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Title page

5A Apolipoprotein Mimetic Peptide Promotes Cholesterol Efflux and Reduces Atherosclerosis in Mice

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Running title page

Running title: Effect of 5A peptide on atherosclerosis

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ABBREVIATIONS: Athero, Atherosclerosis;HDL,, High Density Lipoprotein; LDL, Low Density lipoprotein; RCT, Reverse Cholesterol Transport; apo, apolipoprotein; POPC, Palmitoyl oleoyl phosphatidyl choline; ABC, ATP-binding cassette transporter.

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ABSTRACT

Intravenous administration of apoA-I complexed with phospholipid has been shown to rapidly reduce plaque size in both animal models and in humans. Short synthetic amphipathic peptides can mimic the anti-atherogenic properties of apoA-I and have been proposed as alternative therapeutic agents. In this study, we investigated the atheroprotective effect of the 5A peptide, a bi-helical amphipathic peptide that specifically effluxes cholesterol from cells by the ABCA1 transporter. 5A stimulated a 3.5-fold increase in ABCA1 mediated efflux from cells and a further 2.5-fold increase after complexing it with phospholipid (5A:POPC [1:7 mol/mol]). 5A:POPC but not free 5A was also found to promote cholesterol efflux by ABCG1. When incubated with human serum, 5A-POPC primarily bound to HDL but also to LDL and promoted the transfer of cholesterol from LDL to HDL. 24 hours following IV injection of 5A-POPC (30 mg/kg) into apoE-KO mice, both the cholesterol (181%) and phospholipid (219%) content of HDL significantly increased. By an *in vivo* cholesterol isotope dilution study and by monitoring the flux of cholesterol from radiolabeled macrophages to stool, 5A-POPC treatment was observed to increase reverse cholesterol transport. In three separate studies, 5A when complexed with various phospholipids reduced aortic plaque surface area by 29-53% (n=8 per group, $p<0.02$) in apoE-KO mice. No signs of toxicity from the treatment were observed, during these studies. In summary, 5A promotes cholesterol efflux both *in vitro* and *in vivo* and reduces atherosclerosis in apoE-KO mice, indicating that it may be a useful alternative to apoA-I for HDL therapy.

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INTRODUCTION

High density lipoproteins (HDL) has consistently been shown to be inversely related to cardiovascular disease risk (Remaley et al., 2008). HDL promotes the flux of excess cholesterol from peripheral cells to the liver by the reverse cholesterol transport (RCT) pathway (Duffy and Rader, 2006). The initial step, involves the interaction of apoA-I, the main protein component on HDL, with the ABCA1 transporters in cells, which triggers the removal of cholesterol and phospholipid from cells and the formation of nascent HDL (Remaley et al., 2001). Additional cholesterol can also be effluxed from cells by lipid-rich forms of HDL by the ABCG1 transporter and possibly by other mechanisms (Matsuura et al., 2006).

In addition to promoting RCT, HDL has recently been shown to have numerous other anti-atherogenic properties (Remaley et al., 2008), such as the ability to act as an anti-oxidant , to suppress inflammation and thrombosis , to promote vasodilatation , and to maintain endothelial cell integrity . The *in vivo* role, however, of these other properties of HDL and even the physiologic relevance of RCT has not been definitively established.

Based on the inverse relationship between HDL and the incidence of cardiovascular disease, there has been great interest in developing drugs that raise HDL (Garcia, 2008). Except for niacin there has been limited success in finding small molecule drugs that increase HDL and decrease cardiovascular disease (Barter, 2009). This has prompted the development of a new treatment strategy called HDL Therapy, which

involves the intravenous infusion of exogenous HDL to rapidly stabilize patients with acute coronary syndrome (Sethi et al., 2007; Remaley et al., 2008). Even a single infusion of HDL has been shown in animal studies to reduce plaque size, lipid content and inflammation (Barter, 2009). Two different clinical trials (Nissen et al., 2003; Tardif et al., 2007) have shown that five treatments with either a recombinant form of a mutant form of apoA-I called apoA-I_{Milano} or normal apoA-I purified from human plasma and reconstituted with phospholipid can reduce plaque size to a similar degree achieved with several years of statin therapy (Nanjee et al., 1996; Nissen, 2005).

Short synthetic peptide mimics of apoA-I have also been shown to reduce atherosclerosis in animal models (Bielicki et al., 2010). They are being investigated as possible therapeutic agents, because of the cost and difficulty in producing sufficient quantities of apoA-I (Sethi et al., 2007; Remaley et al., 2008). The most thoroughly investigated apoA-I mimetic peptide is the single helical peptide D-4F and its L-stereoisomer, L-4F, which has been tested in one human clinical trial (Bloedon et al., 2008). Although D-4F can promote cholesterol efflux from cells (Navab et al., 2004), bi-helical peptides have been shown *in vivo* to be superior for promoting cholesterol efflux (Wool et al., 2009). The main anti-atherogenic function of D-4F and L-4F has been proposed to be related to its ability to sequester oxidized lipids (Van Lenten et al., 2008).

In this study, we investigated the effect of 5A peptide treatment on lipid and lipoprotein metabolism in mice and the development of atherosclerosis. The 5A peptide is a bi-

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helical peptide linked with by a proline residue (Sethi et al., 2008). The first amphipathic helix has relatively high lipid affinity, whereas the lipid affinity of the second helix was reduced by substituting 5 non-polar amino acids on the hydrophobic face of the helix with alanine and hence its name. This arrangement of helices was found to reduce its cytotoxicity and to increase its specificity for removing cholesterol from cells by the ABCA1 transporter (Sethi et al., 2008). Intravenous administration of the 5A peptide complexed with phospholipid was found in apoE-KO mice to raise HDL, promote RCT, and reduce atherosclerosis, indicating that 5A shares many of the same beneficial anti-atherogenic features of apoA-I and may be a suitable substitute for apoA-I in HDL Therapy.

METHODS

Peptide Synthesis and Lipid Reconstitution: 5A (DWLKAFYDKVAEKLKEAF- P - DWAKAAYDKAAEKAKEAA) was synthesized by a solid-phase procedure, using an Fmoc protocol on a Biosearch 9600 peptide synthesizer and was purified to greater than 99% purity by reverse-phase HPLC on an Aquapore RP-300 column (Sethi et al., 2008). For experiments with biotinylated 5A, a C6-biotin group was added during the synthesis to N-terminus and biotin-5A was used at a 1 to 10 molar ratio to unlabeled 5A. 5A was reconstituted with lipid at a 1:7 molar ratio with palmitoyl oleoyl phosphatidylcholine (POPC) (5A-POPC) or sphingomyelin and dipalmitoyl phosphatidylcholine (DPPC) (5A-SM/DPCC;1:3.5:3.5 molar ratio), as previously described (Sethi et al., 2008), and delivered intravenously (IV) into the retro-orbital sinus or by intraperitoneal (IP) injection as indicated. Unless otherwise indicated, the dose of

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5A-POPC is based on the protein mass of 5A peptide. POPC liposomes were prepared by sonication (Jonas, 1986).

Mice and diets: Female apoE-KO (Jackson Laboratory, Bar Harbor, ME) were fed a regular rodent chow diet (NIH-07 chow diet: 0.025% cholesterol, 4.5% fat; Ziegler Brothers, Inc., Gardner, PA). All animal studies were approved by the Animal Care and Use Committee from each institution (protocols # H-0050R1, H-0018R1 NHLBI)

Isolation and analysis of lipoproteins: HDL (*d* = 1.063-1.21 g/mL) and LDL (*d* = 1.019-1.063 g/mL) were isolated from human plasma by sequential ultracentrifugation, followed by dialysis at 4°C against PBS, containing 0.01% (wt/vol) EDTA. Lipoproteins were analyzed on a microfluidic-based electrophoresis system, using the P200 protein chip (Agilent Technologies, Santa Clara, CA) on the 2100 Bioanalyzer (Agilent Technologies). Chromatograms from the electrophoresis were used to construct a digital gel image. Human apoA-I was measured by ELISA (WAKO Chemicals, Richmond, VA). A dot blot assay was used to detect Biotin labeled 5A-POPC peptide, by applying samples to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), using a vacuum manifold device (Millipore, Bellerica, MA) and then incubating the membrane with a streptavidin-HRP conjugate (Thermo Scientific, Waltham, MA) for 1 h and HRP Color Development Reagent (Bio-Rad Laboratories, Hercules, CA). For digital quantification, scanned images were analyzed on an ImageQuant v. 5.2 (Molecular Dynamics / GE Healthcare, Pittsburgh, PA).

Analysis of plasma lipids and lipoproteins: Plasma lipoproteins and lipids were measured enzymatically (Wako Chemicals USA, Inc. Richmond, VA) on a ChemWell 2910 analyzer (Awareness Technology, Inc, Palm City, FL). Lipoproteins were analyzed by fast-protein liquid chromatography (FPLC), using two Superose 6 HR 10/30 columns (Amar et al., 1998).

