Supplemental Materials and Methods

Mice.

Male low density lipoprotein receptor deficient ($LDLr^{-/-}$), apolipoprotein B 100 only ($ApoB^{100/100}$) mice were used in this study. These mice were chosen based on previous reports documenting their "human-like" lipoprotein profile[1], atherosclerosis susceptibility [1,2], and responsiveness to dietary fatty acids[2]. All mice were on a mixed background (~75% C57BL/6 and ~25% 129Sv/Jae). To inhibit SCD1, antisense oligonucleotides (ASOs) were given as previously described[3], with minor modifications. Briefly, at 6 weeks of age, mice were switched from rodent chow to one of two synthetic diets containing 12% of energy as primarily either SFA-enriched fat (palm oil) or long chain ω-3 PUFA-enriched fat (fish oil), with 0.1% (w/w) cholesterol added. Please refer to Supplemental Table 1 for quality control analysis of dietary fatty acid composition. We have previously published the effects of SCD1 inhibition in SFA-fed mice[3], and these data are included here strictly for comparative purposes. All experimental animals were sacrificed after 20 weeks of parallel dietary and ASO treatment, except for macrophage experiments where mice were sacrificed after 6 weeks of treatment. Mice were maintained in an American Association for Accreditation of Laboratory Animal Care-approved pathogen-free animal facility, and all experimental protocols were approved by the institutional animal care and use committee at the Wake Forest University School of Medicine.

Plasma Lipid and Lipoprotein Analyses. Plasma samples were collected by submandibular vein puncture at baseline (6 weeks of age chow fed animals), and after 4, 8, or 20 weeks of diet and ASO treatment. Detailed description of plasma lipid analyses has been previously described.[3]

Glucose homeostasis. Intraperitoneal glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed as previously described[3] in mice that had been treated with diet and ASO for 16 weeks.

Quantification of atherosclerosis. *En face* morphometric and biochemical quantification of aortic atherosclerosis was conducted as previously described[3] in mice that had been treated with diet and ASO for 20 weeks.

Hepatic lipid mass and fatty acid composition. Extraction of liver lipids and biochemical analyses were performed as previously described[3] in mice that had been treated with diet and ASO for 20 weeks.

Hepatic ER stress. Total liver homogenates were prepared as previously described[3], and ER stress was measured by immunoblotting using antibodies recognizing CHOP (Cell Signaling Technologies # 2895), protein disulfide isomerase (PDI, Cell Signaling Technologies # 2446), and BIP (Cell Signaling Technologies # 3177).

Peritoneal macrophage isolation and culture. For macrophage studies, mice received diet and ASO treatment for six weeks. Thereafter, thioglycolate-elicited peritoneal macrophages were pooled from five mice per group, and were cultured in 35-mm dishes as previously described.[3] For TLR4 agonist studies, following two hours of culture, adherent macrophage were washed three times with PBS, and subsequently maintained in serum free RPMI-1640 for an additional two hours to dampen basal serum-induced signaling. Thereafter, cells were treated with vehicle (PBS) or 10 ng/ml of the defined TLR 4 agonist[4] Kdo_2-Lipid A for six hours to examine inflammatory gene expression by qPCR as previously described[3], and cytokine secretion was measured using a Bio-Plex mouse cytokine kit (Bio-Rad) using fluorescently labeled microsphere beads and a Bio-Plex suspension array system (Bio-Rad) according the to the manufacturer's instructions. For cholesterol efflux, freshly isolated macrophages were incubated with ^{3}H-cholesterol for 8 hours. Thereafter, cells were gently washed four times with PBS, and then supplemented with 1 ml of efflux medium (serum-free RPMI-1640 and 0.1% fatty-acid-free BSA) in the absence or presence of cholesterol acceptors (10 μg/ml apoA-I or 50 μg/ml HDL) for 6 h at 37°C. To determine cholesterol efflux, the conditioned media were collected and centrifuged for 5 min at 16000 *g* to pellet cellular debris. An aliquot was removed and the radioactivity present in the incubation medium was determined by liquid-scintillation counting. The percentage of radiolabelled cholesterol released (% efflux) was calculated as: (dpm in medium after 6h / dpm in cells at zero time) x 100. For isolated macrophage experiments, the data shown in Figure 3 are representative of one pool of macrophages (n=5 mice per group) split into triplicate plates, and similar results were seen in an independent pool of macrophages (n=5-7 mice per group).

