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Short Term Acyl-CoA:Cholesterol Acyltransferase Inhibition, Combined with Apoprotein A1 Over-expression, Promotes Atherosclerosis Inflammation Resolution in Mice

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ACAT Inhibition, in Combination with APOA1, Promotes Atherosclerosis Resolution

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Non-standard abbreviations: ABCA1, ATP binding cassette transporter subfamily A member 1; ACAT, acyl-CoA:cholesterol acyltransferase; BHK, baby hamster kidney; CCL2, C-C motif chemokine ligand 2; CD36, cluster of differentiation 36; CD68, cluster of differentiation 68; CE, cholesteryl ester; ER, endoplasmic reticulum; FC, free cholesterol; FIZZ1, resistin-like molecule alpha 1; HDL, high-density lipoprotein; HDL-P, HDL particle; HPRT, hypoxanthine-guanine
phosphoribosyltransferase; IL-1β, interleukin 1β; LCM, laser-capture microdissection; OCT, optimal cutting temperature; RT-PCR, real-time polymerase chain reaction; VLDL, very-low density lipoprotein; WD, Western diet. TNFα, tumor necrosis factor-alpha; MRC1, mannose receptor C C-Type 1; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3.
Abstract

Acyl-CoA:cholesterol acyltransferase (ACAT) mediates cellular cholesterol esterification. In atherosclerotic plaque macrophages, ACAT promotes cholesteryl ester accumulation, resulting in foam cell formation and atherosclerosis progression. Its complete inactivation in mice, however, showed toxic effects because of an excess of free cholesterol (FC) in macrophages, which can cause ER stress, cholesterol crystal formation, and inflammasome activation. Our previous studies showed that long-term partial ACAT inhibition, achieved by dietary supplementation with Fujirebio F1394, delays atherosclerosis progression in apoprotein E-deficient (Apoe\(^{-/-}\)) mice by reducing plaque foam cell formation without inflammatory or toxic effects. Here, we determined whether short-term partial inhibition of ACAT, in combination with an enhanced systemic FC acceptor capacity, has synergistic benefits. Thus, we crossbred Apoe\(^{-/-}\) with human apoprotein A1-transgenic (APOA1\(^{tg/tg}\)) mice, which have elevated cholesterol-effluxing high-density lipoprotein particles, and subjected Apoe\(^{-/-}\) and APOA1\(^{tg/tg}/Apoe^{-/-}\) mice to an atherogenic diet to develop advanced plaques. Then mice were either euthanized (baseline) or fed purified standard diet with or without F1394 for four more weeks. Plaques of APOA1\(^{tg/tg}/Apoe^{-/-}\) mice fed F1394 showed a 60% reduction of macrophages accompanied by multiple other benefits, such as reduced inflammation and favorable changes in extracellular composition, in comparison to Apoe\(^{-/-}\) baseline mice. In addition, there was no accumulation of cholesterol crystals or signs of toxicity. Overall, these results show that short-term partial ACAT inhibition, coupled to increased cholesterol efflux capacity, favorably remodels atherosclerosis lesions, supporting the potential of these combined therapies in the treatment of advanced atherosclerosis.
Significance Statement

Short-term pharmacological inhibition of ACAT-mediated cholesterol esterification, in combination with increased free cholesterol efflux acceptors, has positive effects in mice by (1) reducing the inflammatory state of the plaque macrophages, and (2) favoring compositional changes associated with plaque stabilization. These effects occur without toxicity, showing the potential of these combined therapies in the treatment of advanced atherosclerosis.
Introduction

The enzyme acyl:CoA:cholesterol acyltransferase (ACAT) esterifies free cholesterol (FC) to cholesteryl esters (CEs), a key step in intracellular cholesterol storage and hepatic lipoprotein secretion. The mammalian genome encodes two endoplasmic reticulum (ER) enzymes with ACAT activity. While both ACAT isoforms are essential regulators of systemic cholesterol metabolism, ACAT2 is mostly expressed in the liver and small intestine regulating the content of CEs in newly secreted very low-density lipoproteins (VLDL) and chylomicrons, respectively. Most cell types, including macrophages, express ACAT1, which is required for the formation of CEs and lipid droplets (Chang et al., 2009; Lee et al., 2000; Makino et al., 2016). ACAT1 is crucial during the transition of macrophages to foam cells in atherosclerotic plaques, one of the first steps in disease progression that ultimately evolves in the development of coronary artery disease, the main cause of death worldwide (Moore et al., 2013).

Compound F1394 was synthesized and described by Fujirebio Pharmaceuticals as a competitive pan-inhibitor of ACAT activity (Kusunoki et al., 1995b). It showed therapeutic potential by reducing hepatic cholesterol secretion and intestinal cholesterol absorption in preclinical animal models (Aragane et al., 1998; Kusunoki et al., 1995a). Later, our group showed that dietary supplementation with a partial inhibitory dose of F1394 delayed progression of atherosclerotic plaque size in apoprotein E-deficient (Apoe−/−) mice, even in the case of complex preexisting lesions (Kusunoki et al., 2001; Rong et al., 2013). We did not determine, however, effects on the level of inflammation in plaques, an issue of increased clinical relevance in light of the CANTOS trial (Ridker et al., 2017).

