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## The Addition of Medium-Chain Triglycerides to a Purified Fish Oil Based Diet Alters Inflammatory Profiles in Mice

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### Abstract

**Objective**—Parenteral nutrition associated liver disease (PNALD) is a deadly complication of long term parenteral nutrition (PN) use in infants. Fish oil-based lipid emulsion has been shown in recent years to effectively treat PNALD. Alternative fat sources free of essential fatty acids have recently been investigated for health benefits related to decreased inflammatory response. We hypothesized that the addition of medium-chain triglycerides (MCT) to a purified fish oil-based diet would decrease the response to inflammatory challenge in mice, while allowing for sufficient growth and development.

**Materials/Methods**—Six groups of ten adult male C57/B16 mice were pair-fed different dietary treatments for a period of twelve weeks, varying only in fat source (percent calories by weight): 10.84% soybean oil (SOY), 10% coconut oil (HCO), 10% medium-chain triglycerides (MCT), 3% purified fish oil (PFO), 3% purified fish oil with 3% medium-chain triglycerides (50:50 MCT:PFO) and 3% purified fish oil with 7.59% medium-chain triglycerides (70:30 MCT:PFO). An endotoxin challenge was administered to half of the animals in each group at the completion of dietary treatment.

**Results**—All groups demonstrated normal growth throughout the study period. Groups fed MCT and HCO diets demonstrated biochemical essential fatty acid deficiency and decreased IL-6 and

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**Author contributions:** SC, BB, KG, and MP designed the study. SC, AO, PN, VN, MC and EC performed the animal study and tissue preparation / analysis. PM performed statistical analyses. All authors interpreted the data. SC prepared the manuscript. All authors reviewed and approved the final manuscript.

TNF- $\alpha$  response to endotoxin challenge. Groups containing PFO had increased inflammatory response to endotoxin challenge, and the addition of MCT to PFO mitigated this inflammatory response.

**Conclusion**—These results suggest that the addition of MCT to PFO formulations may decrease the host response to inflammatory challenge, which may pose potential for optimized PN formulations. Inclusion of MCT in lipid emulsions given with PN formulations may be of use in therapeutic interventions for disease states resulting from chronic inflammation.

### Keywords

medium-chain triglycerides; fish oil; parenteral nutrition; inflammation

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### Introduction

Essential fatty acids (EFAs), necessary for growth, development, and a variety of biological functions, must be consumed in the diet. Historically, alpha linolenic acid (ALA, n-3 PUFA) and linoleic acid (LA, n-6 PUFA) have been considered the two essential fatty acids. However, recent studies have shown that the downstream metabolic products of ALA and LA, docosahexaenoic acid (DHA) and arachidonic acid (ARA), respectively, are sufficient to sustain growth, development and reproductive function<sup>1-3</sup>

For individuals dependent on parenteral nutrition (PN), EFAs must be provided intravenously as a lipid emulsion. Commercially available lipid formulations in the United States have been exclusively soybean oil-based and contain high levels of LA and lower levels of ALA. Soybean oil-based lipid emulsions are implicated in the development of parenteral nutrition-associated liver disease (PNALD), a progressive and often lethal complication affecting up to 74% of patients dependent on long-term PN<sup>4-7</sup>.

Alternative lipid sources have been investigated as a potential strategy to prevent liver disease in PN-dependent individuals. A fish oil-based lipid emulsion, provided at 1g/kg/day, has been shown to reverse the progression toward liver failure in patients with PNALD<sup>8-10</sup>. However, PN formulations provided with low doses of lipids necessitate higher calories from carbohydrates (dextrose) in order to meet the daily caloric needs of the PN-dependent individual, a consideration that is especially important in developing infants and children. PN formulations high in dextrose predispose patients to hyperglycemia and increased central venous catheter infections, hepatic steatosis, and glycosuria, complications that can lead to significant morbidity and mortality in an already-fragile population<sup>11-13</sup>.

Recently, attention has been given to non-essential fatty acids, so called “EFA-free” lipids, which may be utilized as additives to lipid emulsions in order to augment the total fat calories provided and decrease the requirement for additional dextrose in PN. Ling *et al.* examined the metabolic effects of combinations of EFAs with hydrogenated coconut oil (HCO), an EFA-free lipid source. Rats fed a diet with HCO as the sole source of calories and then given an endotoxin challenge demonstrated a lower inflammatory response, as measured by serum IL-6 and C-reactive protein, when compared to rats fed HCO supplemented with DHA and ARA<sup>14</sup>. These findings are in keeping with Cook *et al.*, who

also demonstrated a decreased systemic inflammatory response in states of essential fatty acid deficiency in rats<sup>15</sup>.

Medium chain triglycerides (MCT) are an alternative source of EFA-free lipids. Studies have shown benefits of MCT oil in the prevention of alcohol-induced liver injury in animal models by a mechanism that is not fully understood but may be related to decreased inflammation<sup>16–18</sup>. Others have demonstrated that dietary supplementation of MCT reduces the degree of endotoxin-induced intestinal and hepatic injury in rats<sup>19,20</sup>. In 2012, the American Society of Parenteral and Enteral Nutrition identified MCT oil as a potentially beneficial additive to lipid emulsions containing soybean and/or fish oil and recommended further research in identifying an optimal ratio of n-3 fatty acids, n-6 fatty acids, and MCT.<sup>21</sup>

The creation of an optimized lipid formulation, which provides sufficient EFAs to sustain growth and development, while modifying the intensity of inflammation, is of high value to the PN-dependent population. Purified fish oil (PFO) is a rich source of DHA and ARA that meets essential fatty acid requirements in rodent models<sup>2,22</sup> and in humans.<sup>23,24</sup> Herein, we utilize a murine model to study several dietary lipids including soybean oil, PFO, HCO and MCT to determine the effects of these lipid sources on growth, serum EFA levels, liver histology, and inflammatory profiles with and without an endotoxin challenge.

