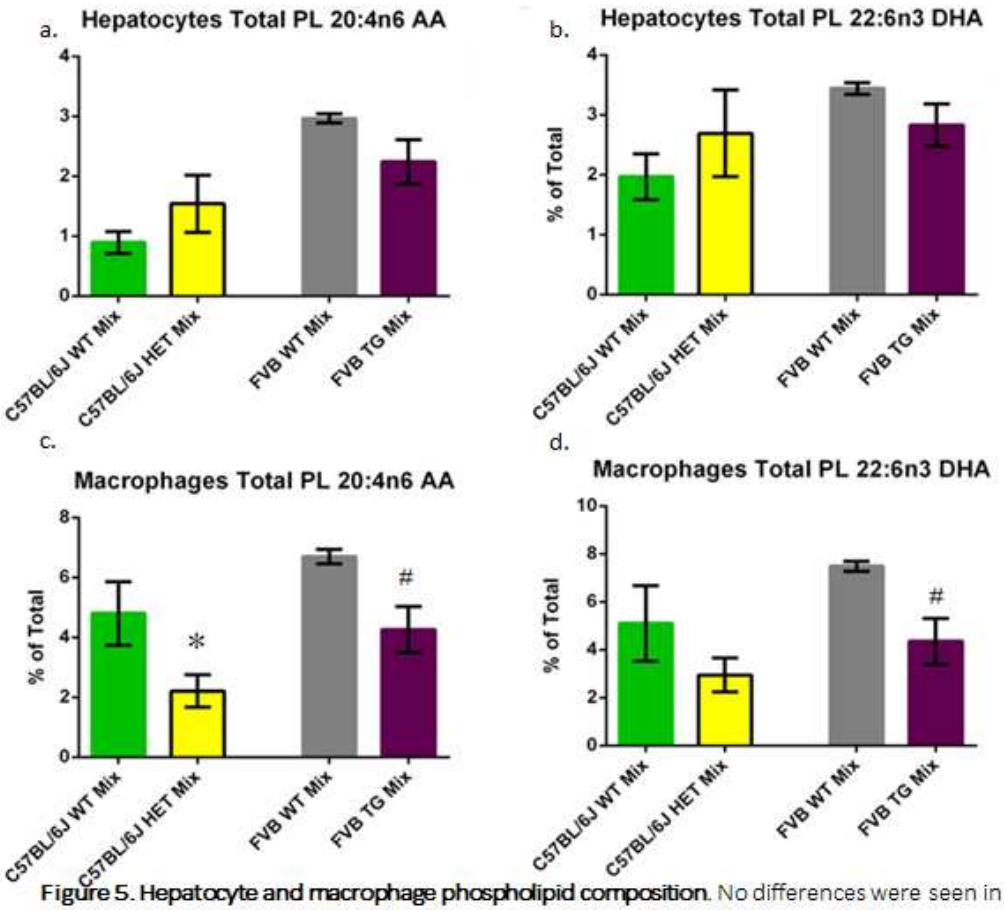

Figure 5. Hepatocyte and macrophage phospholipid composition. No differences were seen in hepatocyte AA levels(a) or DHA levels(b). In macrophages, AA was reduced in HET mice(p=0.0234) and TG mice AA levels were trending higher than WT(p=0.0707)(c). DHA levels in macrophages were not significantly different, however TG mice levels were trending higher than WT(p=0.0945)(d). *p<.05 #p<.1

Study 3: Interaction of *Fads2* genotype and dietary PUFA composition

n6:n3 PUFA imbalance differentially modulates glucose tolerance in Fads2 HET and TG mice

After establishing that both Fads2 genotype and dietary PUFA composition independently influence the effects of high-fat feeding on glucose tolerance, we sought to examine their interaction by evaluating the effect of varying ratios of PUFA in the HFD fed to TG and HET mice compared to their respective WT cohorts. Interestingly, results indicate that diets with a n6:n3 PUFA imbalance (i.e., the ALA or LA diets) abolish the effect of *Fads2* genotype on glucose tolerance observed when dietary n6:n3 PUFAs are balanced 1:1 (MIX diet; Fig. 6)(Supplemental Fig. 5). HET mice with imbalanced diets had higher AUC in GTTs than balanced diets (p=.05) and were equal to WT mice on Mix diet (Fig. 6A). Similar

results were seen in the FVB TG mice. PUFA imbalance lowered AUC from TG mix levels (p=.0073) to WT mix levels (Fig.6B). Likewise, a t-test of the GTT glucose levels shows that HET mice fed an imbalanced PUFA diet was higher than HET mice fed a mixed diet (p=.0072) and that TG mice fed an imbalanced PUFA diet was lower than TG mice fed a mixed diet (p=.0055) (Fig. 6C-D).

