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Refined mackerel oil increases hepatic lipid accumulation and reduces choline and choline-containing metabolites in the liver tissue in mice fed a Western diet

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ABSTRACT

In this study, we aimed to evaluate the impact of consuming refined mackerel oil (MO) from rest raw material on hepatic fat accumulation, glucose tolerance, and metabolomic changes in the liver from male C57BL/6N mice. The mice were fed either a Western diet (WD) or a chow diet, with 30 g or 60 g MO per kg of diet (3% or 6%) for 13 weeks. Body weight, energy intake, and feed efficiency were monitored throughout the experiment. A glucose tolerance test was conducted after 11 weeks, and metabolomic analyses of the liver were performed at termination.

Inclusion of MO in the WD, but not in the chow diet, led to increased liver weight, hepatic lipid accumulation, elevated fasting blood glucose, reduced glucose tolerance, and insulin sensitivity. Hepatic levels of eicosapentaenoic and docosahexaenoic acid increased, but no changes in levels of saturated and monounsaturated fatty acids were observed. The liver metabolomic profile was different between mice fed a WD with or without MO, with a reduction in choline ether lipids, phosphatidylcholines, and sphingomyelins in mice fed MO.

This study demonstrates that supplementing the WD, but not the chow diet, with refined MO accelerates accumulation of hepatic fat droplets and negatively affects blood glucose regulation. The detrimental effects of supplementing a WD with MO were accompanied by increased fat digestibility and overall energy intake, and lower levels of choline and choline-containing metabolites in liver tissue.

1. Introduction

Seafood is the main dietary source of long-chained omega-3 (LC n-3) polyunsaturated fatty acids (PUFAs), and the beneficial health effects of fatty fish intake have largely been attributed to these. With a worldwide growing population and increased pressure on wild fish stocks and marine ingredients, optimized utilization of current LC n-3 sources is needed. Improved utilization of fishery by-products offers the potential for increased sustainability of fisheries and marine food production. The fisheries have the potential to better utilize waste like skin, viscera, and muscle tissue that contain LC n-3 fatty acids, which present a potential source for quality fish oil for human consumption (Zuta et al., 2003) or fish feed additives. Therefore, studies are needed to determine the nutrient and contaminant content of refined fish oil obtained from processing waste and evaluate the potential health effects and risks for consumers. Additionally, the metabolic impact of dietary intake of refined fish oil from processing waste should be examined.

The health effects of omega-3 (n-3) fatty acids in humans have been studied for more than three decades, and the results demonstrate that they are required for healthy growth and development. The intake of seafood is recommended in European and American dietary guidelines, mainly because of the LC n-3 FAs eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA). Several studies have evaluated the dietary intake of PUFAs across different populations in Europe. A systematic review by Sioen et al. (Sioen et al., 2017) reported that the intake of n-3 and n-6 PUFAs among specific population groups in European countries was suboptimal. On average, only 26% of the countries had a mean EPA and DHA intake that met the recommendations from the European Food Safety Authority.

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease globally, with increasing prevalence worldwide. Consumption of n-3 PUFAs has a beneficial effect in preventing and reversing NAFLD in

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both human and experimental animals (Bouzianas et al., 2013; Lee et al., 2020; Valenzuela et al., 2020). Studies in mice investigating the effects of EPA and DHA have reported a reduction in the pathological features of atherogenic high-fat-induced NAFLD as early as after four weeks. EPA has a triacylglycerol (TAG)-reducing effect, whereas DHA has a more suppressive effect on hepatic inflammation and generation of reactive oxygen species (ROS) (Froyland et al., 1997; Rubio-Rodriguez et al., 2010). Fish oil contains high amounts of n-3 PUFAs, which have been reported to suppress hepatosteatosis and fibrosis. Dietary intake of n-3 PUFAs affects hepatic lipid homeostasis by modulating both lipid synthesis and fatty acid β-oxidation and affects gene expression via peroxisome proliferator activated receptor α (PPAR-α), sterol regulatory element-binding protein-1C (SREBP-1c) and carbohydrate responsive element-binding protein (ChREBP) (Kasbi Chadli et al., 2012; Kim et al., 1999), a glucose-responsive transcription factor that is linked to insulin resistance (IR) and hepatic steatosis. Studies have also shown that n-3 PUFAs are involved in controlling glucose homeostasis and affect the development of IR (Kalupahana et al., 2010; Pavlisova et al., 2020; Tapia et al., 2014). Although numerous studies have reported that n-3 fatty acids from fish oil and omega-3 supplements can prevent and possibly reverse the development of NAFLD, there are also studies demonstrating no effects of n-3 PUFAs and even conflicting results where n-3 PUFAs were found to exacerbate hepatic steatosis (Bernhard et al., 2016; Provenzano et al., 2014b; Ruzzin et al., 2010; Shefer-Weinberg et al., 2017; Yamazaki et al., 2007).

This study aims to investigate the health effects and impact on liver metabolomic profile in male C57BL/6N mice following 13 weeks of dietary intake of refined mackerel oil (MO) from rest raw material from processing waste. Mice were fed two doses of refined MO, 3% and 6%, in a Western diet (WD) or a chow diet for 13 weeks. Phenotypic parameters, including body weight, liver weight, and fat mass, were quantified in addition to energy intake, fat digestibility, fatty acid composition in the liver, glucose metabolism, and liver metabolomic profile.