***In vitro* cholesterol efflux assay:** Cholesterol efflux studies were performed as previously described (Remaley et al., 2003), with the following modifications. BHK-mock and BHK stably transfected cells with human ABCA1 cDNA or ABCG1 cDNA (Vaughan and Oram, 2003; Sankaranarayanan et al., 2009) were labeled for 18 h with 1 μCi/mL of ^{3}H-cholesterol in AMEM plus 10% FCS. Transporters were induced with 10nM of mifepristone in DMEM plus 0.2 mg/mL of fatty acid free BSA for 18 h. Cholesterol flux was measured after the addition of media containing an acceptor plus 10nM mifepristone in AMEM with 0.2% fatty acid free BSA. After 6 h, media was collected and filtered (Whatman, 24 well 25 um pore size) and cells were lysed in 0.4mL of 0.1% SDS and 0.1N NaOH. Radioactive counts in media and cell fractions were measured by liquid scintillation counting and results are expressed as % of total counts effluxed per indicated time interval.

***In Vivo* cholesterol efflux assay:** Cholesterol efflux *in vivo* was measured as previously described (Mukhamedova et al., 2008). Briefly, RAW 264.7 macrophage cells were simultaneously radiolabeled and loaded with cholesterol by incubation for 48 h with 29.7 mCi/mL [^{3}H]cholesterol and acetylated LDL (50 μg/mL). Cells were washed,

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incubated for 24 h in serum-free medium, and resuspended in 0.15 M sterile saline at a concentration of 10^7 cells/mL. Cells were injected IP into male C57Bl/6 mice (2 x 10^6 cells containing 5 x 10^6 dpm per mouse) (n=7). Mice were pretreated twice with 5A-POPC (30 mg/kg) 8 h before and within 1h before cell injection. After 24 h, mice were euthanized, and blood, liver, and feces were collected. Aliquots of plasma were counted, and cholesterol from liver and feces was extracted (Folch et al., 1957), cholesterol separated by thin layer chromatography and counted.

Cholesterol isotope dilution RCT assay: RCT was measured in male Sprague-Dawley rats, using a cholesterol isotope dilution assay. Rat (250-300 grams) were purchased with in-dwelling jugular vein cannula (Charles River). A single bolus of 5A-POPC (20mg/mL) was administered IV at 1 mL/min and 30 min following peptide administration, a constant IV infusion (0.1 mL/h) of [2,3-$^{13}C_2$]cholesterol ($^{13}C_2$-C) (99% Cambridge Isotope Labs, Andover, MA) was administered for 5 h. The infusate was prepared by a modification of the method described by Ostlund et al (Ostlund and Matthews, 1993). Briefly, 200 mg of $^{13}C_2$-cholesterol was dissolved into 13 mL of warm USP ethanol and mixed slowly into 200mL of 10% Intralipid (Fresenius-Kabi, Uppsala, Sweden) solution to a final concentration of 1.0 mg/mL $^{13}C_2$-Cholesterol. Food was removed and animals transferred to a clean cage at 7 AM right before starting the infusion. 50-100 μL of heparinized whole blood was collected at 0.5, 2.5, 4, 5.5, 9.5, 25 and 31h from the tail, samples were spun and plasma separated immediately. Following the infusion, animals were individually housed in metabolic cages and total fecal collections were made for the following 5 days.

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Plasma free cholesterol was extracted with ethanol-acetone, acetylated with toluene/pyridine/acetyl chloride, and dissolved in toluene for GC/MS analysis (Agilent 6890 gas chromatograph, J&W Scientific, Folsom, CA). Mass spectrometry was done by electron impact (EI). Selective ion monitoring (SIM) was used with *m/z* 368-370 of the $M_0/M_1/M_2$ mass isotopomers of the molecular ion. The $^{13}C_2$-cholesterol enrichment in M2 (EM2) were calculated by subtracting the %M2 [M2/(M0+M1+M2)] of an unlabeled standard from the %M2 of the sample.

Stool samples were homogenized with water and neutral sterols (NS) and bile acids (BA) were extracted separately under basic and acidic conditions, respectively, in the presence of the internal standards 5α-cholestane and 5β-cholanic acids. The BA extract was split into two fractions. The first, used for compositional analysis by a flame ionization detector was directly subjected to a two-step derivatization: butylation with butanolic HCl followed by silylation by BSTFA-pyridine. The second half was further purified for mass spectrometric analysis with an octadecyl SPE cartridge, selectively eluting primarily deoxycholic acid with a 20% aqueous-methanol solution prior to butylation and silylation. The NS fraction was silylated directly for both compositional and isotope analysis. ^{13}C2-C isotopic enrichments were measured, using GC/C-IR/MS (Thermo Finnegan MAT 253 IR-MS, Bremen, Germany). Enrichments were determined as atom percent excess (APE) by comparison of the unknown samples to a standard curve generated with gravitametrically prepared working lab standards with known enrichments. Molar percent excess (MPE) is calculated as 14.5 or 15 × APE for the

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acetyl or silyl derivative of C, respectively. Compositional analysis and excretion measurement of BA and NS was performed by GC/FID by comparison to the internal standards.

Analysis of Aortic Lesions*.* The left ventricle of the heart was perfused with PBS, followed by a fixative solution (4% paraformaldehyde, 5% sucrose, 20 mM EDTA, pH 7.4). The aorta was dissected from its origin in the heart to the ileal bifurcation and was placed in fixative solution for 18 h, stained with Sudan IV solution for 25 min, and then destained for 25 min in 80% ethanol, and finally washed in water. After removal of any remaining adventitial fat, aortas were cut longitudinally, placed on glass slides embedded with glycerin, covered by a microscope cover glass and sealed with nail polish. Quantification of aortic plaques from the ileal bifurcation to its origin, excluding branching vessels, was performed in a blinded fashion in triplicate, using the Image-Pro Plus version 4.1 software (Media Cybernetics, Inc., MD). Data are reported as % of total aortic surface covered by lesions.

RESULTS

In Vitro effect of 5A-POPC on cholesterol efflux: The effect of phospholipid reconstitution of the 5A peptide on cholesterol efflux was examined in BHK cells stably transfected with ABCA1 (Fig. 1 A, B) or ABCG1 (Fig.1 C, D). As previously described (Sethi et al., 2008), the free 5A peptide promoted cholesterol efflux by ABCA1 (Fig. 1A), but it was ineffective in promoting cholesterol efflux by the ABCG1 transporter (Fig. 1C). Reconstitution of 5A with POPC increased cholesterol efflux from ABCA1 transfected cells by approximately 3-fold (Fig. 1B), but cholesterol efflux from the control cell line increased nearly to the same degree, indicating that most of the increase was independent of ABCA1. After subtracting the non-ABCA1 component of cholesterol efflux detected from the control cell line, Vmax for cholesterol efflux by ABCA1 (12±1%/18h) was found not to significantly change after reconstitution of 5A with phospholipid, but it did lower the Km for cholesterol efflux from 6.17 ± 0.98 to 1.22 ±0.51 μM. In contrast to free 5A peptide, 5A-POPC was also capable of promoting cholesterol efflux from ABCG1 transfected cells (Fig. 1D), but again after subtraction of the cholesterol efflux results from the control cell line, only about 20±1.5% of the increase in cholesterol efflux after phospholipid reconstitution was due to ABCG1.

Ex Vivo effect of 5A-POPC on plasma lipoproteins: Reconstitution of 5A with POPC resulted in only a single peak containing both peptide and phospholipid, which migrated on FPLC slightly before HDL (Fig. 2A, 2B). When biotin labeled 5A was reconstituted with POPC and incubated with human plasma at 37^{o} C for 45 min and separated by FPLC, approximately 65% of 5A was found bound to HDL, with the remaining bound to

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LDL and a small amount to VLDL (Fig. 2C). Incubation of 5A-POPC with plasma did not significantly shift the elution position of human apoA-I to either lower or higher molecular weight fractions (Fig. 2D). Treatment of plasma with 5A-POPC was also associated with a considerable increase in the size of both HDL and LDL, as observed by FPLC (Fig. 2E, F). The 5A-POPC treatment increased 11-fold the phospholipid content of HDL (Fig. E), while the cholesterol content of HDL increased by approximately 26% and the cholesterol content of LDL decreased by approximately 18% (Fig. 2F).

The *ex vivo* effect of 5A-POPC treatment on isolated HDL and LDL and plasma lipoproteins was also examined after separation by electrophoresis and staining with a dye sensitive to phospholipids (Fig. 3). 5A-POPC was found to migrate in a pre-beta position (Lane 1) before the two major HDL subfractions observed in the alpha position for isolated human HDL (Lane 2). When 5A-POPC was incubated with HDL, it appeared to form a major band in the alpha position, as well as some slower migrating bands in the pre-beta region (Lane 3). When 5A-POPC was incubated with isolated LDL (Lane 5), it appeared to fuse with LDL, leaving only a faint residual band in the pre-beta region. Finally, when 5A-POPC was added to plasma (Lane 6), it increased the intensity of the band in the LDL region and slowed its migration, but the primary change was a marked increase in the intensity of a lipoprotein band in the same region as isolated 5A-POPC and an increase in the intensity of a band in the pre-beta position

In Vivo effect of 5A-POPC on plasma lipoproteins: 5A-POPC (30mg/kg) was injected IV into apoE-KO mice and plasma lipoproteins were monitored over time by FPLC (Fig.

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4). 5A-POPC was found by 3 h to decrease LDL-C by approximately 17% and increased HDL-C by 29% (Fig. 4A-B). By 24 h, LDL-C returned to baseline but HDL-C further increased to 149% above baseline. Cholesteryl ester was found by 3 h to increase in LDL by approximately 13% and at 24 h to decrease by 42%, whereas for HDL it increased by 75% and 181%, at 3 h and 24 h, respectively (Fig. 4C-D). The major change, however, in HDL composition was in phospholipids (Fig. 4E-F). At 3 h phospholipid content of HDL increased by 72% and at 24 h by 219%.