These interactive effects of *Fads2* genotype and dietary PUFA composition differ from what would be expected from the results of the first 2 studies, and those postulated based on the human SNP data in the literature. Specifically, it has been postulated that higher FADS2 activity might augment the pathogenic effects of a high-n6 PUFA modern diet, as well as the putative benefits of dietary n3-PUFA intake in humans [25]. Based on this hypothesis and the results from studies 1 and 2, HET mice fed ALA diet would be least glucose intolerant, and TG mice LA diet would be most glucose intolerant. However, results indicate that in the presence of a HFD, hypothesized effects of *Fads2* genotype on glucose tolerance only manifest when dietary n6:n3 PUFA intake is balanced. How disrupting this balance by increasing either ALA or LA intake abolishes genotype effects is unclear, and merits further investigation.

High n6 diet increases hepatic macrophage accumulation in Fads2-HET mice

Given the association between *Fads2* genotype and hepatic macrophage accumulation in mice fed the mixed diet, we determined if altering dietary n6:n3 PUFA balance affected this parameter (Fig. 6). Results indicated that HET mice fed LA diet had more hepatic macrophages than HET mice fed a Mix or ALA diet (p=.0058; Fig. 6E), but no other significant differences were observed in the TG mouse cohorts(Fig. 6F). While higher hepatic macrophage levels in HET-LA mice are consistent with the trends for higher levels in WT-LA mice and the putative proinflammatory effect of n6-PUFAs, the absence of any effect in TG suggest a perplexing inverse relationship between dietary n6-PUFA intake and *Fads2* expression. Moreover, a similar effect of the ALA and LA diets on glucose intolerance in both TG and HET cohorts dissociates this outcome from the putative influence of hepatic macrophage content seen in other cohorts. As discussed above, measurement of hepatic macrophage phenotype (M1 vs. M2

polarization) and associated inflammatory cytokines would be beneficial in furthering investigating the complex links between hepatic inflammation, dietary PUFA composition and Fads2 genotype.