2. Materials and methods

2.1. Experimental design and diets

The animal experiment was approved by the Norwegian Animal Research Authority (FOTS ID number 15230). The experimental protocols and animal handling were performed following the European Convention for the Protection of Animals used for scientific purposes and national guidelines. Charles River wildtype C57BL/6N male mice were acclimated for one week to standard temperature conditions (20–22 °C) with a 12 h light and dark cycle. Sixty mice were assigned to six groups (n = 10/group) and fed experimental diets for 13 weeks. The experimental diets were a chow diet, chow diet with 30 g/kg refined MO (Chow 3% MO), chow diet with 60 g/kg refined MO (Chow 6% MO) (Table A.1), and a WD, WD with 30 g/kg refined MO (WD 3% MO) and WD with 60 g/kg refined MO (WD 6% MO) (Table 1). The chow diet was a "semi-purified diet", which is readily available on the commercial market. The diets were provided in pellet form and prepared by Ssniff Spezialdiäten GmbH (Soest, Germany). The mice were housed individually, and their body weights were recorded once per week and fresh feed was provided three times per week during the experimental period. Body composition measurements were performed by non-invasive scanning (Bruker Minispec LF50 Body Composition Analyzer mq7.5; Bruker Optic GmbH, Germany), as described earlier (Halldorsdottir et al., 2009). Mice were sacrificed under isoflurane anesthesia (Isoba vet, Denmark). Blood was collected from cardiac puncture into EDTA-coated tubes. Red blood cells and plasma were separated by centrifugation and stored at −80 °C for further analyses. Organs were dissected out, weighed, and snap frozen in liquid nitrogen. Liver tissue for histology was fixated in 4% formaldehyde and processed as described earlier (Bernhard et al., 2016). Over the course of the experiment, it became necessary to euthanize three mice due to hair loss and skin issues. Adhering to the principles outlined in the animal care guidelines, their body weight had exhibited a reduction that exceeded the predetermined limits, indicating a decline in their overall well-being.

Table 1
Diet composition for western diets (WD) used in the experiment.

Component (g/kg diet)	WD	WD 3% MO	WD 6% MO
Fat	*200*	*200*	*200*
Soybean oil	13	13	13
Corn oil	7	7	7
Vegetable shortening	60	60	60
Milk fat	60	60	60
Lard	60	30	0
Mackerel oil (MO)	0	30	60
Cholesterol	1.5	1.5	1.5
Protein (casein)	*200*	*200*	*200*
Carbohydrate	*491*	*491*	*491*
Sucrose	80	80	80
Maltodextrin	100	100	100
Corn starch	311	311	311
Fiber[1]	50	50	50
Vitamin and mineral mix[2]	52	52	52
Choline Bitartrate	2.5	2.5	2.5
L-Cystine	3	3	3
Energy (kcal/100 g)*	470	470	470

* Analyzed values, energy content shown in kcal/100 g feed.
[1] All diets were added 25 g/kg inulin and 25 g/kg cellulose.
[2] All diets were added 40 g/kg Mineral mix: SDS, AIN93 G mineral mix and 12 g/kg Vitamin mix: SDS, AIN93VX NCR95 compliant.

2.2. Refined MO

The refined MO from processing waste used in the experimental diets was obtained as a gift from Epax AS (Ålesund, Norway) and stored in a cool, dry place until analysis. The oil was then analyzed for the presence of persistent organic pollutants (POPs) (Table A.2), oxidation parameters (Table A.3), fat-soluble vitamins (Table A.4), and fatty acid composition (Table A.5).

Analyses of the POPs were performed as described earlier (Berntssen et al., 2010). The concentrations of dioxins (PCDDs), furans (PCDFs), the sum of dioxins and furans (PCDD/Fs), non-ortho PCBs, mono-ortho PCBs, sum of dioxin-like (dl)-PCBs, and the sum of PCDD/Fs + dl-PCBs were determined using an upper bound approach (Table A.2).

Oxidation parameters and sensory analysis were performed by Møreforskning AS (Ålesund, Norway). Free fatty acids (FFAs), peroxide value (PV), anisidine value (AV), and sensory analysis were performed on the refined MO sample. FFA content was determined using the titration method. PV and AV were measured using standard procedures. Sensory analysis was performed by a trained panel of individuals who evaluated the oil for any off-flavors or odors. The concentrations of fat-soluble vitamins (vitamin D, α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, vitamin A1, and A2) were determined using high-performance liquid chromatography with diode array detection.

2.3. Energy intake, feed efficiency, and fat digestibility

All mice had *ad libitum* access to feed during the experiment. The feed intake was monitored throughout the experiment, and total energy intake and feed efficiency were calculated. In week three of the experiment, fecal fat content was measured in feces collected from cages during that week.

2.4. Glucose tolerance test (GTT)

An oral glucose tolerance test was performed on all animals after 11 weeks on the experimental diets. After 6 h of fasting, all mice received 3 mg glucose per gram of lean body mass by oral gavage. Blood glucose was measured before and 15-, 30-, 60-, and 120-minutes following

glucose administration. Glucose levels were measured in blood from the tail vein using a glucometer (Ascensia Contour, Norway). Blood samples were collected at baseline and 15- and 120 min after the administration of glucose, and insulin levels were quantified in plasma with the EIA-3439 kit, as described in the manufacturer's instructions (DRG Diagnostics GmbH).

2.5. Fatty acids in liver tissue and histology examination

The quantification of fatty acids in the refined MO (Table A.5), experimental diets, and liver tissues was performed as described earlier (Liisberg et al., 2016). The known and unknown fatty acids analyzed were summarized and represented total fat content. Paraffin-embedded liver sections were stained with hematoxylin and eosin (H&E), and micrographs were taken using a NanoZoomer S60 digital slide scanner (Hamamatsu, Japan). Representative micrographs were taken at 20x enlargement using NDP view software.