## Methods

### Animals and diets

Adult male C57/Bl6 mice (Jackson Laboratories, Bar Harbor ME) were housed five to a cage and maintained in a climate-controlled facility with a 12:12-hour light-dark cycle for a period of four days of acclimation prior to experimentation. During this period, animals had free access to water and a standard rodent chow diet. Experimental protocols were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. Animals were then assigned by cage to one of the following six dietary treatment groups (10 animals per group) based on fat content by weight: 10.84% soybean oil (SOY), 10% coconut oil (HCO), 10% medium-chain triglycerides (MCT), 3% purified fish oil (PFO), 3% purified fish oil with 3% medium-chain triglycerides (50:50 MCT:PFO) and 3% purified fish oil with 7.59% medium-chain triglycerides (70:30 MCT:PFO). Purified fish oil was derived primarily from sardine oil from which all saturated fat and fats with carbon lengths less than 18 were removed, donated by Pronova BioPharma / BASF (Lysaker, Norway). Medium-chain triglyceride (MCT) oil was purchased from Health and Sport, LLC (Amston, CT). All diets were formulated by or purchased from Dyets, Inc. (Bethlehem, PA) and provided in pelleted form. Equivalent amounts of casein, L-cystine, sucrose, dyetrose (a version of dextrose used by the manufacturer which allows the diet to be easily pelleted), and vitamin and mineral mixtures were included in all diets. Slight variations in corn starch and cellulose were necessary to make the diets as nearly isocaloric as possible. Total kcal/kg was slightly lower in the PFO and 50:50 MCT:PFO diets. Table 1 lists the composition of the six different diets.

## Experimental design

Animals were pair-fed the experimental diets in groups of 5 for a period of 12 weeks. Individual body weight and total cage food intake were recorded every three days for the duration of the study period. Food intake was controlled by the lowest food intake among the six groups at each time to ensure that there were no differences in food intake across the six dietary groups. Blood samples were obtained from each animal prior to initiation of dietary treatment and again at 2, 4, 6, 9, and 12 weeks after diet initiation. At the end of 12 weeks, animals in each dietary group were divided into two subgroups: one group received an intraperitoneal (IP) injection of lipopolysaccharide (LPS) 150 µg in saline ( /µL) and the second group received an IP saline injection of equivalent volume. Four hours after receiving LPS or saline, blood samples were obtained via retro-orbital venous access and animals were euthanized. Livers were immediately harvested and lobes were divided into three sections: one was prepared for H&E staining as described by Lillie<sup>25</sup>, a second for oil red O staining as described by Bancroft<sup>26</sup>, and a third was snap frozen and stored at -80C for later analysis. Liver slides were reviewed by two independent blinded board-certified pathologists for qualitative analysis, and there were no discrepancies in interpretation between the two pathologists.

## Analytical methods

Total lipid from plasma was extracted into chloroform / methanol (2:1) by the method of Folch *et al.*<sup>27</sup> Before the extraction, 30 µL of a 1mg/mL solution of diheptadecanoyl phosphatidylcholine and 30µL of a 1mg/ml solution of triheptadecanoyl glycerol (Nu-Check Prep, Elysian, NY) in chloroform-methanol (1:1, vol/vol) were added as an internal standard to all samples. Plasma phospholipids and triglycerides were separated and transmethylated, and fatty acid methyl ester profiles were acquired by gas chromatography as previously described.<sup>2</sup>

Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels were determined using commercially available ELISA kits from R&D Systems (Minneapolis, MN) and Invitrogen (Camarillo, CA), respectively, according to the manufacturers' instructions. All samples were prepared and analyzed in duplicate.

## Statistical analyses

Results are presented as mean± SEM for each outcome (all fatty acid profiles and inflammatory markers). A generalized linear mixed model was used to assess the statistical significance of differences in mean outcome after adjusting for baseline levels among the different dietary groups over time.<sup>28</sup> The covariance matrix of the parameter estimates was computed using a sandwich estimator, and further adjusted for small sample size by the method of Morel, Bokossa and Neerchal<sup>29</sup>. Significance for all analysis was defined as *P* 0.05. The type I error rate was controlled by limiting within-group comparisons to those specified *a priori*, and only when the diet-by-time interaction was significant. All analysis was performed with GraphPad Prism® v6, La Jolla, CA and SAS® v9, Cary, NC.

## Results

### Food intake and body weight

There were no differences in food intake among the six groups for the duration of the study period. Average food intake per group remained consistent at approximately 3–4 g/mouse/day for 12 weeks. Animals in all dietary groups gained weight throughout the study. Baseline weights ranged from 23 to 24 g, and at the end of 12 weeks final weights ranged from 29 to 31 g (Figure 1). No significant differences in weight were noted among the six groups. No animal demonstrated physical signs of EFA deficiency (EFAD), such as scaly dermatitis, hair loss, or skin atrophy.

