Very-long-chain ω-3 fatty acid supplements and adipose tissue functions: a randomized controlled trial[1]

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ABSTRACT

Background: Increased omega-3 (n–3) fatty acid consumption is reported to benefit patients with metabolic syndrome, possibly due to improved adipose tissue function.

Objective: We tested the effects of high-dose, very-long-chain ω-3 fatty acids on adipose tissue inflammation and insulin regulation of lipolysis.

Design: A double-blind, placebo-controlled study compared 6 mo of 3.9 g eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)/d (4.2 g total ω-3/d; $n = 12$) with a placebo (4.2 g oleate/d; $n = 9$) in insulin-resistant adults. Before and after treatment, the volunteers underwent adipose tissue biopsies to measure the total ($CD68^+$), pro- ($CD14^+$ = M1), and anti- ($CD206^+$ = M2) inflammatory macrophages, crown-like structures, and senescent cells, as well as a 2-step pancreatic clamping with a [U-^{13}C]palmitate infusion to determine the insulin concentration needed to suppress palmitate flux by 50% ($IC_{50(palmitate)}f$).

Results: In the ω-3 group, the EPA and DHA contributions to plasma free fatty acids increased ($P = 0.0003$ and $P = 0.003$, respectively), as did the EPA and DHA content in adipose tissue ($P < 0.0001$ and $P < 0.0001$, respectively). Despite increases in adipose and plasma EPA and DHA in the ω-3 group, there were no significant changes in the $IC_{50(palmitate)}f$ (19 ± 2 compared with 24 ± 3 μIU/mL), adipose macrophages (total: 31 ± 2/100 adipocytes compared with 33 ± 2/100 adipocytes; $CD14^+$: 13 ± 2/100 adipocytes compared with 14 ± 2/100 adipocytes; $CD206^+$: 28 ± 2/100 adipocytes compared with 29 ± 3/100 adipocytes), crown-like structures (1 ± 0/10 images compared with 1 ± 0/10 images), or senescent cells (4% ± 1% compared with 4% ± 1%). There were no changes in these outcomes in the placebo group.

Conclusions: Six months of high-dose ω-3 supplementation raised plasma and adipose ω-3 fatty acid concentrations but had no beneficial effects on adipose tissue lipolysis or inflammation in insulin-resistant adults. This trial was registered at clinicaltrials.gov as NCT01686568. *Am J Clin Nutr* 2017;105:1552–8.

Keywords: EPA, DHA, macrophage, lipolysis, inflammation, insulin resistance

INTRODUCTION

Adipose tissue has many functions that help maintain a healthy metabolic state, including its role to release free fatty acids (FFAs) for utilization by lean tissues. Insulin is a major regulator of this process; it inhibits FFA release into circulation, thereby enhancing insulin's ability to promote glucose disposal (1). If adipose tissue has a diminished response to insulin, as is the case with insulin resistance and type 2 diabetes, excess FFAs are released, primarily from upper-body subcutaneous adipose tissue (2, 3). Elevated circulating FFA concentrations are associated with metabolic abnormalities, including muscle and liver insulin resistance (4–6) and impaired insulin secretion by pancreatic β cells (7, 8).

Adipose tissue also secretes cytokines. A majority of these are secreted from stromovascular cells, including senescent cells (9) and/or classically activated macrophages (10) that infiltrate adipose tissue. These proinflammatory cells accumulate with adipose tissue expansion (11, 12). Like FFAs, excess proinflammatory cytokines can inhibit insulin signaling, which can exaggerate the negative impact of excess FFAs on systemic metabolism.

By focusing therapeutic strategies on maintaining and promoting healthy adipose tissue function, the signs and symptoms of unhealthy metabolic function can theoretically be improved. Research has focused on changes in dietary fatty acids given the high potential for the achievable efficacy of such an intervention. Fatty acid composition of an individual's diet is reflected in tissue lipid content (13, 14) and can thus modify adipose tissue function. Fish oils that contain the ω-3 fatty acids EPA and DHA have shown promise to prevent or improve insulin resistance in cell cultures (15) and animal models (16–19). Although data from human studies are conflicting (20–24), evidence suggests that ω-3 supplements reduce adipose macrophage infiltration in adults with metabolic syndrome (25).