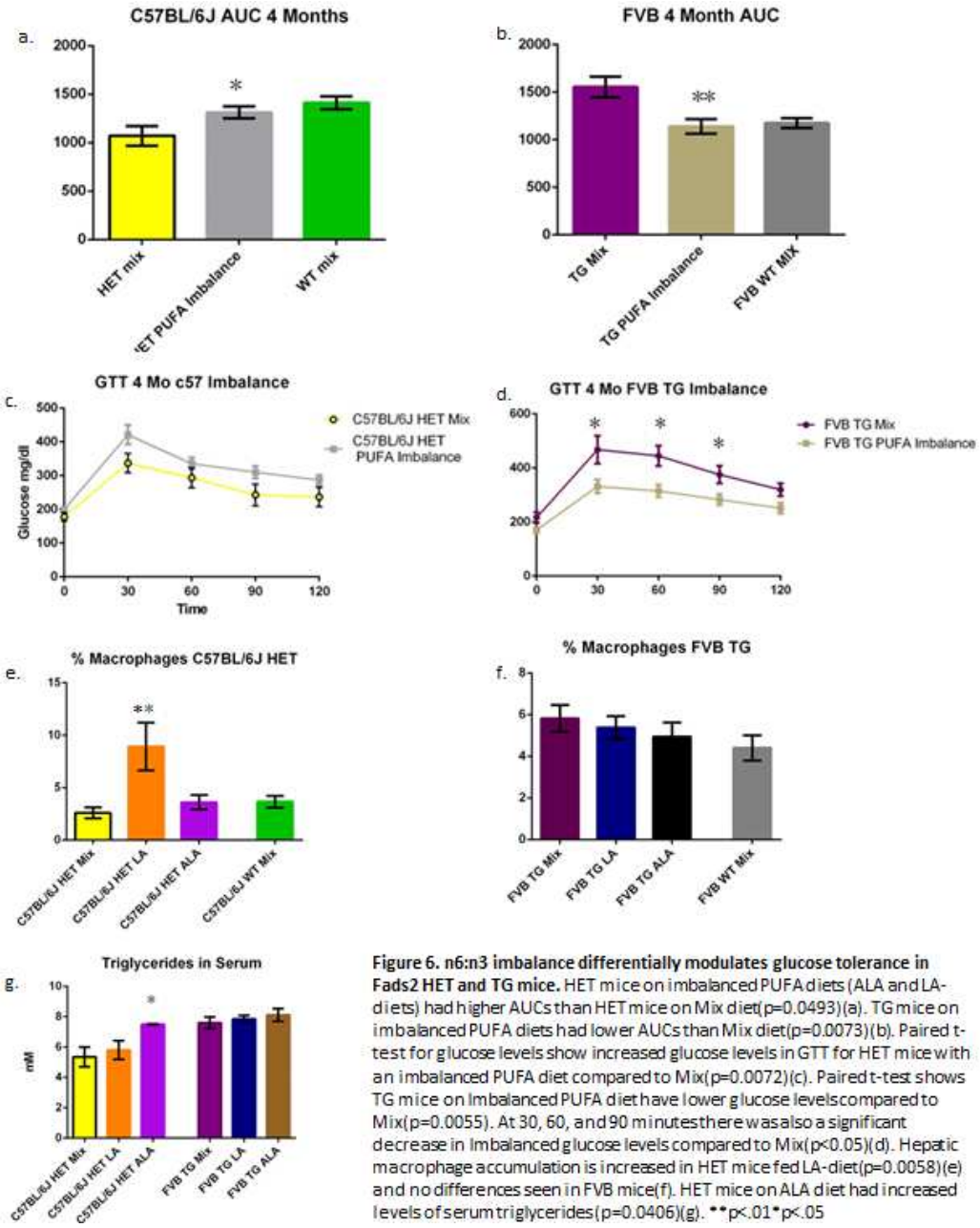

Figure 6. n6:n3 imbalance differentially modulates glucose tolerance in Fads2 HET and TG mice. HET mice on imbalanced PUFA diets (ALA and LA-diets) had higher AUCs than HET mice on Mix diet($p=0.0493$)(a). TG mice on imbalanced PUFA diets had lower AUCs than Mix diet($p=0.0073$)(b). Paired t-test for glucose levels show increased glucose levels in GTT for HET mice with an imbalanced PUFA diet compared to Mix($p=0.0072$)(c). Paired t-test shows TG mice on Imbalanced PUFA diet have lower glucose levels compared to Mix($p=0.0055$). At 30, 60, and 90 minutes there was also a significant decrease in Imbalanced glucose levels compared to Mix($p<0.05$)(d). Hepatic macrophage accumulation is increased in HET mice fed LA-diet($p=0.0058$)(e) and no differences seen in FVB mice(f). HET mice on ALA diet had increased levels of serum triglycerides($p=0.0406$)(g). $^{**}p<.01$ $^{*}p<.05$

High n3 diet increases serum triglycerides in Fads2-HET mice

Interestingly, HET mice fed ALA diet increased serum triglycerides compared to HET fed mix diet while no effects of dietary PUFA composition were observed in TG mice (Fig. 6G). No changes in serum

triglycerides were seen in WT mice fed HFD with different n6:n3 PUFA (study 1) or different levels of *Fads2* expression when n6:n3 PUFA intake was balanced (study 2). Therefore, it is incongruous that HET-ALA mice had increased serum triglycerides compared to LA and MIX. This might explain why HET-ALA mice developed more glucose intolerance than Het-Mix fed mice, but is inconsistent with findings from the HET-LA mice who were similarly glucose intolerant (Supplemental Fig. 5) . Therefore, there is no clear association between serum triglycerides and glucose intolerance in context of dietary PUFA composition and Fads2 genotype in the presence of high-fat feeding.

n6:n3 imbalance has no effect on liver fat accumulation in Fads2-HET or -TG mice

Comparable to both *Fads2* expression and PUFA ratio, there was no effect on liver fat accumulation in HET or TG mice fed LA or ALA diets (Supplemental Fig. 6). This further dissociates hepatic steatosis from glucose intolerance under the experimental conditions investigated.

Macrophage and hepatocyte phospholipids follow diet in HET and TG mice

HET and TG mice were equivalent to WT mice fed varying n6:n3 diets, regarding phospholipid composition. For macrophage phospholipid composition, HET and TG mice fed LA diet had the highest levels of AA and the lowest levels of DHA. Hepatocyte phospholipid composition was consistent with macrophages, with HET and TG mice on LA diet having the highest AA levels and lowest DHA levels. Mice on the ALA diet had the lowest levels of AA in macrophage and hepatocyte phospholipids, and had the highest levels of DHA in both cell phospholipids. HET and TG mice fed the Mix diet were in between LA and ALA diet for AA and DHA levels in both macrophages and hepatocytes (Supplemental Fig.6). As discussed above, these results indicate that changes in phospholipid composition in macrophage and hepatocytes contribute very little towards aggregate glucose intolerance, at least in HET and TG mice.