### Fatty acid profiles

Animals in both MCT and HCO groups had biochemical EFAD, as defined by triene:tetraene ratios  $>0.2$ , by 2 weeks of dietary treatment, and these ratios continued to significantly increase for both groups over the remaining 10 weeks of treatment (HCO  $P<0.0001$ ; MCT  $P=0.003$ ). None of the groups fed diets containing PFO or SOY demonstrated biochemical EFAD at any point. At 12 weeks, HCO-fed animals showed increased triene:tetraene ratios relative to MCT-fed animals (mean (95% CI) 0.40 (0.17, 0.63)) indicating a slightly greater degree of EFAD in the HCO group (Figure 2).

The increased triene:tetraene ratio in HCO-fed animals is due to higher mead acid (20:3n9) in the HCO group despite lower ARA in the MCT group compared to the HCO group at 12 weeks ( $5.21\pm0.45$  and  $6.57\pm0.78$ , respectively,  $P=0.15$ ) (Figure 3). This slight increase in Mead acid is presumably attributable in part to the presence of substantial stearic acid (18:0), a Mead acid precursor found in HCO and not in MCT oil.

At 2 weeks of dietary treatment, significant differences among treatment groups were observed in plasma levels of various n-6 and n-3 fatty acids. SOY animals had only modest decreases in linoleic acid (LA, 18:2n6) from baseline ( $-2.4\pm2.9$ ;  $P=0.40$ ), compared to decreases on the order of 11% of total nmol in HCO and MCT groups ( $P<0.0001$  for each), and even greater decreases on the order of 19% of total nmol in all groups containing PFO ( $P<0.0001$  for each). Dihomo-gamma-linolenic acid (20:3n6) was similar among SOY, HCO and MCT, and present in very low levels in all groups containing both MCT and PFO. Arachidonic acid (ARA, 20:4n6) decreased by week 2 in all groups containing PFO ( $P<0.001$ ), while unchanged from baseline for both SOY ( $P=0.81$ ) and MCT ( $P=0.60$ ). HCO experienced a modest decrease from baseline ( $P=0.04$ ). No significant changes were observed from baseline to week 2 for alpha linolenic acid (ALA, 18:3n3). Docosahexaenoic acid (DHA, 22:6n3) increased by week 2 to  $9.50\pm1.27\%$  of total nmol in animals on the PFO diet ( $P=0.008$ ) while remaining constant in all other groups. Eicosapentaenoic acid (EPA, 20:5n3) increased in all groups containing PFO ( $P<0.05$ ) while being barely detectable in the non-PFO containing groups (Figure 4).

At 12 weeks, mice fed HCO and MCT still had detectable levels of LA, dihomogammalinolenic acid, and ARA, despite continued treatment with a diet deplete of these essential fatty acids. In fact, ARA levels in HCO and MCT groups were higher than in any PFO containing group. PFO, 50:50 MCT:PFO, and 70:30 MCT:PFO groups demonstrated

low levels of all n-6 fatty acids measured at 12 weeks. ARA was higher in SOY than all other groups at 12 weeks, significantly so when compared to the PFO groups ( $P<0.05$ ). Although DHA and EPA were lower in all non-PFO containing groups, DHA levels were preserved in SOY and MCT, with only a modest decrease from baseline in HCO ( $-1.96\pm 0.93$ ;  $P=0.04$ ), despite the lack of n-3 fatty acids in MCT and HCO and low n-3 source in SOY. Figures 3 and 4 show n-3 and n-6 fatty acid levels, respectively.

### IL-6 and TNF- $\alpha$

Measurement of IL-6 and TNF- $\alpha$  was performed on serum samples from all animals after 12 weeks of dietary treatment to establish a baseline. Baseline levels of IL-6 and TNF- $\alpha$  were found to be just at the detection threshold with no differences between dietary groups. After saline treatment, no differences were seen in either IL-6 or TNF- $\alpha$  from baseline in any dietary group (data not shown). After LPS treatment, IL-6 and TNF- $\alpha$  levels increased significantly in all groups. Animals fed PFO or 50:50 MCT:PFO diets were found to have the highest mean level of IL-6 when compared to all other dietary groups. Animals in the 50:50 MCT:PFO or PFO groups had highest levels of TNF- $\alpha$  relative to the other groups. IL-6 and TNF- $\alpha$  were lowest in MCT, though not significantly different than HCO, 70:30 MCT:PFO or SOY. Figure 5 shows the IL-6 and TNF- $\alpha$  levels, respectively, in all animals treated with LPS.

### Liver analysis

All liver specimens were stained for H&E and oil red O and analyzed. All MCT and HCO livers demonstrated significant peri-portal and mid-zonal micro- and macrosteatosis, representing the increased de novo lipogenesis of essential fatty acid deficiency. SOY livers showed rare to mild mid-zonal microsteatosis to a much lesser degree than MCT or HCO livers. PFO, 50:50 MCT:PFO and 70:30 MCT:PFO livers appeared grossly normal without evidence of hepatosteatosis. Among all groups, livers of animals treated with saline injection had glycogen deposits throughout the tissue, which were notably absent in livers of animals treated with LPS. Administration of LPS was not noted to have an effect on degree of steatosis or presence of inflammatory cells in any group. Representative histological sections are presented in Figure 6.