Our objective was to test whether very-long-chain ω-3 fatty acids improve insulin-mediated suppression of adipose tissue

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lipolysis and the proinflammatory cellular composition of adipose in insulin-resistant adults with excess body fat. We hypothesized that 6 mo of ω-3 supplements would improve insulin-mediated suppression of adipose tissue lipolysis and reduce proinflammatory cells (i.e., macrophages, crown-like structures, and senescent cells) in adipose tissue compared with the placebo group.

METHODS

This protocol was an addendum to a Mayo Clinic Institutional Review Board–approved study. The primary results of this trial, which was conducted at Mayo Clinic in Rochester (MN) between January 2013 and October 2014, have been published by Lalia et al. (26).

Study design

This prospective, randomized, placebo-controlled, double-blind study evaluated the effects of 6 mo of ω-3 supplements on metabolic health in insulin-resistant (HOMA-IR: ≥2.6), overweight or obese [BMI (in kg/m^2): ≥25.0] adults aged 18–65 y. Inclusion and exclusion criteria for recruitment have been published (26). Briefly, potential volunteers were excluded if they were taking medication or supplements or had disorders or diseases (e.g. diabetes) known to affect the outcome measures. Volunteers were weight stable and did not engage in ≥30 min of structured exercise >2 d/wk. Participants were instructed not to systematically alter dietary and physical activity habits throughout their participation in the study. After screening and baseline measurements, the participants were randomly assigned to groups based on a table prepared by a statistician. All study staff and laboratory staff remained blinded to the intervention until the completion of the trial and sample analyses. Those randomly assigned to the ω-3 group were provided with oral supplements containing 3.9 g EPA+DHA/d, with small amounts of α-linolenic, moroctic (stearidonic), eicosatetraenoic, heneicosapentaenoic, and docosapentaenoic acids. The total amount was 4.2 g ω-3 fatty acids/d. Participants randomly assigned to the placebo group were provided with 4.2 g oleic acid/d. Compliance to the intervention was determined by a pill count every 4 wk. Before and after the intervention participants underwent measurements of body composition and insulin regulation of adipose tissue lipolysis. They also had abdominal subcutaneous adipose tissue biopsies for measurement of fatty acid content, adipocyte size, and macrophage and senescent cell content. The trial ended when all participants had either completed their study visits or confirmed that they were unable to continue participation.

Body composition

Body composition was analyzed with DXA (Lunar DPX-L; Lunar Radiation). Abdominal fat areas were measured from the average of 3 images taken at 1-cm intervals centered on the umbilicus with a Signa 3.0 Tesla MRI scanner (GE Health care) and analyzed by a single, trained technician using Analyze Software System (Mayo Clinic Biomedical Imaging Resource). These analyses were used to estimate mass of lower- and upper-body subcutaneous and visceral fat (27). We also used an image analysis tool to measure the anterior-posterior and lateral diameters of the abdomen in ≥2 MRI images to allow us to calculate waist circumference using the formula for an ellipse (waist circumference was not measured as part of the entry criteria for this study).

Insulin regulation of adipose tissue lipolysis

One of our prespecified primary outcome measures was the sensitivity of adipose tissue lipolysis to insulin suppression, which was calculated as the insulin concentration needed to suppress palmitate appearance rates (i.e., flux) by 50% ($IC_{50(palmitate)}$f) (28). Palmitate flux was measured in the overnight postabsorptive (i.e., basal) state and during a 2-step euglycemic, pancreatic clamp that was also used to measure insulin regulation of glucose metabolism (26). In brief, participants followed a weight-maintaining diet providing 20% protein, 50% carbohydrates, and 30% fat for 3 d. On the third day of the diet, they were admitted in the evening to the Clinical Research and Trials Unit and fasted overnight. For the pancreatic clamp we infused somatostatin (60 ng · kg^{-1} · min^{-1}), glucagon (0.65 ng · kg^{-1} · min^{-1}), and human growth hormone (3 ng · kg^{-1} · min^{-1}). The insulin infusion rates were 0.6 and 2.3 mU · kg fat free mass^{-1} · min^{-1} for steps 1 and 2, respectively. Euglycemia was maintained at ∼5.0 mmol/L by infusing 40% dextrose. We infused [U-^{13}C]palmitate (330 nmol/min) beginning 1 h before starting the clamp to measure basal palmitate flux and used a smaller amount (60 nmol/min) during the last hour of each of the two 3-h steps of the clamp to account for the anticipated suppression of lipolysis by insulin.