SUMMARY AND CONCLUSIONS

The present studies suggest that in the context of moderate (WT) *Fads2* expression, increasing n3-ALA intake may attenuate glucose intolerance induced by a high-fat/high-sucrose diet, while higher n6-LA intake augments glucose intolerance. These effects tend to correlate with weight gain on the HFD independent of food intake by mechanisms that remain to be established. Higher intake of n6 -PUFA promoted hepatic macrophage accumulation and increased AA levels in macrophage and hepatocyte phospholipid composition, which might also contribute to glucose intolerance by promoting hepatic inflammatory signaling. Conversely, lower membrane AA in ALA-diet macrophage and hepatocyte phospholipid composition might also contribute to improved glucose tolerance in the presence of a HFD.

Our studies also clearly demonstrate an effect of *Fads2* genotype on glucose intolerance induced by high-fat feeding. When dietary n6:n3 PUFAs are balanced (Mix), higher *Fads2* expression augments HFD-induced glucose intolerance, while lower *Fads2* expression attenuates it. While weight gain was not affected by *Fads2* genotype under these conditions, hepatic macrophage accumulation tended to parallel effects on glucose intolerance, suggesting potential links between Fads2 expression, hepatic inflammation, and metabolic risk when n6:n3 PUFA intake is balanced. Interestingly, an imbalance of dietary n6:n3 PUFA intake (ALA or LA diet) reverses the effect of *Fads2* genotype on glucose intolerance observed when n6:n3 PUFA intake is balanced. The mechanism(s) responsible for this effect are unclear, but are unlikely to be explained solely by changes in serum triglycerides, hepatic steatosis or macrophage accumulation. Moreover, these results suggest that the balance dietary n6:n3 PUFA intake, rather n6 vs. n3 intake, governs the effect of *Fads2* genotype on metabolic risk.

Taken together, these studies demonstrate that no single mechanism is responsible for exerting the effects of *Fads2* or dietary PUFA intake on HFD-induced glucose intolerance. Hepatic inflammation emerged as a key regulator of metabolic phenotype induced by both *Fads2* genotype and PUFA intake,

but more complex mechanisms appear to be responsible for their interactive effects on glucose tolerance. Elucidating these mechanisms merits further investigation, which might provide new insights necessary to advance our understanding of how common *FADS2* haplotypes and dietary PUFAs modulate metabolic risk in humans.

Future directions, ongoing studies and limitations

Western Blotting

Western blotting of macrophage proteins to determine levels of iNOS and arginase in order to categorized phenotypic M1/M2 polarization of macrophages is currently underway. In hepatic tissue, quantification of pIRS-1 and pJNK will be done in order to assess the canonical signaling pathway involved in insulin resistance induced by lipotoxic and inflammatory stimuli.

Analysis of hepatic free AA, DHA and eicosanoids

LC/MS analysis of unesterified AA, DHA and their oxidized derivatives might reveal distribution patterns more consistent with *Fads2* genotype than the presented phospholipid compositional analysis. If hypothesized trends are seen (AA and eicosanoids in TG>WT>HET), we will follow up with a relevant cytokine array on liver homogenates (RayBio).

Limitations

Assessment of glucose tolerance did not involve direct measurement of insulin resistance or action.

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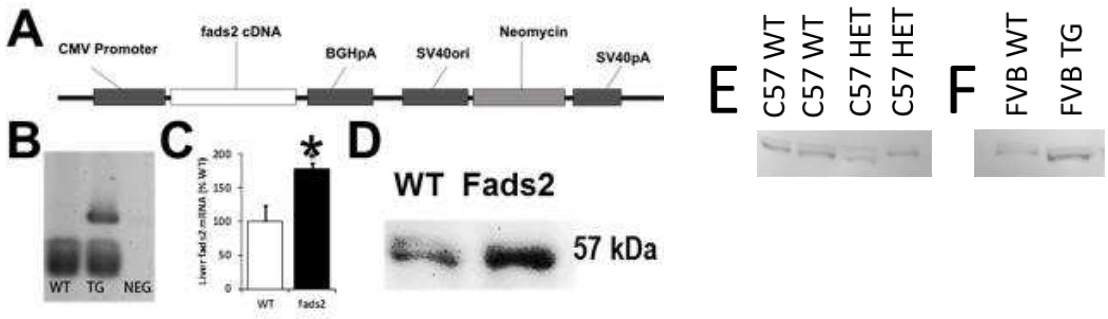

Supplemental Figure 1. A. CMV-Fads2 expression cassette used to generate *Fads2*-TG mice. B. Representative blot of transgene expression from F3 TG mice. Elevated hepatic *Fads2* mRNA (C) and D6D protein in chow-fed Fads2-TG vs. FVB WT mice. <Figures A-D provided by Dr. Chicco> E. Representative D6D protein blots of liver homogenates from C57BL/6J WT (first two lanes) and Fads2-HET mice (second two lanes) from the MIX diet group in the present study. F. Representative D6D blots of liver homogenates from FVB WT (first lane) and *Fads2*-TG (second lane) mice from the MIX diet group in the present study.