In the previous studies, F1394 did not show cellular or tissue toxicity (Aragane et al., 1998; Kusunoki et al., 2001; Rong et al., 2013; Rong et al., 2005), unlike the genetic ablation of ACAT1 either globally or in myeloid cells (Accad et al., 2000; Fazio et al., 2001), indicating that the partial, rather than complete ACAT inhibition could have therapeutic applications. The
absence of toxicity in the plaques of F1394-treated mice implied that partial ACAT inhibition resulted in a level of cellular FC that could be safely maintained by the efflux capacity of high-density lipoprotein (HDL) particles (HDL-Ps) (Rong et al., 2013), thereby preventing the activation of either ER stress (Feng et al., 2003) or inflammasomes (Duewell et al., 2010). Consistent with this is finding that in the ACAT-inhibited mice, both FC and CE plaque contents were reduced compared to control Apoe<sup>-/-</sup> mice (Rong et al., 2013). Therefore, we reasoned that if the naturally low HDL-P concentrations in Apoe<sup>-/-</sup> mice were increased concurrent with ACAT inhibition, we would observe even greater benefits on reducing atherosclerosis. Besides the observations reviewed above, an additional cause for optimism came from previous studies by us (Feig et al., 2011b; Rong et al., 2001) and others (Shah et al., 2001; Son et al., 2016; Tangirala et al., 1999) showing that raising HDL-P levels, by increasing the production of its key protein apoprotein A1 (Apoa1), in mouse models of atherosclerosis resulted in several beneficial changes in preexisting plaques, even without ACAT inhibition (Barrett et al., 2019).

In the current study, we sought to determine whether short-term dietary supplementation with the ACAT inhibitor F1394 stimulated the resolution of inflammation in advanced plaques and if the raising of HDL-P could potentiate such an effect. For this purpose, we used Apoe<sup>-/-</sup> mice, as well as human APOA1-transgenic mice (APOA1<sup>tg/tg</sup>) crossed to Apoe<sup>-/-</sup> mice (APOA1<sup>tg/tg/Apoe<sup>-/-</sup></sup>). All mice were fed Western-type diet (WD) until they developed advanced plaques. Then, they were fed standard diet for four weeks (with or without F1394 compound) to lower plasma cholesterol levels. F1394-treated mice showed an improvement in various histological and gene expression markers in atherosclerotic plaques. Notably, these positive effects were greater in the APOA1<sup>tg/tg/Apoe<sup>-/-</sup></sup> mice treated with F1394, consistent with enhanced FC efflux capacity resulting from their elevated plasma HDL-P concentration.
Materials and Methods

Animals and diets

All mice used in this study were on the C57BL/6 background and obtained from Jackson Laboratories (Bar Harbor, Maine). All animal experiments were performed with protocols previously approved by the NYU School of Medicine Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals adopted and promulgated by the U.S. National Institutes of Health. Apoe<sup>-/-</sup> and APOA1<sup>tg/tg</sup> mice were crossbred to generate APOA1<sup>tg/tg</sup>/Apoe<sup>-/-</sup> mice. Male and female Apoe<sup>-/-</sup> and APOA1<sup>tg/tg</sup>/Apoe<sup>-/-</sup> mice were weaned at the age of three weeks and caged in groups of three to five mice. At the age of four weeks, mice were fed WD containing 21% w/w lard and 0.15% cholesterol (Dyets Inc, Bethlehem, PA). Due to the intrinsic resistance of APOA1<sup>tg/tg</sup>/Apoe<sup>-/-</sup> mice to develop atherosclerotic lesions when compared to Apoe<sup>-/-</sup> mice (Choudhury et al., 2004), we fed WD to these two strains 16 and 12 weeks, respectively. After this period, one or two mice from each cage were sacrificed as the baseline group, while their siblings were switched to a purified standard chow diet (control group) or purified standard chow diet containing 900 mg/kg of Fujirebio compound F1394 (ACAT inhibitor group) for four extra weeks before tissue harvest.

Mice were euthanized at the completion of the dietary interventions with a peritoneal injection of ketamine/xylazine. Blood was collected in EDTA-containing tubes via cardiac puncture for plasma analyses. Mice were then perfused with 10% sucrose in saline solution (0.9% NaCl in water) for at least three minutes. Aortic roots were embedded in optimal cutting temperature (OCT) compound (Sakura, Torrance, CA), and OCT blocks immediately frozen at -80°C.

Aortic Analysis – Immunostaining and microscopy
Serial sections of 6-µm thickness were collected and mounted on glass slides. We detected the amount of the cluster of differentiation 68 (CD68)+ cells in the aortic lesions with a rat anti-mouse CD68 antibody (Bio-Rad, CA), as previously described (Yu et al., 2017). Briefly, sections were fixed and permeabilized with 100% ice-cold acetone, blocked, and stained with anti-CD68 antibody. The primary antibody was detected after the incubation with biotinylated anti-rat IgG secondary antibody (Vector Laboratories, CA), and visualized using the Vectastain ABC kit (Vector Laboratories). Slides were counterstained with hematoxylin/eosin (Sigma), dehydrated in an ethanol gradient and xylene (Fisher Scientific, NH), and mounted with coverslips using Permount (Fisher Scientific).

Total collagen content in lesions was detected with a picrosirius red staining. Briefly, glass slides were fixed using 10% formalin solution (Sigma) for 10 minutes at room temperature, washed with PBS, and stained with picrosirius red solution (Polysciences, Inc., PA) for one hour. Stained slides were washed, dehydrated with ethanol and xylene, and mounted with coverslips using Permount (Fisher Scientific). We localized the position and area of the atherosclerotic lesion by using the bright field, and determined the presence of collagen in the plaque by using polarized light microscopy. Polarized light images were taken after applying background correction, as done previously (Peled et al., 2017). For the visualization of cholesterol crystals, we fixed the slides using 10% formalin solution (Sigma) for 10 minutes at room temperature, washed with PBS. Nuclei were stained using a solution containing 1 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI) for 5 minutes at room temperature. To set up the background correction for the cholesterol crystals, we utilized a sample exposed to acetone for 5 minutes in which all the cholesterol crystals were dissolved, as done in the past (Josefs et al., 2020). Crystals were visualized using a 10x N.A. 0.3 plan lens on Zeiss AxiObserver AxioObserver.Z1 microscope with crossed polarizing filters and an Axiocam 503 Monochrome camera with Zen 2.6 software.
Bright-field images of stained sections were taken at 10x magnification using a Leica microscope. Polarized light microscopy images were taken using DAPI as a counterstain. Morphometric analyses were performed using ImageProPlus 7 (Micro Optical Solutions LCC, MA).

