Original Research Article

Anionic nanoliposomes reduced atherosclerosis progression in Low Density Lipoprotein Receptor (*LDLR*) deficient mice fed a high fat diet¹

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Abstract

Atherosclerosis is a systemic disease characterized by the deposition of cholesterol and inflammatory cells within the arterial wall. Removal of cholesterol from the vessel wall may have an impact on the size and composition of atherosclerotic lesions. Anionic phospholipids or liposome vesicles composed of a lipid bilayer such as nanoliposomes have been suggested as treatments for dyslipidemia. In this study, we investigated the effect of anionic nanoliposomes on atherosclerosis in a mouse model. Low-density lipoprotein receptor knockout mice (*Ldlr*^{-/-}) were fed with an atherosclerosis promoting high fat and cholesterol (HFC) diet for 12 weeks. Anionic nanoliposomes including hydrogenated soy phosphatidylcholine (HSPC) and distearoyl phosphatidylglycerol (DSPG) (molar ratio: 1:3) were injected intravenously into HFC-fed *Ldlr*^{-/-} mice once a week for 4 weeks. Mice receiving nanoliposomes had significantly reduced atherosclerosis within the aortic arch as assessed by Sudan IV staining area ($p=0.007$), and reduced intima/media ratio ($p=0.030$) and greater collagen deposition within atherosclerosis plaques within the brachiocephalic artery ($p=0.007$), compared to control mice. Administration of nanoliposomes enhanced markers of reverse cholesterol transport (RCT) and increased markers of plaque stability in HFC-fed *Ldlr*^{-/-} mice. Reduced cholesterol accumulation was observed in the liver along with the up-regulation of the major genes involved in the efflux of cholesterol such as hepatic ATP-binding cassette transporters (ABC) including *Abc-a1*, *Abc-g1*, *Abc-g5* and *Abc-g8*, Scavenger receptor class B, member 1 (*Scarb1*) and Liver X receptor alpha (*Lxr*)- α . Lecithin Cholesterol Acyltransferase activity within the plasma was also increased in mice receiving nanoliposomes. Anionic nanoliposome administration reduced atherosclerosis in HFC-fed *Ldlr*^{-/-} mice by promoting RCT and upregulating the ABC-A1/ABC-G1 pathway.

Key words: Atherosclerosis, Anionic nanoliposome, Low Density Lipoprotein Receptor (LDLR) deficient mice, reverse cholesterol transport, ATP-binding cassette transporters.

Nonstandard Abbreviations and Acronyms

ABC	ATP-binding cassette transporters
BCA	Brachiocephalic arch
CVD	Cardiovascular disease
HDL-C	High density-lipoprotein cholesterol
HFC	High fat/cholesterol
LCAT	Lecithin acyl transferase
LDL-C	Low density lipoprotein cholesterol
<i>Ldlr</i> ^{-/-}	Low density lipoprotein receptor deficient
ORO	Oil Red O
RCT	Reverse cholesterol transport
TG	Triglycerides

Introduction

Atherosclerosis is a multi-factorial disease of the large arteries and the leading cause of morbidity and mortality in the developed world (Heron, 2007). Dyslipidemia, which is characterised by elevated levels of plasma low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) and low levels of high-density lipoproteins (HDL-C) is a major causative factor in atherosclerosis (Blum and Levy, 1987; Steinberg, 2005). Atherosclerosis is believed to be initiated by the focal infiltration and

retention of apolipoprotein B (apoB)- containing lipoproteins, like LDL, lipoprotein(a) and triglyceride-rich remnant lipoproteins (TRLs) in the sub-endothelial matrix of arteries (Tabas et al., 2007). The liver is the only organ from which substantial net excretion of cholesterol can occur, either directly or after conversion to bile acids (Dietschy et al., 1993). The efflux and transport of cholesterol from the peripheral tissues to the liver for ultimate intestinal excretion is known as reverse cholesterol transport (RCT) and is a major pathway responsible for maintaining whole body cholesterol homeostasis (Khera and Rader, 2010). The ATP-binding cassette (ABC) transporter protein super family members are critical in the RCT pathway through promoting the efflux of cellular cholesterol to HDL from peripheral cells [8]. Subsequently, the enzyme Lecithin cholesterol acyltransferase (LCAT) catalyses the esterification of free cholesterol in plasma lipoproteins, which is carried as cholesterol ester (CE) in the core of the HDL particle to the liver. Strategies that stimulate RCT have potential as novel treatments for atherosclerosis.