### Discussion

The incorporation of fish oil into PN formulations has emerged as a viable approach to treat parenteral nutrition-associated liver injury. However, critics of the use of fish oil monotherapy have speculated that fish oil alone may be insufficient to support growth and prevent EFAD. Short-term animal studies have shown appropriate growth and prevention of EFAD after dietary treatment with native fish oil in mice when given as at least 20% of total calories, which supplies sufficient ARA to complement the very limited amount of linoleic acid found in fish oil.<sup>2</sup> Nehra *et al.* demonstrated that animals fed a diet providing DHA and ARA as the sole source of fat in at a 20:1 ratio (similar to that which is found in native fish oil) at 2.1% of total calories is sufficient to sustain reproduction over several generations in mice.<sup>3</sup> To our knowledge, this present study is the first study to demonstrate that dietary

treatment with PFO alone long-term (over 12 weeks) sustains growth and prevents EFAD in mice.

Our study shows an increase in IL-6 and TNF- $\alpha$  following treatment with PFO-based diets and subsequent endotoxin challenge compared to HCO- and soy-based diets. This increased inflammatory response is mitigated by the addition of MCT at 50% of fat calories. These findings are similar to those published by Ling *et al.*, who demonstrated that the addition of DHA and ARA to an HCO-based diet leads to increased IL-6 and C-reactive protein (CRP) after endotoxin administration. However, this is the first study to demonstrate that the inflammatory response is mitigated by the addition of MCT to PFO. The increase in the systemic inflammatory response noted when using either PFO or only two of the active components (DHA and ARA) is unexpected given the multitude of evidence that standard fish oil preparations have been shown to be anti-inflammatory.<sup>30,31</sup> Other studies have shown a decrease in pro-inflammatory markers with fish oil treatment. Endres *et al.* demonstrated that the production of IL-1 and TNF- $\alpha$  are decreased after endotoxin administration in healthy adults whose diet was supplemented with 18g of fish oil for a period of six weeks.<sup>32</sup> It is important to note that previous studies have often utilized native fish oil as a supplement in much smaller amounts than studied by Endres *et al.* and that the supplements are taken with a normal diet containing substantial amounts of other fats.<sup>32–34</sup> In native or unpurified fish oil, EPA and DHA are present at 25–30% of total fatty acids and ARA at approximately 0.5–1% with the remaining majority of fatty acids as saturated fatty acids.<sup>35</sup> A potential mechanism for the changes seen in inflammatory potential in the present study relates to the principal fatty acids that underlie inflammation. ARA is presumed to be pro-inflammatory through its production of 2-series eicosanoids like prostaglandin and prostacyclin and 4 series leukotrienes.<sup>15,36</sup> In contrast, EPA and DHA are anti-inflammatory in part due to the production of 3-series prostanoids and 5-series leukotrienes, and DHLA is presumed to be anti-inflammatory from its production of 1-series prostanoids<sup>37</sup>. In this study, while ARA levels are high and EPA levels low in the SOY group, DHLA levels are also higher in PFO-containing groups compared to those not containing PFO. Moreover a state of essential fatty acid deficiency in itself, as with HCO and MCT, has been shown to lead to increased mortality in humans due to infection, presumably because of the underlying decreased response to endotoxin challenge.<sup>38</sup> In contrast, groups containing PFO have the highest EPA and DHA and lowest ARA, which should severely down regulate inflammation; notably, these groups have essentially no DHLA. Whether the effect of DHLA would be enough to change the degree of inflammation vis-à-vis PFO or whether EPA and DHA alone in these amounts might have opposite effects cannot be determined in this study. Furthermore the increase in systemic inflammatory response also reflects an increased immune response, which, under certain circumstances, could be beneficial as in the correction of essential fatty acid deficiency. Whatever the ultimate explanation, these results should give pause to the potential effects of purifying fish oil without consideration of the potential fatty acid interactions that this might entail. At least provisionally the dilution of PFO by 50% MCT seems to provide the optimal liver histology with the best inflammatory profile in response to endotoxin and would clinically allow the provision of substantial fat calories to reduce the carbohydrate burden.

In conclusion, we have shown that long-term treatment with PFO as the sole source of fat calories sustains growth and prevents EFAD in a murine model. In addition, these results suggest that the addition of MCT to PFO formulations may decrease the host response to inflammatory challenge, though further investigation is warranted. Inclusion of MCT in lipid emulsions given with PN formulations may be of use in therapeutic interventions for disease states resulting from chronic inflammation.

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## Abbreviations

<b>EFA</b>	essential fatty acid
<b>PUFA</b>	polyunsaturated fatty acid
<b>ALA</b>	alpha linolenic acid
<b>LA</b>	linoleic acid
<b>DHA</b>	docosahexaenoic acid
<b>ARA</b>	arachidonic acid
<b>PN</b>	parenteral nutrition
<b>PNALD</b>	parenteral nutrition associated liver disease
<b>HCO</b>	hydrogenated coconut oil
<b>MCT</b>	medium-chain tryglycerides
<b>PFO</b>	purified fish oil
<b>IP</b>	intraperitoneal
<b>LPS</b>	lipopolysaccharide
<b>IL-6</b>	interleukin 6
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor alpha
<b>EFAD</b>	essential fatty acid deficiency