Our results are based on a single arterial site of analysis, namely the proximal aorta. This and its related site (aortic root) are often considered as surrogates for human atherosclerosis, based on histopathological studies, though there are also a number of differences, such as the lack of plaque rupture (Nakashima et al., 1994).

Aortic Analysis – Laser capture microdissection and RT-PCR analysis

Total mRNA from CD68+ cells was isolated from atherosclerotic plaques using laser capture microdissection (LCM), as previously described (Yu et al., 2017). Briefly, aortic root sections were stained with hematoxylin/eosin in RNAse-free conditions to isolate the CD68+ cells using consecutive guiding sections previously stained for CD68+ cells (described above). CD68+ cells from each sample were pooled, purified, and total mRNA was isolated using the PicoPure Kit (ThermoFisher Scientific). The quality and quantity of the RNA samples were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was converted to cDNA and amplified using the WT-Ovation Pico RNA Amplification Kit (NuGEN, San Carlos, CA). Real-time polymerase chain reaction (RT-PCR) analyses were performed using 5 ng of amplified cDNA in the Quantstudio 7 Flex (Applied Biosystems).

Quantitative RT-PCR was performed with Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA), and Taqman primer/probe mixes for mouse monocyte C-C motif chemokine ligand 2 (CCL2), tumor necrosis factor-alpha (TNFα), ATP binding cassette transporter subfamily A member 1 (ABCA1), resistin-like molecule alpha 1 (FIZZ1), mannose
receptor C C-Type 1 (MRC1), cluster of differentiation 36 (CD36), NLR family pyrin domain containing 3 (NLRP3), and interleukin 1β (IL-1β). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a housekeeping control gene. Gene expression was calculated using the ΔΔCt method.

**Plasma cholesterol concentration**

Plasma cholesterol measurements were performed using standard colorimetric assays (Wako).

**Plasma HDL concentration**

HDL-P concentration was measured by calibrated differential ion mobility analysis (Hutchins et al., 2014; Vaisar et al., 2020). Total HDL-P number was obtained by summing the concentrations of the three main HDL subspecies (small, medium, and large HDL).

**Cholesterol Efflux Assays**

Cholesterol efflux capacity of the serum HDL was measured after precipitation of apoB-containing lipoproteins with polyethylene glycol (Sigma). Confluent J774 macrophages were stimulated with cyclic AMP for 24 hours to upregulate ABCA1 expression and incubated with 0.5 µCi [³H]cholesterol/mL. Cells were exposed to the different mouse plasma for 4 hours to measure the cellular efflux of cholesterol to HDL. All steps were performed in the presence of the 4 µg/mL of the ACAT inhibitor Sandoz S9318 (Sigma) to promote cholesterol efflux.

Alternatively, baby hamster kidney (BHK) cells over-expressing human ABCA1 (ABCA1-BHK) under the regulation of mifepristone (10 nM) were used to measure ABCA1-dependent cholesterol efflux in the presence of 0.5 µCi [³H]cholesterol/mL during 24 hour incubation. Next, cells were exposed to the different mouse plasmas for 4 hours to measure the cellular efflux of cholesterol to HDL (n = 3/group). We calculated the ABCA1-specific cholesterol efflux capacity by calculating the differences between ABCA1-BHK incubated in the absence (control cells) and
the presence of mifepristone (ABCA1 overexpression). All experiments were performed the same day to avoid variation.

**Study outcomes and statistical analyses**

Data are expressed as mean ± standard deviation (SD). For the animal study, the primary objective was to determine the effect of the genotype (**Apoe**⁻/⁻ and **APOA1**tg/tg/Apoe⁻/⁻ mice) and the experimental group (Baseline, Control diet, ACAT inhibitor diet) on CD68+ cell content. Total cholesterol, lesion area, collagen content, cholesterol crystal content, and mRNA abundances of CCL2, NLRP3, IL-1β, FIZZ1, MRC1, CD36 and ABCA1 were assessed as secondary outcomes. We conducted two-way ANOVA with interaction to assess the association among outcome, genotype, and experimental group. If the interaction term was non-significant, the model was then re-fitted with only main effects. The pair-wise comparisons among study groups were implemented using t-test. The Benjamini-Hochberg's method was adopted to adjust for the multiple comparison in the pair-wise assessment, and the analyses of secondary outcomes. The significance level was set to 5%, and statistical analyses were performed in software program R (4.0.3).

For the determination of plasma HDL-P and cholesterol efflux assays, we conducted one-way ANOVA with Dunnett’s multiple comparisons test in GraphPad Prism 9, with a significance level set to 5%.
Results

**Effects of a modest reduction of hypercholesterolemia and concurrent treatment with F1394 on atherosclerosis regression in Apoe<sup>-/-</sup> and APOA<sup>Tg/Tg</sup>/Apoe<sup>-/-</sup> mice**

We wished to determine whether short-term ACAT inhibition, in combination with a modest reduction of plasma cholesterol and enhanced cholesterol efflux acceptor capacity, would favor the regression of inflammation of clinically-relevant advanced, complex atherosclerotic lesions in Apoe<sup>-/-</sup> mice. To achieve such lesions, which contain lipid cores and cholesterol crystal clefts (American Heart Association lesion class IV (Stary et al., 1995)), we fed Apoe<sup>-/-</sup> mice 12 weeks with WD. Because APOA<sup>Tg/Tg</sup>/Apoe<sup>-/-</sup> mice show intrinsic resistance to develop atherosclerotic lesions, we fed these mice WD 16 weeks to obtain atherosclerotic plaques of comparable size, based on our previous studies (Choudhury et al., 2004; Rong et al., 2001). To establish baseline plaque characteristics before the interventions, subsets of mice were sacrificed after either 12 (Apoe<sup>-/-</sup>) or 16 (APOA<sup>Tg/Tg</sup>/Apoe<sup>-/-</sup>) weeks of WD feeding. The rest of the mice were randomly divided into groups consuming either a purified standard chow diet (control diet) or a purified standard chow diet containing 900 mg/kg of the ACAT inhibitor F1394 for four weeks (Figure 1A). Standard chow was used to cause a modest reduction in plasma cholesterol levels, as would occur in patients improving their diet after diagnosis of coronary artery disease. Although F1394 will inhibit ACAT in liver and intestine, previous studies (Kusunoki et al., 2001) have shown the atherosclerosis effects to be likely attributable to ACAT inhibition in plaque macrophages, consistent with finding a reduction in aortic cholesterol ester content (Rong et al., 2013).