Statins (HMG-CoA reductase inhibitors) are the primary cholesterol-modifying drugs used in the therapeutic management of cardiovascular disease (CVD). Statin monotherapy leads to a relative reduction in cardiovascular events of ~30% (Baigent et al., 2005; Cholesterol Treatment Trialists et al., 2008). Statins cannot be tolerated by a significant number of patients due to side effects, particularly myopathy. Moreover, there is a considerable residual cardiovascular risk that remains after statin treatment, even in those attaining therapeutic LDL-C goals (Sampson et al., 2012). Hence, intensive research is currently being undertaken to identify additional lipid-modifying medications.

Phospholipids have been highlighted as potential agents for the treatment of dyslipidemia (Pandey and Sparks, 2008), and anionic phospholipids such as phosphatidylinositol have been shown to interact with the receptors central to the metabolism, secretion and clearance of lipoproteins (Sparks et al., 2008; Stamler et al., 2000). Previous studies suggest that liposomes comprised of at least 75% anionic phospholipids can coalesce with LDL-C, leading to the formation of complexes that are cleared via the LDL receptors (LDLR) in hepatocytes or macrophages (Amin et al., 2002; Greenspan et al., 1995). Furthermore, cholesterol-poor liposomes were reported to interact with plasma lipoproteins and to serve as a sink or carrier, especially for LDL-C.

Nanoliposomes are novel targeted drug delivery systems that consists of phospholipid bilayers (Mamot et al., 2005). Previous studies in animal models have successfully utilised immunoliposomal drug delivery systems, for example, anti-P selectin antibody-conjugated liposomes containing vascular endothelial growth factor (VEGF) (Scott et al., 2009) and lectin-like oxidised low-density lipoprotein receptor (LOX)-1 (Saito et al., 2011). A recent study suggested that active targeting of nanoliposomes to LDL particles enhances the liposome-lipoprotein interaction to promote plasma cholesterol clearance, probably due to the rapid uptake of nanoliposomes by the liver (Sahebkar et al., 2015). In that study, a single intravenous dose of apoB-targeted anionic nanoliposomes reduced serum levels of LDL-C, apoB, non-HDL-C, total cholesterol (TC) and triglycerides (TG), while elevating the concentration of HDL-C (Sahebkar et al., 2015). The lipid-lowering effect of the nanoliposomes was found to last for at least 48 hours and could potentially inhibit atherosclerosis progression.

In the present study, we aimed to evaluate the anti-atherosclerotic effects of a nanoliposomal formulation using a mouse model of established dyslipidemia. The primary outcome measure for the study was atherosclerosis severity assessed by plaque burden within the aortic arch

measured by *en face* staining. Secondary outcome measures included: (i) histological assessment of atherosclerotic plaque morphology; (ii) measurements of the aortic and liver expression of genes involved in RCT; (iii) assessment of the circulating lipid profile; and (iv) analysis of circulating inflammatory markers.

Methods

Preparation and characterization of nanoliposomes

Conventional liposomes were prepared from hydrogenated soy phosphatidylcholine (HSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol (DSPG) (HSPC/DSPG molar ratio: 1:3) using the thin lipid-film hydration method. After vacuum and freeze drying, the obtained thin lipid film was hydrated with histidine (10mM)/sucrose (10%). The resultant multilamellar dispersions were reduced in size using vortexing, sonication with three cycles of high-pressure homogenization using an EmulsiFlex-C3 apparatus (Avestin, Canada) (Sahebkar A, 2015; Sahebkar et al., 2014). Hydrodynamic diameters of the nanoliposomes and the polydispersity index of the preparation were measured in triplicate using a Dynamic Light Scattering Instrument (Nano-ZS; Malvern, UK). The zeta potential of nanoliposomal preparations were measured on the same apparatus using the zeta potential mode as the average of 20 measurements. The Bartlett test was used to measure the phospholipid content of the nanoliposomal preparations, as previously described in details (Bartlett, 1959; Sahebkar et al., 2014) (*Supplementary data*).