2.6. Total lipid analysis

Total lipids were extracted from the refined MO and the experimental diets with chloroform: methanol, 2:1 (v/v). After filtration and centrifugation, methyl esters were prepared by boron trifluoride (12% BF3 in methanol) and separated on a Thermo Finnegan Trace 2000 GC (Chrompak Ltd., Middelburg, Netherlands), as described earlier (Fjaere et al., 2014).

2.7. Liver metabolomic profiling and metabolomics data analyses

A global untargeted metabolite profile was determined in individual liver samples of 5–8 randomly assigned mice from each experimental group, to identify the possible effects of refined MO on the liver metabolism. We conducted analyses on the fatty acid composition and metabolomic data using samples collected from 10 mice that were assigned to the control diets – 5 from the chow group and 5 from the WD group. Additionally, we analyzed 32 samples from mice exposed to varying levels of refined mackerel oil, with 16 samples taken from both the chow and WD groups, and at two different concentrations: 3% and 6% MO. The liver samples analyzed originate from the same experiment that produced the physiological data presented. The untargeted metabolomic analysis was performed by Biocrates metabolomics health (Innsbruck, Austria). Extracts were separated into non-polar and polar fractions and prepared for GC–MS and LC-MS/MS analyses after protein precipitation. The MxP global profiling data was normalized against the median in a pool of reference samples analyzed in parallel through the entire analytical process to compensate for inter-and intra-instrumental variation. All data from the liver metabolomic profiling were processed and analyzed using Qlucore Omics Explorer 3.5 (Qlucore AB, Lund, Sweden).

2.8. Statistical analyses

All results are expressed as mean ± SEM. Normal distribution and homogeneity of variance were evaluated in all data before applicable statistics were performed. Dixon's Q-test was performed on all data to screen for outliers. All data related to WD and chow fed mice were analyzed separately using one-way ANOVA, followed by a post-hoc Fisher's least significant difference (LSD) test. Statistical significances between mice fed the WDs are denoted as #, $p < 0.05$ comparing 3% or 6% MO with WD; * $p < 0.05$ comparing 3% MO with 6% MO. Figures and statistical analyses were performed using GraphPad Prism v6 (GraphPad Software Inc.).

3. Results

3.1. C57BL/6N mice fed a WD with refined MO increases body weight, energy intake, and fat digestibility

Mice were fed a WD or a modified WD with refined MO from processing waste (Table 1) for 13 weeks. Compared to the WD-fed mice, the mice fed a WD with 3% MO and 6% MO inclusion had a significantly higher body weight after 13 weeks on the experimental diets (Fig. 1A). The weight gain in WD 6% MO-fed mice was accompanied by an increased accumulated energy intake compared to mice fed the WD (Fig. 1B). A tendency towards higher feed efficiency was observed in mice fed 3% MO compared to mice fed the WD (Fig. 1C). In week three of the experiment, mice that were given 3% and 6% of refined MO had a decrease in total fecal fat content (Fig. 1D). This decrease may contribute to the observed differences in body weight gain between mice on different diets. It is worth noting that there were no differences in the total amount of feces during the week of collection.

3.2. Refined MO in a WD does not affect total fat mass but increases liver weight and hepatic lipid accumulation

Body composition was measured after 10 weeks on the experimental diets. Despite an increase in body weight, no significant increase in fat mass was observed in mice fed 3% and 6% refined MO compared to WD measured by non-invasive scanning (Fig. 2A). Although no difference in total fat mass was observed, a lower epididymal white adipose tissue (eWAT) weight was recorded in mice fed 3% of MO at the termination of the study. No differences were observed in inguinal white adipose tissue (iWAT) weight (Fig. 2B–C), but 3% and 6 % refined MO increased liver weight (Fig. 2D). The H&E-stained liver sections revealed presence of larger lipid droplets in mice fed 3% and 6% refined MO compared to mice fed the WD (Fig. 2E), and fatty acid measurement confirmed that the amount of fatty acids was increased in both groups fed WD with MO (Fig. 2F).

3.3. Refined MO increases fasted blood glucose and reduces glucose tolerance

Increased liver fat and altered glucose homeostasis are closely connected. The glucose homeostasis was evaluated by performing a glucose tolerance test (GTT). After eleven weeks on experimental diets, blood glucose levels were measured in animals feed-deprived for six hours. Higher fasted blood glucose levels were observed in mice given 3% and 6% of MO in a WD (Fig. 3A). Further, blood glucose levels were significantly higher 30-, 60-, and 120 min after the glucose challenge (Fig. 3B) in mice fed a WD with MO, leading to an increased area under the curve and demonstrating that MO in the diet reduces glucose tolerance (Fig. 3C). Plasma insulin levels were higher in 6-h feed-deprived animals and 120 min after glucose challenge in mice fed MO (3% and 6%) compared to mice fed the standard WD (Fig. 3D-E).

3.4. The intake of MO increases PUFAs and n-3 fatty acid levels in the liver, but does not affect the saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs)

The n-3 PUFAs EPA and DHA are reported to improve body weight development, NAFLD, and insulin sensitivity in several animal studies. No significant changes in the levels of liver SFAs and MUFAs in mice fed MO were observed (Fig. 4A and B), but the inclusion of refined MO in the diet increased the levels of PUFAs (Fig. 4C). The levels of n-6 PUFAs did not differ between the groups, but as expected, increased levels of n-3 PUFAs were observed (Fig. 4D-E). The deposition levels of EPA and DHA were of interest, as the experimental diets contained relatively low amounts of fish oil in comparison to earlier animal experiments, where fish oil largely replaced dietary oils in the experimental diets. Significant