For this study, we used a comparable number of male and female mice in each experimental group (n = 5 to 7 gender/group). We did not observe gender differences among groups, as both Apoe<sup>-/-</sup> and APOA<sup>Tg/Tg</sup>/Apoe<sup>-/-</sup> male and female mice responded similarly to our experimental...
interventions (diet switch and F1394 treatment) for all our analytical outcomes (data not separately shown). Total plasma cholesterol concentrations were comparable between baseline mice independently of their genotype. The experimental group showed a significant effect on the total cholesterol level (adjusted P = 0.001) although no effect by genotype was established (adjusted P = 0.46). Mice switched to control diet for four weeks showed a reduction in plasma cholesterol levels in both genotypes, independent of the presence of F1394 (Figure 1B). We observed a trend towards increasing in total cholesterol in $APOA1^{tg/tg}/Apoe^{-/-}$ mice in comparison to $Apoe^{-/-}$ controls, which could be explained by increased cholesterol in the HDL fraction (Supplementary Figure 1), as originally described by Plump and colleagues (Plump et al., 1994).

Next, we quantified the atherosclerotic lesion area present at the level of the aortic root in all of the groups. Both $Apoe^{-/-}$ and $APOA1^{tg/tg}/Apoe^{-/-}$ mice developed lesions of similar size, independent of the plasma cholesterol concentrations or F1394 treatment (Figure 1C, D). The size of an atherosclerotic lesion, however, is determined by the net contributions of individual components, particularly macrophages and extracellular matrix. In this regard, we have reported major decreases in plaque macrophage content without a change in the total area because of counterbalancing increases in collagen content (e.g., (Parathath et al., 2011)). Thus, we evaluated the amount of CD68+ cells (a marker of macrophages and macrophage foam cells) to estimate the inflammatory cell content of the plaque. Our analysis detected a significant interaction by two-way ANOVA between the genotype and experimental group factors (P = 0.02). Pair-wise comparisons are represented in Figure 1E. As expected from previous studies of atherosclerosis progression (e.g.(Choudhury et al., 2004; Plump et al., 1994; Rong et al., 2001; Rubin et al., 1991)), that baseline $APOA1^{tg/tg}/Apoe^{-/-}$ mice had a nearly 35% reduction in macrophage plaque content when compared to baseline $Apoe^{-/-}$ mice (Figure 1E). In comparison to $Apoe^{-/-}$ baseline mice, mice fed F1394 diet showed with reductions in CD68+ area of approximately 30% in $Apoe^{-/-}$ mice and 60% in $APOA1^{tg/tg}/Apoe^{-/-}$ mice (Figure 1E).
Effects of a modest reduction of hypercholesterolemia and concurrent treatment with F1394 on plaque macrophage inflammatory status in Apoe<sup>−/−</sup> and APOA<sub>1</sub>tg/tg/Apoe<sup>−/−</sup> mice

The results in Figure 1 showed the changes in macrophage content in each group. As we have reviewed, atherosclerosis regression typically involves the dampening of macrophage inflammation (Moore et al., 2013). To evaluate the inflammatory status of plaque macrophages, we isolated CD68+ cells from atherosclerotic lesions using LCM. Total mRNA was isolated and used to quantify the expression of target genes related to inflammation and macrophage polarization by RT-PCR.

As shown in Table 1, the markers of inflammation characteristic of the pro-inflammatory M1 phenotype showed a numerical trend towards the lowest levels in the plaque macrophages from APOA<sub>1</sub>tg/tg/Apoe<sup>−/−</sup> mice fed control diet and the ACAT inhibitor F1394. In the case of markers associated with the M2, inflammation-resolving, phenotype such as Fizz1, Mcr1, and Cd36, we observed that APOA<sub>1</sub>tg/tg/Apoe<sup>−/−</sup> mice treated with ACAT inhibitor F1394 showed the highest expression values. None of these measurements, however, reached statistical significance by two-way ANOVA analysis (Table 2).

Overall, these results indicate that the partial ACAT inhibition achieved by F1394 tilted the balance of inflammatory to anti-inflammatory features in APOA<sub>1</sub>tg/tg/Apoe<sup>−/−</sup> mice towards the state that we have found to be characteristic of plaques undergoing atherosclerosis resolution (Rahman and Fisher, 2018).

Effects of a modest reduction of hypercholesterolemia and concurrent treatment with F1394 on plaque extracellular features in Apoe<sup>−/−</sup> and APOA<sub>1</sub>tg/tg/Apoe<sup>−/−</sup> mice

One of the hallmarks of inflammation resolution is the accumulation of collagen in the plaque (e.g., (Feig et al., 2011a)), which in humans is thought to stabilize atherosclerotic lesions preventing their rupture (Libby and Aikawa, 2002). We quantified the presence of collagen in
plaques stained with picrosirius red by polarized light microscopy followed by image analysis (Figure 2A). Collagen content showed a numerical trend towards increasing in mice switched to chow diet, regardless of their genotype, though statistical significance was not reached (Table 2). This increase was more pronounced in mice treated with F1394, especially in $\text{APOA1}^{\text{tg/tg}}/\text{Apoe}^{-/-}$ mice fed F1394 (Figure 2B).