Table 1. Nutritional Information of Diets. Mix-diet(a). LA-diet(b). ALA-diet(c).

a.

Teklad Custom Research Diet Data Sheet

TD.150554 21% Fat Diet (50:50 LA/LNA, 14% CCB)

Formula	g/Kg
Casein, "Vitamin-Free" Test	195.0
DL-Methionine	3.0
Sucrose	341.46
Corn Starch	150.0
Corn Oil	25.0
Flaxseed Oil	45.0
Cocoa Butter	140.0
Cholesterol	1.5
Cellulose	50.0
Mineral Mix, AIN-76 (170915)	35.0
Calcium Carbonate	4.0
Vitamin Mix, Teklad (40060)	10.0
Ethoxyquin, antioxidant	0.04

Footnote
A modification of TD.88137/TD.09376 basal mix to use a mixture of corn oil, flaxseed oil and cocoa butter as fat sources. Total cholesterol of 0.15%.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	17.9	15.7
Carbohydrate	48.5	42.7
Fat	21.0	41.6
Kcal/g 4.5		

[1]Values are calculated from ingredient analysis or manufacturer data

Teklad Diets are designed & manufactured for research purposes only.

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- askanutritionist@harlan.com

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harlan
www.harlan.com

b.

Teklad Custom Research Diet Data Sheet

TD.150552 21% Fat Diet (7% CO, 14% CCB, Y)

Formula	g/Kg
Casein, "Vitamin-Free" Test	195.0
DL-Methionine	3.0
Sucrose	341.36
Corn Starch	150.0
Corn Oil	70.0
Cocoa Butter	140.0
Cholesterol	1.5
Cellulose	50.0
Mineral Mix, AIN-76 (170915)	35.0
Calcium Carbonate	4.0
Vitamin Mix, Teklad (40060)	10.0
Ethoxyquin, antioxidant	0.04
Yellow Food Color	0.1

Footnote
A modification of TD.88137/TD.09376 basal mix to use a mixture of corn oil and cocoa butter as fat sources. Total cholesterol of 0.15%. Yellow food coloring added.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	17.9	15.7
Carbohydrate	48.5	42.7
Fat	21.0	41.6
Kcal/g 4.5		

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c.

Teklad Custom Research Diet Data Sheet

TD.150553 21% Fat Diet (7% FSO, 14% CCB, O)

Formula	g/Kg
Casein, "Vitamin-Free" Test	195.0
DL-Methionine	3.0
Sucrose	341.36
Corn Starch	150.0
Flaxseed Oil	70.0
Cocoa Butter	140.0
Cholesterol	1.5
Cellulose	50.0
Mineral Mix, AIN-76 (170915)	35.0
Calcium Carbonate	4.0
Vitamin Mix, Teklad (40060)	10.0
Ethoxyquin, antioxidant	0.04
Orange Food Color	0.1

Footnote
A modification of TD.88137/TD.09376 basal mix to use a mixture of flaxseed oil and cocoa butter as fat sources. Total cholesterol of 0.15%. Orange food coloring added.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	17.9	15.7
Carbohydrate	48.5	42.7
Fat	21.0	41.6
Kcal/g 4.5		