SCD Activity Assay. Hepatic SCD1 activity was measured in liver microsomes as previously described[3], in mice that had been treated with diet and ASO for 20 weeks.

Quantitative Real-Time PCR (qPCR). RNA extraction and qPCR was conducted as previously described[3] on pooled samples (n=5 per group). Messenger RNA levels for each gene represent the amount relative to the amount in the control ASO treated SFA diet-fed group, which was arbitrarily standardized to 100%. Primers used for qPCR have been previously described.[3]

Statistical Analysis. Data are expressed as the mean ± standard error of the mean (SEM). All data were analyzed using two-way analysis of variance (ANOVA) using diet and ASO treatment as individual variables, followed by Student's t tests for post hoc analysis. Differences were considered significant at $p < 0.05$. All analyses were performed using JMP version 5.0.12 (SAS Institute; Cary, NC) software.

Supplemental Results

Dietary fish oil and SCD1 ASO treatment reduce SCD1 expression in a tissue-specific manner. To specifically inhibit SCD1 activity, we utilized antisense oligonucleotide (ASO) treatment as previously described[3] in low density lipoprotein receptor knockout (LDLr $^{-/-}$), apolipoprotein B only (ApoB$^{100/100}$) mice, a mouse model of LDL-driven atherosclerosis. Mice were fed either a SFA-rich or a ω-3 PUFA-rich diet for 20 weeks. Regardless of diet, SCD1 mRNA levels were reduced by 99% in the liver and 78-93% in adipose tissue in mice treated with SCD1 ASO (Supplemental Figure 1, A and B). In agreement, hepatic SCD1 activity was reduced by > 95% after 20 weeks of SCD1 ASO treatment in mice fed the SFA-rich diet (Supplemental Figure 1C). As has been previously described[5,6], when compared to SFA-feeding, dietary ω-3 PUFA supplementation resulted in a 75% reduction in hepatic SCD1 mRNA levels in control ASO treated mice (Supplemental Figure 1A). This dietary fish oil-driven reduction of SCD1 mRNA occcured only in the liver, and not in adipose tissue (Supplemental Figure 1B) or macrophages (Figure 3C), indicative of tissue-specific regulation.

Combination therapy of dietary fish oil and SCD1 ASO prevents diet-induced obesity and insulin resistance. We previously demonstrated that ASO-mediated inhibition of SCD1 prevented diet-induced obesity and insulin resistance in LDLr $^{-/-}$, ApoB$^{100/100}$ mice fed a SFA-rich diet.[3] Likewise, it has been demonstrated that dietary fish oil supplementation can similarly improve obesity and insulin resistance.[7,8] Therefore, we set out to determine if the combination therapy of dietary fish oil and

SCD1 ASO could act synergistically to further ameliorate diet-induced obesity and insulin resistance. In agreement with previous reports[3], SCD1 inhibition prevented diet-induced obesity in LDLr $^{-/-}$, ApoB$^{100/100}$ mice fed either diet (Supplemental Figure 2, A and B). Following 20 weeks of treatment, epididymal fat pad mass was reduced by ~85% in mice treated with SCD1 ASO fed the SFA-rich diet, compared to control ASO treated mice (Supplemental Figure 2B). In agreement with previous reports[7,8], dietary fish oil supplementation in control ASO-treated mice significantly reduced body weight gain, compared to control ASO-treated mice fed a SFA-rich diet (Supplemental Figure 2, A and B). It is important to point out that the effects of dietary fish oil and SCD1 ASO were not synergistic in reducing body weight. In addition to dramatic effects of adiposity, SCD1 ASO treatment significantly improved glucose tolerance in mice fed either diet (Supplemental Figure 2C). In control ASO treated mice, dietary fish oil modestly improved glucose tolerance, compared to SFA-feeding (Supplemental Figure 2C), regardless of ASO treatment. Furthermore, during insulin tolerance tests (ITT), SCD1 ASO treated mice had enhanced blood glucose disposal, compared to control ASO treated mice on either diet, indicating enhanced insulin sensitivity (Supplemental Figure 2D). However, the effects of dietary fish oil and SCD1 ASO were not synergistic in improving glucose and insulin tolerance. Collectively, these data support the notion[9-21] that SCD1 ASO treatment alone is extremely effective in the prevention of diet-induced obesity and insulin resistance, and that SCD1 inhibition in the presence of dietary fish oil does not provide synergistic protection.