Animal study

Male *Ldlr*^{-/-} mice (C57BL/6J background, Jackson Laboratory, Bar Harbor, Maine) were housed under a 12:12-h light-dark cycle (relative humidity: 55–60%; temperature: 22±1°C) and were given standard chow and water *ad libitum*. At 3 months of age mice (n=10) were commenced on a high fat and high cholesterol (HFC) diet (cholesterol 1.25%, total fat 24.50%, ghee 45%; SF08-043, Specialty Feeds, Perth, Western Australia) for 12 weeks. After 12 weeks mice were weighed and split into 2 groups of equal body weight. The 2 groups of mice were subjected to the following interventions: Group 1, administered with 100 µmol/kg of nanoliposomes (n=5), and Group 2, administered with saline (vehicle control; n=5). Nanoliposomes or vehicle control were administered *via* tail vein injections once a week for a total of 4 weeks. All solutions were sterilized by passing through a 0.22 µm filter and all intravenous injections were carried out under sterile conditions. At the end of the experiment, mice were sacrificed by CO₂ asphyxiation and blood was collected by cardiac puncture. The aorta was flushed with 5 mL of phosphate buffered saline (PBS, pH 7.4) introduced through the left ventricle at physiological pressure. The aorta was dissected free from surrounding fat and aortic arches were separated and stored in Optimum Cutting Temperature (OCT, ProSciTech, Australia) compound at -80°C. The remaining aortic tissues were placed in RNALater (Ambion) and stored at -80°C until needed. Livers were excised immediately after sacrifice, weighed and sectioned (100–150 mg/section) for storage at -80°C for the following analysis: one portion was snap frozen in OCT to be used for histology, another portion was submerged in RNALater and stored at -80°C until assays were performed and the third portion of liver was snap frozen in OCT for lipid analysis. This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). The protocol was approved by the Animal Ethics Committee of James Cook University. No animals died during the experimental procedures prior to experiment termination.

Plasma and tissue analysis

Blood was collected from each mouse before starting the HFC diet and following 12 weeks of the HFC diet via retro-orbital bleeding (200 μ L), and at sacrifice via cardiac puncture following 4 weeks of liposome administration. Blood was collected into tubes for separation of platelet poor plasma (EDTA coated tubes, BD Microtainer) and serum (heparin coated tubes, BD Microtainer) (Krishna et al., 2012). Samples were stored at -80°C until assays were performed. Plasma TC (Wako, Japan), HDL-C (Wako, Japan), LDL-C (Crystal Chem, USA) and TG (Abcam) levels were assayed using commercially available kits according to the manufacturer's instructions. Liver (100 mg) tissue was homogenised in Dounce homogeniser and processed to assess TC using commercially available kits (Cell Biolabs Inc.) according to the manufacturer's instructions. LCAT activity was assessed in plasma using a commercially available fluorometric kit as per the manufacturer's instructions (Cell Biolabs Inc). Briefly, platelet poor plasma was incubated with a fluorescent substrate, and the fluorescence intensity of the intact substrate was measured by a fluorescence spectrophotometer at 342nm excitation and 400nm emission using a POLARStar Omega™ fluorescence plate reader (BMG Labtech). The LCAT phospholipase activity in the plasma was expressed as relative fluoresce units (RFU).

Processing of aortas

Aortas were cleared of fat and the brachiocephalic artery (BCA) (with part of the right subclavian artery attached for accurate orientation), heart and the aortic branches including the iliac bifurcation, harvested. The BCA was cut away from the aortic arch and fixed overnight in 10% buffered formaldehyde in PBS (Sigma-Aldrich) prior to embedding in paraffin. The aortic arch and thoracic aorta were stored in OCT (ProSciTech); and the supra-renal and infra-renal aortic segments were stored in RNA Later (Ambion).

Assessment of aortic atherosclerosis severity

Plaque area was quantified as previously described (Krishna et al., 2012). Briefly, all of the aortic arch segments stored in OCT (ProSciTech) from each of the 2 groups were cleared of excess fat under a dissecting microscope. After removing the adventitia and adipose tissue, the aorta was placed in 10% neutral buffered formalin overnight. The aortic arch was then opened lengthwise through the lesser curvature and pinned flat *en face* in a wax bottomed dissecting pan. The tissue was stained for 15 mins with 0.5% Sudan IV solution (ProSciTech) in acetone and 70% ethanol (1:1). The tissue was then placed in 80% ethanol for 5 mins and washed gently with water for several minutes. Digital images of the stained samples were acquired and staining quantified as a percentage of the total tissue area. We have previously reported excellent reproducibility of this technique (Krishna et al., 2015; Krishna et al., 2012).