In Vivo effect of 5A-POPC on RCT: The *in vivo* effect of 5A-POPC on RCT was examined with three different experimental approaches. In the first study, after an IV bolus of 5A-POPC (30mg/kg), plasma was removed at various times and was tested for its ability to promote cholesterol efflux from ABCA1 and ABCG1 transfected BHK cells (Fig. 5). Treatment with 5A-POPC was found to increase cholesterol efflux by approximately 15±5% at 6 h and by 20±8% by 24 h. 5A-POPC treatment had no significant effect on cholesterol efflux by ABCG1 at 6 h but increased ABCG1 cholesterol efflux by approximately 47±13% at 24 h. No increase in non-specific cholesterol efflux was observed from the mock transfected control cell line after treatment with 5A-POPC.

The effect of 5A-POPC on RCT from peripheral tissues was also tested in rats by a cholesterol isotope dilution study (Fig. 6), which monitors the efflux of unlabeled cholesterol from peripheral tissues, which results in the dilution of the plasma tracer ^{13}C-cholesterol that is continuously intravenously infused. Treatment with 5A-POPC (40

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mg/Kg) increased the efflux of cholesterol into the plasma from peripheral tissues by approximately 20±4% at the low dose (40 mg/kg) and by 65±7% at the high dose (100 mg/kg) (Fig. 6A). No significant change was observed in fecal excretion of neutral sterols or bile acids after 5A-POPC treatment (Fig. 6B).

IP treatment of mice with 5A-POPC (30 mg/kg) was found to promote cholesterol efflux from intraperitoneal RAW 264.7 macrophages labeled with ^{3}H-cholesterol (Fig. 7). The C57Bl/6 mice were treated with 5A-POPC 8 h before and again within 1h of injection of macrophages. 24 h after IP injection of the radiolabeled macrophages, the 5A-POPC treatment increased the appearance of radioactive plasma cholesterol by 43±8% over control mice. 5A-POPC treatment also led to an increase in the excretion of the radiotracer as fecal sterols and bile acids after 24 h by 42±6% and 500±40%, respectively. The amount of radiolabeled cholesterol in the liver increased by 13±6% but was not statistically significant (data not shown).

Effect of 5A-POPC treatment on atherosclerosis: The effect of 5A-POPC treatment on atherosclerosis in ApoE-KO mice on a normal chow diet was tested in 3 different experiments. In the first study (Fig. 8A), we compared the protection provided by 5A-POPC versus POPC liposomes alone. Four month old mice were treated IV three times a week (Monday, Wednesday and Friday), with 5A-POPC (30 mg/kg 5A plus 1.25 mg POPC) or POPC liposomes (1.25 mg of POPC) for 13 weeks. Compared to the POPC treated control group, mice treated with 5A-POPC showed 29% less aortic lesion area

(6.6± 0.5 % vs. 9.3± 1.2 %, *p*=0.05). No significant difference was observed from the 5A-POPC or POPC treatment on liver or renal function tests or body weight (Table 1).

In a second study, 8-month old mice, with advanced atherosclerotic plaques, were treated with 5A-POPC (30 mg/kg, IV) three times a week for 13 weeks (Fig 8B) and compared to saline treated mice. At the end of the study, the mice were found to have approximately 4-fold more atherosclerosis than what was observed in the first study of the younger mice, but the 5A-POPC treated mice showed 30% less aortic surface coverage than saline injected mice (28.8± 4.7% vs. 41.1± 4.8%, *p*=0.03). In a third study (Fig 8C), 4-month old mice were treated with a different lipid formulation of 5A. Based on the observation that nascent HDL is enriched in SM, which can lead to increased efflux of cholesterol from cells (Liu et al., 2003), 5A was reconstituted with sphingomyelin and DPPC (5A:SM:DPPC; 1:3.5:3.5 molar ratio), and it was injected IP three times a week for 13 weeks. In this study control mice received only saline injections. Compared to the control group, 54% less plaque surface coverage of the aorta was observed with the 5A treatment (3.2± 0.4% vs. 6.9± 0.6 %, *p*=0.001).

DISCUSSION

ApoA-I preparations used to treat patients have first been reconstituted with phospholipid to mimic nascent type HDL (Nissen, 2005; Tardif et al., 2007), which is particularly good in promoting cholesterol efflux , especially by ABCA1 (Mukhamedova et al., 2007). We, therefore, first tested the effect of reconstituting 5A with phospholipid on cholesterol efflux (Fig. 1). Compared to the free 5A peptide, no difference was between lipid-free 5A and 5A-POPC in the Vmax for cholesterol efflux by ABCA1,

although the phospholipid addition appeared to increase the affinity of the peptide complex to the ABCA1 transporter based on its lower Km (Fig. 1). A similar finding has recently been made for another amphipathic peptide (Bielicki et al., 2010). These results suggest that either the 5A-POPC complex can directly interact with the ABCA1 transporter in the same way that pre-β HDL has recently been proposed to be both a product and substrate of the ABCA1 transporter (Duong et al., 2008) or that 5A can dissociate from the complex and then interact with the transporter. A potential advantage of reconstituting 5A with phospholipid is that it also increased cholesterol efflux by the ABCG1 transporter, which unlike ABCA1 prefers phospholipid rich acceptor particles (Cavelier et al., 2006). The addition of phospholipid to 5A caused even a larger increase in cholesterol efflux from the mock transfected control cells, which most likely is occurring by an aqueous diffusion process, which also requires phospholipid rich acceptor particles (Yancey et al., 2003).

Ex vivo experiments with human plasma revealed that the 5A peptide has a preference for interacting with HDL, but it can also bind to LDL, depending on the relative concentration of the two lipoproteins (Fig. 2, 3). Interestingly, it appeared to transfer cholesterol from LDL particles to HDL, but the main effect of 5A-POPC treatment of plasma was a marked increase in cholesterol and in particular the phospholipid content of HDL (Fig 2, 3). The phospholipid content of HDL from mice treated with 5A-POPC was also increased (Fig. 4). Enrichment of phospholipid on HDL has been shown to increase the capacity of HDL for removing cholesterol from cells (Davidson et al., 1994;

Yancey et al., 2003; Silva et al., 2008), which may have accounted for the increase ability of serum from treated mice to promote more cholesterol efflux (Fig. 5).

Using two different approaches, 5A-POPC treatment was shown to increase the *in vivo* flux of cholesterol (Fig 6-7). 5A-POPC mobilized radiolabeled cholesterol from peritoneal macrophages and increased the excretion of fecal neutral sterols and bile salts (Fig. 7). Similarly, 5A-POPC was also found to increase the global efflux of cholesterol from peripheral tissues, as measured in the cholesterol isotope dilution study, but it did not appear to increase overall fecal neutral sterol excretion of sterols or bile salts by this method (Fig. 6). This difference may be due to differences in the experimental details of these two studies, such as dose, route and number of 5A treatments and animal model. Multiple treatments with 5A-POPC may also be needed to deliver enough cholesterol to liver to then up regulate the expression of hepatic genes that facilitate the excretion of fecal sterols and bile salts. It is also likely that the cholesterol isotope dilution method is relatively insensitive to the flux of cholesterol from macrophages, because of its small pool size compared to the whole body. Mobilization of cholesterol from macrophages and redistribution to other tissues, however, may still be sufficient for reducing atherosclerosis, without increased fecal loss of sterols (Remaley et al., 2008). For example, it has been shown in mice that acute infusion of HDL mobilizes cholesterol from peripheral tissues, which will reduce atherosclerosis, but does not increased the level of fecal sterols (Alam et al., 2001).

Treatment of mice with 5A was found to reduce atherosclerosis (Fig. 8) comparable to what has been previously observed with apoA-I and D-4F (Kawashiri et al., 2002). Reconstitution of 5A with sphingomyelin and DPPC appeared to be even more effective than when 5A was reconstituted with POPC (Fig. 8), although it was delivered by IP injection instead of IV like the other treatments. More studies are needed to investigate the route of delivery and the effect of different types and amounts of phospholipids on their ability to alter the anti-atherogenic properties of amphipathic peptides. One study has already reported that different types of phospholipids affect the ability of apoA-I to reduce inflammation in endothelial cells (Baker et al., 2000). It should also be noted that we only examined progression of atherosclerosis and that in the future regression models will also have to be used to determine if 5A and related peptides can reduce existing disease, which is more relevant to how these peptides would likely be used to treat patients.

Recently, a single helical peptide called ATI-5261, which also promotes cholesterol efflux by ABCA1, was also found to reduce atherosclerosis in mice (Bielicki et al., 2010). Although the results from ATI-5261 and the 5A peptide are consistent with the concept that these peptides reduce atherosclerosis in mice, because of their ability to promote cholesterol efflux, it does not exclude other possibilities. In fact, 5A was recently observed to be as potent as apoA-I in reducing inflammation in a rabbit collar model (Tabet et al., 2009). In this study, it was found that 5A decreased the expression of VCAM and ICAM on endothelial cells and decreased ROS production in endothelial cells (Tabet et al., 2009). 5A has also been found to reduce the activation of the

adhesion protein CD11 on monocytes (Murphy et al., 2008). Cholesterol efflux from cells, such as macrophages, has been shown to decrease their inflammatory phenotype (Yvan-Charvet et al.; deGoma et al., 2008), so the ability of 5A to promote cholesterol efflux can perhaps still be related to its anti-inflammatory properties. The D-4F peptide has also been shown to reduce platelet activation (Buga et al., 2010), enhance the anti-oxidant properties of HDL and to sequester oxidized lipids (Van Lenten et al., 2008). The 5A peptide has not been tested for these other anti-atherogenic properties, but given the fact that the key structural motif that appears to be necessary for all these processes is the presence of an amphipathic helix, these properties may be shared by other amphipathic peptides.