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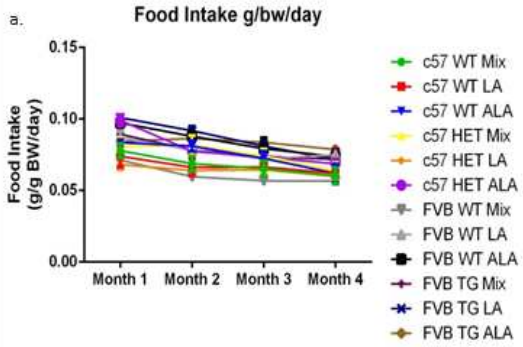
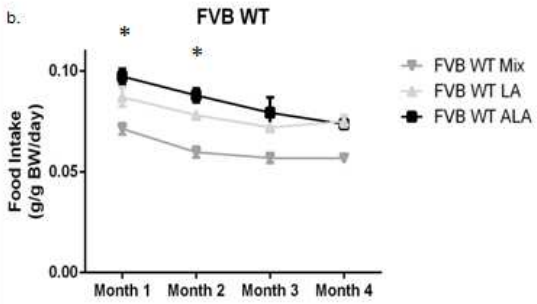
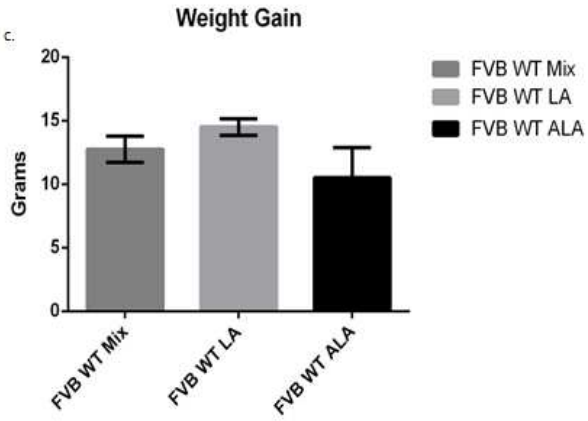
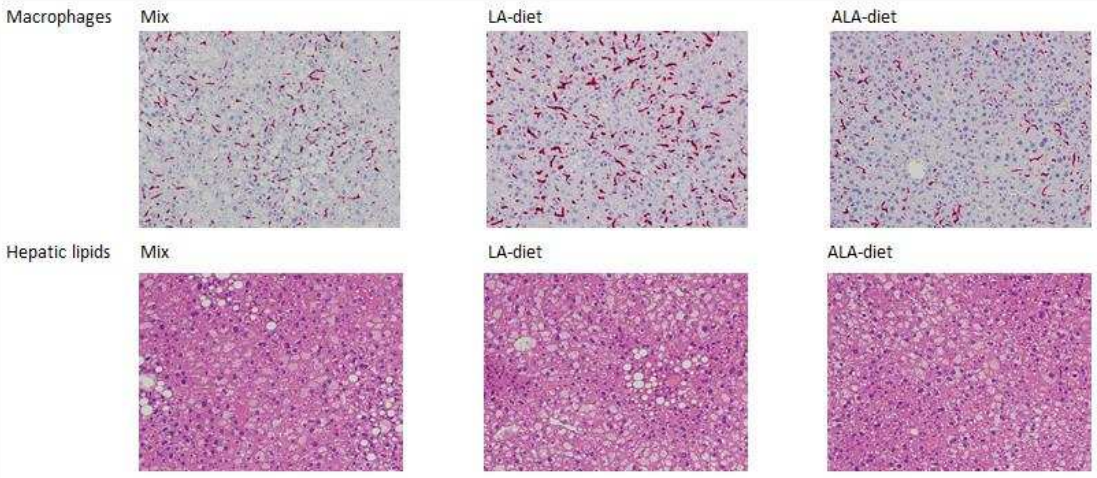


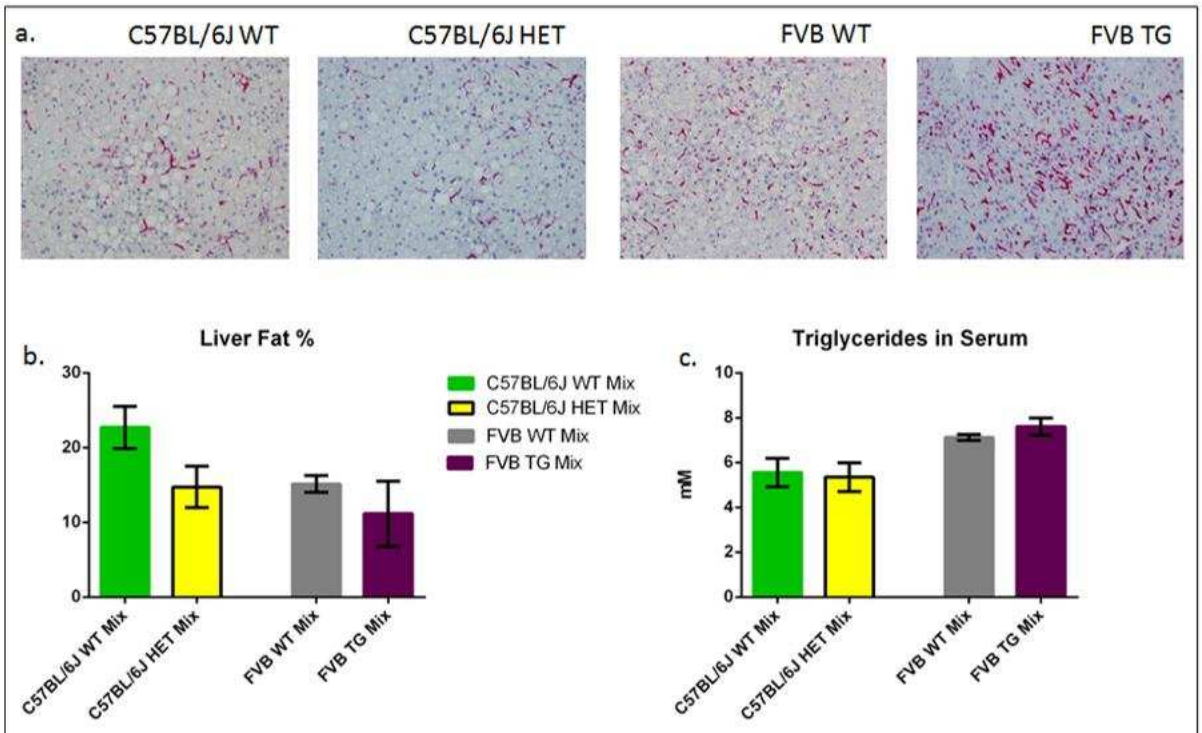
Supplemental Figure 2. Food intake had no effect on weight gained. Food intake expressed as grams/gram body weight/day(a). FVB WT mice on Mix diet were the only group that had significantly altered food intake. This occurred during month 1 and month 2 of the diet($p<.05$)(b). Weight gain was not affected because of this decrease(c).