During the clamp, arterialized blood samples were taken from a catheter placed in retrograde fashion into a hand vein by using the heated-box technique (29). Blood samples were collected at 10-min intervals for the last 30 min of the basal state and each stage of the clamp for measurement of plasma insulin, glucose, and FFA concentrations. Plasma insulin and glucose (26), as well as FFA concentrations and palmitate enrichments (30), were measured as previously described.

Subcutaneous adipose tissue sampling and analysis

At least 1 wk after the pancreatic clamp study, the participants were again admitted to the Clinical Research and Trials Unit and provided a standardized meal before an overnight fast. The next morning an abdominal subcutaneous adipose tissue biopsy was collected under local anesthesia by using a sterile technique. The samples were analyzed for adipocyte size (31). Another one of our prespecified primary outcome measurements was the tissue burden of senescent cells, which we measured by staining for the percentage of positive cells with senescence-associated β-galactosidase activity (32). Our third, prespecified, primary outcome measurement was immunohistochemistry assessments of macrophage burden [total (CD68), M1 (CD14), and M2 (CD206) macrophages/100 adipocytes], as well as the number of crown-like structures/10 images (33).

Materials

[U-^{13}C]palmitate was purchased from Isotec (SigmaAldrich).

Power and statistical analysis

We measured the combined biological and methodologic variability for adipose tissue content for total (CD68), M1 (CD14), and M2 (CD206) macrophages by using immunohistochemistry. Spencer et al. (25) found that adipose tissue macrophages (CD68) were reduced by ~30% with ω-3 fatty acid supplements. Based on our known adipose macrophage assay reproducibility, our sample size provided >80% power to detect a 30% reduction in total adipose macrophages with a $P < 0.05$.

Insulin-mediated glucose disposal is 2–3 times greater in lean than in upper-body-obese adults, and the difference in IC_{50} for insulin-mediated suppression of lipolysis is of similar magnitude (34). Our goal was to detect smaller, yet clinically relevant, effects of ω-3 fatty acids on adipose tissue insulin resistance, such as those observed with a 10% weight loss due to a comprehensive lifestyle intervention. In a previous study that used this approach to help obese adults with insulin resistance, we found that insulin-suppressed fatty acid flux improved by 38% ± 46% and insulin-stimulated glucose disposal increased by 30% (35). Although not "normal," we estimated that this degree of improvement would be clinically meaningful, in line with the observations of Magkos et al. (36). We calculated that with 12 participants in the ω-3 group and 9 in the placebo group we had >80% power to detect a 38% difference in $IC_{50(palmitate)}$f with an α at 0.05.

The baseline categorical variables (e.g., sex and race/ethnicity) were tested for between-group differences by using Fisher's exact test. Continuous variables (e.g., age and body composition parameters) with normal distribution were compared by using *t* tests, and nonnormally distributed data were analyzed with Wilcoxon's rank-sums tests. By using the multivariate repeated-measures platform, 2-factor repeated-measures ANOVA tested for group differences in changes in body composition parameters across 6 mo of intervention. This analytic approach also tested whether the changes in adipose tissue outcome measures differed between groups. If data were not normally distributed, the data were log transformed. Post hoc analyses were conducted to test whether EPA and DHA concentrations in plasma and subcutaneous abdominal adipose tissue in response to intervention explained variation in outcome measurements of adipose tissue lipolysis insulin sensitivity and inflammatory markers postintervention. Analyses were run with JMP 10.0.0 (SAS Institute), and significance was set at $P \leq 0.05$. Values are expressed as means ± SDs.