**DHLA** dihomogamma-linolenic acid  
**CRP** C-reactive protein

## Animal Weight Before and After Dietary Treatment

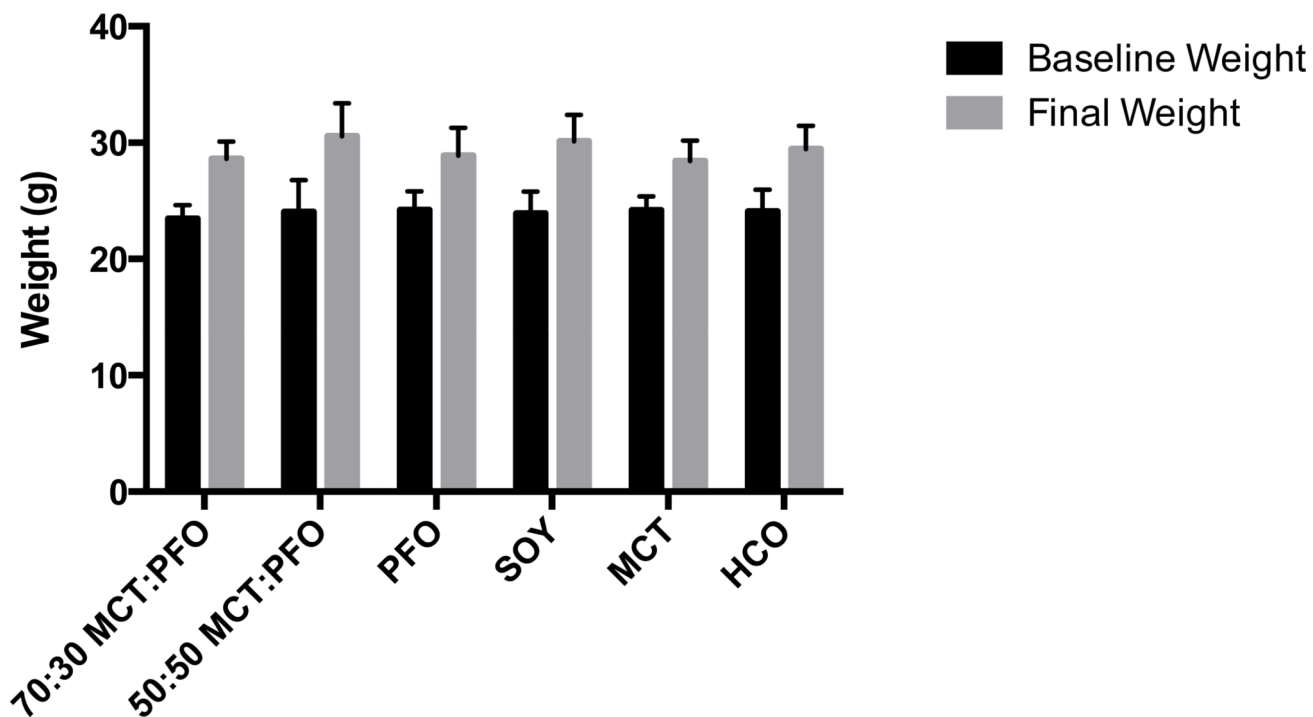
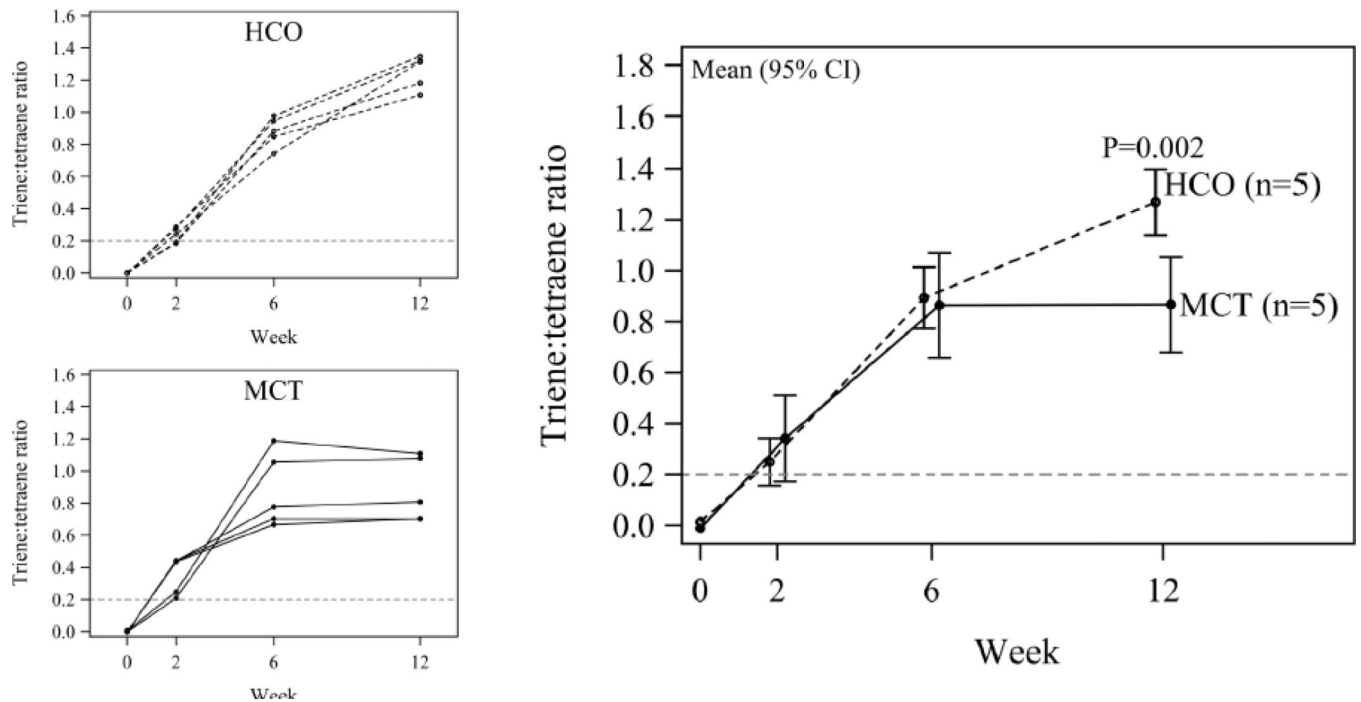


