Fish Oil Promotes Macrophage Reverse Cholesterol Transport in Mice

Tomoyuki Nishimoto, Michael A. Pellizzon, Masakazu Aihara, Ioannis M. Stylianou, Jeffery T. Billheimer, George Rothblat, Daniel J. Rader

Objective—Fish oil (FO), and specifically omega 3 fatty acids, has favorable effects on cardiovascular outcomes. The aim of this study was to investigate the effects of FO on the process of macrophage reverse cholesterol transport (RCT) in an in vivo mouse model.

Methods and Results—C57BL/6J mice were fed a FO diet, whereas control mice were fed diets containing alternative sources of fats, soybean oil (SO), and coconut oil (CO) for 4 weeks. Macrophage RCT was assessed by injecting [3H]cholesterol-labeled J774 macrophages intraperitoneally into mice. After 48 hours, tissues were harvested and feces were collected. An increase in the excretion of macrophage-derived [3H]-tracer recovered in fecal neutral sterols for FO-fed mice was observed (273% versus SO and 182% versus CO). FO also decreased [3H]-tracer in hepatic cholesteryl ester compared to SO and CO by 76% and 56%, respectively. To specifically determine the effect of FO on the fate of HDL-derived cholesterol, mice fed FO or SO diets were injected with HDL labeled with [3H]cholesteryl oleate, and the disappearance of [3H]-tracer from blood and its excretion in feces was measured. There was no significant difference in the fractional catabolic rate of [3H]cholesteryl oleate-HDL between the 2 groups. However, there was a 242% increase in the excretion of HDL-derived [3H]-tracer recovered in fecal neutral sterols in FO-fed mice, concordant with significantly increased expression of hepatic Abcg5 and Abcg8 mRNA.

Conclusion—As measured by this tracer-based assay, FO promoted reverse cholesterol transport, primarily by enhancement of the hepatic excretion of macrophage-derived and HDL-derived cholesterol. (Arterioscler Thromb Vasc Biol. 2009;29:1502-1508.)

Key Words: HDL ■ atherosclerosis ■ nutrition

It is well-known that nutritional factors influence the incidence of cardiovascular disease. Dietary fat is considered one of the most important factors associated with the development of atherosclerosis. Saturated fatty acids increase plasma LDL-cholesterol (LDL-C) and promote the progression of atherosclerosis. Alternatively, unsaturated fatty acids are thought to protect against the progression of atherosclerosis. For example, linoleic acid reduces atherosclerosis by lowering plasma LDL-C. Omega-3 fatty acids such as docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA), abundant in fish oil (FO), reduce clinical cardiovascular complications of atherosclerotic disease.

Several mechanisms have been proposed by which FO reduces cardiovascular events, including triglyceride-lowering effects, antiinflammatory effects, antithrombotic effects, and antiarrhythmic effects.

Reverse cholesterol transport (RCT), by which cholesterol is transported from peripheral macrophages to HDL-based acceptors in the liver for excretion in the bile, is thought to be a protective mechanism against atherosclerosis. FO may promote this process by increasing cholesterol efflux from macrophages and increasing hepatic uptake of HDL-cholesterol (HDL-C). In this study, we evaluated the effect of FO on in vivo macrophage RCT using previously established methods for RCT in mice. We found that FO significantly increased macrophage-to-feces RCT. Additional studies suggest that at least part of the mechanism is via reduction in esterification and promotion in the biliary excretion of HDL-derived cholesterol by the liver.

Methods

Materials

RPMI 1640 were purchased from Invitrogen. [1,2-3H]cholesterol and [1,2,3-3H]cholesteryl oleate were purchased from Perkin-Elmer Life Science. Other reagents without citation in this article were purchased from Fisher Scientific.

Animals and Diets

Female C57BL/6j mice (Jackson Laboratories, Bar Harbor, Me), 13 to 15 weeks old, were housed in polycarbonate cages (4 to 6 animals in each cage) with a 12-hour light/dark cycle. The room temperature...
was maintained at 22±1°C and humidity at 30% to 50%. All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

All diets were based on the AIN-93 mol/L formula and were purchased from Research Diets Inc. The low soybean diet (L SO) contained 9% energy from soybean oil and matched the AIN-93 mol/L formula except that it contained 1.25 g cholesterol/3850 kcals. Three high-fat diets, HSO, CO, and FO diets, contained 1.25 g cholesterol/3851 kcal and 42% energy from fat with 5% energy from soybean oil as a source of essential fatty acids and the remaining fat as soybean oil, hydrogenated coconut oil, and menhaden oil, respectively. Unlike other oils, menhaden oil contains 0.4% (w/w) cholesterol; consequently less cholesterol was added to prepare the FO diet. The fatty acid profile for each diet is provided in supplemental Table I (available online at http://atvb.ahajournals.org).

Diet Treatment

After a 2-week introductory feeding period with the L SO diet, mice were fasted for 4 hours and blood was obtained to determine plasma lipid levels. Mice were divided into 3 groups (n=5 to 6/group) with equal mean plasma total cholesterol (TC) levels and fed either, HSO, CO, or FO diets. In addition, 1 group of mice was fed L SO diet throughout the experiment.

In Vivo RCT With J774 Macrophages

RCT studies were performed as previously described. A more detailed description of the methods used can be found in the supplemental materials.

Ex Vivo Cholesterol Efflux From J774 Macrophages

J774 macrophages were used to study the ability of serum, from diet-fed mice, to promote cholesterol efflux ex vivo as previously described. A more detailed description of the methods used can be found in the supplemental materials.

Ex Vivo Cholesterol Efflux From Mouse Peritoneal Macrophages

Mouse peritoneal macrophages (MPMs) from diet-fed mice were used to test their ability to efflux cholesterol to defined acceptors ex vivo. A more detailed description of the methods used can be found in the supplemental materials.