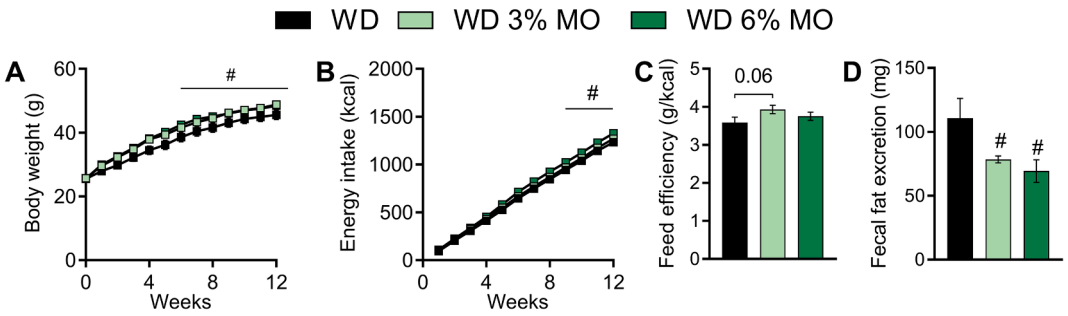

Fig. 1. Effect of refined MO on body weight, energy intake, feed efficiency, and fecal fat content. (a) Body weight development during 13 weeks of feeding. (b) Total energy intake during the feeding experiment was calculated from the amount of feed eaten during the experiment. (c) Feed efficiency was calculated based on body weight gain and total energy intake. (d) The total fecal fat excretion in feces collected during the third week of the experiment. All results are presented as mean ± SEM (n = 8–10), and statistical significances are denoted with symbols as follow: # $p < 0.05$ compared to WD; * $p < 0.05$ comparing WD 3% MO to WD 6% MO.

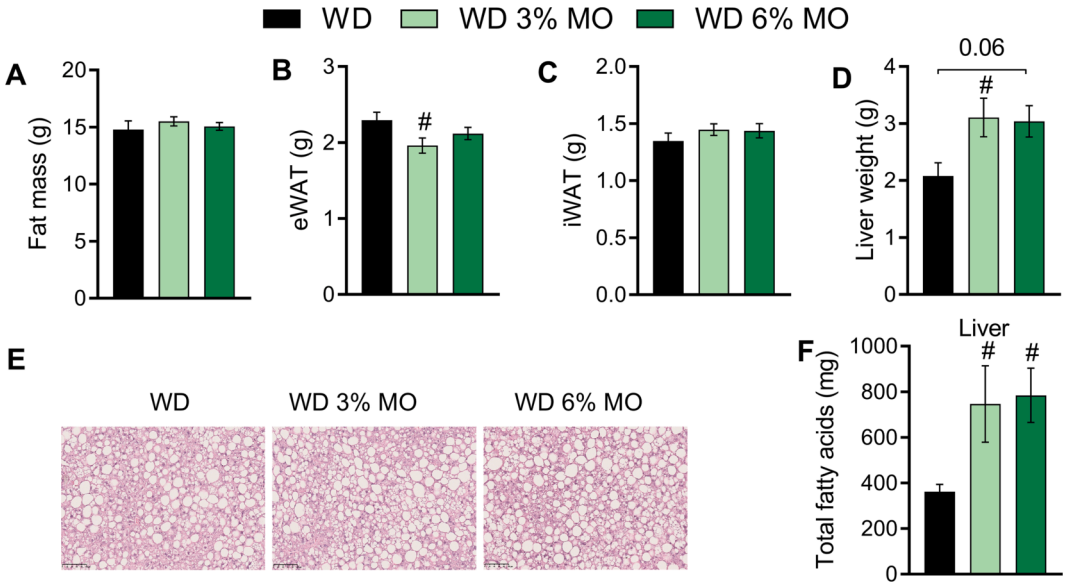

Fig. 2. Effects of refined MO on fat mass, liver weight, and liver morphology. (a) Total fat mass evaluated by MRI scan after 10 weeks on the experimental diet, (b) eWAT weight, (c) iWAT weight, (d) liver weight, (e) representative micrographs of liver tissue and (f) total fatty acid content in the liver after the 13-week intervention period. All results are presented as mean ± SEM (n = 8–10), and statistical significances are denoted with symbols as follow: # $p < 0.05$ compared to WD; * $p < 0.05$ comparing WD 3% MO to WD 6% MO.

higher levels of EPA and DHA were seen in the liver after 13 weeks, and mice fed the highest level of MO (6% MO) had almost twice the levels of n-3 fatty acids as mice fed the lower dose (3% MO) (Fig. 4E). Consequently, the n-3/n-6 ratio in liver tissue increased with the addition of MO in the WD (Fig. 4F).

3.5. Liver metabolites in mice fed WD spiked with MO revealed lower levels of choline ether lipids, phosphatidylcholines, and sphingomyelins compared to mice fed WD

Mice fed 3% and 6% MO in a WD had a distinct metabolic profile in the liver, and the PCA plot including all metabolites revealed a separation (across PC2) compared to mice fed a WD without MO (Figure A.1). Ninety-seven metabolites showed significant ($q < 0.05$) differences in abundance levels when comparing all groups, including a cluster of metabolites related to choline ether lipids, phosphatidylcholines, sphingomyelins, phosphatidylinositol (PI), diacylglycerols (DAGs), and TAGs (Fig. 5). Overall, lower levels of choline ether lipids, phosphatidylcholines, and sphingomyelins were observed in the liver tissue when MO was included in the diet, and the lowest levels were observed in mice fed the highest dose of MO (6% MO). In contrast, the levels of several DAGs and long-chained TAGs were significantly higher in the MO-fed mice (Fig. 5).