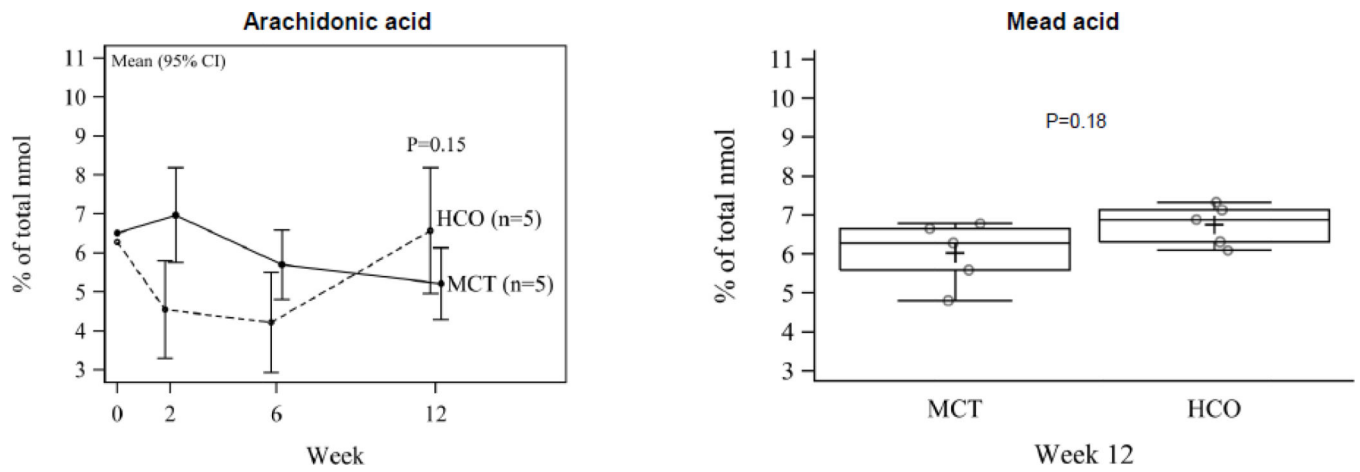
Figure 1.

Animal weight by treatment group. All groups gained weight during the study period. There were no significant weight differences between treatment groups at the beginning or the end of treatment.