HDL Labeling and Turnover

Mouse HDL was isolated from plasma of chow-fed C57BL/6J mice by differential ultracentrifugation, and HDL (1.063<d<1.21) was labeled with [3H]cholesterol oleate according to the methods described previously with slight modifications. A more detailed description of the methods used can be found in the supplemental materials.

mRNA Gene Expression Analysis

For RNA expression analysis, livers from the macrophage RCT study were removed and pieces were soaked in RNA reagent (Qiagen). The excised small intestines, from HDL turnover study, were washed by ice-cold PBS, and then divided into 6 parts with equal length (duodenum, upper jejunum, mid jejunum, lower jejunum, upper ileum, and lower ileum). Total RNA was isolated from liver or small intestine using EZ1 RNA Tissue Mini Kit (Qiagen). cDNA was produced from total RNA via reverse transcription using

Table. Plasma Lipids of Diet-Fed Mice

<table>
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Values are given as mean mg/dL ±SD; n=6 mice per group. ANOVA indicates a significant difference between groups for all lipids (P<0.01). Sig indicates the statistical significance where different letters indicate significantly different among the high-fat diet groups determined by Tukey HSD (alpha=0.05). The same letter indicates no significant difference between groups. Two-tailed tests were performed to examine differences between low-fat L SO and high-fat L SO diets (*P<0.05, **P<0.01).

Superscript First strand synthesis system Kit (Invitrogen). Quantification of mRNA expression using TagMan or SYBR green assay systems was performed using an ABI 7300 Real Time thermocycler (Applied Biosystems). Abundance of genes was determined after correcting for loading to Gapdh expression (DCI), and relative expression attributable to diet conditions is compared to the L SO group.

Statistical Analyses

Results are presented as the mean±SD. One-way ANOVA (P<0.05) followed by Tukey HSD-test (a=0.05) was used for detecting the statistical differences among high-fat diet groups (L SO, CO and FO). Student t test was used for detecting differences between L SO and L SO diets to understand the influence of high-fat diet. All analyses were performed using JMP v7.0 (SAS Institute).

Results

Plasma Lipids

Body weight and food intake of mice fed different diets were similar throughout the study (data not shown). Plasma lipids on the different diets are shown in the Table. Compared with the control L SO, the higher level of soybean oil, L SO diet, did not change the plasma total cholesterol (TC) level but it increased HDL-cholesterol (HDL-C) levels and decreased non–HDL-C, triglyceride (TG), and nonesterified fatty acid (NEFA) levels. The CO diet significantly increased TC,
HDL-C, non–HDL-C, TG, and NEFA levels compared with the HSO diet. Finally, compared with the other high-fat diets, the FO diet significantly decreased plasma HDL-C levels (13% and 36% compared to HSO and CO, respectively) and NEFA levels (20% and 61% compared to HSO and CO, respectively), and compared with the CO diet the FO diet significantly reduced non–HDL-C (23%) and TG (62%) levels.

**Macrophage RCT**

Comparisons among the high-fat diet groups with regard to indices of RCT (Figure 1A) indicate that the plasma [3H]-tracer in FO diet–fed mice was similar to that in HSO diet–fed mice at all time points and significantly less than that in CO diet–fed mice at 48 hours. With regard to fecal neutral sterols, FO substantially increased (by 273% versus HSO, and by 182% versus CO) the excretion of macrophage-derived [3H]-tracer. FO decreased [3H]-tracer recovered in hepatic cholesteryl ester by 76% and 56% compared to HSO and CO, respectively, although there were no differences in [3H]-tracer recovered in hepatic free cholesterol. The reduction in [3H]-tracer in recovered hepatic cholesteryl ester was reflected by a similar reduction in hepatic cholesteryl ester mass in FO diet–fed mice (Figure 1A).

A comparison between the LSO with the HSO diet (Figure 1B) showed no effect on the macrophage-derived [3H]-tracer in plasma. However, the HSO diet had significantly decreased [3H]-tracer in hepatic cholesteryl ester after 48 hours.

**Figure 1.** Macrophage RCT in mice. Mice were fed LSO, HSO, CO, and FO diets (n=6 per each group). A, Comparison of high-fat diet groups. ANOVA was performed for each lipid measurement, and if significance was observed (P<0.01) then Tukey HSD was applied to test for significance between all groups. Different letters indicate significant differences between diets (Tukey HSD, alpha=0.05), absence of letters indicates no significant difference between groups. B, Comparison of low-fat versus high-fat diet (soybean oil). *Significant difference between LSO and HSO by Student t test (P<0.001).
The LSO diet indicates a higher level of cholesterol efflux capacity of serum compared to the HSO diet. Comparing the FO and CO diets significantly increased the cholesterol efflux relative to HSO. Interestingly, macrophages from FO-fed mice did not significantly increase cholesterol efflux relative to HSO diet. Alternatively, in the presence of cAMP, both the FO and CO diets significantly increased the cholesterol efflux capacity of serum compared to the HSO diet. Comparing the LSO and HSO diets indicates that a higher level of soybean oil increased the cholesterol efflux capacity of serum compared to HSO. There were no significant differences between LSO and HSO by t test. Comparisons between high-fat groups was performed by ANOVA, if significance was observed (P < 0.01) then Tukey HSD was applied to test for significance between all groups. Different letters indicate significant differences between diets (Tukey HSD, alpha = 0.05), whereas absence of letters indicates no significant difference between groups.

**Macrophage Cholesterol Efflux**

Macrophage cholesterol efflux is the first, and one of the most important, steps in the RCT pathway. The effects of the FO diet on cholesterol efflux from macrophages ex vivo were investigated in 2 different ways. First, we examined the ability of serum obtained from mice on the FO and other diets to promote cholesterol efflux from J774 macrophages (Figure 2). Comparisons among the high-fat diet groups indicate that, in the absence of cAMP (which upregulates ABCA1), the FO diet did not increase the cholesterol efflux capacity of serum, whereas the CO diet significantly increased it compared to the HSO diet. Alternatively, in the presence of cAMP, both the FO and CO diets significantly increased the cholesterol efflux capacity of serum compared to the LSO diet. Comparing the LSO and HSO diets indicates that a higher level of soybean oil increased the cholesterol efflux capacity of serum either with or without cAMP.