Histological evaluation of the BCA by hematoxylin and eosin, Van Giesson's and picrosirius red staining

The BCA was dissected out to provide a 'Y'-shaped piece of vessel comprising the origin of right subclavian artery and right carotid artery. Serial, 5 μ m thick, sections of the paraffin embedded BCA tissue were prepared for multiple histological assessments. The BCA segments were oriented to obtain sections starting from proximal (near the aortic arch) to distal (towards the right subclavian artery). Morphometric analysis was performed on BCA sections obtained at 120 μ m intervals from 0 to 480 μ m distal to the aortic arch. Hematoxylin and eosin (ProSciTech) staining was performed on 5 μ m sections to assess the extent of plaque deposition in the BCA. Histological sections were examined by light microscopy to evaluate the overall architecture. All slides were analyzed at the same magnification (400x) using a Nikon Eclipse 50i microscope fitted with a CCD Camera (*DSFi1*) and digital images

were captured to a PC supported with NIS Elements (*version F2.30*). BCA sections were also stained with a Van Giesson's staining (EVG, ProSciTech) protocol for elastin. The areas enclosed by the external elastic lamina (EEL), internal elastic lamina (IEL) and lumen were measured using image analysis software (Adobe®Photoshop® CS5 Extended version 12, Adobe Systems Incorporated). These measurements were used to derive the total vessel area enclosed by the EEL and the area inside the IEL. The area inside the IEL was subtracted from the area inside the EEL to provide a measure of media area. The intima-media ratio was obtained by dividing the intimal area by the medial area (%). All analyses were carried out by an observer blinded to the group allocation, and 4 sections per mouse were quantified for each of the groups. We observed that these measurements could be repeated with good inter-observer reproducibility (Concordance correlation coefficient=0.99; Coefficient of variation, COV=3.40%, N=5).

Sections were similarly processed for staining with 0.05% saturated picosirius red (ProSciTech) for collagen content and imaged under a circularly polarised light using a Zeiss microscope fitted with a CCD camera (Carl Zeiss MicroImaging, Germany; Diagnostic Instruments, MI). Digital images were captured to a microcomputer supported with *Zeiss AxioVision version 4.8.2* software. Collagen content was expressed as percentage of stained area of the total section area. Sections were photographed with an identical exposure setting for each section. The percentage of the field area that birefringence was determined for 4 to 5 high power fields for at least 3 sections per mouse (N=5 samples/group), and the mean value was calculated for each region following previously published protocols (Krishna et al., 2015). We have previously published that these measurements could be repeated with good intra-observer reproducibility (Intra-observer COV= 2.68%, N=6) (Krishna et al., 2015).

Immunohistochemistry of the BCA

Immunohistochemical (IHC) analysis was performed as previously described (Gaspari et al., 2013). Paraffin sections were de-paraffinised and hydrated. Antigen retrieval was performed in a microwave using citrate buffer (Antigen Retriever, Sigma) and subsequently cooled to room temperature and rinsed for 5 minutes in tap water. Endogenous peroxidase activity was blocked by incubating the sections in BLOXALL™ Blocking Solution (Vector Labs) for 10 minutes. Sections were incubated overnight at 4°C with primary antibodies (MOMA-2 and α -Smooth muscle actin, 1:200 dilutions; Abcam). Sections were then rinsed in PBS and incubated with biotinylated secondary antibodies (biotinylated anti-Rat IgG and biotinylated anti-Rabbit IgG, 1:500 dilutions; Vector Labs) followed by incubation with avidin-biotin peroxidase complex solution (VECTASTAIN *Elite* ABC KIT, Vector Labs). Finally, DAB substrate chromogen system (ImmPACT™ DAB KIT, Vector Labs) was used for visualization and sections were counter stained with haematoxylin (Vector Labs) before being examined under a light microscope (Nikon, Diagnostic Inc, USA). Three to 4 sections from each BCA were analyzed for staining location and the percentage staining in the total section area assessed. We have previously shown that these protocols can be performed with excellent reproducibility (Krishna et al., 2015; Krishna et al., 2012).