RESULTS

Subject characteristics

Twenty-seven of the 31 participants enrolled in the study and provided written, informed consent to undergo the lipolysis measures and adipose biopsies in addition to the methods of the main study (26). Six participants completed measurements at baseline but did not return for the second studies; therefore, their data were not included in this analysis. The 12 participants randomly assigned to the ω-3 group and 9 participants randomly assigned to the placebo group who completed the intervention and studies were included in this analysis. **Figure 1** presents these details in a flow diagram. **Table 1** presents subject characteristics. Six participants did not have a complete MRI scan at baseline and/or postintervention and are missing upper-body subcutaneous and visceral fat data. The 2 groups had generally similar characteristics at baseline. At baseline all participants had ≥1 and, on average, 2 metabolic syndrome criteria according to NIH guidelines for diagnosis of metabolic syndrome. The most prevalent risk factors were a large waist circumference (76%; 101 ± 7 cm) and hypertriglyceridemia (48%; 151 ± 68 mg/dL). We found that abdominal subcutaneous adipocyte size was greater in the placebo group than in the ω-3 group at baseline ($P = 0.02$, not adjusted for multiple post hoc comparisons of baseline characteristics). However, adipocyte size was not different between groups postintervention, and the change in size from baseline was also not different between groups (Table 1). Fat-free mass, upper-body subcutaneous fat mass, and visceral fat mass also did not differ across the intervention or between groups. However, BMI (+0.7; $P = 0.03$), percentage of body fat (0.9%; $P = 0.009$), and leg fat mass (0.5 kg; $P = 0.02$) increased for participants in both groups at the end of the intervention, but the changes were not different between groups (Table 1).

Plasma and adipose tissue fatty acid content

Fasting plasma total FFA concentrations did not change in either group ($P = 0.42$); however, the FFA profile changed in a manner consistent with the intervention. The EPA and DHA contributions to plasma FFAs increased dramatically in the ω-3 group and did not change in the placebo group (**Table 2**). The interaction effects in EPA ($P = 0.0002$) and DHA ($P = 0.0004$) reflected the more robust increases in these plasma FFAs in the ω-3 group compared with the minimal change in the placebo group. The percentage of adipose tissue FFAs as EPA ($P < 0.0001$) and DHA ($P < 0.0001$) likewise increased substantially in the ω-3 group but not in the placebo group (Table 2).

Adipose tissue lipolysis and inflammation

Despite the increases in tissue and plasma EPA and DHA content and despite robust statistical power to detect clinically meaningful changes in $IC_{50(palmitate)}$f, the between-group differences in response to the 6-mo intervention were not different (Table 2). Furthermore, there were no improvements and no trends for improvements in adipose tissue markers of inflammation, including senescent cells; total, pro- or anti-inflammatory macrophages; and crown-like structures (Table 2).

DISCUSSION

Metabolic syndrome, specifically systemic insulin resistance, may be a consequence of adipose tissue abnormalities. Failure of insulin to normally inhibit adipose tissue lipolysis can result in elevated FFAs, a known contributor to insulin resistance (4, 37). It has been proposed that proinflammatory cytokines produced by senescent cells and macrophages infiltrating adipose tissue contribute to local and systemic inflammation (38). Therefore, interventions targeting adipose tissue inflammation, such as supplements of the ω-3 polyunsaturated fatty acids EPA and DHA (25, 39), could improve systemic metabolism. However, the insulin-sensitizing effects of ω-3 fatty acids are still in