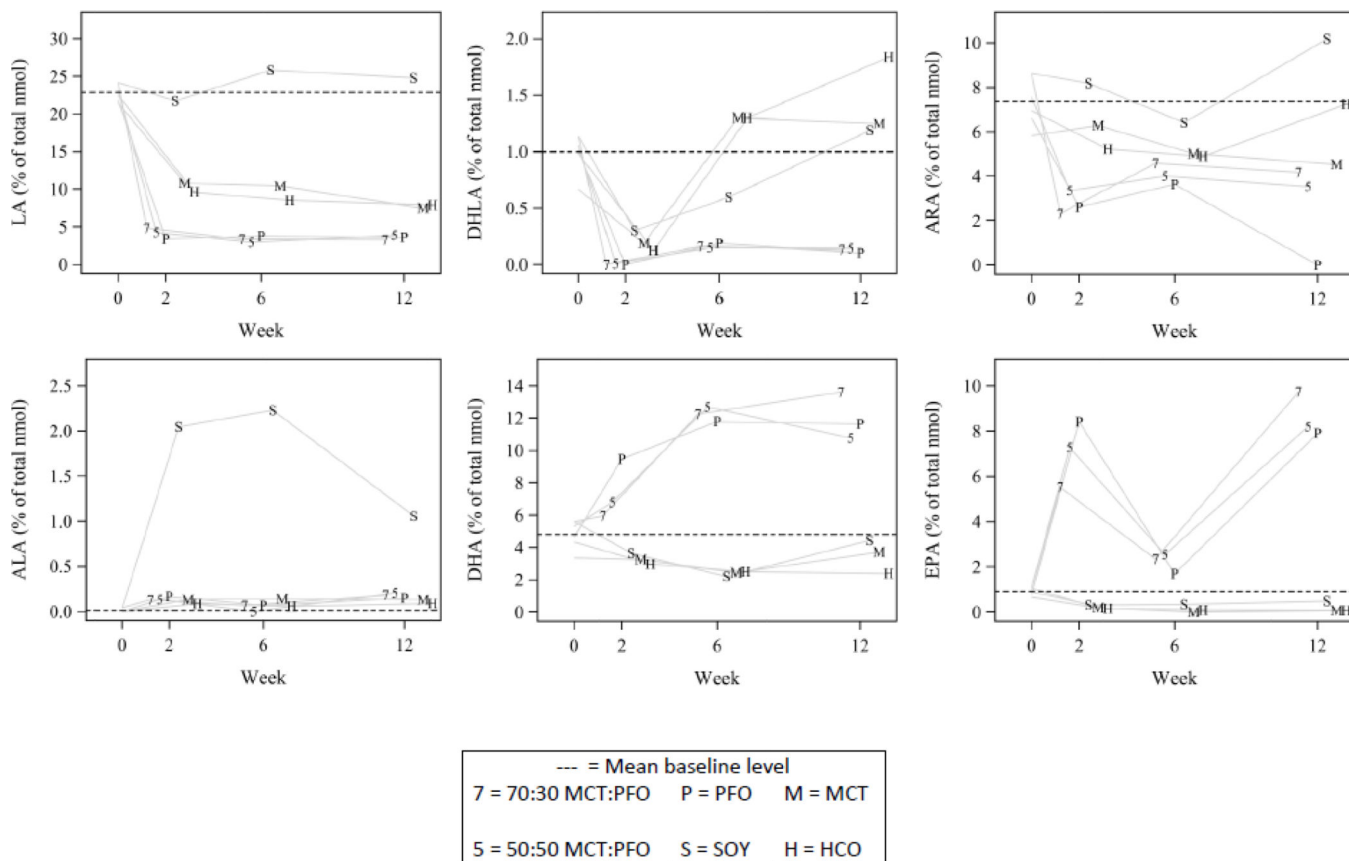


**Figure 2.**

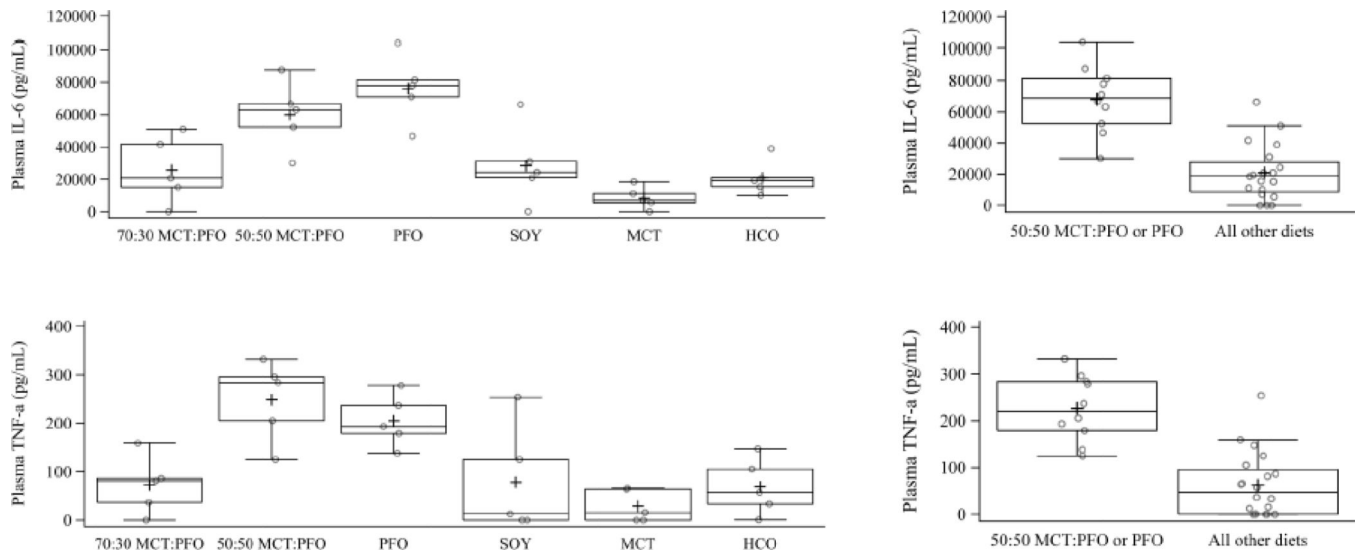
Triene:tetraene ratio at weeks 0, 2, 6 and 12. Shown is the trajectory for each animal over time for HCO and MCT separately (left), as well as aggregated by diet (right; mean and 95% confidence interval from a generalized linear model). The week-by-diet interaction is statistically significant ( $P=0.003$ ). At 12 weeks, HCO-fed animals showed increased triene:tetraene ratios relative to MCT-fed animals, indicating a slightly greater degree of EFAD in the HCO group.



**Figure 3.** Arachidonic acid over time (mean, 95% CI) and mead acid at week 12 (median, IQR). ARA is lower and mead acid is higher in the MCT group compared to the HCO group at 12 weeks.