Combined therapy of dietary fish oil and SCD1 ASO prevents diet-induced hepatic steatosis. Mice lacking SCD1 are protected against diet- and genetically-induced hepatic steatosis.[13,14,17,20,21] Likewise, dietary ω-3 PUFA supplementation protects against hepatic steatosis in a number of experimental models.[6,22] It remains possible that the anti-steatotic actions of fish oil may be attributable in part to its ability to downregulate the hepatic expression of SCD1 (Supplemental Figure 1A,[6,7]). As expected, in mice fed a SFA-rich diet, SCD1 ASO treatment alone resulted in striking reductions in hepatic neutral lipid accumulation (Supplemental Figure 4, A and B). In fact, in SFA-fed mice, SCD1 inhibition reduced hepatic TG by 93% (Supplemental Figure 3A) and hepatic cholesteryl ester mass (CE) by 81% (Supplemental Figure 3B), compared to control ASO treated mice. Likewise, dietary fish oil supplementation decreased hepatic TG by 93-96% on both diets (Supplemental Figure 3A). After 20 weeks of treatment, the rank order for the groups in regards to hepatic TG levels was: SFA-fed/Control ASO (1409 mg/g protein) > Fish-fed/Control ASO (462 mg/g protein) > SFA-fed/SCD1 ASO (96 mg/g protein) > Fish-fed/SCD1 ASO (19 mg/g protein). In contrast to neutral lipids, hepatic phospholipid mass was not different among the four groups (Supplemental Figure 3C). As expected[3], SCD1 ASO treatment caused a striking decrease in the oleate (18:1) to stearate (18:0) ratio in hepatic TG in both diet groups (Figure 3D). Dietary fish oil alone also caused a significant reduction in 18:1 to 18:0 ratio, compared to the dietary SFA group (Supplemental Figure 3D), in agreement with the previously described fish oil-driven reductions in hepatic SCD1 activity (Supplemental Figure 1C). In addition, dietary fish oil supplementation resulted in expected ω-3 PUFA-enrichment in hepatic TG (Supplemental Figure 3F). Interestingly, ASO-mediated inhibition of SCD1 in mice fed a

fish oil diet caused significantly more ω-3 PUFAs to be incorporated into hepatic TG, compared to control ASO treated mice. The reduction in hepatic TG seen with both dietary fish oil and SCD1 inhibition is likely due in part to both of these treatments causing robust downregulation of genes involved in *de novo* fatty acid synthesis.[6,9] In support of this, both dietary fish oil and SCD1 ASO treatment caused dramatic decreases in lipogenic genes including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and mitochondrial glycerol-3-phosphate acyltransferase (mGPAT) to a similar degree (Supplemental Figure 3E). Unlike genetic deletion of SCD1 in mice fed a very low fat diet,[23] SCD1 ASO treatment did not alter the expression of lecithin:cholesterol acyltransferase (LCAT) expression in mice fed either diet (Supplemental Figure 3E). Interestingly, both dietary fish oil and SCD1 ASO treatment caused an 81-86% decrease in hepatic expression of 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase) (Supplemental Figure 3E). As previously described[3], SCD1 ASO treatment results in a ~2.2-fold increase in hepatic cholesterol 7alpha-hydroxylase (Cyp7α) expression, regardless of diet (Supplemental Figure 3E). Collectively, these data support previous observations that demonstrate both dietary fish oil and SCD1 inhibition are efficacious in the prevention hepatic steatosis.[6,13,14,17,20,21]

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Supplemental Table I.