Early studies using an $\text{Acat1}$-deficient mouse model showed that either the complete absence of this enzyme or its selective deficiency in macrophages promoted atherosclerosis progression (Fazio et al., 2001). Some of these effects were attributed to the formation of cholesterol crystals, which occurs spontaneously when excessive cholesterol monohydrate accumulates (Grebe and Latz, 2013). In turn, this accumulation activates the NLRP3 inflammasome, thereby increasing inflammation (Duewell et al., 2010). To determine whether the partial inhibition of ACAT also affects the formation of cholesterol crystals in plaques, we evaluated their presence by using confocal reflection microscopy (Figure 2C). Our quantification did not show any significant changes in cholesterol crystal accumulation under any treatment or genotype (Figure 2D).

**Effects of increased APOA1 expression on cholesterol efflux capacity in plasma**

The above results show a number of benefits to plaque macrophages by combining the inhibition of cholesterol esterification by F1394, and an increase in HDL-P resulting from the overexpression of human APOA1. To test for an association with these benefits with cholesterol efflux capacity of the plasma, given the differences in HDL levels, we isolated plasma from $\text{Apoe}^{-/-}$, $\text{APOA1}^{\text{tg/tg}}$ and $\text{APOA1}^{\text{tg/tg}}/\text{Apoe}^{-/-}$ mice, and performed the following in vitro analyses.

First, we quantified the total HDL-P concentration in plasma samples. $\text{Apoe}^{-/-}$ mice have intrinsic low HDL-P (Plump et al., 1994), but when crossed with $\text{APOA1}^{\text{tg/tg}}$ mice, HDL-P number in $\text{APOA1}^{\text{tg/tg}}/\text{Apoe}^{-/-}$ mice is restored (Figure 3A).
Next, we quantified the cholesterol efflux capacity in apoB-depleted plasma samples using an established assay, in which murine J774 macrophages were loaded with radiolabeled cholesterol (Borja et al., 2015). As shown in Figure 3B, cholesterol efflux results paralleled the concentrations of HDL-P in each mouse strain. Low levels of efflux were observed in the samples from Apoe$^{-/-}$ mice, with relatively higher levels in the samples from the APOA1$^{tg/tg}$/Apoe$^{-/-}$ and APOA1$^{tg/tg}$ mice (Figure 3B). To evaluate whether this enhanced efflux was through the ABCA1-mediated pathway, the major efflux pathway utilized by foam cells (Adorni et al., 2007), we performed assays in an established system for this purpose (Borja et al., 2015). Thus, BHK cells over-expressing ABCA1 (ABCA1-BHK) were used for efflux assays using the aforementioned mouse plasma samples. Again, we observed that plasma isolated from both lines of mice over-expressing the human APOA1 transgene favored cholesterol efflux in comparison to plasma isolated from Apoe$^{-/-}$ mice (Figure 3C).

The results in vitro indicate that the beneficial changes in plaque macrophage phenotype in vivo were also associated with the increase in cholesterol efflux capacity due to the raised level of HDL-P concentration in APOA1$^{tg/tg}$/Apoe$^{-/-}$ versus Apoe$^{-/-}$ mice. Our in vitro analyses show that this increased capacity was mediated in part by ABCA1, which also contributes to cholesterol efflux in vivo (Westerterp et al., 2014). Therefore, for completeness, we measured the level of Abca1 expression in LCM-isolated plaque macrophages processed as in Table 1, although we did not observe changes between groups (Figure 3D and Table 2). Abca1 expression is induced by the accumulation of FC (reviewed in (Baldan et al., 2009)). These results, then, likely reflect the ability of the increase in HDL-P concentration to unload the excess FC that would be expected to accumulate when ACAT is inhibited, thereby removing the stimulus for the upregulation of macrophage Abca1 gene expression.
Discussion

Cholesterol efflux from macrophages is a complex process involving the interplay among extracellular cholesterol acceptors (HDL/apoA1), membrane transporters ABCA1/G1, intracellular CE hydrolases, and acylCoA:transferases (ACATs) (Westerterp et al., 2014). As FC accumulates in macrophages, it is esterified to CEs by ACAT and foam cells form. Over time, foam cells accumulate in atherosclerotic plaques, and local inflammation increases. This disease progression has been thought to be amenable to ACAT inhibition, which would prevent the conversion of FC to CE, thereby allowing the removal of FC by efflux pathways mediated by HDL, as CE cannot be effluxed. One major drawback of this strategy is that complete absence of ACAT activity apparently overwhelms the efflux capacity of HDL, which would result in ER stress (Feng et al., 2003) and the formation of cholesterol crystals (Duewell et al., 2010; Fazio et al., 2001), both possibilities expected to result in even greater plaque inflammation.

In one of our previous studies, we tested the effects of partial inhibition of ACAT activity, to see if we could overcome this drawback and observe benefits on reducing atherosclerosis. Partial inhibition in Apoe−/− mice was achieved by supplementation of the WD with a 900mg/kg diet of F1394 compound during 17 weeks, which resulted in ~70% reduction in macrophage ACAT activity. This intervention reduced atherosclerosis progression without showing detrimental effects (Kusunoki et al., 2001). Because most patients have significant atherosclerosis at time risk reduction is initiated (Robinson et al., 2018), we were interested in extending these results by studying the potential for partial inhibition of ACAT to have beneficial effects on preexisting plaques. In our first study towards this goal, Apoe−/− mice were first fed WD to develop advanced lesions, then some were fed for an additional 14 weeks a chow diet that either contained or did not, F1394 (Rong et al., 2013). In the non-F1394 group, there was a subsequent increase in plaque macrophage content, which was prevented in the F1394 group, again, with no detrimental effects.
In the present study, we wished to improve the effectiveness of F1394 on preexisting atherosclerosis and hypothesized that this could be achieved by increasing extracellular cholesterol acceptor capacity, concurrent with F1394 treatment. The rationale for this was based on the expected consequences of inhibiting cholesterol esterification in macrophages. While this inhibition would suppress foam cell formation, cholesterol ordinarily destined for esterification would increase the content of FC, which can have adverse effects on macrophages in atherosclerotic plaques. By providing additional FC acceptors, we would potentially augment the protection already afforded by native levels of HDL-Ps, and if so, this would be manifested by observable benefits to preexisting atherosclerosis in a shorter time period than in previous studies. This reasoning is also consistent with the speculation by Fazio et al. (Fazio et al., 2001) that adverse effects on plaque macrophages when cholesterol esterification was blocked could be offset by interventions that would promote FC efflux.