In summary, the 5A peptide, which was designed to specifically efflux cholesterol by the ABCA1 transporter, was found when injected into mice to increase HDL, particularly its phospholipid concentration, to increase cholesterol efflux from macrophages, as well as from other peripheral tissues and to reduce atherosclerosis. These features and its apparent lack of toxicity make it a potential substitute for apoA-I in HDL Therapy. Additional structure-function studies of 5A and related peptides may not only enhance the anti-atherogenic effects of these peptides, but may also lead to a better understanding of which properties of HDL are most important for reducing atherosclerosis.

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References

Alam K, Meidell RS and Spady DK (2001) Effect of up-regulating individual steps in the reverse cholesterol transport pathway on reverse cholesterol transport in normolipidemic mice. *J Biol Chem* **276**:15641-15649.

Amar MJ, Dugi KA, Haudenschild CC, Shamburek RD, Foger B, Chase M, Bensadoun A, Hoyt RF, Jr., Brewer HB, Jr. and Santamarina-Fojo S (1998) Hepatic lipase facilitates the selective uptake of cholesteryl esters from remnant lipoproteins in apoE-deficient mice. *J Lipid Res* **39**:2436-2442.

Baker PW, Rye KA, Gamble JR, Vadas MA and Barter PJ (2000) Phospholipid composition of reconstituted high density lipoproteins influences their ability to inhibit endothelial cell adhesion molecule expression. *J Lipid Res* **41**:1261-1267.

Barter P (2009) Lessons learned from the Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial. *Am J Cardiol* **104**:10E-15E.

Bielicki JK, Zhang H, Cortez Y, Zheng Y, Narayanaswami V, Patel A, Johansson J and Azhar S (2010) A new HDL mimetic peptide that stimulates cellular cholesterol efflux with high efficiency greatly reduces atherosclerosis in mice. *J Lipid Res*., Epub

Bloedon LT, Dunbar R, Duffy D, Pinell-Salles P, Norris R, DeGroot BJ, Movva R, Navab M, Fogelman AM and Rader DJ (2008) Safety, pharmacokinetics, and pharmacodynamics of oral apoA-I mimetic peptide D-4F in high-risk cardiovascular patients. *J Lipid Res* **49**:1344-1352.

Buga GM, Navab M, Imaizumi S, Reddy ST, Yekta B, Hough G, Chanslor S, Anantharamaiah GM and Fogelman AM (2010) L-4F alters hyperlipidemic (but not healthy) mouse plasma to reduce platelet aggregation. *Arterioscler Thromb Vasc Biol* **30**:283-289.

Cavelier C, Lorenzi I, Rohrer L and von Eckardstein A (2006) Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1. *Biochim Biophys Acta* **1761**:655-666.

Davidson WS, Lund-Katz S, Johnson WJ, Anantharamaiah GM, Palgunachari MN, Segrest JP, Rothblat GH and Phillips MC (1994) The influence of apolipoprotein structure on the efflux of cellular free cholesterol to high density lipoprotein. *J Biol Chem* **269**:22975-22982.

deGoma EM, deGoma RL and Rader DJ (2008) Beyond high-density lipoprotein cholesterol levels evaluating high-density lipoprotein function as influenced by novel therapeutic approaches. *J Am Coll Cardiol* **51**:2199-2211.

Duffy D and Rader DJ (2006) Emerging therapies targeting high-density lipoprotein metabolism and reverse cholesterol transport. *Circulation* **113**:1140-1150.

Duong PT, Weibel GL, Lund-Katz S, Rothblat GH and Phillips MC (2008) Characterization and properties of pre beta-HDL particles formed by ABCA1-mediated cellular lipid efflux to apoA-I. *J Lipid Res* **49**:1006-1014.

Folch J, Lees M and Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**:497-509.

Garcia RA (2008) Pharmacological therapies for raising HDL cholesterol beyond synthetic small molecules. *Curr Opin Investig Drugs* **9**:274-280.

Jonas A (1986) Reconstitution of high-density lipoproteins. *Methods Enzymol* **128**:553-582.

Kawashiri MA, Zhang Y, Pure E and Rader DJ (2002) Combined effects of cholesterol reduction and apolipoprotein A-I expression on atherosclerosis in LDL receptor deficient mice. *Atherosclerosis* **165**:15-22.

Liu L, Bortnick AE, Nickel M, Dhanasekaran P, Subbaiah PV, Lund-Katz S, Rothblat GH and Phillips MC (2003) Effects of apolipoprotein A-I on ATP-binding cassette transporter A1-mediated efflux of macrophage phospholipid and cholesterol: formation of nascent high density lipoprotein particles. *J Biol Chem* **278**:42976-42984.

Matsuura F, Wang N, Chen W, Jiang XC and Tall AR (2006) HDL from CETP-deficient subjects shows enhanced ability to promote cholesterol efflux from macrophages in an apoE- and ABCG1-dependent pathway. *J Clin Invest* **116**:1435-1442.

Mukhamedova N, Escher G, D'Souza W, Tchoua U, Grant A, Krozowski Z, Bukrinsky M and Sviridov D (2008) Enhancing apolipoprotein A-I-dependent cholesterol efflux elevates cholesterol export from macrophages in vivo. *J Lipid Res* **49**:2312-2322.

Mukhamedova N, Fu Y, Bukrinsky M, Remaley AT and Sviridov D (2007) The role of different regions of ATP-binding cassette transporter A1 in cholesterol efflux. *Biochemistry* **46**:9388-9398.

Murphy AJ, Woollard KJ, Hoang A, Mukhamedova N, Stirzaker RA, McCormick SP, Remaley AT, Sviridov D and Chin-Dusting J (2008) High-density lipoprotein reduces the human monocyte inflammatory response. *Arterioscler Thromb Vasc Biol* **28**:2071-2077.

Nanjee MN, Crouse JR, King JM, Hovorka R, Rees SE, Carson ER, Morgenthaler JJ, Lerch P and Miller NE (1996) Effects of intravenous infusion of lipid-free apo A-I in humans. *Arterioscler Thromb Vasc Biol* **16**:1203-1214.

Navab M, Anantharamaiah GM, Reddy ST, Hama S, Hough G, Grijalva VR, Wagner AC, Frank JS, Datta G, Garber D and Fogelman AM (2004) Oral D-4F causes formation of pre-beta high-density lipoprotein and improves high-density lipoprotein-mediated cholesterol efflux and reverse cholesterol transport from macrophages in apolipoprotein E-null mice. *Circulation* **109**:3215-3220.

Nissen SE (2005) Effect of intensive lipid lowering on progression of coronary atherosclerosis: evidence for an early benefit from the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial. *Am J Cardiol* **96**:61F-68F.

Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM, Lauer MA, Sheldon WS, Grines CL, Halpern S, Crowe T, Blankenship JC and Kerensky R (2003) Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA* **290**:2292-2300.

Ostlund RE, Jr. and Matthews DE (1993) [13C]cholesterol as a tracer for studies of cholesterol metabolism in humans. *J Lipid Res* **34**:1825-1831.

Remaley AT, Amar M and Sviridov D (2008) HDL-replacement therapy: mechanism of action, types of agents and potential clinical indications. *Expert Rev Cardiovasc Ther* **6**:1203-1215.

Remaley AT, Stonik JA, Demosky SJ, Neufeld EB, Bocharov AV, Vishnyakova TG, Eggerman TL, Patterson AP, Duverger NJ, Santamarina-Fojo S and Brewer HB, Jr. (2001) Apolipoprotein specificity for lipid efflux by the human ABCAI transporter. *Biochem Biophys Res Commun* **280**:818-823.

Remaley AT, Thomas F, Stonik JA, Demosky SJ, Bark SE, Neufeld EB, Bocharov AV, Vishnyakova TG, Patterson AP, Eggerman TL, Santamarina-Fojo S and Brewer HB (2003) Synthetic amphipathic helical peptides promote lipid efflux from cells by an ABCA1-dependent and an ABCA1-independent pathway. *J Lipid Res* **44**:828-836.

Sankaranarayanan S, Oram JF, Asztalos BF, Vaughan AM, Lund-Katz S, Adorni MP, Phillips MC and Rothblat GH (2009) Effects of acceptor composition and mechanism of ABCG1-mediated cellular free cholesterol efflux. *J Lipid Res* **50**:275-284.

Sethi AA, Amar M, Shamburek RD and Remaley AT (2007) Apolipoprotein AI mimetic peptides: possible new agents for the treatment of atherosclerosis. *Curr Opin Investig Drugs* **8**:201-212.

Sethi AA, Stonik JA, Thomas F, Demosky SJ, Amar M, Neufeld E, Brewer HB, Davidson WS, D'Souza W, Sviridov D and Remaley AT (2008) Asymmetry in the lipid affinity of bihelical amphipathic peptides. A structural determinant for the specificity of ABCA1-dependent cholesterol efflux by peptides. *J Biol Chem* **283**:32273-32282.