mRNA expression analysis

Total RNA was extracted using an RNA extraction kit (Qiagen) from liver and aortic tissue samples stored in RNA Later (Qiagen). Contaminant DNA was removed with RNase free DNase I (1U/sample; Qiagen). mRNA expression levels were assessed by quantitative Real-Time PCR (q-RT-PCR) using Rotor Gene Q. RT² qPCR Primer Assays (Qiagen; listed in *Supplementary Table S1*) were used to determine aortic expression of the following genes: ATP-binding cassette transporter A, member 1 (*Abca1*; PPM03952F), ABC member G1

(*Abcg1*; PPM03895A), ABC member G5 (*Abcg5*; PPM03948A), ABC member G8 (*Abcg8*; PPM26893A), Scavenger receptor class B, member 1 (*Scarb1*; PPM05368C) and Liver X receptor alpha (*Lxr-α*; PPM05356F), as previously described (Biros et al., 2012). The relative expression of these genes were calculated by using the concentration-Ct-standard curve method and normalized using the average expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; QT01658692) for each sample using the Rotor-Gene Q operating software version 2.0.24. The QuantiTect SYBR® Green one-step RT-PCR Kit (Qiagen) was used according to the manufacturer's instructions and all reactions were independently repeated in duplicate.

Liver histology

Hepatic lipid deposition was assessed in samples stored in OCT by Oil Red O (ORO) staining. Briefly, liver tissue was sectioned at 5 µm thickness and fixed for 10 min in 60% isopropanol. Sections were stained with 0.35% ORO (Sigma) in 60% isopropanol for 30 min and washed with 60% isopropanol, counterstained with Gill's hematoxylin (ProSciTech) and washed with acetic acid solution (4%). Tissue was then mounted with aqueous solution and photographed immediately. To obtain an overview of intra-tissue lipid accumulation, a minimum of 3 sections from different depths of the tissue were collected during sectioning and the staining value was averaged across the sections. The area of positive staining for ORO was calculated as a percentage of total section area using Adobe software (*version CS5*) from three to five views per 3 sections from each animal (Mehlem et al., 2013).

Spleen cell preparation and analysis

Single cell suspensions were prepared from whole spleens by disrupting the spleen using a 26-gauge needle and forceps in ice-chilled MACS separation buffer (pH 7.2, Miltenyi Biotec) containing PBS (pH 7.4), 0.5% bovine serum albumin (BSA), 2mM EDTA and 0.09% azide. The resulting cell suspension was treated with red blood cell lysis buffer (BioLegend). Cells were pre-incubated for 10 min with anti-CD16/32 antibodies (BD Pharmingen) to prevent Fc receptor binding before addition of surface staining antibodies (listed in *Supplementary Table S2*). Surface staining was performed in darkness for 30 minutes at 4°C in MACS buffer. Cells were then washed with MACS buffer and acquired on a CyAn ADP flow cytometer (Beckman Coulter) using the Summit 4.3 software (Beckman Coulter). Debris were excluded and viable splenocytes were identified by forward/side scatter profile and by propidium iodide (PI) exclusion. A forward scatter-area against forward scatter-linear gate was used to exclude doublets from the analysis. Monocytes were identified by their expression of CD11b and Ly-6C and lack of expression of Ly-6G, NK1.1, B220 and βTCR surface proteins. The population included Ly-6C^{hi} and Ly-6C^{low} monocyte subsets and Ly-6C^{negative} macrophages. The CD11b⁺ Ly6G^{low}Ly6B.2^{hi} cells corresponded to Ly6C^{hi} monocytes that represent the inflammatory subtype and the CD11b⁺ Ly6G^{low} Ly6B.2^{low} cells corresponded to Ly6C^{low} subtypes. The gating strategy is illustrated in *Supplementary Figure S1*. Flow cytometry data were analysed using Kaluza analysis software (V 1.2, Beckman Coulter). Analysis was performed with the operator blinded to the origin of experimental samples.

Statistical Analyses

The D'Agostino and Pearson test was used to test the normality of the data. Results are expressed as median and inter quartiles range (IQR) since data were not normally distributed. Comparisons between groups were made using Mann-Whitney U-test. The Wilcoxon matched pairs signed rank test was used to compare parameters measured in the same mice at different time points. Percent changes in body weight over time were calculated as 100*[(Final weight/Weight prior to treatment)-1]. For all analyses, differences between

groups were considered to be statistically significant if $p < 0.05$. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc., USA).