Thus, the present protocol now entailed partial inhibition of ACAT activity using the F1394 compound in the chow diet, as before, but now only for four weeks (vs. the 14 weeks in (Rong et al., 2013)). In addition, we compared the effects of the treatment in Apoe⁻/⁻ mice to those in Apoe⁻/⁻ mice crossed with APOA1tg/tg/Apoe⁻/⁻ mice, which increases the level of cholesterol efflux-mediating HDL-Ps (Choudhury et al., 2004; Plump et al., 1994; Rubin et al., 1991). Our major findings provide strong support for our hypothesis by showing that the combination of the ACAT inhibitor and APOA1 overexpression had greater benefits than either intervention alone and that these were achieved in a shorter period than we previously observed. In addition, the shorter intervention with the ACAT inhibitor did not affect plasma cholesterol levels (Figure 1B), as happens with longer feeding periods (Kusunoki et al., 2001; Rong et al., 2013). Thus, rather than confounding the interpretation of the changes in atherosclerosis by altering lipids systemically, the results with partial inhibition by F1394 highlight its effects on plaque macrophages, the primary storage pool of CE in atherosclerosis.
Tall’s group reported back in 2010 that hypercholesterolemia favors myelopoiesis, and that the presence of cholesterol acceptors such as HDL can suppress this process (Yvan-Charvet et al., 2010). We measured total white blood cells, neutrophils, and monocytes levels at the moment of the sacrifice, and we observed a trend towards increased cell numbers in Apoe−/− baseline mice in comparison to the other experimental groups (Supplementary Figure 2). These results led us to discard alterations in myelopoiesis as the driver of the effects we observed upon ACAT inhibition.

The benefits we observed were primarily in the areas of plaque macrophage content and inflammation status, together with plaque extracellular composition. That the largest relative reduction in plaque macrophage content was in APOA1tg/tg/Apoe−/− mice fed F1394 was expected, based on our previous studies. First, we have shown macrophage emigration from plaques is inversely associated with plaque cholesterol content (Feig et al., 2011b), which is reduced by increasing HDL-P levels (Feig et al., 2011b; Hewing et al., 2014). Consistent with these findings, the present data show that cholesterol efflux capacity was higher in the plasma of APOA1tg/tg/Apoe−/− vs. Apoe−/− mice (Figure 3). Second, by inhibiting esterification, F1394 shifts the plaque macrophage cholesterol pool from CE to FC (Rong et al., 2013), which can be ordinarily removed, but even more efficiently by the intrinsic increased efflux capacity of APOA1tg/tg/Apoe−/− mice.

Turning to inflammation, there is a wide range of macrophage phenotypes in plaques, from pro-inflammatory M1 to anti-inflammatory M2 (Amengual and Barrett, 2019; Rahman and Fisher, 2018). When compared to the other experimental groups, APOA1tg/tg/Apoe−/− mice fed control diet or F1394 exhibited a numerical trend towards a down-regulation of the expression of markers associated with the M1 state. On the contrary, APOA1tg/tg/Apoe−/− mice fed F1394 showed a trend towards an up-regulation of M2 markers (Table 1), suggesting a shift in plaque macrophages from predominately a pro- to an anti-inflammatory state. These results are in
accordance with our previous data, where we showed that M2 macrophages are enriched in regressing plaques, where they play a key role in favorable plaque remodeling (reviewed in (Moore et al., 2018)).

This remodeling includes an increase in the collagen content of plaques (Figure 2), thought to be a sign of stability in human plaques e.g. (Libby and Aikawa, 2002). Collagen content showed a numerical trend towards an increase upon F1394 feeding, which was more pronounced in the APOA1<sup>tg/tg</sup>/Apoel<sup>−/−</sup> mice, consistent with their larger M2 and smaller M1 populations of plaque macrophages, which have increased collagen synthesis and degrading activities, respectively (reviewed in (Amengual and Barrett, 2019; Rahman and Fisher, 2018)). Another compositional aspect is cholesterol crystal content. In mouse models of atherosclerosis, either globally deficient in ACAT1 (Accad et al., 2000) activity or transplanted with bone marrow from Acat1<sup>−/−</sup> mice (Fazio et al., 2001), macrophages exhibited toxicity and increased inflammation. This not only reflected that FC efflux mechanisms were exceeded, but also that the concentration of macrophage FC rose sufficiently high to cause ER stress (Feng et al., 2003) and inflammasome-activation by cholesterol crystals (Duewell et al., 2010).

As we have previously shown, cholesterol loading reprograms vascular smooth muscle cells (VSMC) to a macrophage-like foam cell state (Rong et al., 2003). In our model, these cells may have been affected by F1394 in ways similar to bona-fide macrophage foam cells. However, at the time aortae from the baseline mice were harvested, the numbers of VSMC foam cells would be expected to be few (Misra et al., 2018), and that cholesterol efflux via the ABCA1 transporter may have been limited, given reports showing that VSMC express low levels of ABCA1 (Choi et al., 2009). Future experiments using VSMC lineage marked mice will be needed for definitive investigations of whether VSMC and macrophage foam cells respond differentially to ACAT inhibition.
Notably, we found no evidence of increased cholesterol crystals in any of the groups, in line with our previous studies (Kusunoki et al., 2001; Rong et al., 2013). This is likely because partial inhibition of ACAT does not result in a level of macrophage FC that overwhelms even the native levels of HDL-P in \textit{Apoe}^{-/-} mice, let alone the higher levels in \textit{APOA1}^{tg/tg}/\textit{Apoe}^{-/-} mice. This lack of exacerbation of plaque cholesterol crystals by partial inhibition of ACAT activity is also consistent with the macrophage inflammatory markers (Table 1).