Silva RA, Huang R, Morris J, Fang J, Gracheva EO, Ren G, Kontush A, Jerome WG, Rye KA and Davidson WS (2008) Structure of apolipoprotein A-I in spherical high

density lipoproteins of different sizes. *Proc Natl Acad Sci U S A* **105**:12176-12181.

Tabet F, Remaley AT, Segaliny AI, Millet J, Yan L, Nakhla S, Barter PJ, Rye KA and Lambert G (2009) The 5A Apolipoprotein A-I Mimetic Peptide Displays Antiinflammatory and Antioxidant Properties In Vivo and In Vitro. *Arterioscler Thromb Vasc Biol*.

Tardif JC, Gregoire J, L'Allier PL, Ibrahim R, Lesperance J, Heinonen TM, Kouz S, Berry C, Basser R, Lavoie MA, Guertin MC and Rodes-Cabau J (2007) Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial. *JAMA* **297**:1675-1682.

Van Lenten BJ, Wagner AC, Jung CL, Ruchala P, Waring AJ, Lehrer RI, Watson AD, Hama S, Navab M, Anantharamaiah GM and Fogelman AM (2008) Anti-inflammatory apoA-I-mimetic peptides bind oxidized lipids with much higher affinity than human apoA-I. *J Lipid Res* **49**:2302-2311.

Vaughan AM and Oram JF (2003) ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. *J Lipid Res* **44**:1373-1380.

Wool GD, Vaisar T, Reardon CA and Getz GS (2009) An apoA-I mimetic peptide containing a proline residue has greater in vivo HDL binding and anti-inflammatory ability than the 4F peptide. *J Lipid Res* **50**:1889-1900.

Yancey PG, Bortnick AE, Kellner-Weibel G, de la Llera-Moya M, Phillips MC and Rothblat GH (2003) Importance of different pathways of cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol* **23**:712-719.

Yvan-Charvet L, Wang N and Tall AR Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. *Arterioscler Thromb Vasc Biol* **30**:139-143.

Footnotes

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Legends for figures

Fig 1. Effect of 5A on cholesterol efflux. Free 5A **(Panel A, C)** or 5A-POPC **(Panel B, D)** at the indicated doses were used to efflux cholesterol from ABCA1 **(Panel A, B)** or ABCG1 **(Panel C, D)** transfected BHK cells. Contribution of cholesterol efflux from the transporter (∆) was calculated after subtraction of cholesterol efflux from the transfected cell line (■) from the mock transfected control cell line (●). Results are expressed as the mean ±1SD of triplicates.

Fig 2. Effect of *ex vivo* incubation of 5A-POPC on plasma lipoproteins after separation by FPLC. Panel A: Protein absorbance FPLC profile of 5A reconstituted with POPC (1:7 molar ratio). **Panel B:** Phospholipid FPLC profile of 5A reconstituted with POPC (1:7 molar ratio). **Panel C:** FPLC profile of biotinylated 5A-POPC (5 μg /mL final concentration) incubated with plasma for 45min at 37^{o}C and separated by FPLC. **Panel D:** ApoA-I FPLC profile of plasma before (dashed line) and after (solid line) incubation with 5A-POPC (5 μg /mL final concentration) for 45min at 37^{o}C. **Panel E:** Phospholipid FPLC profile of plasma before (dashed line) and after (solid line) incubation with 5A-POPC (5 μg/mL final concentration) for 45min at 37^{o}C. **Panel F:** Total cholesterol FPLC profile of plasma before (dashed line) and after (solid line) incubation with 5A-POPC (5 μg /mL final concentration) for 45min at 37^{o}C.

Fig 3. Effect of *ex vivo* incubation of 5A-POPC on plasma lipoproteins after separation by electrophoresis. 5A-POPC (5 μg /mL final dose) was incubated for

45min at 37^{o}C with either HDL or LDL isolated by density gradient ultracentrifugation or with plasma and then separated by electrophoresis on the 2100 Bioanalyzer. **Lanes 1 and 8:** 5A-POPC, **Lane 2:** HDL, **Lane 3:** HDL plus 5A-POPC, **Lane 4:** LDL, **Lane 5:** LDL plus 5A-POPC, **Lane 6:** plasma, **Lane 7:** plasma plus 5A-POPC. Horizontal lines indicate location of internal control.

Fig 4. Effect of *In Vivo* treatment of 5A-POC on plasma lipid and lipoproteins. ApoE-KO mice (n=5) were injected IV with 5A-POPC (30 mg/kg) and pooled plasma collected at baseline (dashed lines), 3 h post injection (gray solid lines) and 24 h post injection (black solid lines) were separated by FPLC and analyzed for total cholesterol (**Panel A**), cholesteryl esters (**Panel C**) and phospholipids (**Panel E**). **Panels B, D and F** are an enlargement of the HDL peak from panels A, C and E, respectively.

Fig 5. Effect of *In Vivo* treatment of 5A-POC on cholesterol efflux by plasma. ApoE-KO mice (n=6) were injected IV with 5A-POPC (30 mg/kg) and pooled plasma collected at baseline (black bars), 1 h (white bars), 6 h (gray bars), and 24 h post injection (stripped bars) were tested for cholesterol efflux from control mock transfected cells, ABCA1 transfected or ABCG1 transfected cells. Results represent the mean ±1 SD of quadruplicates. ***** indicates $p<0.05$ compared to baseline result.

Fig 6. Effect of 5A-POPC treatment on *In Vivo* cholesterol efflux from total tissues. **Panel A:** Cholesterol efflux from tissues in rats (n=4) injected IV with the indicated dose of 5A-POPC was determined from isotope dilution curves. **Panel B:** Fecal sterol

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excretion was determined for neutral sterols (open bars) and bile acids (solid bars) for rats treated with the indicated dose of 5A-POPC. Results represent the mean ±1 SD of triplicates. ***** indicates $p<0.05$ compared to the 0 dose treatment result.

Fig 7. Effect of 5A-POPC treatment on *In Vivo* cholesterol efflux from macrophages: C57Bl/6 mice (n=7) were injected IP with RAW 264.7 macrophage radiolabeled with ^{3}H-cholesterol. 5A-POPC (30 mg/kg) was injected IP 8 h before and within 1 hour before cell injection. 24h after cell injection, plasma was monitored for radioactive counts (**Panel A**), and stool was monitored for fecal sterols (**Panel B**) and fecal bile acids (**Panel C**). Results represent the mean ±1 SD of seven replicates. *indicates $p<0.01$ compared to the untreated control group.

Fig 8. Effect of 5A-POPC treatment on atherosclerosis: Each set of data is represented by a scattered dot plot (on the left) and a box and whiskers plot (on the right). 4-month old ApoE-KO mice (n=8) treated I.V. with POPC or 5A-POPC (30 mg/kg) (**Panel A**) or 8-month old apoE-KO mice (n=8) treated I.V. with Saline or 5A-POPC (30 mg/kg) (**Panel B**) on a normal chow diet were injected every Monday, Wednesday and Friday for 13 weeks and then analyzed for % surface area coverage of aortic plaque. In **panel C**, 4-month old ApoE-KO mice (n=8) on a normal chow diet were injected IP with 5A-SM/DPCC (30 mg/kg) or Saline every Monday, Wednesday and Friday for 13 weeks and then analyzed for % surface area coverage of aortic plaque.

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Tables

Table 1.

	POPC	5A-POPC	*p* value
AST (U/L)	**85 ± 10**	**81 ± 13**	**0.8**
ALT (U/L)	**38 ± 5**	**39 ± 2**	**0.9**
LDH (U/L)	**252 ± 18**	**274 ± 39**	**0.62**
Creatinine (mg/dL)	**0.14 ± 0.02**	**0.11 ± 0.03**	**0.3**
BUN (mg/dL)	**19 ± 0.5**	**19 ± 1**	**0.83**
Glucose (mg/dL)	**135 ± 2**	**144 ± 9**	**0.48**
Weight (g)	**26 ± 2**	**24 ± 0.5**	**0.2**

Table 1. Effect of 5A-POPC treatment of plasma chemistry profile and body weight. 4-month old ApoE-KO mice (n=8) were injected IV with 5A-POPC (30 mg/kg) every Monday, Wednesday and Friday for 13 weeks, weighed and then plasma was analyzed for the above parameters. Results represent the mean ±1 SD of eight replicates.

Fig 1.