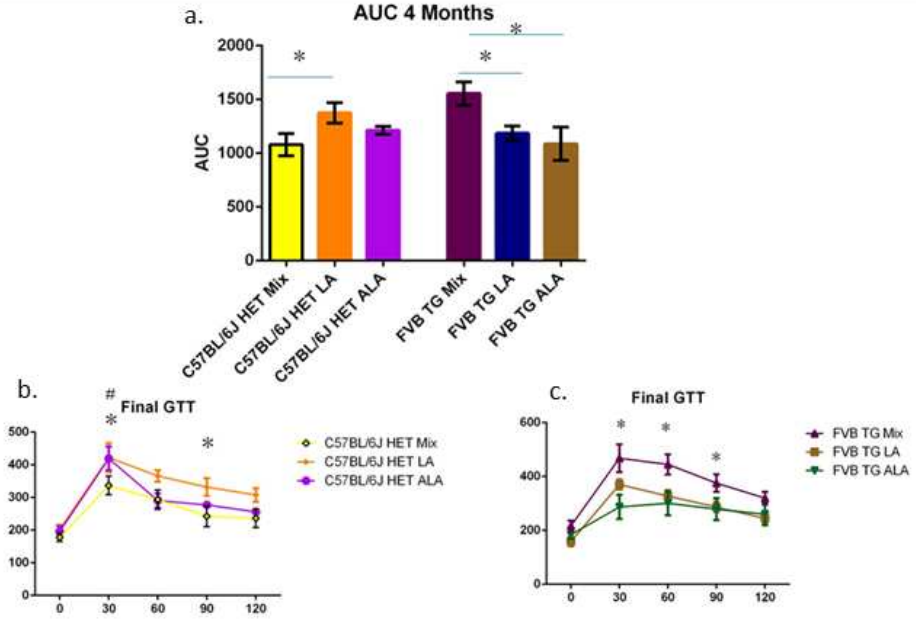




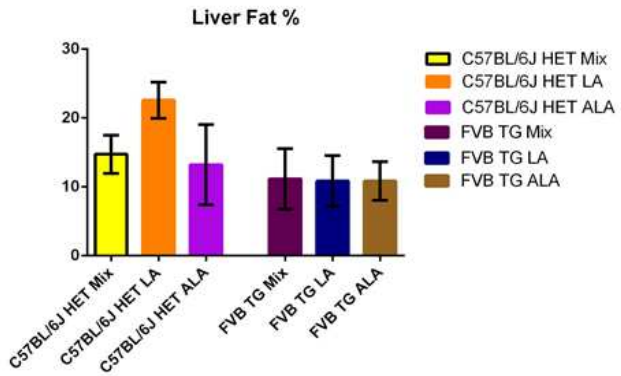
Supplemental Figure 3. LA-diet increases macrophage accumulation, while no differences in hepatic lipid droplets occur between diets. Images shown are from FVB WT mice. Macrophage accumulation(top) is higher in mice fed LA-diet. Hepatic lipid droplets (bottom) were not different between Mix-diet(left) LA-diet(center) or ALA-diet(right). All images are at 200x magnification.