Secondly, we examined the ability of macrophages isolated from mice fed the experimental diets to efflux cholesterol to defined acceptors; pooled serum (from control chow-fed C57BL/6J mice; 2%) or human Apo-A-I (100 μg/mL; Figure 3). Among the high-fat diet groups, there were no differences with regard to cholesterol efflux from MPMs using mouse serum as the acceptor (Figure 3A). Similarly, using human Apo-A-I as the acceptor, macrophages from FO-fed mice did not significantly increase cholesterol efflux relative to HSO. Interestingly, CO-fed mice had decreased cholesterol efflux compared with macrophages from FO-fed mice (Figure 3B). No differences were observed between the LSO and HSO groups in cholesterol efflux from peritoneal macrophages.

Specific activities of the [3H]-cholesterol in MPM were 48 471 ± 10 865 (LSO), 57 612 ± 8000 (CO), 47 003 ± 6809 (FO), and 49 519 ± 11 948 (HSO), cpm/6809 (FO), and 49 519 ± 11 948 (HSO), cpm/8000 (CO), 47 003 ± 6809 (FO), and 49 519 ± 11 948 (HSO), cpm/

**Fate of HDL-Derived Cholesteryl Ester**

Another key step in RCT is the hepatic uptake and excretion of HDL-derived cholesterol. To determine whether the FO diet may have promoted these steps, a HDL turnover study using [3H]-cholesteryl ester labeled HDL in mice fed the LSO, HSO, and FO diets was performed including tracing the excretion of the labeled cholesterol in the feces (Figure 4). There was no difference between HSO and FO in the plasma FCR of HDL-CE. However, there was a 242% increase in the excretion of HDL-derived [3H]-tracer recovered in fecal neutral sterols in FO diet–fed mice compared to HSO-fed mice (P < 0.0001). As observed in the macrophage RCT.
study, FO did not affect the [3H]-tracer recovered in fecal bile acids. Similarly, as in the RCT study, FO significantly decreased [3H]-tracer in hepatic cholesteryl ester by 79% compared to HSO (P < 0.0001). Comparing LSO and HSO indicates that a higher level of soybean oil decreased the FCR of HDL-CE (P = 0.004).

**Hepatic mRNA Expression Analysis**

The fish oil diet significantly increased the [3H]-tracer recovered in fecal neutral sterols but not in fecal bile acid in both the RCT and HDL turnover studies. FO markedly decreased [3H]-tracer recovered in hepatic cholesteryl ester in both experiments. These results suggest that FO inhibits the esterification of macrophage and HDL-derived cholesterol and increases the excretion of neutral sterols from liver to bile and possibly from bile to feces. Several liver genes that influence cholesterol esterification and biliary cholesterol excretion were therefore analyzed by RT-QPCR (Figure 5).

Consistent with a previous report, 13 FO significantly decreased mRNA of hepatic triglyceride synthesis related genes (Srebp-1c, Fas, and Scd1) and increased hepatic expression of Acox which catalyzes β-oxidation of fatty acids. The reduction in Scd1 expression, in particular, could result in reduced esterification of HDL-derived cholesterol, consistent with our observations and potentially leading to enhanced excretion into the bile.

In addition, FO did not affect mRNA levels of Scarb1, which is consistent with the HDL-CE turnover data. Western blot analysis also did not show a change (data not shown). The FO diet showed a small, but not significant increase in Abca1 relative to the other high-fat diets, whereas Abcg1 expression was unchanged among the different diet groups. ABCG5 and ABCG8 play a critical role in biliary excretion of neutral sterols. Comparisons among the high-fat diet groups show FO significantly increased hepatic expression of both Abcg5 and Abcg8 but not Cyp7a1. Compared to the control LSO diet, the HSO diet had little effect on hepatic expression of Abcg5 and Abcg8.

Finally, NPC1L1 plays a crucial role in intestinal absorption of neutral sterols. In the small intestine, a higher level of soybean oil (LSO diet) significantly decreased Npc1l1 mRNA levels compared with the HSO diet (P = 0.0029) (Figure 5B). FO further decreased Npc1l1 expression in the small intestine compared to HSO, although the additional decrease was not significant (P = 0.23). The significant increase in hepatic Abcg5/R8 and potential decrease in intestinal Npc1l1 mRNA may account for the increased fecal excretion of macrophage-derived cholesterol.

**Discussion**

This study shows that a diet high in omega-3 fatty acids substantially increased the fecal excretion of macrophage-de-
rived and HDL-derived [3H]cholesterol, compared with a diet high in soybean oil as well as a low-fat diet. The fish oil diet reduced hepatic esterification of HDL-derived cholesterol, increased the fecal excretion of HDL-derived cholesterol, upregulated hepatic Abcg5 and Abcg8, and downregulated intestinal Npc1l1, all of which may have contributed to the increased rate of RCT.

Marmillot et al reported that FO feeding in rats enhanced the ability of their HDL to promote cholesterol efflux from J774 cells. In the present study, the serum from FO-fed mice promoted cAMP-dependent cholesterol efflux (but not basal efflux) from J774 cells, compared with that from μSO fed mice. Because the addition of cAMP to the cells causes an increase in ABCA1 at the cell surface, our results suggest that serum from FO-fed mice has a greater capacity to promote efflux of cellular cholesterol through the ABCA1-dependent pathway. Serum from CO-fed mice also increased efflux compared to μSO both in the absence and presence of cAMP. Because 2% serum is used as an acceptor, the increase is probably attributable to the 38% increase in HDL in the CO-fed mice. We also tested the relative ability of MPMs isolated from mice fed different diets to efflux cholesterol. There was no difference in efflux to pooled mouse serum from FO-fed mice to efflux cholesterol.

In the absence of compelling data to suggest that the FO diet promoted cholesterol efflux from macrophages, we hypothesized that the effect of the FO diet on RCT was attributable to effects downstream from the efflux step. To evaluate later steps involved in RCT which may be affected by FO, we performed an HDL turnover study using [3H]cholesteryl olate-labeled HDL. There was no change in FCR of plasma HDL-CE between FO and μSO diet–fed mice and no change in hepatic Scarb1 expression. Le Morvan et al reported that FO-fed mice had an increased FCR and hepatic uptake of HDL-CE, accompanied by an increase in Scarb1 expression in FO-fed mice, in contrast with data presented here. The differences in these results may be attributable to the difference in the diet composition: Le Morvan et al used a grain-based basal diet, whereas here, a purified AIN-93 mol/L basal diet was used with added cholesterol. Vasadani et al reported that hepatic Scarb1 was unchanged by a semisynthetic diet containing EPA and DHA in LDL receptor–deficient mice, which is consistent with observations made here. However, despite the unchanged turnover of HDL-CE in FO-fed mice, FO substantially increased the fecal excretion of HDL-derived cholesterol to a similar extent as was observed in the macrophage RCT study. These results suggest that FO promotes in vivo macrophage RCT at least in part via an enhancement of the excretion of HDL-derived cholesterol from liver to feces.