3.6. Mice fed refined MO in a chow diet does not increase liver weight or accumulation of fatty acids in the liver and does not affect insulin sensitivity

Refined MO exaggerated WD-induced increase in liver weight and fatty acid accumulation in the liver, and therefore, we next investigated whether MO had the same effects in mice fed a diet with a lower fat content. For this purpose, a chow diet was used. The chow diet used had 12% fat, and refined MO was added in the same amount as for the WD, 3% or 6% MO per kg diet (Table A.1). No differences in body weight, energy intake, feed efficiency or fecal fat excretion were observed (Figure A.2 A-D). Further, the masses of total fat, adipose tissue depots and liver were unchanged (Figure A.2 E-H). MO did not lead to increased accumulation of hepatic fat when included in a chow diet, and in line with this measurements of glucose tolerance were unchanged (Figure A.2 I-P) However, the liver metabolic profile in mice fed 3% or 6% MO in a chow diet revealed more than hundred significantly changed metabolites. As observed in WD fed mice, a separation across PC2 in liver metabolites also was observed in mice fed a chow diet supplemented with MO (Figure A.3). The metabolomic profile in the

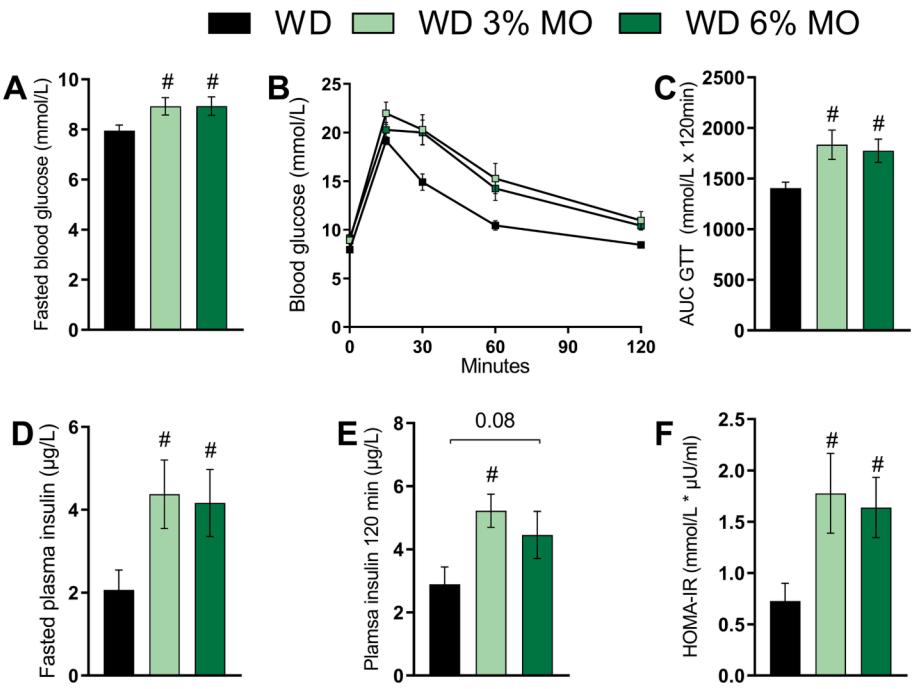

Fig. 3. Blood glucose, plasma insulin, and HOMA-IR during the oral glucose tolerance test (GTT) in mice fed the representative diets for eight weeks. (a) Fasted (6 h) blood glucose levels, (b) the GTT curve with blood glucose measurements before (0) and 15, 30, 60, and 120 min after glucose administration. (c) The area under the GTT curve. (d) Fasted (6 h) plasma insulin levels and (e) plasma insulin levels 120 min after glucose administration. (f) HOMA-IR calculated from fasted plasma insulin levels and blood glucose (6 h). All results are presented as mean ± SEM (n = 8–10), and statistical significances are denoted with symbols as follow: # $p < 0.05$ compared to WD; * $p < 0.05$ comparing WD 3% MO to WD 6% MO.

liver from mice fed refined MO in a chow diet demonstrated lower levels of individual choline ether lipids and sphingomyelins, in accordance with mice fed MO in a WD, but not phosphatidylcholines (Figure A.4).

4. Discussion

Here, we aimed to evaluate the metabolic effect of dietary intake of refined MO and how it affects accumulation of hepatic fat in male C57BL/6JN mice. Further, we evaluated the fatty acid composition and metabolic profile in the liver with increasing doses of MO in the diet.

In this experiment, we demonstrated that dietary intake of 3% or 6% of refined MO in a WD increases body weight, liver weight, and fat accumulation in the liver. Our results contradict earlier studies using LC n-3 PUFAs in both mice and humans (Di Minno et al., 2012; Flachs et al., 2005; Oosting et al., 2010). Unlike earlier studies using other fish oils, we observed that mice fed the highest dose of refined MO had an increased energy intake during the 13-week experiment. Increasing the amount of refined MO in the diet led to reduced fecal fat excretion, and together with higher energy intake in mice fed the highest dose of MO, this could partly explain the increase in body weight in mice fed MO. Although there was no difference in total fat mass, mice fed MO had reduced insulin sensitivity and increased accumulation of fatty acids in the liver.

Although several studies have shown a preventive effect on NAFLD development in animal studies (Bouzianas et al., 2013; Valenzuela et al., 2020), Popescu et al. have demonstrated that fish oil must be combined with a diet with limited amounts of calories, defined as a normocaloric diet by the authors, to reverse NAFLD (Popescu et al., 2013). Earlier studies on C57BL/6J mice have shown a greater hepatic TAG-reducing effect on EPA than on DHA in atherogenic high-fat diet-induced NAFLD (Suzuki-Kemuriyama et al., 2016). Further, EPA are far more efficient than DHA in reducing plasma TAG levels and hepatic lipid droplets in rats (Froyland et al., 1997). The relatively high DHA/EPA ratio in MO may, at least in part, explain the lack of the TAG-reducing effect from MO.