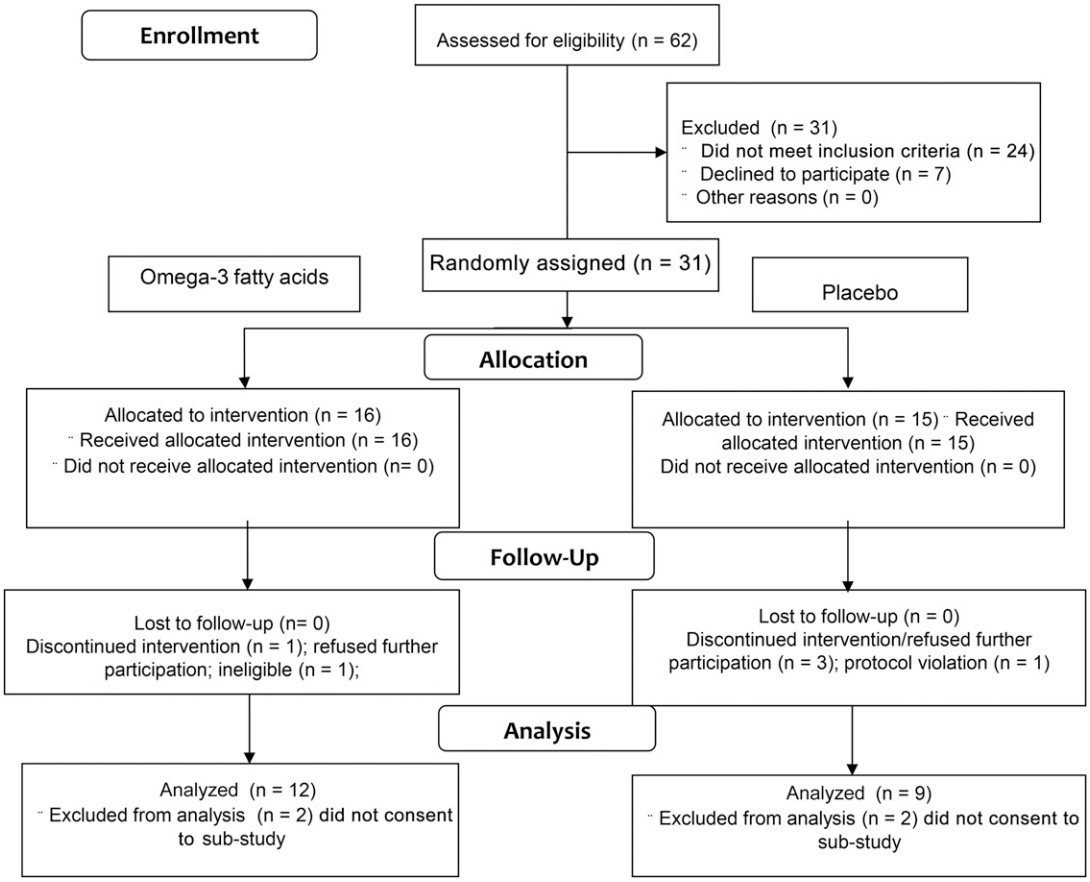

FIGURE 1 Flow diagram of the study participants.

debate. As part of this double-blind, placebo-controlled, 6-mo intervention using maximal FDA-approved doses of EPA and DHA given to insulin-resistant adults, we studied the insulin regulation of adipose tissue lipolysis and adipose inflammation. We found significant increases in EPA and DHA in plasma and subcutaneous adipose tissue in the group supplemented with very-long-chain ω-3 fatty acids but no concurrent changes in adipose tissue insulin sensitivity or inflammation.

TABLE 1
Subject characteristics[1]