Fatty acid composition of diets (% of total FA)

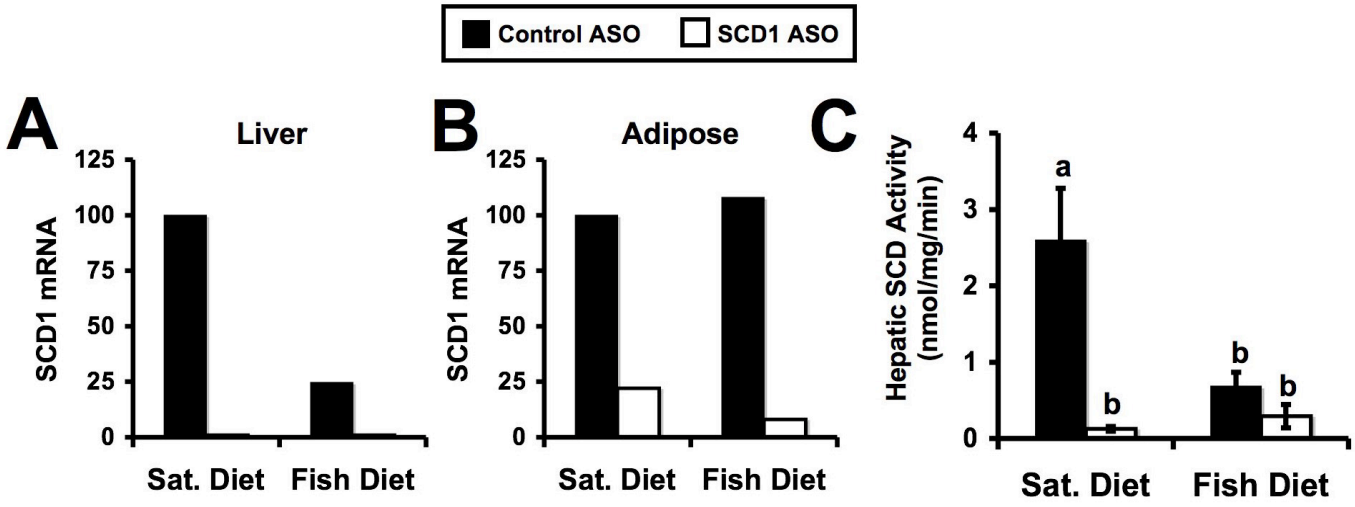
	12:0	14:0	16:0	16:1	18:0	18:1 (n-9)	18:2 (n-6)	18:3 (n-3)	20:0	20:4 (n-6)	20:5 (n-3)	22:6 (n-3)
Saturated Diet	2.0	6.4	31.7	0.7	6.1	24.4	17.9	0.5	0.2	0.5	0.5	0.5
Fish Oil Diet	0.1	6.1	20.4	10.3	4.5	13.5	6.4	0.3	0.1	0.9	10.4	8.5