Overall, we have extended our previous studies by showing that increasing the concentration of HDL-Ps at the same time of inhibiting ACAT activity results in a number of beneficial changes in atherosclerotic plaques in a relatively short time. These positive findings need to be considered in the context of the status of ACAT as a therapeutic clinical target in atherosclerosis. Once considered promising, it started to fall out of favor after the mouse ACAT knockout studies were published, and slid further after negative intravascular ultrasound (IVUS) studies of patients with atherosclerosis who were treated with ACAT inhibitors (Nissen et al., 2006; Tardif et al., 2004).

On one hand, the aforementioned mouse knock out studies imply that complete deficiency of ACAT1, at least when combined with APOE and LDLR deficiencies either in all tissues (Accad et al., 2000) or in transplanted bone marrow myeloid cells in \textit{Ldlr}^{-/-} recipient mice (Fazio et al., 2001) is too extreme. On the other hand, in another study, \textit{Apoe}^{-/-} mice with advanced atherosclerosis and macrophage/neutrophil deficiency of ACAT1 (compared to \textit{Apoe}^{-/-} controls) exhibit reduced plaque macrophage content and inflammation (Melton et al., 2019), just as we found. However, myeloid ACAT1 deficiency reduced overall survival by approximately 25% in comparison to \textit{Apoe}^{-/-} controls (Melton et al., 2019), suggesting that there may be negative effects when ACAT1 is absent, even in myeloid cells, and that such effects are avoided by only inhibiting ACAT activity partially.
Turning to the clinical studies, it is not clear what the tissue-level of ACAT inhibition achieved was, so direct comparisons to the pre-clinical studies, including the present one, are difficult. Nevertheless, it is interesting to recall that in the editorial (Fazio and Linton, 2006) accompanying one of the IVUS trials (Nissen et al., 2006), the authors wrote: “If there is a clinical future for ACAT inhibitors, it may be to combine them with therapies that increase the number of acceptor particles in the plaque, leading to the export of FC from macrophages into the HDL pathway”. The present data certainly provide support for this suggestion.

Another point arising from the IVUS studies, which were focused on the assessment of stable atherosclerotic plaques, is that the negative results do not preclude the potential for success of the short-term use of an ACAT inhibitor concurrent with increasing HDL-P levels. Given that currently tested are short term infusions of HDL or HDL-like particles in acute coronary syndrome patients (reviewed in (Di Bartolo et al., 2018)), it is tempting to suggest that an ACAT inhibitor in combination with HDL-Ps would be an effective approach to favorably modify the culprit plaques by reducing their inflammation and increasing their stability.
Acknowledgments

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Authorship contributions

Participated in research design: J.A. E.A.F


Wrote or contributed to the writing of the manuscript: J.A., E.A.F.
References


Footnotes

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Legends for Figures

Figure 1. (A) Experimental design. Apoe<sup>−/−</sup> and APOA1<sup>tg/tg</sup>/Apoe<sup>−/−</sup> male and female mice (n = 5 to 7 gender/group) were fed Western diet for 12 or 16 weeks, respectively. After this period, a subset of mice was sacrificed (Baseline group, black symbols) while the rest of the mice were fed chow diet (Control group, blue symbols) or chow diet containing 900 mg/kg of the ACAT inhibitor F1394 (ACAT inhibitor group, red symbols) for 4 extra weeks. (B) Total cholesterol plasma levels. (C) Histological sections at the level of the aortic root stained for CD68 (red) and counterstained with hematoxylin. (D) Lesion area quantification, and (E) relative CD68+ cell content in plaques. Values are means ± SD, n = 8 to 13 mice/group. The pair-wise comparison in figure 1E were evaluated using t-test with Benjamini-Hochberg adjustment. * adjusted P < 0.05, *** adjusted P < 0.005, **** adjusted P < 0.0001.

Figure 2. Collagen and cholesterol crystal contents in atherosclerotic lesions. Apoe<sup>−/−</sup> and APOA1<sup>tg/tg</sup>/Apoe<sup>−/−</sup> male and female mice (n = 2 to 5 gender/group) were fed Western diet for 12 or 16 weeks, respectively. After this period, a subset of mice was sacrificed (Baseline group, black symbols) while the rest of the mice were fed chow diet (Control group, blue symbols) or chow diet containing 900 mg/kg of the ACAT inhibitor F1394 (ACAT inhibitor group, red symbols) for 4 extra weeks. Histological analyses were performed on lesions present at the level of the aortic root at the moment of the sacrifice. (A) Collagen content in the plaque, (B) and its quantification. Upper panels show polarized microscopy pictures depicting collagen (red), bottom panels show bright field images. (C) Cholesterol crystal (green) content in the plaque. Nuclei are stained with DAPI (Blue). (D) Cholesterol crystal content quantification. Values are means ± SD, n = 4 to 13 mice/group. Size bar; 100 µm.