	ω-3 (n = 12)		Placebo (n = 9)		P		
	Baseline	Postintervention	Baseline	Postintervention	Group	Time	Interaction
Sex, M/F	4/8	—	2/7	—	—	—	—
Race/ethnicity, % Caucasian	92	—	89	—	—	—	—
Age, y	36 ± 11	—	34 ± 9	—	—	—	—
BMI, kg/m^2	34.4 ± 3.6	35.2 ± 4.4	34.7 ± 5.0	35.4 ± 5.0	0.88	0.03	0.82
Fat-free mass, kg	57.4 ± 9.0	57.7 ± 8.9	55.0 ± 9.2	55.6 ± 9.1	0.55	0.33	0.70
Body fat, %	42.2 ± 7.5	43.2 ± 8.0	42.7 ± 7.0	43.5 ± 7.2	0.91	0.009	0.83
Visceral fat, kg	2.5 ± 0.9[2]	3.1 ± 1.2[3]	3.0 ± 1.4[4]	2.8 ± 1.1[4]	0.92	0.73	0.06
Upper-body subcutaneous fat							
Fat mass, kg	25.7 ± 6.2[2]	26.6 ± 7.6[3]	25.3 ± 9.1[4]	27.6 ± 7.5[4]	0.75	0.16	0.81
Adipocyte size, μg lipid/cell	0.87 ± 0.25	1.00 ± 0.34	1.12 ± 0.18	1.14 ± 0.27	0.06	0.30	0.45
Lower-body fat							
Fat mass, kg	12.8 ± 4.3	13.3 ± 4.9	14.4 ± 4.4	13.7 ± 4.7	0.83	0.02	0.90

[1] Values are means ± SDs, unless otherwise noted, for subject characteristics for both intervention groups supplemented with ω-3 fatty acids or a placebo at baseline and postintervention, along with analysis results from multivariate models with repeated measures.
[2] n = 9.
[3] n = 8.
[4] n = 7.

TABLE 2
Plasma and adipose tissue responses to ω-3 fatty acid or placebo supplements[1]

	ω-3 (n = 12)		Placebo (n = 9)		P		
	Baseline	Postintervention	Baseline	Postintervention	Group	Time	Interaction
Plasma, % of total FFAs							
EPA	0.95 ± 0.22	6.0 ± 0.92	1.2 ± 0.27	1.1 ± 0.19	0.002	0.0006	0.0002
DHA	0.89 ± 0.23	3.5 ± 0.84	1.2 ± 0.39	0.90 ± 0.18	0.12	0.002	0.0004
Adipose tissue, % of total FFAs							
EPA	0.06 ± 0.00	0.19 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.0002	<0.0001	<0.0001
DHA	0.14 ± 0.01	0.28 ± 0.02	0.15 ± 0.01	0.16 ± 0.02	0.03	<0.0001	<0.0001
$IC_{50(palmitate)}f$[2]	19 ± 2	24 ± 3	29 ± 4[3]	27 ± 6[3]	0.17	0.94	0.11
Senescent cells, %	4 ± 1	4 ± 1	4 ± 3	4 ± 2	0.84	0.65	0.68
Macrophages, n/100 adipocytes							
Total (CD68)	31 ± 2	33 ± 2	33 ± 2	31 ± 2	0.97	0.74	0.25
M1 (CD14)	13 ± 2	14 ± 2	13 ± 1	12 ± 2	0.49	0.93	0.49
M2 (CD206)	28 ± 2	29 ± 3	29 ± 2	29 ± 2	0.84	0.78	0.87
Crown-like structures (n/10 images)	1 ± 0	1 ± 0	1 ± 1	1 ± 0	0.33	0.77	0.31

[1] Values are means ± SEs for effects of intervention groups' ω-3 fatty acid or placebo supplement on insulin regulation of lipolysis and inflammation markers in subcutaneous abdominal adipose tissue, along with analysis results from multivariate models with repeated measures. FFA, free fatty acid.

[2] $IC_{50(palmitate)}f$: the insulin concentration (μIU/mL) resulting in a 50% suppression of palmitate FFA flux is a measure of insulin-mediated suppression of adipose tissue lipolysis.

[3] n = 8.

Increased EPA and DHA intake is well known to improve hypertriglyceridemia (25, 26, 40). However, other features of metabolic syndrome, such as insulin sensitivity, do not consistently improve with increased very-long-chain ω-3 intake (25, 41, 42). We previously reported that, although skeletal muscle insulin sensitivity and pancreatic β cell insulin secretion did not change, hepatic insulin sensitivity improved with ω-3 supplements (26). By measuring FFA kinetics before and during the pancreatic clamp, we also could determine insulin sensitivity of adipose tissue lipolysis. Our finding that in vivo regulation of adipose tissue lipolysis by insulin was unchanged helps put into perspective previous work showing no change in gene expression of lipolysis enzymes in adipose tissue after ω-3 supplementation (41). Together, these findings suggest that even an extended duration of high-dose, very-long-chain ω-3 fatty acids will not improve adipose tissue insulin sensitivity with regard to FFA metabolism.