Our results suggest several complementary mechanisms of this effect of FO on the biliary and fecal excretion of HDL-derived cholesterol. First, we demonstrate a striking reduction in macrophage- and HDL-derived [3H]-tracer in hepatic cholesteryl ester of FO-fed mice relative to both CO and μSO diets, which increases the pool of unesterified cholesterol available for biliary excretion. This observation with the tracer studies is consistent with a significant decrease in hepatic cholesteryl ester mass in FO-fed mice. Omega-3 fatty acids such as EPA and DHA decreased hepatic cholesteryl ester compared with omega-6 fatty acids in LDL receptor–deficient mice. EPA and DHA are poor substrates for the ACAT reaction. In addition, FO feeding inhibited Fas expression, as previously shown, which can cause a depletion of endogenous substrates for acyl-CoA cholesterol acyl transferase (ACAT). Furthermore, SCD1 depletion causes a reduction in not only hepatic triglyceride but also hepatic cholesteryl ester levels. In this study FO significantly decreased Scd1 mRNA expression in liver, which may contribute to the depletion of cholesteryl ester in the liver. Thus, in FO-fed mice, macrophage- and HDL-derived free cholesterol is less effectively esterified, making it more available for biliary excretion.

A second mechanism to explain our finding is that the FO diet significantly increased hepatic mRNA levels of Abcg5/g8, key proteins which regulate hepatic cholesterol secretion into bile. LXR agonists induce Abcg5/g8 expression. Recently, Uehara et al reported that EPA decreased LXR regulated genes, Abcg1 and Abca1, in an in vitro study. However, other LXR regulated genes, Abcg5/g8 and Cyp7a1, were not affected by a diet with 10% FO (w/w) in an in vivo rat model compared to those fed the same diet with olive oil. The FO diet used here contains 0.15% cholesterol which in the presence of the FO diet could have resulted in increased levels of unesterified cholesterol in the liver and increased generation of oxysterols, which could serve to activate LXR. Furthermore, PPARα agonists modestly increased Abcg5/g8 expression. EPA and DHA are more potent PPARα agonists compared with monounsaturated or saturated fatty acid. Indeed, in our study the FO diet increased hepatic mRNA expression of Acox, a classic target gene of PPARα. Furthermore, we observed a significant correlation between Abcg5/g8 and Acox mRNA expression. Although further investigation is needed to clarify the mechanism by which FO increases Abcg5/g8 expression, activation of LXR or PPARα are among possible mechanisms.

A third potential mechanism is reduced intestinal reabsorption of biliary HDL-derived cholesterol attributable to downregulation of intestinal Npc1l1. We have shown that ezetimibe, which inhibits Npc1l1, promotes the fecal excretion of macrophage and HDL-derived cholesterol. Our data here suggest that the FO diet reduced the expression of Npc1l1. Although the mechanism of this observation is unknown, PPARα agonists have been shown to downregulate Npc1l1 leading to increased macrophage RCT, and it is possible that FO could influence Npc1l1 expression through this mechanism.

In conclusion, our data indicate that a FO diet promoted the excretion of macrophage- and HDL-derived cholesterol into the feces in mice, primarily because of increased hepatic fecal excretion of HDL-derived cholesterol as a result of several complementary mechanisms. We suggest that this effect of
fish oils could contribute to their antiatherosclerotic and cardioprotective properties.

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Disclosures
None.

References
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### Supplemental Table 1. Fatty acid profile for fat sources in all diets

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Fatty acids with less than 0.1% (w/w) were not shown; fatty acid data was calculated from data provided by Research Diets, Inc.
Supplement Material

Methods

In vivo RCT with J774 macrophages

RCT studies were performed as previously described\textsuperscript{11, 12}. Briefly, mice received intraperitoneal injections of cholesterol-loaded J774 cells labeled with \(^{3}\text{H}\)cholesterol. Blood was collected at 6, 24, and 48hrs, and the plasma was counted in a liquid scintillation counter (LSC). At 48hrs after injection, mice were exsanguinated and perfused with ice-cold PBS, and portions of the liver were flash-frozen for lipid extraction or soaked in RNA\textit{later} reagent (Qiagen, Valencia, CA) for mRNA gene expression analysis. Liver lipids were extracted according to the procedure of Bligh and Dyer\textsuperscript{13}. Briefly, 50mg of tissue was homogenized in water, and lipids were extracted with a 2:1 (v/v) mixture of chloroform/methanol. The lipid layer was collected, evaporated, re-suspended in toluene, and counted in an LSC (total cholesterol). Cholesterol ester was separated from free cholesterol by thin layer chromatography using hexane/ethyl ether/acetic acid (85/15/0.5, v/v/v), visualized by iodine vapor and scraped off, then counted in an LSC. The total feces collected from 0 to 48hrs were weighed and soaked in water (2ml H\textsubscript{2}O/100mg feces) overnight at 4°C. An equal volume of ethanol was added the next day, and the samples were homogenized. To extract the \(^{3}\text{H}\)cholesterol and \(^{3}\text{H}\)bile acid fractions, 1ml of homogenized sample was combined with 1ml ethanol and 200\textmu l NaOH. The samples were saponified at 95°C for 2hrs and cooled to room temperature, then cholesterol was extracted three times with 4.5ml hexane. The extracts were pooled, evaporated, re-suspended in toluene, and then counted in an LSC. To extract bile acids, the remaining aqueous portion of the feces was acidified with concentrated HCl and then extracted three times with 4.5ml ethyl acetate. The extracts were pooled together, evaporated, re-suspended in ethyl acetate, and counted in an LSC.