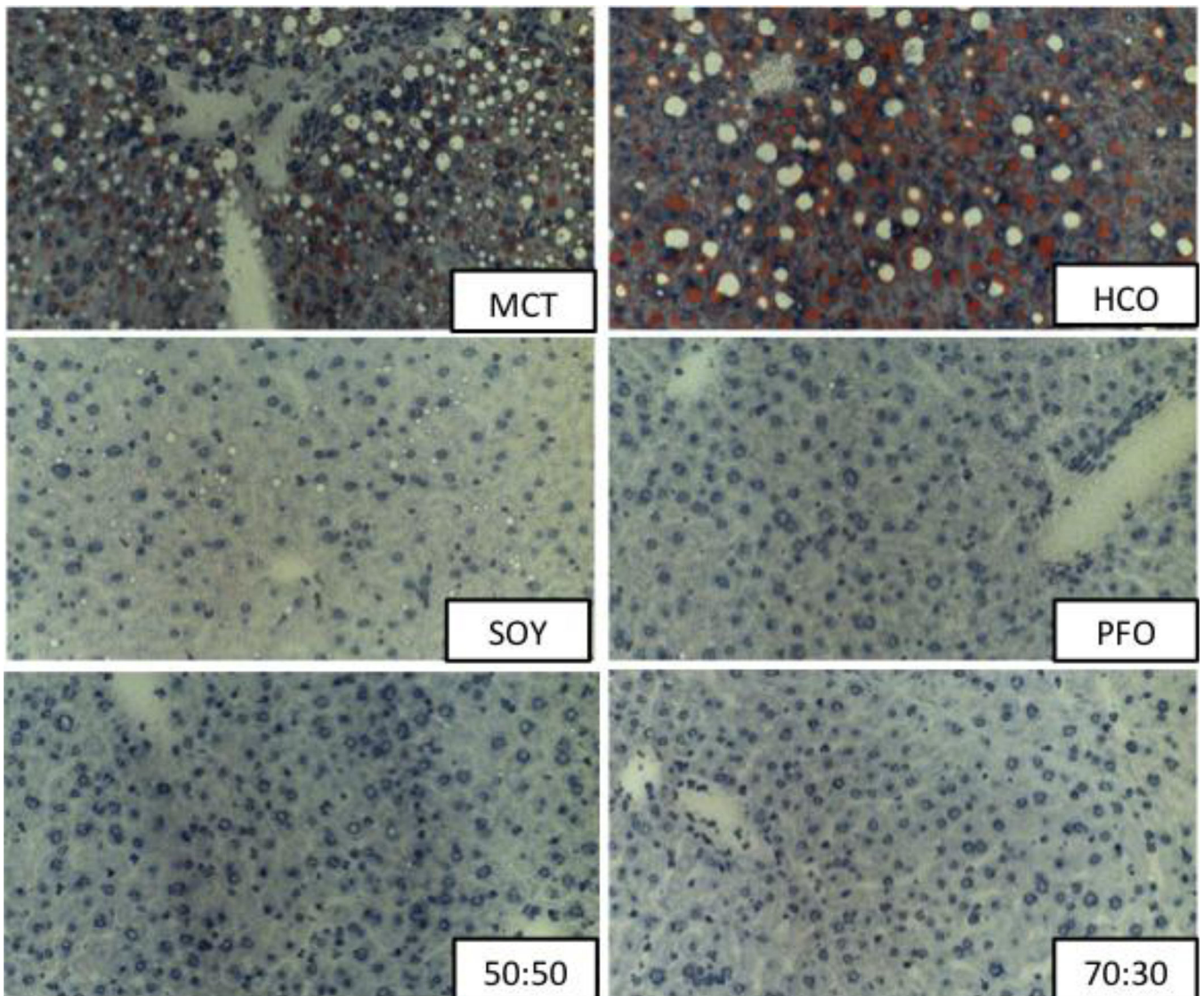
**Figure 4.** Omega-3 and omega-6 fatty acid concentrations. Shown are unadjusted means at weeks 0, 2, 6 and 12. The horizontal dashed reference line indicates the baseline mean. Data are shifted left or right of the week label to spread the data to enhance readability



**Figure 5.**

IL-6 and TNF- $\alpha$  at week 12. Diets 70:30 MCT:PFO, soy, MCT, and HCO are not different from one another (IL-6  $P=0.30$ ; TNF- $\alpha$   $P=0.69$ ). The 50:50 MCT:PFO and PFO diets are higher in both IL-6 and TNF- $\alpha$  than all other diets combined ( $P<0.0001$  for each). Results are based on analysis of variance.





**Figure 6.**

Liver samples stained for Oil Red O. All MCT and HCO livers demonstrated significant peri-portal and mid-zonal micro- and macrosteatosis. SOY livers showed rare to mild mid-zonal microsteatosis. PFO, 50:50 MCT:PFO and 70:30 MCT:PFO livers appeared normal without evidence of hepatosteatosis.

Table 1

Dietary compositions (grams per kg)

	MCT	HCO	SOY	PFO	50:50 MCT:PFO	70:30 MCT:PFO
Casein	140	140	140	140	140	140
L-Cystine	1.8	1.8	1.8	1.8	1.8	1.8
Sucrose	100	100	100	100	100	100
Cornstarch	405.68	405.68	405.68	481.58	451.58	405.68
Dyetrose	155	155	155	155	155	155
Medium Chain Triglycerides	108.4	0	0	0	30	75.9
Purified Fish Oil	0	0	0	30	30	30
Soybean Oil	0	0	100	0	0	0
Hydrogenated Coconut Oil	0	100	0	0	0	0
t-Butylhydroquinone	0.02	0.02	0.02	0.02	0.02	0.02
Cellulose	41.6	50	50	44.1	44.1	44.1
Mineral Mix #210050	35	35	35	35	35	35
Vitamin Mix #310025	10	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Total kcal per kg	3925.7	3925.9	3925.9	3569.2	3710.2	3925.9

Dietary compositions (grams per kg). Equivalent amounts of casein, L-cystine, sucrose, dyetrose (a version of dextrose used by the manufacturer which allows the diet to be easily pelleted), and vitamin and mineral mixtures were included in all diets. Slight variations in corn starch and cellulose were necessary to make the diets as nearly isocaloric as possible. Total kcal/kg was slightly lower in the PFO and 50:50 MCT:PFO diets.