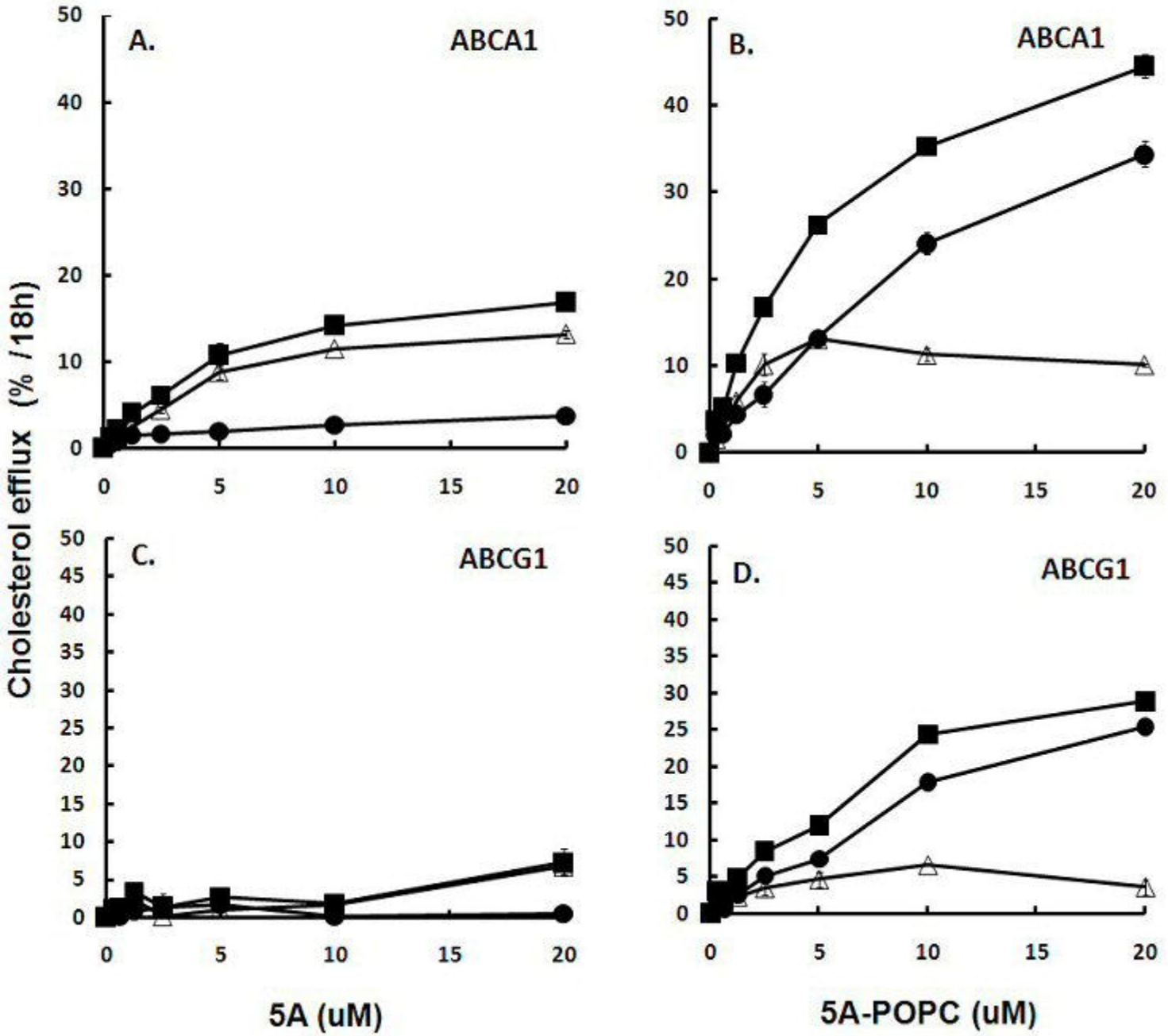

Fig 2.

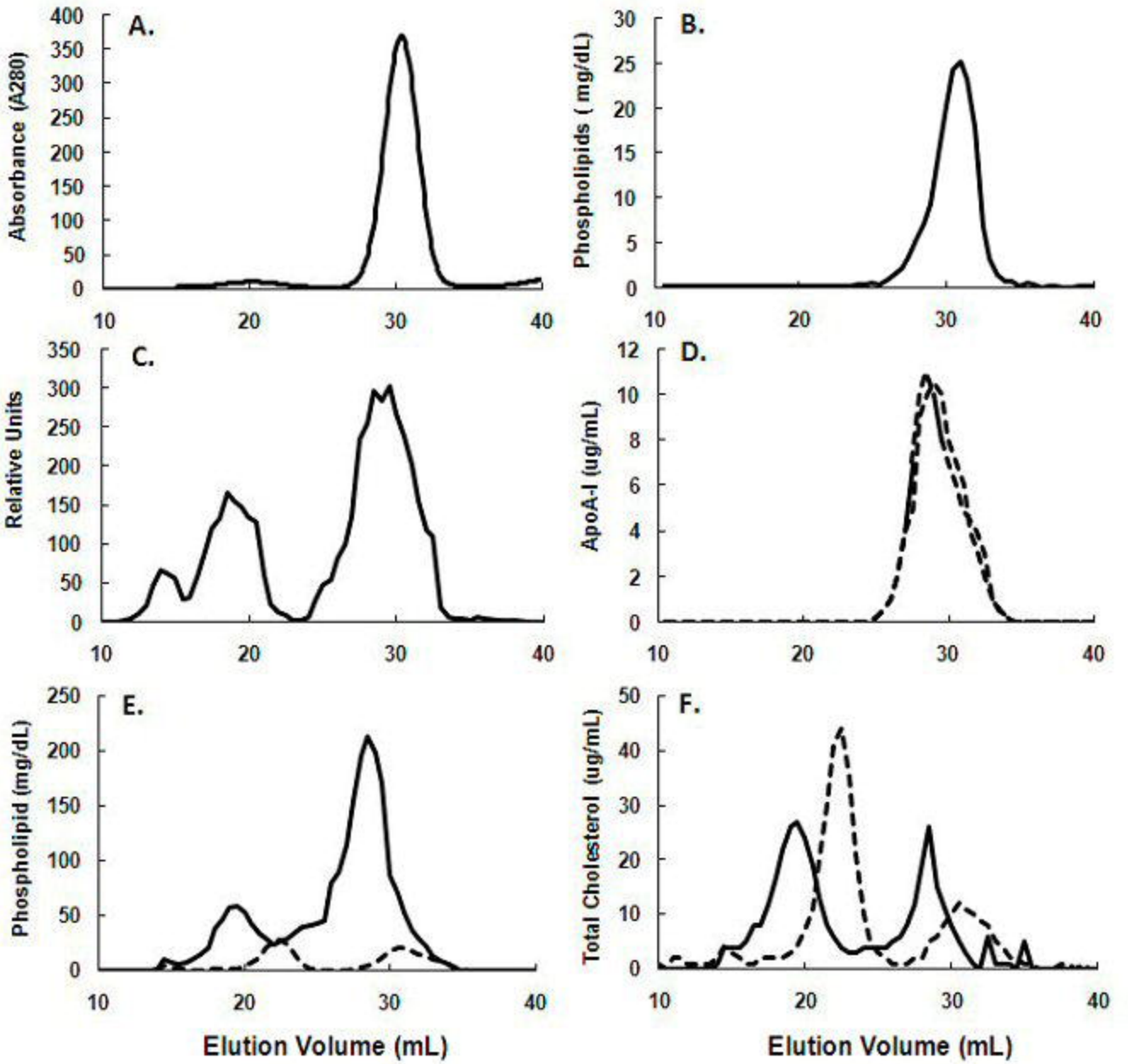
A.
Absorbance (A280)
400
350
300
250
200
150
100
50
0
10
20
30
40
B.
Phospholipids (mg/dL)
30
25
20
15
10
5
0
C.
Relative Units
350
300
250
200
150
100
50
D.
ApoA-I (ug/mL)
12
10
8
6
4
2
E.
Phospholipid (mg/dL)
250
200
150
100
50
F.
Total Cholesterol (ug/mL)
50
40
30
20
10
Elution Volume (mL)
Elution Volume (mL)

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Fig 3.