NAFLD includes conditions ranging from nonalcoholic steatohepatitis (NASH) to benign hepatosteatosis. It is hypothesized that NASH follows a multi-hit model, where the first hit involves excessive neutral lipid accumulation, and the second hit is characterized by hepatic insulin resistance, stress, and elevated levels of the hepatic enzyme Alanine Aminotransferase (ALT) (Jump et al., 2016). The glucose tolerance test revealed that mice fed refined MO had reduced glucose tolerance, combined with increasing fasted blood glucose, and elevated fasted insulin levels, compared to mice fed the WD. However, no increase in plasma ALT or aspartate aminotransferase (AST) was observed. Thus, the evidence of n-3 fatty acids' preventive effects on type 2 diabetes is not conclusive. Three extensive review studies concluded that n-3 fatty acids could have a positive effect on blood glucose regulation but none of the studies were conclusive (Flachs et al., 2014; Lombardo & Chicco, 2006; Poudyal et al., 2011), and the effects of n-3 fatty acids on diabetes type 2 vary depending on the doses of n-3 and if participants were diabetic or nondiabetic. Most of the studies included in the mentioned systematic reviews, with a couple of exceptions, lack information about the source of the fish oil or the source of the supplements used in their studies.

The macronutrient composition in the background diet may influence the effects of fish oil. Yamazaki et al. showed that whereas fish oil prevented sucrose-induced fatty liver, safflower oil-induced fatty liver was exacerbated by fish oil (Yamazaki et al., 2007). The potential aggravating effect of fish oil in a safflower oil-induced fatty liver was explained by the increased mRNA expression of the peroxisome proliferator-activated receptor-gamma and CD36 in these mice. In our study, the background diet, and especially the amount of dietary fat content, was shown to be important for the dietary effect of refined MO. When MO was included in a standard chow diet with relative low-fat content, no adverse effects on liver weight, lipid accumulation in the liver, or glucose regulation were observed. The inconsistent effects of including MO in a chow diet and a WD, suggest that the dietary macronutrient composition influences the metabolic effects of MO. A possible interpretation is that MO potentially could amplify a negative effect when the liver is already being excessively challenged, which is suggested in other studies involving other stressors combined with experimental diets containing LC n-3 fatty acids or PUFAs (Nanji, 2004; Provenzano et al., 2014a). A similar pattern was observed in previous studies where n-3 PUFAs aggravated α-HBCD hepatotoxicity in female BALB/c mice (Bernhard et al., 2016), and dietary EPA augmented TAG accumulation in mice with impaired mitochondrial fatty acid oxidation (Du et al., 2013). No certain conclusion regarding the mechanism of

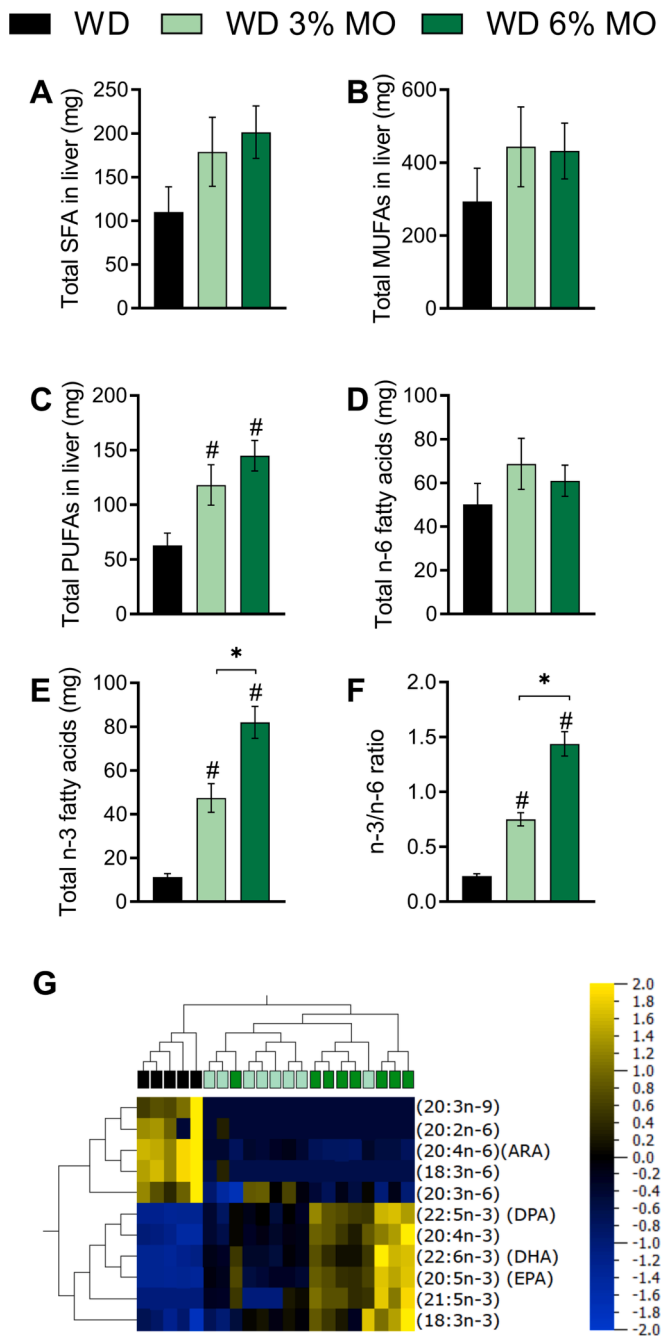

Fig. 4. Fatty acids in the liver of mice fed WD, WD 3% MO, and WD 6% MO for 13 weeks (n = 5–8). (a) Sum SFA, (b) sum MUFAs, (c) sum PUFAs, (d) sum n-6 fatty acids, (e) sum n-3 fatty acids, (f) n-3/n-6 ratio in liver tissue, (g) heatmaps of regulated fatty acids between the experimental groups (q-value below 0.05) (n = 5–8). The color scale of the heat map indicates a change from the mean in the normalized data (mean = 0, variance = 1). All results are presented as mean ± SEM (n = 8–10), and statistical significances are denoted with symbols as follow: # $p < 0.05$ compared to WD; * $p < 0.05$ comparing WD 3% MO to WD 6% MO.

action of n-3 fatty acids and a possible interaction related to the macronutrient composition of the diet can be drawn from these data.