Ex vivo cholesterol efflux from J774 macrophages

J774 macrophages were used to study the ability of serum, from diet-fed mice, to promote cholesterol efflux \textit{ex vivo}. J774 cells were seeded on 24-well plates at the density of 1.5 x 10\textsuperscript{5} cells/well in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). After two days,
cells were washed with PBS and labeled by incubation in RPMI 1640 supplemented with 1% FBS containing \[^3\text{H}\]cholesterol (3µCi/ml) and Ac-LDL (50µg/ml) for 24hrs. Following the labeling period, cells were washed with PBS and incubated with RPMI 1640 containing 0.2% (w/v) BSA (Sigma, St. Louis) in the presence or absence of 0.3mM 8-(4-chlorophenylthio)-cAMP (CPT-cAMP; Sigma) for 16hrs. The cells were again washed with PBS and cholesterol efflux was initiated by the addition of RPMI-0.2% BSA in the presence or absence (background) of individual serum (2%) from mice fed the experimental diets for three weeks in the \textit{in vivo} RCT study. After 4hrs incubation, aliquots of the medium were centrifuged to remove the floating cells and the supernatants were counted in a LSC. The cells were extracted with isopropyl alcohol, evaporated under nitrogen gas, re-suspended in toluene, and counted in an LSC. Cholesterol efflux was expressed as percentage of total tritium radioactivity presented in the cells plus the effluxed medium.

\textit{Ex vivo cholesterol efflux from mouse peritoneal macrophages (MPMs)}

MPMs from diet-fed mice were used to test their ability to efflux cholesterol to defined acceptors \textit{ex vivo}. MPMs were prepared as described previously from six week diet-fed mice\textsuperscript{14}. Briefly, mice were intraperitoneally injected with 1ml of 10% thioglycollate broth (Sigma). Four days after injection, the mice were euthanized and macrophages were isolated by peritoneal lavage with ice-cold PBS. Cells were seeded on 24-well plates at a density of 1 x 10\textsuperscript{6} cells/well in RPMI 1640 supplemented with 10% FBS. Four hours after seeding, cells were washed three times with MEM containing 25mM HEPES to remove non-adherent cells. Cells were labeled by incubation in RPMI 1640 supplemented with 2.5% FBS containing \[^3\text{H}\]cholesterol (3µCi/ml) for 5hrs. Cells were washed twice with MEM-25mM HEPES and incubated with RPMI 1640-0.2% BSA for 2hrs. Cholesterol efflux was determined as described above, except that we used pooled serum (2%) from C57BL/6J mice fed a chow-diet (Rodent Diet #5010, PMI Nutrition International, St. Louis, MO) or human apoA-I (Sigma; 100µg/ml) as the acceptor.

\textit{HDL Labeling and turnover}

Mouse HDL was isolated from plasma of chow-fed C57BL/6J mice by differential ultracentrifugation. HDL (1.063<d<1.21) was labeled with \[^3\text{H}\]cholesterol oleate according to
the methods described previously with slight modifications\textsuperscript{15}. Briefly, 1mCi of $[^3]$H]cholesterol oleate was evaporated under nitrogen gas and dissolved in ethanol. This solution was added into mouse HDL (e.q. 8mg protein) containing human lipoprotein deficiency serum (e.q. 200mg protein: 1.2 % ethanol f.c.) and incubated for 24hrs at 37°C. The labeled solution was dialyzed with 0.15M NaCl/0.4mM EDTA (pH 7.4), it was subjected to differential ultracentrifugation to isolate the labeled-HDL (1.063<d<1.21). The labeled HDL was dialyzed with 0.15M NaCl/0.4mM EDTA (pH 7.4) and kept in 4°C until used for the HDL turnover study. The specific activity of labeled HDL was 46cpm/ng protein.

$[^3]$H]cholesterol oleate-labeled HDL was intravenously injected via tail vein (4,700,000 cpm/mouse) under anesthesia to LSO, HSO and FO diet fed mice. Blood was collected at 2 min, 1, 2, 6, 9, 24, and 48hrs, and the plasma was counted in an LSC. The determination of radioactivity in the feces and liver was performed by the method described in the macrophage RCT study. Fractional catabolic rate (FCR) for HDL-cholesteryl ester (HDL-CE) was assessed using a multi-compartmental model as described previously\textsuperscript{16}. The SAAMII program was used to fit the model to the observed tracer data using a weighted least-squares approach to determine the best fit. Additionally, the small intestine was removed, and soaked in RNaAlater reagent (Qiagen, Valencia, CA) for mRNA gene expression analysis.
Results

Plasma lipids

Body weight and food intake of mice fed different diets were similar throughout the study (data not shown). Plasma lipids on the different diets are shown in Table 1. Compared with the control LSO, the higher level of soybean oil, HSO diet, did not change the plasma total cholesterol (TC) level but it increased HDL-cholesterol (HDL-C) levels and decreased non-HDL-C, triglyceride (TG), and non-esterified fatty acid (NEFA) levels. The CO diet significantly increased TC, HDL-C, non-HDL-C, TG, and NEFA levels compared with the HSO diet. Finally, compared with the other high fat diets the FO diet significantly decreased plasma HDL-C levels (13% and 36% compared to HSO and CO respectively) and NEFA levels (20% and 61% compared to HSO and CO respectively), and compared with the CO diet the FO diet significantly reduced non-HDL-C (23%) and TG (62%) levels.

Macrophage RCT

Comparisons among the high-fat diet groups with regard to indices of RCT (Fig. 1A) indicate that the plasma $[^3]$H-tracer in FO diet-fed mice was similar to that in HSO diet-fed mice at all time points and significantly less than that in CO diet-fed mice at 48 hrs. With regard to fecal neutral sterols, FO substantially increased (by 273% vs. SO, and by 182% vs. CO) the excretion of macrophage-derived $[^3]$H-tracer. FO decreased $[^3]$H-tracer recovered in hepatic cholesteryl ester by 76% and 56% compared to HSO and CO, respectively, although there were no differences in $[^3]$H-tracer recovered in hepatic free cholesterol. The reduction in $[^3]$H-tracer in recovered hepatic cholesteryl ester was reflected by a similar reduction in hepatic cholesteryl ester mass in FO diet-fed mice (Fig. 1A).