Saturated Diet

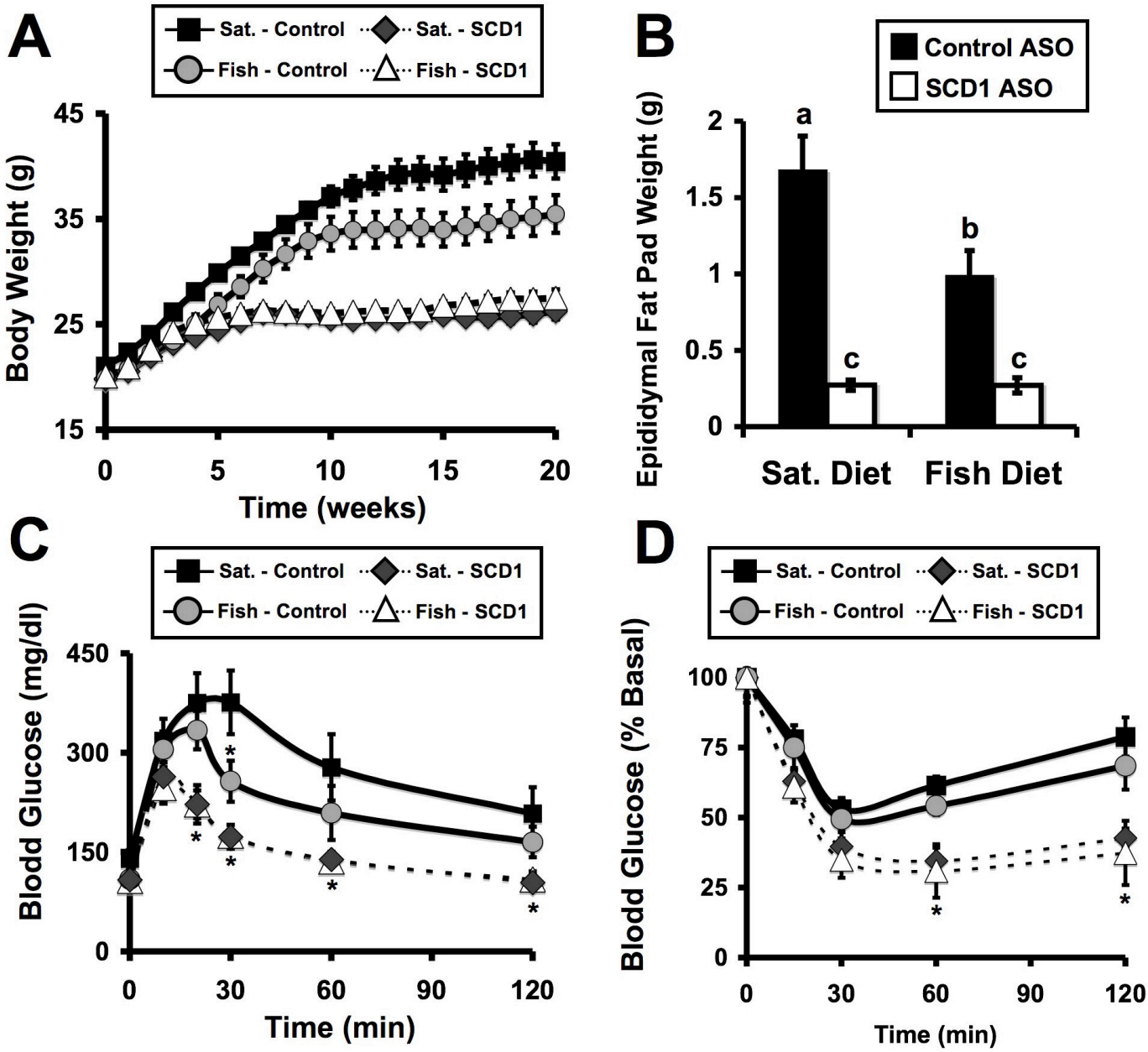
	g / 100 g	Fat (g)	Carbohydrate (g)	Protein (g)	Cholesterol (mg)	Phytosterol (mg)	α-tocopherol (mg)
Sat. Blend (AC Humko)	4.00	4.00				1.60	0.76
Fish Oil (Omega Protein)	0.22	0.22			1.09		0.02
Casein	8.00			8.00			
Lactalbumin	4.00			4.00			
Dextrin	17.00		17.00				
Sucrose	17.00		17.00				
Wheat Flour, Self-Rising	35.00	0.35	25.90	3.15		1.93	0.53
Alphacel (Fiber)	7.13						
Crystalline Cholesterol	0.10				100.00		
Vitamin Mixture, Teklad	2.50		1.03				30.28
Hegsted Salt Mixture	5.00						
β-sitosterol	0.03					32.00	
Tenox 20A (antioxidant)	0.004						
Vitamin E 5-67	0.002						1.34
TOTAL	100	4.6	60.9	15.2	101.1	35.5	33.7
Kcal		40.4	243.7	60.6			
% of Energy		12	71	17			