Liver metabolomic profiling was conducted to identify the metabolites impacted by dietary intake of MO. The PCA indicated that the effect of the diet on liver metabolites was relatively small and other unknown factors influenced the separation observed along PC1. Nevertheless, the metabolic profile of the liver showed a general reduction in choline ether lipids, phosphatidylcholines, and sphingomyelins in mice that were fed refined MO. (Best et al., 1936), who observed fatty livers in mice fed a choline-deficient diet, recognized choline as essential for mice. A connection between choline deficiency and the development of fatty liver and NAFLD in humans was established many years ago (Corbin & Zeisel, 2012). A high-fat diet has been shown to aggravate choline deficiency and thus increase choline requirement (Jin et al., 2019). Choline is an essential mineral and must be supplied by our diet despite a small endogenous production, mostly as phosphatidylcholine (PC). PC is required for assembly of VLDL particles and secretion of TAGs from the liver. Hence, choline deficiency results in TAG and DAG accumulation within the hepatocyte (Duric et al., 2012). The metabolic liver profile of mice fed refined MO indicates that intake of refined MO could potentially increase the requirement for choline in the diet. Although the metabolic profiles of mice fed refined MO in a chow-based diet demonstrated significantly lower levels of several choline ether lipids, no clear pattern was observed for PC or sphingomyelin levels. The potential effects of PC in prevention of NAFLD have been previously described (Duric et al., 2012), and several studies have demonstrated a critical role of choline (Jump et al., 2016; Lombardo & Chicco, 2006; Suzuki-Kemuriyama et al., 2016) in reducing VLDL secretion and, consequently, promoting TAG accumulation in the liver (Yao & Vance, 1990). During the refinement of the fish oil, non-lipid-remains and phospholipids were removed, thus the refined oil used in this experiment contains purely TAG lipids. Generally, our results indicate that the dietary intake of refined MO combined with a WD increases the requirement for choline, which accelerates accumulation of hepatic fat in mice fed an obesogenic diet. Lower levels of choline-containing metabolites in the liver, in addition to a high fat intake, could imply that the choline required for the export of triglycerides into very low-density lipoproteins in the liver is not met, and thereby potentially affect the distribution of fat from the liver.

Erucic acid (22:1n-9) is naturally present in the marine food chain and in the lipids of fish and shellfish. Higher levels of erucic acids are found in fish fillets with high lipid levels (Sissener et al., 2018). Dietary intake of erucic acid has been shown to partially hamper beta-oxidation in rodent experiments (Chen et al., 2020; Flatmark et al., 1983). However, in a concentration well above the tolerable daily intake (TDI) of 7 mg/kg body weight established, based on a no observed adverse effect level of 0.7 g/kg body weight per day for lipidosis in newborn piglets and young rats (Chain et al., 2016). Despite the relatively high levels of erucic acids in the refined MO, the final concentration in the experimental diet is below the TDI and is likely not the causative component in increased hepatic fat accumulation; however, more data is needed to be conclusive. Earlier animal studies observed an obesogenic effect of seafood, which has been connected to dietary exposure to fat-soluble persistent organic pollutants (Ibrahim et al., 2011; Ruzzin et al., 2010). The concentration of persistent organic pollutants (POPs) in the refined oil utilized in this experiment was low and based on previous studies conducted with rodents using fish oil, it is highly unlikely that the observed increase in liver lipid accumulation and decreased insulin sensitivity in the mice were a result of these levels of POPs. The refinement process dramatically reduced the levels of PCDD, PCDF, dl-PCB, and PCB6 compared to the crude MO, and the detectable levels in the oil are well below the maximum levels set for human consumption in both the EU and Norway (Table A.2). Despite the low levels of fat-soluble contaminants in the refined oil, the possible effects of contaminants could not be fully excluded. A diet with the inclusion of refined MO altered the fatty acid composition and significantly reduced the n-6: n-3 ratio in the liver tissue of mice fed MO, which parallels similar experiments using diets with inclusion of seafood (Albracht-Schulte et al., 2018; Liisberg et al., 2016). However, in contrast to earlier studies (Bernhard et al., 2016), no significant reduction in SFAs and MUFAs levels was observed in mice fed refined MO compared to WD.