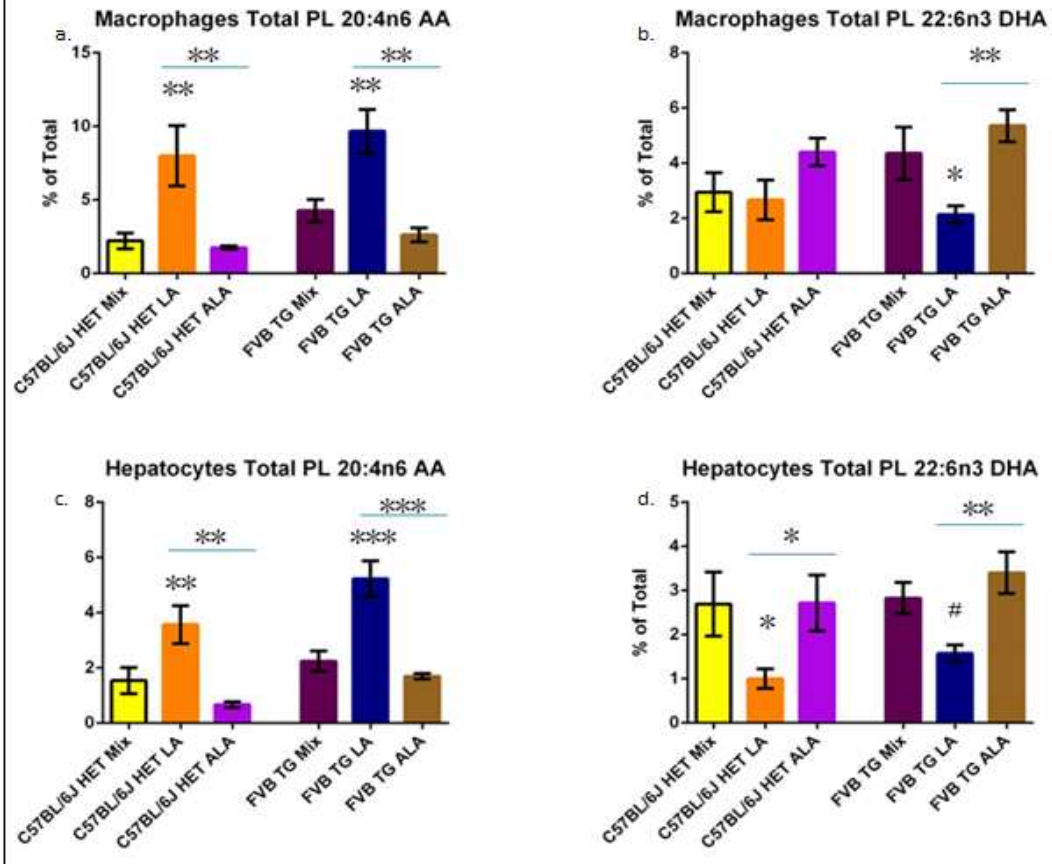
Supplemental Figure 4. *Fads2* genotype predicts hepatic macrophage accumulation, but has no effect on liver fat or serum triglycerides. Decreased *Fads2* expression (HET) lowered hepatic macrophage accumulation while TG expression increased macrophage accumulation(a). *Fads2* had no clear effect on liver fat accumulation(b) or serum triglyceride levels(c).



Supplemental Figure 5. PUFA imbalance differentially modulates glucose tolerance in Fads2 HET and TG mice. AUC is increased for HET mice on LA-diet(p=0.0435) and not significantly increased in ALA-diet(a). Similar results are seen in GTTs. At thirty minutes, glucose levels of HET Mix mice are lower than LA(p=0.0292) and trending lower than ALA(p=0.0627) and at 90 minutes HET Mix is lower than LA (p=0.0228) (b). At 30 minutes, TG mice on Mix diet are higher than LA(p=0.0157) and ALA(p=0.0028). Results are similar at 60 minutes (Mix vs LA p=0.0036, Mix vs ALA p=0.0006) and 90 minutes (Mix vs LA p=0.0274, Mix vs ALA p=0.0207)(c).



Supplemental Figure 6. Liver fat accumulation in HET and TG. No significant differences or trends(p<.1) were observed in HET or TG mice on experimental diets.



Supplemental Figure 7. Macrophage and hepatocyte phospholipid composition. Macrophage AA phospholipid levels are higher in HET mice fed LA-diet compared to Mix(p=0.0015) and ALA(p=0.006). TG mice fed LA-diet also had higher levels compared to Mix(p=0.0028) and ALA(p=0.0002)(a). DHA levels in macrophages were not significantly different in HET mice, but TG mice fed LA-diet had lower levels than Mix(p=0.0227) and ALA-diet(p=0.0016)(b). Hepatocytes were very similar, with AA levels higher in LA-diet compared to Mix(p=0.001) and ALA-diet(.0061). In TG mice, LA-diet fed mice had higher levels of AA compared to Mix(p=0.0001) and ALA-diet(p=0.0001)(c). Hepatic DHA levels were lower in HET mice on LA-diet compared to Mix(p=0.0292) and ALA-diet(p=0.0138), and TG mice on LA-diet had trending levels lower than Mix(p=0.061), and lower levels than ALA-diet(p=0.0081)