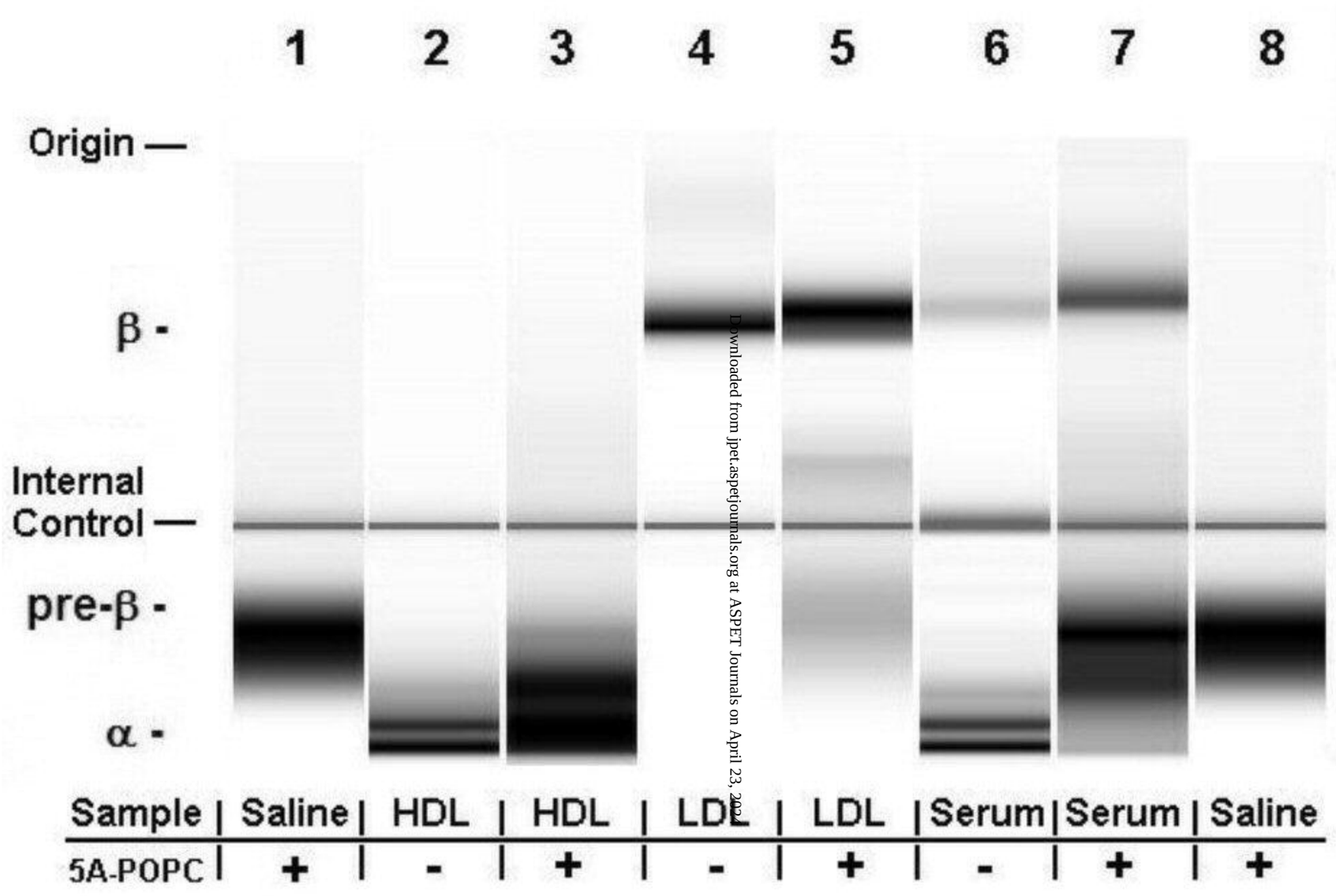


Fig 4.

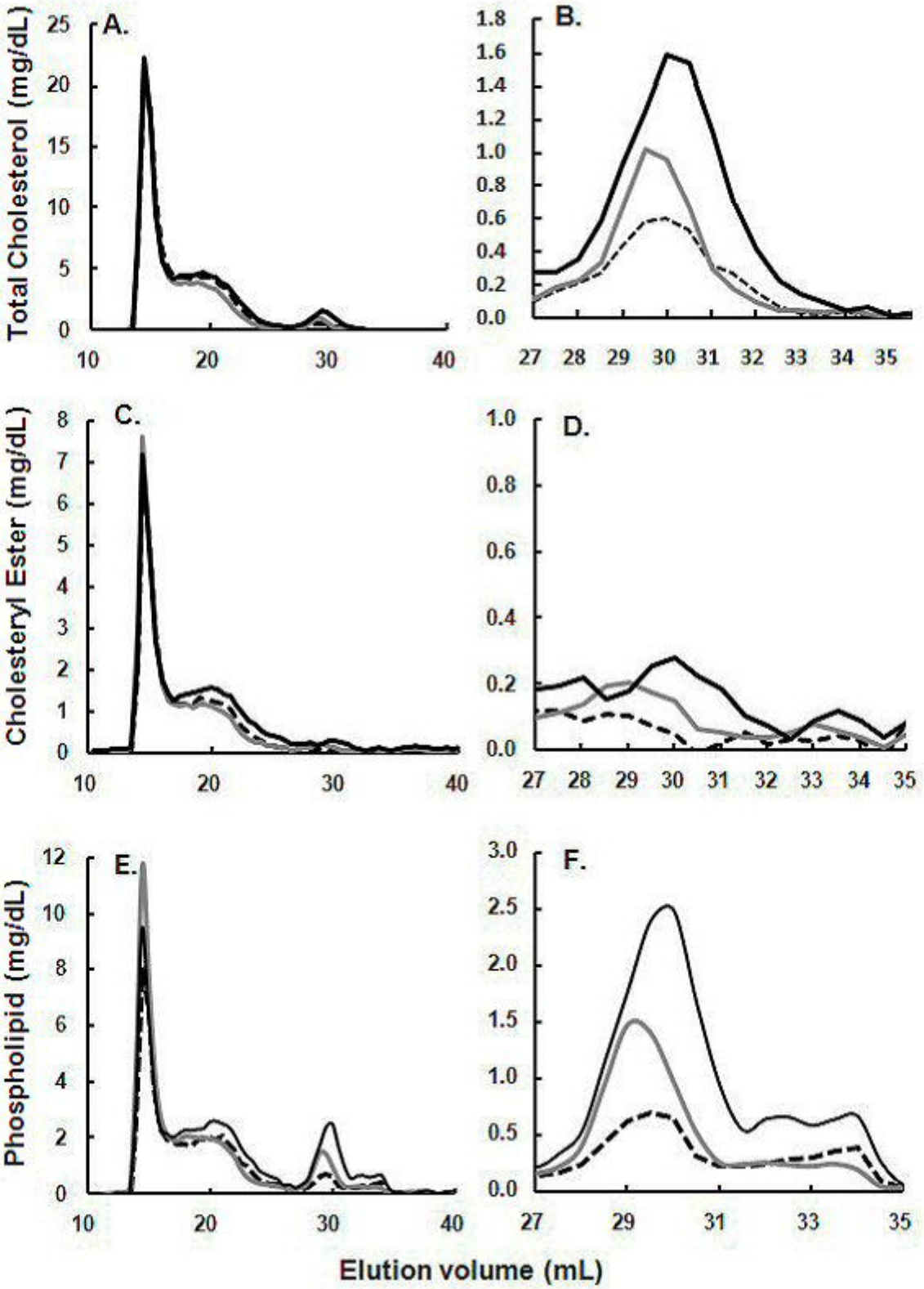

A.
B.
C.
D.
E.
F.
Total Cholesterol (mg/dL)
Cholesteryl Ester (mg/dL)
Phospholipid (mg/dL)
Elution volume (mL)

Fig 5.

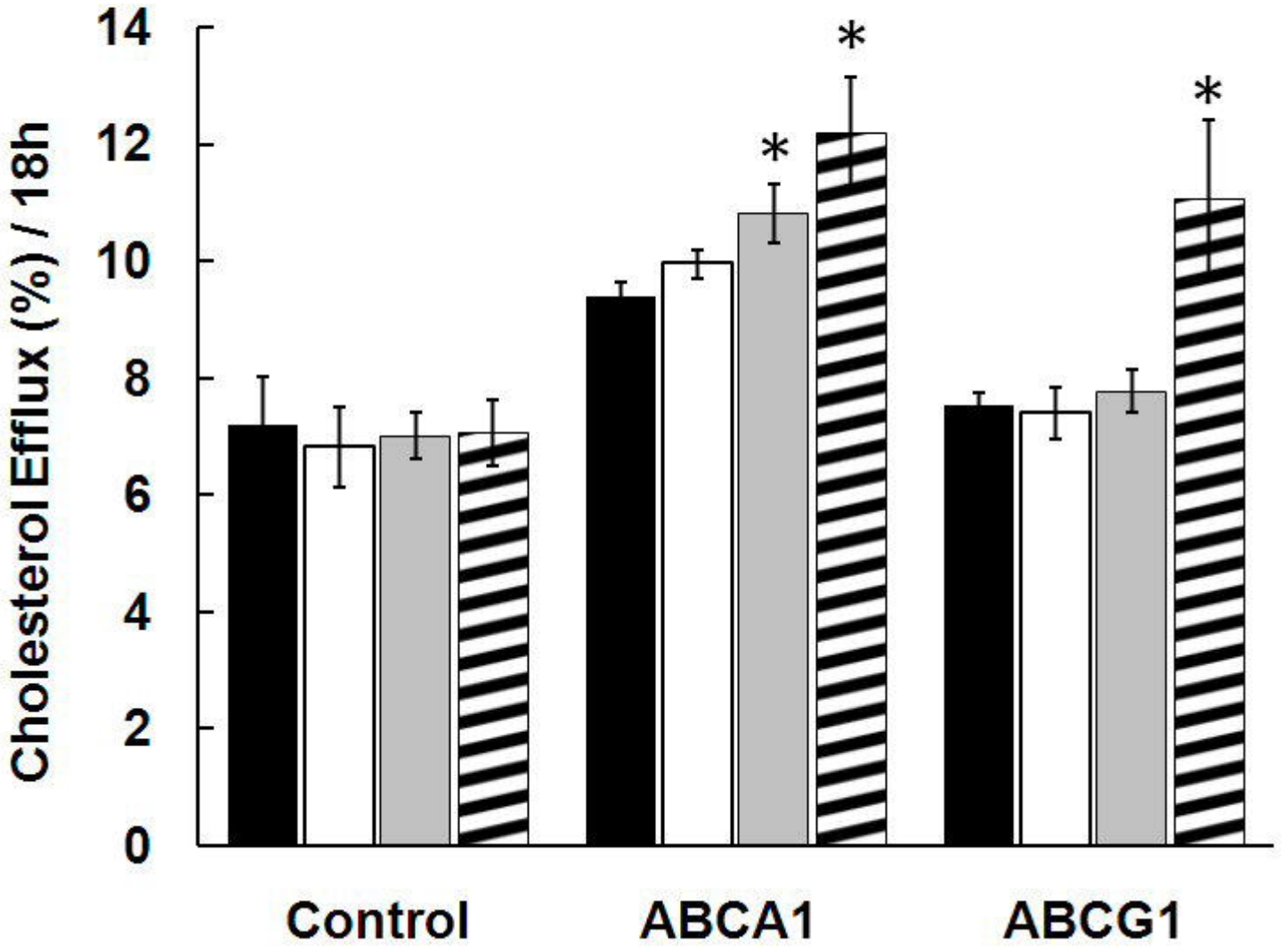


Fig 6.