A comparison between the LSO with the HSO diet (Fig. 1B) shows there is no affect on the macrophage-derived $[^3]$H-tracer in plasma. However, the HSO diet had significantly decreased $[^3]$H-tracer in hepatic cholesteryl ester after 48hrs.

Macrophage cholesterol efflux

Additional experiments were performed to determine the mechanisms by which the FO diet increased macrophage RCT. Macrophage cholesterol efflux is the first, and one of the most
important steps in the RCT pathway. The effects of the FO diet on cholesterol efflux from macrophages *ex vivo* were investigated in two different ways. First, we examined the ability of serum obtained from mice on the FO and other diets to promote cholesterol efflux from J774 macrophages (Fig. 2). Comparisons among the high-fat diet groups indicate that, in the absence of cAMP (which up-regulates ABCA1), the FO diet did not increase the cholesterol efflux capacity of serum, while the CO diet significantly increased it compared to the *H*SO diet. Alternatively, in the presence of cAMP, both the FO and CO diets significantly increased the cholesterol efflux capacity of serum compared to the *H*SO diet. Comparing the *L*SO and *H*SO diets indicates that a higher level of soybean oil increased the cholesterol efflux capacity of serum either with or without cAMP.

Secondly, we examined the ability of macrophages isolated from mice fed the experimental diets to efflux cholesterol to defined acceptors; pooled serum (from control chow-fed C57BL/6J mice; 2%) or human ApoA-I (100 µg/ml) (Fig. 3). Among the high-fat diet groups, there were no differences with regard to cholesterol efflux from MPMs using mouse serum as the acceptor (Fig. 3A). Similarly, using human ApoA-I as the acceptor, macrophages from FO-fed mice did not significantly increase cholesterol efflux relative to *H*SO. Interestingly CO-fed mice had *decreased* cholesterol efflux compared with macrophages from FO-fed mice (Fig. 3B). No differences were observed between the *L*SO and *H*SO groups in cholesterol efflux from peritoneal macrophages.

Specific activities of the [3H]-cholesterol in MPM were determined by summing cellular and media [3H]-counts. The specific activities were 48,471 ± 10,865 (*H*SO), 57,612 ± 8,000 (CO), 47,003 ± 6,809 (FO), and 49,519 ± 11,948 (*L*SO), cpm/1 × 10^6 cells. The CO group was significantly higher than the *H*SO (P<0.05) and FO (P<0.01) groups.

*Fate of HDL-derived cholesteryl ester*

Another key step in RCT is the hepatic uptake and excretion of HDL-derived cholesterol. To determine whether the FO-diet may have promoted these steps, a HDL turnover study using [3H]-cholesteryl ester labeled HDL in mice fed the *L*SO, *H*SO, and FO diets was performed including tracing the excretion of the labeled cholesterol in the feces (Fig 4). There was no difference between *H*SO and FO in the plasma FCR of HDL-CE. However, there was a 242% increase in the excretion of HDL-derived [3H]-tracer recovered in fecal neutral sterols in FO diet-
fed mice compared to \text{H}SO-fed mice (p<0.0001). As observed in the macrophage RCT study, FO did not affect the $[^3\text{H}]$-tracer recovered in fecal bile acids. Similarly, as in the RCT study, FO significantly decreased $[^3\text{H}]$-tracer in hepatic cholesteryl ester by 79\% compared to \text{H}SO (p<0.0001). Comparing \text{L}SO and \text{H}SO indicates that a higher level of soybean oil decreased the FCR of HDL-CE (p=0.004).

Hepatic mRNA expression analysis

The fish oil diet significantly increased the $[^3\text{H}]$-tracer recovered in fecal neutral sterols but not in fecal bile acid in both the RCT and HDL turnover studies. FO markedly decreased $[^3\text{H}]$-tracer recovered in hepatic cholesteryl ester in both experiments. These results suggest that FO inhibits the esterification of macrophage and HDL-derived cholesterol and increases the excretion of neutral sterols from liver to bile and possibly from bile to feces. Several liver genes that influence cholesterol esterification and biliary cholesterol excretion were therefore analyzed by RT-QPCR (Fig 5).

Consistent with previous reports\textsuperscript{17, 18} FO significantly decreased mRNA of hepatic triglyceride synthesis related genes (\textit{Srebp-1c}, \textit{Fas}, and \textit{Scd1}) and increased hepatic expression of \textit{Acox} which catalyzes $\beta$-oxidation of fatty acids. The reduction in \textit{Scd1} expression, in particular, could result in reduced esterification of HDL-derived cholesterol\textsuperscript{19}, consistent with our observations and potentially leading to enhanced excretion into the bile.

In addition, FO did not affect mRNA levels of \textit{Sr-b1}, which is consistent with the HDL-CE turnover data. Western blot analysis also did not show a change (data not shown). The FO diet showed a small, but not significant increase in \textit{Abca1} relative to the other high-fat diets, while and \textit{Abcg1} expression was unchanged among the different diet groups.

\textit{Abcg5} and \textit{Abcg8} play a critical role in biliary excretion of neutral sterols\textsuperscript{20}. Comparisons among the high-fat diet groups show FO significantly increased hepatic expression of both \textit{Abcg5} and \textit{Abcg8}, but not \textit{Cyp7a1}. Compared to the control \text{L}SO diet, the \text{H}SO diet had little effect on hepatic expression of \textit{Abcg5} and \textit{Abcg8}.

Finally, NPC1L1 plays a crucial role in intestinal absorption of neutral sterols\textsuperscript{21}. In the small intestine, a higher level of soybean oil (\text{H}SO diet) significantly decreased \textit{Npc1l1} mRNA levels compared with the \text{L}SO diet (p=0.0029) (Fig 5B). FO further decreased \textit{Npc1l1} expression in the small intestine compared to \text{H}SO, although the additional decrease was not significant.
(p=0.23). The significant increase in hepatic Abcg5/g8 and potential decrease in intestinal Npc1l1 mRNA may account for the increased fecal excretion of macrophage and HDL-derived cholesterol.
Discussion

This study shows that a diet high in fish oil/omega-3 fatty acids, substantially increased the fecal excretion of macrophage-derived and HDL-derived [\( ^{3} \)H]cholesterol, compared with diets high in soybean oil or, as well as with a low fat diet where as there was no difference in fecal excretion between CO and the SO diets. This is the first in vivo evidence demonstrating that fish oil increases in vivo macrophage RCT. We show that the fish oil diet reduces hepatic esterification of HDL-derived cholesterol, increases the fecal excretion of HDL-derived cholesterol, and up-regulates hepatic Abcg5 and Abcg8 mRNA. It is also possible that FO decreases Npc1l1 mRNA in the intestine, although the decrease is not much greater than that observed in other high-fat diets.