Fish Oil Diet

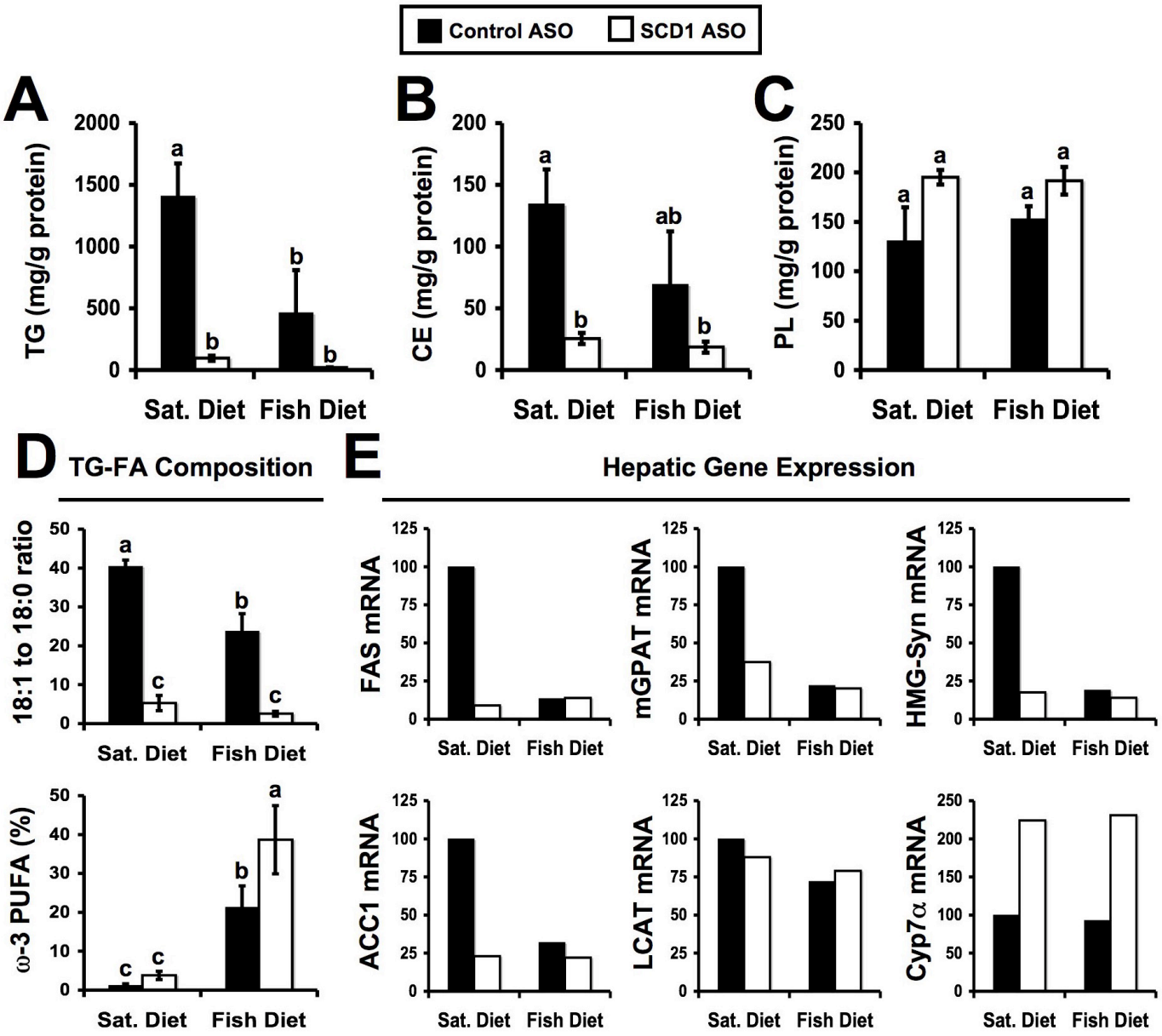
	g / 100 g	Fat (g)	Carbohydrate (g)	Protein (g)	Cholesterol (mg)	Phytosterol (mg)	α-tocopherol (mg)
Sat. Blend (AC Humko)	0.00					1.60	0.76
Fish Oil (Omega Protein)	4.20	4.20			20.83		0.02
Casein	8.00			8.00			
Lactalbumin	4.00			4.00			
Dextrin	17.00		17.00				
Sucrose	17.00		17.00				
Wheat Flour, Self-Rising	35.00	0.35	25.90	3.15		1.93	0.53
Alphacel (Fiber)	7.13						
Crystalline Cholesterol	0.08				80.00		
Vitamin Mixture, Teklad	2.50		1.03				30.28
Hegsted Salt Mixture	5.00						
β-sitosterol	0.03					32.00	
Tenox 20A (antioxidant)	0.004						
Vitamin E 5-67	0.002						1.34
TOTAL	100	4.6	60.9	15.2	101.1	35.5	33.7
Kcal		40.4	243.7	60.6			
% of Energy		12	71	17			