Overall, this study suggests that dietary intake of refined MO increases the dietary requirement of choline leading to hepatic lipid accumulation when refined MO intake is combined with a WD. Our

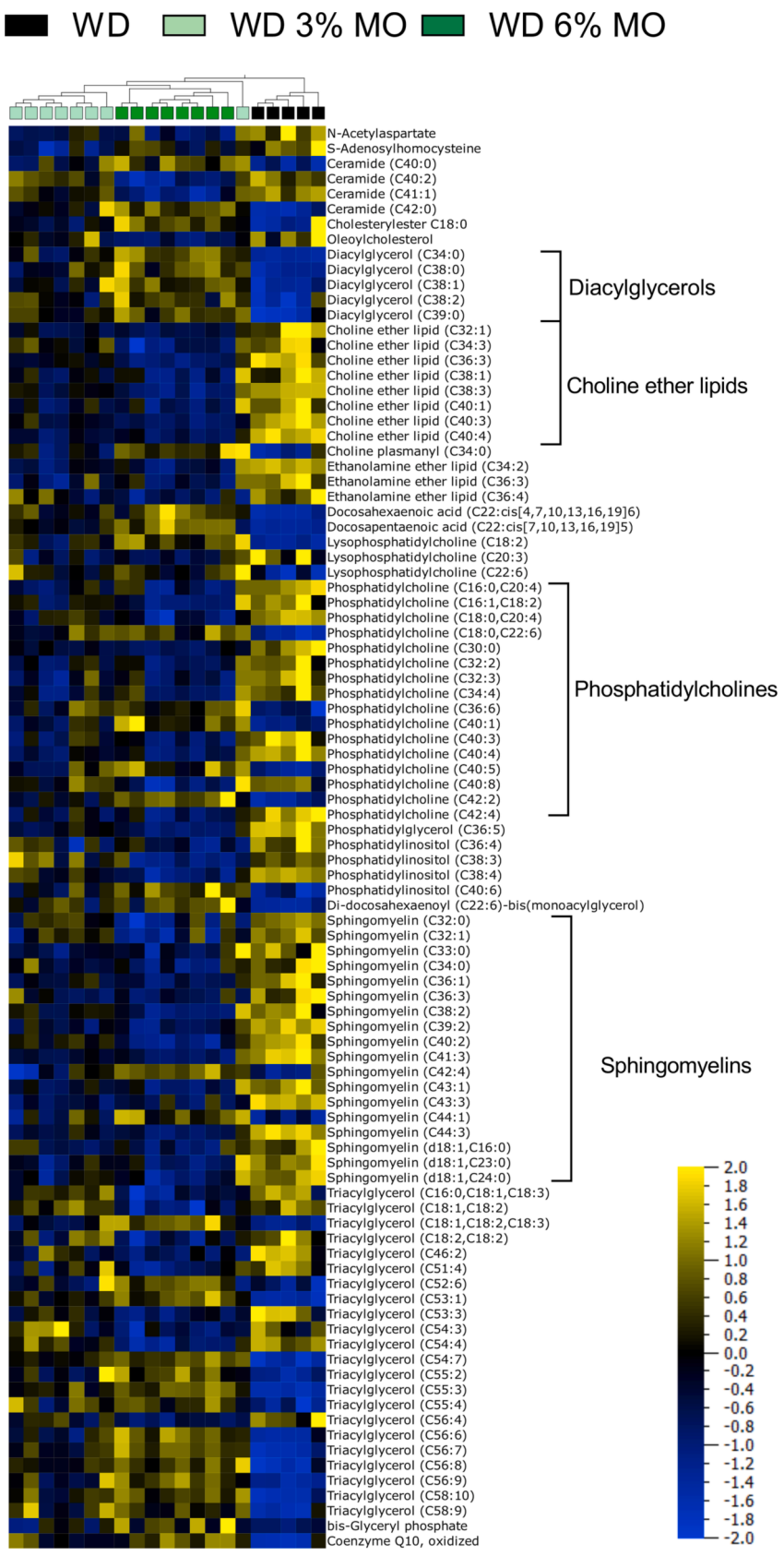

Fig. 5. Metabolomic analysis in the liver tissue of mice fed WD, WD 3% MO, and WD 6% MO for 13 weeks (n = 5–8). Heatmap and hierarchical clustering of liver metabolites significantly different between all groups (q-value < 0.05). Color scale of the heat map indicates change from mean in the normalized data (mean = 0, variance = 1). Data were visualized, and statistical tests were performed using a Qlucore Omics Explorer.

results suggest that the increased choline requirement does not cause any negative metabolic effects if the background diet contains relatively low levels of dietary fat. Further studies are needed to confirm whether enhanced liver weight, increased lipid accumulation in the liver, and reduced insulin sensitivity could be prevented or reversed by increased choline content in the diet.

5. Conclusion

Our results demonstrate that dietary intake of refined MO combined with a WD enhances body weight and lipid accumulation in the liver. Increased fat accumulation in the liver was observed together with reduced overall insulin sensitivity. These findings stand in contrast to the results of several prior studies involving fish oil and n-3 concentrates. The fatty acid profile in the liver demonstrated increased accumulation of PUFAs and n-3 fatty acids; however, no compensatory reductions in SFA or MUFA were observed. Liver metabolic profiling revealed an increased accumulation of fatty acids in the liver and lower levels of choline and choline-derived metabolites. Overall, this study indicates that dietary intake of MO in the diet increases the requirement for choline. Choline plays a crucial role as a component of several membrane phospholipids. During refining processes, choline levels may diminish due to the extraction of phospholipids. Conversely, MO supplementation combined with lower dietary fat content did not affect body weight, fat accumulation in the liver, or insulin sensitivity in these mice, which highlights the importance of the background diet when evaluating the effect of omega-3 PUFA supplements or fatty acids on NAFLD development and insulin sensitivity. These results indicate that the refinement process potentially diminishes the beneficial effects of MO. Hence, it is essential to undertake additional research to ascertain these effects prior to the use of refined MO made from processing waste for human consumption.

Author Contributions

E.F. designed the experiments, E.F. and L.S.M. wrote the manuscript and prepared the figures. E.F., L.S.M., J.R., A.B., L.F., and L.M. performed the experiments. All authors contributed to the data analysis, interpreted the results, edited, revised, and contributed to finalizing the manuscript. In addition, all authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113450.

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