Marmillot et al. reported that FO-feeding in rats enhanced the ability of their HDL to promote cholesterol efflux from J774 cells\(^8\). In the present study, the serum from FO-fed mice promoted cAMP-dependent cholesterol efflux (but not basal efflux) from J774 cells, compared with that from \( ^{1} \)SO fed mice. Since the addition of cAMP to the cells causes an increase in ABCA1 at the cell surface\(^22\), our results suggest that serum from FO-fed mice has a greater capacity to promote efflux of cellular cholesterol through the ABCA1-dependent pathway. Serum from CO-fed mice also increased efflux compared to \( ^{1} \)SO both is absence and presence of cAMP. Since 2% serum is used as an acceptor the increase is probably due to the 38% increase in HDL in the CO –fed mice (Table 1).

We also tested the relative ability of MPMs isolated from mice fed different diets to efflux cholesterol. There was no difference in efflux to mouse serum from MPM derived from the different diet groups. When apoA1 was used as the acceptor there was no difference in the FO group but a significant decrease in CO vs. \( ^{1} \)SO. There was an 18% increase in tritiated cholesterol label of CO-MPM versus the other groups which might suggest an increase in cellular cholesterol in a pool not available for efflux to apoA-1. Alternatively, ABCA1 mediates efflux of cholesterol to apoA-1 and its gene expression is increased by PPAR agonist working through LXR. Since saturated fatty acids are poor agonists of PPAR relative to unsaturated fatty acids one might expect a decrease in ABCA1 expression\(^23,24\). The FO diet did not result in an increase in plasma tracer in the in vivo macrophage RCT study. It should be noted that plasma tracer is not an indicator of macrophage efflux but a snapshot in time which is reflective of both input of tracer into the plasma as well as its removal from plasma. The increase in tracer in the
CO-fed mice is likely due to reduced turnover of the effluxed cholesterol. Increase in plasma tracer has also been observed in Sr-b1-/- and Ldr-/- mice transduced with CETP due to decreased plasma clearance.\textsuperscript{25, 26}

In the absence of compelling data to suggest that the FO diet promoted cholesterol efflux from macrophages, we hypothesized that the effect of the FO diet on RCT was due to effects downstream from the efflux step. To evaluate later steps involved in RCT which may be affected by FO, we performed an HDL turnover study using \( ^{3}H \)cholesteryl oleate-labeled HDL. There was no change in FCR of plasma HDL-CE between FO and \( ^{14}C \)SO diet-fed mice and no change in hepatic Sr-1 expression. Le Morvan \textit{et al.} reported that FO-fed mice had an increased FCR and hepatic uptake of HDL-CE, accompanied by an increase in Sr-b1 expression in FO-fed mice\textsuperscript{9}, in contrast with data presented here. The differences in these results may be due to the difference in the diet composition; Le Morvan \textit{et al} used a grain-based basal diet, whereas here, a purified AIN-93M basal diet was used with added cholesterol. Vasandani \textit{et al.}\textsuperscript{27} reported that hepatic SR-BI was unchanged by a semi-synthetic diet containing EPA and DHA in LDL receptor deficient mice, which is consistent with observations made here. However, despite the unchanged turnover of HDL-CE in FO-fed mice, FO substantially increased the fecal excretion of HDL-derived cholesterol to a similar extent as was observed in the macrophage RCT study. These results suggest that FO promotes \textit{in vivo} macrophage RCT at least in part via an enhancement of the excretion of HDL-derived cholesterol from liver to feces.

Our results suggest several complementary mechanisms of this effect of FO on the biliary and fecal excretion of HDL-derived cholesterol. First, we demonstrate a striking reduction in macrophage- and HDL-derived \( ^{3}H \)-tracer in hepatic cholesteryl ester of FO-fed mice relative to both CO and \( ^{14}C \)SO diets, which increases the pool of unesterified cholesterol available for biliary excretion. This observation with the tracer studies is consistent with a significant decrease in hepatic cholesteryl ester mass in FO-fed mice. Vasandani \textit{et al.} also reported that omega-3 fatty acids such as EPA and DHA decreased hepatic cholesteryl ester compared with omega-6 fatty acids in LDL receptor deficient mice\textsuperscript{27}. EPA and DHA are poor substrates for the ACAT reaction.\textsuperscript{28} In addition, FO feeding inhibited \textit{Fas} expression, as previously shown\textsuperscript{27}, which can cause a depletion of endogenous substrates for acyl-CoA cholesterol acyl transferase (ACAT). Furthermore, Miyazaki \textit{et al.} reported that SCD1 depletion causes a reduction in not only hepatic triglyceride but also hepatic cholesteryl ester levels.\textsuperscript{19} In this study FO significantly decreased
Scd1 mRNA expression in liver, a finding also reported by other investigators²⁹, which may contribute to the depletion of cholesteryl ester in the liver. Thus, in FO-fed mice, macrophage- and HDL-derived free cholesterol is less effectively esterified, making it more available for biliary excretion.