Figure 3. Plasma obtained from APOA1<sup>tg/tg</sup>/Apoe<sup>−/−</sup> mice facilitates cholesterol efflux in cell culture. (A) HDL particle (HDL-P) concentration in plasma of age and diet matched Apoe<sup>−/−</sup>,
APOA1<sup>tg/tg</sup>/Apoe<sup>-/-</sup> and APOA1<sup>tg/tg</sup> male mice (n=3/genotype). (B) Cholesterol efflux capacity to the isolated HDL fraction from the aforementioned mouse genotypes using J774 macrophages, or (C) BHK cells over-expressing ABCA1 (ABCA1-BHK). (D) mRNA levels of ABCA1 in Apoe<sup>-/-</sup> and APOA1<sup>tg/tg</sup>/Apoe<sup>-/-</sup> mice fed Western diet for 12 or 16 weeks, respectively. After this period, a subset of mice was sacrificed (Baseline group, black) while the rest of the mice were fed chow diet (Control group, blue) or chow diet containing 900 mg/kg of the ACAT inhibitor F1394 (ACAT inhibitor group, red) for 4 extra weeks. Total mRNA was isolated from CD68+ cells in atherosclerotic lesions using laser capture microdissection (LCM) on lesions present at the level of the aortic root. Values are means ± SD. HDL-P and cholesterol efflux assays were performed in three different mice/genotype, in triplicate. Gene expression values represent n = 5 to 9 mice/group. For figures 3A to C, statistical differences were measured using one-way ANOVA with Dunnett’s multiple comparisons test. ** P < 0.01, *** P < 0.005, **** P < 0.0001.
Table 1. Gene expression changes on plaque CD68+ cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group, (sample size)</th>
<th>CCL2</th>
<th>NLRP3</th>
<th>IL-1β</th>
<th>FIZZ1</th>
<th>MRC1</th>
<th>CD36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoe⁻/⁻ mice</td>
<td>Baseline, (7)</td>
<td>1.00 ± 0.91</td>
<td>1.00 ± 0.65</td>
<td>1.00 ± 1.14</td>
<td>1.00 ± 1.39</td>
<td>1.00 ± 1.22</td>
<td>1.00 ± 0.75</td>
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<tr>
<td></td>
<td>Control diet, (5)</td>
<td>1.09 ± 0.50</td>
<td>2.07 ± 2.35</td>
<td>0.57 ± 0.54</td>
<td>0.02 ± 0.02</td>
<td>0.41 ± 0.50</td>
<td>2.03 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>ACAT inhibitor diet, (6)</td>
<td>1.06 ± 1.73</td>
<td>1.01 ± 1.54</td>
<td>0.32 ± 0.34</td>
<td>0.01 ± 0.01</td>
<td>0.32 ± 0.28</td>
<td>2.35 ± 1.23</td>
</tr>
<tr>
<td>APOA1tg/tg /</td>
<td>Baseline, (7)</td>
<td>1.82 ± 1.34</td>
<td>1.09 ± 2.29</td>
<td>0.42 ± 0.41</td>
<td>0.02 ± 0.02</td>
<td>0.99 ± 0.93</td>
<td>1.49 ± 1.06</td>
</tr>
<tr>
<td>Apoe⁻/⁻ mice</td>
<td>Control diet, (7)</td>
<td>0.56 ± 0.57</td>
<td>0.94 ± 2.09</td>
<td>0.35 ± 0.19</td>
<td>1.14 ± 2.77</td>
<td>0.59 ± 0.41</td>
<td>2.51 ± 1.21</td>
</tr>
<tr>
<td></td>
<td>ACAT inhibitor diet, (7)</td>
<td>0.38 ± 0.54</td>
<td>0.02 ± 0.01</td>
<td>0.07 ± 0.10</td>
<td>10.66 ± 15.49</td>
<td>2.97 ± 3.44</td>
<td>3.11 ± 1.77</td>
</tr>
</tbody>
</table>

Apoe⁻/⁻ and APOA1tg/tg/Apoe⁻/⁻ male and female mice were fed Western diet for 12 or 16 weeks, respectively. After this period, a subset of mice was sacrificed (Baseline) while the rest of the mice were fed chow diet (Control diet) or chow diet containing 900 mg/kg of the ACAT inhibitor F1394 (ACAT inhibitor diet for 4 extra weeks. Total mRNA was isolated from CD68+ cells in atherosclerotic lesions using laser capture microdissection (LCM) on lesions present at the level of the aortic root. The expression of pro-inflammatory gene markers and anti-inflammatory gene markers are represented. HPRT was used as housekeeping control gene.
Table 2. Adjusted P values from two-way ANOVA model for the secondary outcomes derived from the animal study (Figure 1A).

<table>
<thead>
<tr>
<th>Secondary outcome</th>
<th>Genotype effect</th>
<th>Experimental group effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>0.46</td>
<td>0.001</td>
</tr>
<tr>
<td>Lesion area</td>
<td>0.89</td>
<td>0.72</td>
</tr>
<tr>
<td>Collagen content</td>
<td>0.29</td>
<td>0.35</td>
</tr>
<tr>
<td>Cholesterol crystal content</td>
<td>0.60</td>
<td>0.89</td>
</tr>
<tr>
<td>CCL2</td>
<td>0.72</td>
<td>0.34</td>
</tr>
<tr>
<td>NLRP3</td>
<td>0.22</td>
<td>0.32</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>FIZZ1</td>
<td>0.35</td>
<td>0.29</td>
</tr>
<tr>
<td>MRC1</td>
<td>0.29</td>
<td>0.34</td>
</tr>
<tr>
<td>CD36</td>
<td>0.30</td>
<td>0.08</td>
</tr>
<tr>
<td>ABCA1</td>
<td>0.54</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Interaction for all the models was not significant, and the results presented were from model with only main effects. The multiple comparisons were adjusted by Benjamini-Hochberg procedure, and the adjusted P values were presented.
Figure 1

A. Experimental Design

Baseline Control ACAT inhibitor

Chow – Control

Chow – ACAT inhibitor

Western Diet

12 weeks

16 weeks

B. Total Cholesterol

Total Cholesterol

mg/dL

Apoe⁻ mice

APOA1tg/tg/Apoe⁻ mice

Baseline

Control

ACAT inhibitor

C. Lesion Area

Lesion Area

μm²

D. CD68+ cell content

CD68+ cell content

% in lesion

Apoe⁻ mice

APOA1tg/tg/Apoe⁻ mice
Figure 2

A

Baseline Control ACAT inhibitor

Apoe−/− mice

APOA1tg/tg/Apoe−/− mice

B

Collagen content

Baseline Control ACAT inhibitor

% in lesion

0 5 10 15 20

Apoe−/− mice APOA1tg/tg/Apoe−/− mice

C

Baseline Control ACAT inhibitor

D

Cholesterol crystal content

% in lesion

0 5 10 15 20

Δapo−/− mice ΔAPOA1Δapo/Apoe−/− mice
Figure 3

A. HDL particle concentration

B. In J774 macrophages

C. In ABCA1-BHK cells

D. ABCA1

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