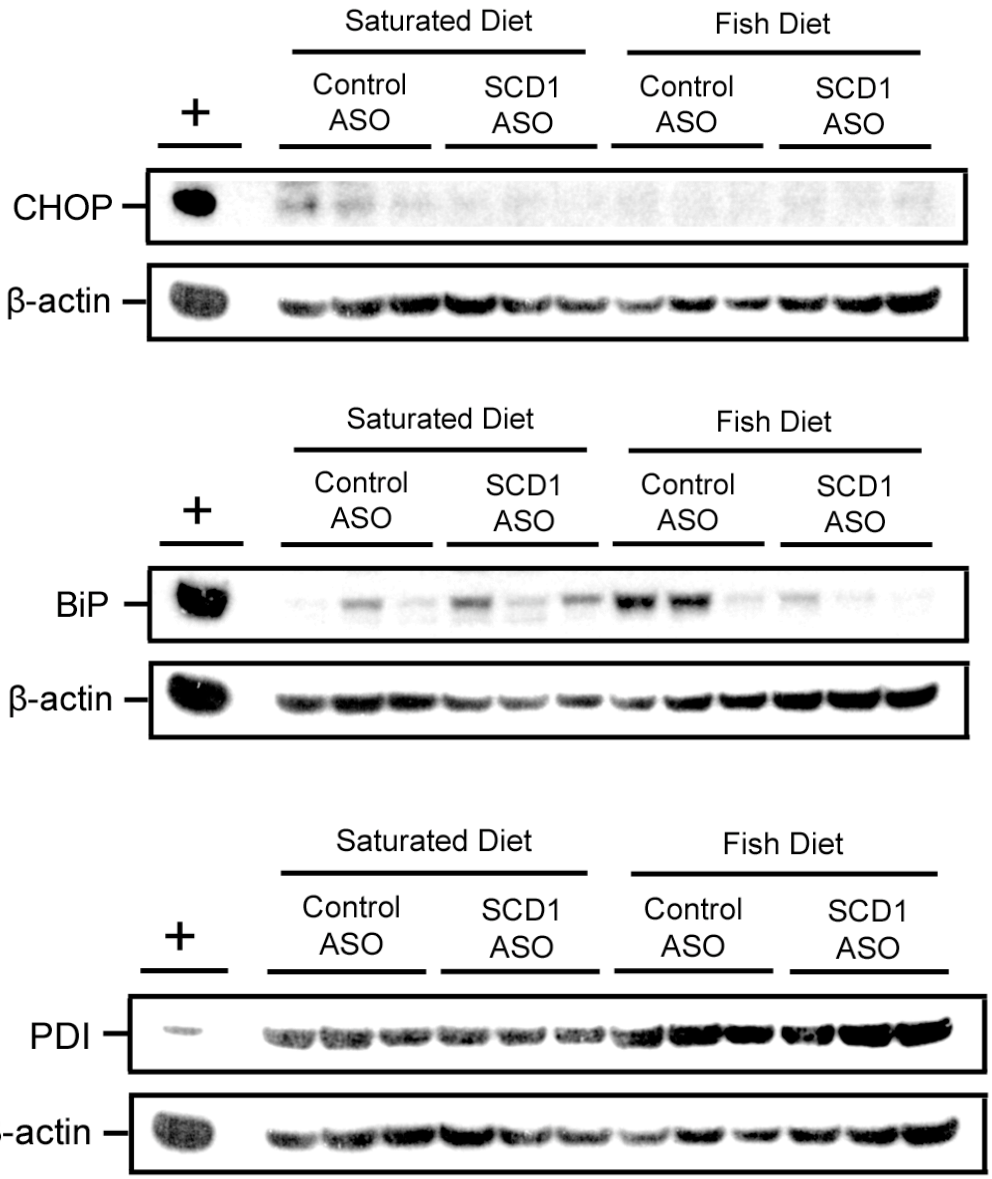
Supplemental Figure 1. Dietary and ASO-mediated regulation of SCD1 expression and function. Male LDLr $^{-/-}$, ApoB$^{100/100}$ mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω-3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a non-targeting control ASO ■ or SCD1 ASO □. Relative quantification of SCD1 mRNA levels in the liver **(A)** or epididymal adipose tissue **(B)** was conducted by real-time qPCR, and normalized to cyclophilin. Data shown in panels **A** and **B** represent pooled RNA samples with n=5 mice per group. **C.** Hepatic SCD1 activity, data represent the mean ± SEM from 5 mice per group, and values not sharing a common superscript differ significantly ($p < 0.05$).