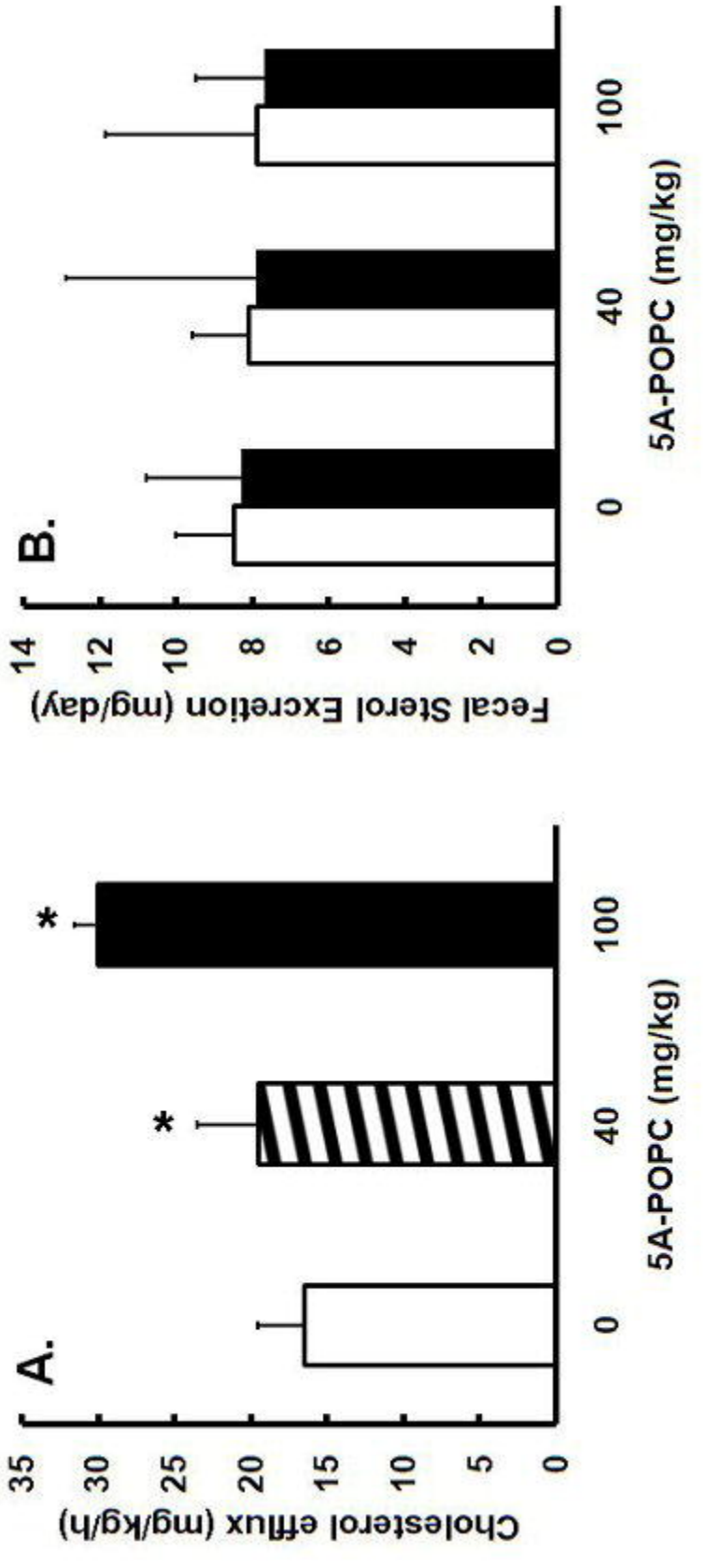

A.
Cholesterol efflux (mg/kg/h)
35
30
25
20
15
10
5
0
*
*
0
40
100
5A-POPC (mg/kg)
B.
Fecal Sterol Excretion (mg/day)
14
12
10
8
6
4
2
0
0
40
100
5A-POPC (mg/kg)

Fig 7.

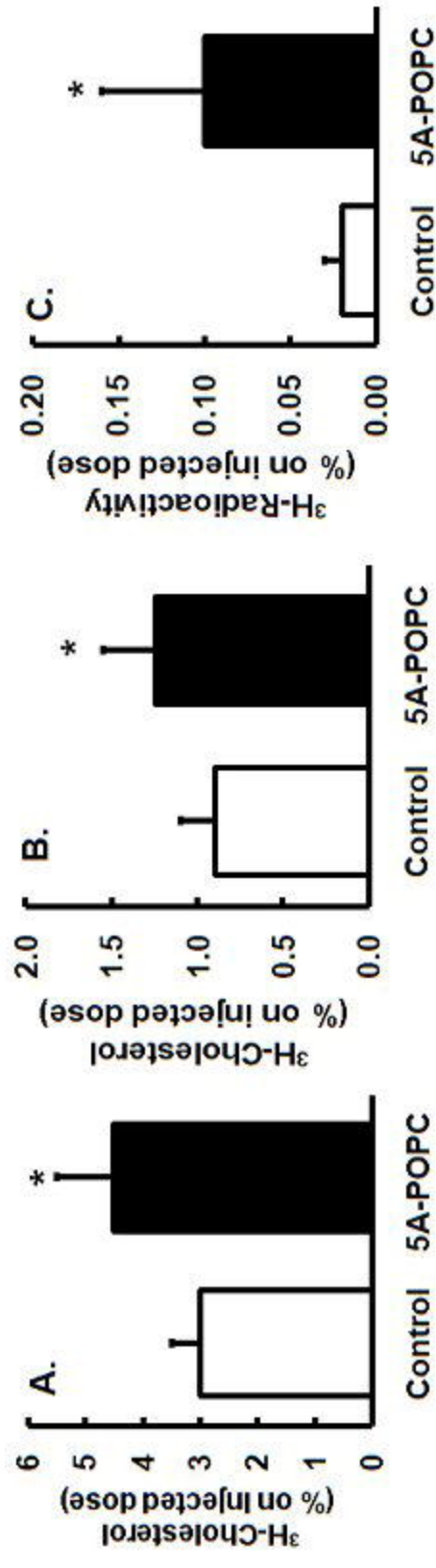
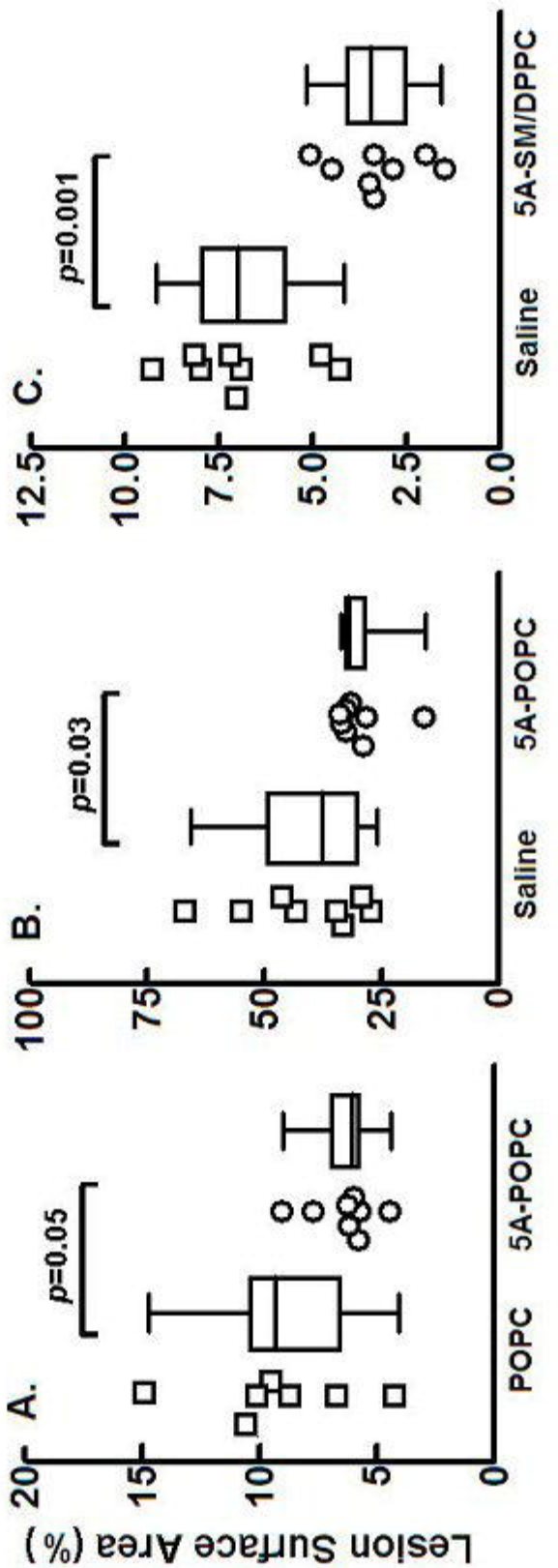

A.
B.
C.
3H-Cholesterol
(% on injected dose)
3H-Radioactivity
(% on injected dose)
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
0.00
0.05
0.10
0.15
0.20
*
Control
5A-POPC

Fig 8.


Lesion Surface Area (%)
A.
20
15
10
5
0
p=0.05
POPC
5A-POPC
B.
100
75
50
25
0
p=0.03
Saline
5A-POPC
C.
12.5
10.0
7.5
5.0
2.5
0.0
p=0.001
Saline
5A-SM/DPPC