A second mechanism to explain our finding is that the FO diet significantly increased hepatic mRNA levels of Abcg5/g8, key proteins which regulate hepatic cholesterol secretion into bile³⁰. Wu et al. reported that hepatic Abcg5/g8 overexpression in mice increased cholesterol excretion into bile and feces²⁰. LXR agonists induce Abcg5/g8 expression³¹. Recently, Uehara et al. reported that EPA decreased LXR regulated genes, Abcg1 and Abca1, in an in vitro study³². However, Pawar et al. reported that other LXR regulated genes, Abcg5/g8 and Cyp7a1, were not affected by a diet with 10% FO (w/w) in an in vivo rat model compared to those fed the same diet with olive oil³³. The FO diet used here contains 0.15% cholesterol which in the presence of the fish oil diet could have resulted in increased levels of unesterified cholesterol in the liver and increased generation of oxysterols, which could serve to activate LXR. Furthermore, several investigators reported that PPARα agonists modestly increased Abcg5/g8 expression³⁴,³⁵. EPA and DHA are more potent PPARα agonists compared with mono-unsaturated or saturated fatty acids¹⁸,³⁶. Indeed, in this study FO increased hepatic mRNA expression of Acox, which is a classic target gene of PPARα. Furthermore we observed a significant correlation between Abcg5/g8 and Acox mRNA expression. Although further investigation is needed to clarify the mechanism by which FO increases Abcg5/g8 expression, activation of LXR and/or PPARα by FO are among probable mechanisms.

A third potential mechanism is reduced intestinal re-absorption of biliary HDL-derived cholesterol due to downregulation of intestinal Npc1l1. We have shown that ezetimibe, which inhibits Npc1l1, promotes the fecal excretion of macrophage and HDL-derived cholesterol (Briand et al., 2009 in press). Our data here suggest that the FO diet reduced the expression of Npc1l1. While the mechanism of this observation is unknown, PPAR delta agonists downregulate Npc1l1 as shown by van der Veen et al., 2005 ³⁷ and Briand et al., 2009 (in press), and it is possible that FO could influence Npc1l1 expression through this mechanism.

A limitation of this study is that high doses of omega-3 fatty acids were used. Four capsules of Lovaza, which is the highest recommended dose for treating hypertriglyceridaemia, provides 3.4 g of EPA+DHA, constituting 1.6% energy for a 2000 kcal diet in a clinical situation.
In the present study, the FO diet provided about 8% energy as EPA+DHA, which is five times higher than that of a clinical situation. Consequently, the clinical relevance of these observations requires further studies.

In conclusion, our data indicate that a FO diet promotes the excretion of macrophage- and HDL-derived cholesterol into the feces in mice, which is primarily due to increased hepatic fecal excretion of HDL-derived cholesterol due to several mechanisms. We suggest that this could contribute to the anti-atherosclerotic effects of FO.
Acknowledgement

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Disclosure: None
References


Table 1: Plasma lipids of diet fed mice

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Values are given as mean mg/dL +/- SD N=6 mice per group. ANOVA indicates a significant difference between groups for all lipids (p<0.01). Levels indicates the significance levels where different letters indicate significantly different high-fat diet groups determined by Tukey’s HSD (alpha=0.05). Two-tailed tests were performed to examine differences between low-fat LSO and high-fat HSO diets ( * p<0.05, ** p<0.01).
Figure Legends

**Fig. 1: Macrophage RCT in mice**
Mice were fed $^{1}$SO, $^{11}$SO, CO and FO diets (N=6 per each group). [A] Comparison of high-fat diet groups. ANOVA was performed for each lipid measurement, and if significance was observed (p<0.01) then Tukey’s HSD was applied to test for significance between all groups. Different letters indicate significant differences between diets (Tukey’s HSD, alpha=0.05), absence of letters indicates no significant difference between groups. [B] Comparison of low-fat versus high-fat diet (soybean oil). * Indicates significant different between $^{1}$SO and $^{11}$SO by Student’s t-test (p<0.001).

**Fig. 2: Cholesterol efflux**
cAMP-dependent cholesterol efflux from J774 cells to serum from mice fed $^{1}$SO, $^{11}$SO, CO and FO diets (N=6 per each group). * Indicates significant different between $^{1}$SO and $^{11}$SO by Student’s t-test (p<0.01). Comparison of high-fat diet groups was performed by ANOVA, and if significance was observed (p<0.01) then Tukey’s HSD was applied to test for significance between all groups. Different letters indicate significant differences between diets (Tukey’s HSD alpha=0.05), whereas absence of letters indicates no significant difference between groups.

**Fig. 3: Cholesterol efflux in MPM**
Mice were fed $^{1}$SO, $^{11}$SO, CO and FO diets for 6 wks then injected with thioglycollate broth. After 4 days, MPM were collected and individually seeded (N=4 per each group). Cells were then incubated with or without pooled mouse serum [A] or human APOA-1 [B]. There were no significant differences between $^{1}$SO and $^{11}$SO by t-test. Comparisons between high-fat groups was performed by ANOVA, if significance was observed (p<0.01) then Tukey’s HSD was applied to test for significance between all groups. Different letters indicate significant differences between diets (Tukey’s HSD, alpha=0.05).

**Fig. 4: HDL kinetics in mice**
Mice were fed $^{1}$SO, $^{11}$SO or FO diets (N=5 per each group) for 4 weeks received intravenously injections of $[^3]$H- cholesterol oleate labeled HDL. Fractional catabolic rate (FCR) for plasma
HDL-CE was calculated based on the disappearance of [3H]-tracer from plasma of each group. The FCR was transformed by a multiplying by a factor of 10 for scale purposes. * Indicates significant different between LSO and HSO by Student’s t-test (p<0.01). Different letters between the high-fat groups HSO and FO also indicates a significant difference by Student’s t-test (p<0.01).

**Fig. 5: mRNA abundance in [A] liver and [B] small intestine from mice kept on each of the four diets.** Data are presented relative to HSO, which is set at 1.0 for each gene. The symbol *indicates a significant different between LSO and HSO by Student’s t-test (p<0.01). Statistical comparison of the high-fat diet groups was performed by ANOVA, and if a significant difference was observed (p<0.01) then Tukey’s HSD was applied. Different letters indicate significant differences between diets (Tukey’s HSD alpha=0.05), whereas absence of letters indicates no significant difference between groups. A post-hoc non-parametric analysis (Wilcoxon / Kruskal-Wallis test) was performed for the genes Fas, Scd1, Abca1, and Abcg1 (Barlett test p<0.001) since their variation was not normally distributed. Under this analysis Fas and Scd1 were significantly lower in FO than in the other three high fat diets (p<0.005) as denoted by the symbol †.