Supplemental Figure 2. Combination therapy of dietary fish oil and SCD1 ASO prevents diet-induced obesity and insulin resistance in LDLr$^{-/-}$Apob$^{100/100}$ mice. Starting at six weeks of age, mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω-3 fatty acids (Fish) for a period up to 20 weeks in conjunction with biweekly injections (25 mg/kg) of either a non-targeting control (control) or SCD1 ASO (SCD1). **(A)** Body weight, and **(B)** epididymal fat pad mass of mice following 20 weeks of diet and ASO treatment. Data in panel **B** represent the mean ± SEM from 8-15 mice per group, and values not sharing a common superscript differ significantly (p<0.05). Glucose tolerance tests **(C)** and insulin tolerance tests **(D)** were performed following 16 weeks of diet and ASO treatment. Data shown in panels **C** and **D** represent the mean ± SEM from 5 mice per group, * = significantly different than the Sat.-Control group within each time point (p<0.05).



Supplemental Figure 3. Combined therapy of dietary fish oil and SCD1 ASO prevents diet-induced hepatic steatosis in LDLr $^{-/-}$, ApoB$^{100/100}$ mice. Starting at six weeks of age, male mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω-3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a non-targeting control ASO (Control) or SCD1 ASO (SCD1). Hepatic lipid mass measurements were conducted for **A.** triglyceride (TG), **B.** cholesteryl ester (CE), and **C.** phospholipids (PL). Data shown in panels **A-C** represent the mean ± SEM from 6-8 mice per group; values not sharing a common superscript differ significantly ($p<0.05$). **D.** Fatty acid (FA) composition [18:1 to 18:0 ratio or % of total FA as long chain ω-3 (eicosapentaenoic and decosahexaenoic) fatty acids] of hepatic triglycerides (TG-FA). Data shown is panel **D** represents the mean ± SEM from 6-8 mice per group, and values not sharing a common superscript differ significantly ($p<0.05$). (E) Hepatic gene expression was measured in pooled samples (n=5 per pool) by qPCR, and normalized to cyclophilin. The mRNAs examined include: fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC1), mitochondrial glycerol-3-phosphate acyltransferase-1 (mGPAT), lecithin:cholesterol acyltransferase (LCAT), 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-Syn), cholesterol 7alpha-hydroxylase (Cyp7α).



Supplemental Figure 4. Hepatic endoplasmic reticulum (ER) stress is not altered by diet or ASO treatment. Starting at six weeks of age, male mice were fed diets containing 0.1% (w/w) cholesterol enriched in either saturated (Sat.) or long chain ω-3 fatty acids (Fish) for 20 weeks in conjunction with biweekly injections (25 mg/kg) of a non-targeting control ASO (Control) or SCD1 ASO (SCD1). ER stress was quantified by immunoblotting as described in materials and methods. The positive control (+) for this experiment represents 25 μg of protein lysate from tunicamycin treated (10 μg/ml for 10 hours) elicited peritoneal macrophages, which exhibit marked ER stress.

Supplemental Table II. Plasma cytokine levels following 20 weeks of diet and ASO treatment. Data represents the mean ± SEM (n = 5); N.D. = values not detectable. Values not sharing a common superscript differ significantly ($p < 0.05$).

Plasma Cytokine Levels (pg/ml)

	Sat. - Control	Sat. - SCD1	Fish - Control	Fish - SCD1
IL-1β	30.5 ± 18.8^{a}	8.3 ± 3.5^{a}	N.D.	6.6 ± 4.3^{a}
IL-6	12.9 ± 4.8^{a}	5.4 ± 1.6^{ab}	7.0 ± 2.5^{ab}	0.9 ± 0.9^{b}
IL-12-p40	411.4 ± 39.7^{ab}	448.7 ± 34.3^{a}	246.2 ± 31.2^{c}	343.3 ± 25.0^{bc}
KC	205.3 ± 67.0^{a}	142.8 ± 16.3^{a}	132.9 ± 28.9^{a}	107.6± 11.0^{a}
MCP-1	101.9 ± 7.5^{a}	100.9 ± 14.6^{a}	65.9 ± 5.5^{b}	90.5 ± 14.8^{ab}
MIP-1α	10.5 ± 10.5^{a}	2.4 ± 2.4^{a}	N.D.	5.0 ± 5.0^{a}
TNFα	10.5 ± 10.5^{a}	1.2 ± 1.2^{a}	N.D.	2.5 ± 2.5^{a}
RANTES	12.6 ± 1.3^{ab}	18.2 ± 5.2^{a}	5.4 ± 1.1^{b}	21.8 ± 4.8^{a}