In addition to FFAs, adipose tissue is the major tissue involved in release of adipokines that are linked to inflammation and insulin resistance. DHA and EPA are thought to induce anti-inflammatory actions by reducing proinflammatory cytokines in adipose tissue. Several investigators have reported decreased gene expression of proteins in the proinflammatory pathway (e.g., monocyte chemoattractant protein-1, IL-6) in cultured adipocytes (43) and animal models (44). In humans, however, neither expression of inflammation-related genes in subcutaneous adipose tissue (25, 41) nor circulating markers of inflammation (25, 41) consistently improve with ω-3 fatty acid treatment. However, Spencer et al. (25) found decreases in total macrophage (pro- and anti-inflammatory) and crown-like structure content in subcutaneous adipose tissue after EPA and DHA supplementation. The baseline abdominal adipose tissue EPA and DHA content we observed is consistent with those previously reported in femoral subcutaneous adipose tissue (45), and the changes in adipose EPA and DHA content were in line with those of Spencer et al. (25). Although we confirmed no changes in the circulating inflammatory markers leptin, adiponectin, C-reactive protein, or IL-6 (26), we could not replicate the improvements in adipose tissue inflammatory markers with a higher dose of DHA and EPA supplementation for a longer duration (Table 2). The conflicting results may be due to recruitment criteria; Spencer et al. (25) recruited participants with features of metabolic syndrome and found those with the greatest number of adipose tissue macrophages at baseline had the greatest decrease with treatment. Our participants were also insulin resistant and carried excess weight but may have had less marked metabolic abnormalities and were ~10 y younger.

Our study is limited to the effects of very-long-chain ω-3 supplements on adipose tissue insulin resistance and abdominal subcutaneous adipose tissue inflammation. For practical reasons we did not measure inflammatory responses in visceral fat, a depot known to be inherently more immune-cell infiltrated than subcutaneous fat. Some investigators have reported positive effects of ω-3 fatty acid supplements on visceral fat (46, 47), and others have noted that greater self-reported ω-3 fatty acid intakes are negatively associated with visceral fat and percentage of body fat in healthy children (48). We cannot know whether there might have been a beneficial effect of ω-3 fatty acids on visceral fat inflammation in our participants, but if there was it did not improve inflammatory markers (26) or insulin sensitivity. We previously reported the results with regard to skeletal muscle and liver insulin sensitivity (26), but we did not measure inflammatory markers in these tissues. Proinflammatory macrophage infiltration and/or cytokine production in skeletal muscle and the liver could contribute to systemic insulin resistance and inflammation (49). Effects of ω-3 FFA supplementation may have varied effects on features of metabolic syndrome depending on disease severity. For example, adults with type 2 diabetes (41) and those with ≥3 features of metabolic syndrome (25) are reported to have improvements in adipose tissue health and function, whereas those with 1–2 components of metabolic syndrome, such as the participants in

the current study, do not. To date, there is insufficient evidence from clinical trials to clearly define which stage in the progression of metabolic syndrome might benefit from ω-3 supplement therapies. However, we note that although 33% of US adults have ≥3 features of metabolic syndrome (50), this would indicate that an even larger portion of US adults have ≤2 metabolic syndrome features, and our findings more likely apply to this group.

In summary, high-dose ω-3 supplementation for 6 mo, sufficient to raise plasma and adipose tissue ω-3 FFAs, had no beneficial effects on insulin-mediated suppression of lipolysis or adipose tissue inflammation in insulin-resistant, overweight and obese adults. Our study does not support the hypothesis that very-long-chain ω-3 fatty acids can improve the features of metabolic syndrome via actions on regulation of adipose tissue insulin sensitivity and reducing inflammation, which is thought to be a potential underlying cause of this disorder.

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