Results

Nanoliposome administration limited the severity of atherosclerosis in *Ldlr*^{-/-} mice

All mice completed the study and tolerated the administration of nanoliposomes or vehicle control. There were no differences in the amount of food consumed between the groups or the physical appearance of the mice as a result of the nanoliposome administration. Body weight did not differ significantly between the groups at the end of the experiment ($p = 0.055$; **Table 1**). At the end of the experiment, the severity of atherosclerosis within the aortic arch was evaluated by *en face* staining. Median staining area of the control mice was 57.16% (IQR 48.63-65.79%), and was significantly smaller in mice receiving nanoliposomes (20.74%, IQR: 10.37-35.88; $p = 0.007$; **Fig. 1 A, B**; *Supplementary Figure S2*).

Atherosclerotic plaques within the BCA were smaller with greater smooth muscle cell and collagen content in mice receiving nanoliposomes

Assessment of hematoxylin and eosin stained sections of the BCA suggested that atherosclerosis was more severe in control mice than those receiving nanoliposomes (**Fig 1. C, D**). Estimated plaque area within the BCA was ~4-fold lower in mice receiving nanoliposomes than controls [estimated median plaque area: 15.76% (IQR 10.11-49.41) vs. 65.28% (IQR 55.21-79.33%), respectively, $p = 0.007$; **Fig 2. A-C**], with a concurrently lower median intima/media ratio [nanoliposomes: 1.54 (IQR 1.26-3.58); vehicle control: 5.09, (IQR 3.66-8.75), $p = 0.030$; **Fig 2. D**]. Subsequent immunohistochemical analysis using α -smooth muscle actin antibody suggested that the advanced fibrous plaques had smooth muscle cells within the fibrous cap (*Supplementary Figure S3*). Mice receiving nanoliposomes had greater α -smooth muscle actin staining area within the examined atherosclerotic plaques than did animals receiving the vehicle control ($p = 0.032$). Quantification by computerized image analysis suggested that the liposome administration favored more stable atherosclerosis as indicated by the higher collagen content in the atherosclerotic plaques (**Fig 3. A**). Mice receiving nanoliposomes had significantly higher median collagen birefringence under polarized light (2.95% IQR 2.30-6.03, control; 9.53%, IQR 6.13-16.20, nanoliposome; $p = 0.031$; **Fig. 3. B**).

Nanoliposome administration reduced the number of pro-inflammatory monocytes within BCA atherosclerotic plaques and spleen in *Ldlr*^{-/-} mice

As inflammation plays a central role in atherosclerosis, we assessed the numbers of inflammatory cells in atherosclerotic plaques within the BCA. IHC analyses suggested that mice receiving nanoliposomes had significantly reduced median macrophage staining area within the BCA samples compared to controls (18.91%, IQR 14.08-29.64, vs. 8.95%, IQR 4.21-11.59, $p = 0.015$; **Fig. 4. B, D**). The spleen plays an important role in the control of immune function. Mature monocytes reside in the sub-capsular red pulp of the spleen, where they are rapidly deployed in response to inflammatory signals and have been implicated in atherosclerosis (Potteaux et al., 2015). We performed flow cytometric analysis of freshly isolated spleens. Monocytes were defined as side scatter-low forward scatter high cells that expressed high levels of myeloid antigens 7/4 and CD11b but were negative for Ly6G, a neutrophil marker. The CD11b⁺ Ly6G^{low}7/4^{hi} cells correspond to Ly6C^{hi} monocytes that represent the pro-inflammatory subtype and the CD11b⁺ Ly6G^{low}7/4^{low} cells correspond to Ly6C^{low} subtypes. Ly6C^{hi} monocytes are recruited to sites of inflammation in mice and differentiate into M1 macrophages, which secrete pro-inflammatory cytokines (Meng et al.,

2014). Mice that received nanoliposomes (median 1.25×10^5 , IQR $1.14 \times 10^5 - 1.34 \times 10^5$) had significantly lower numbers of Ly-6C^{hi}/CD11b⁺ macrophages than controls (median 4.60×10^5 , IQR $1.93 \times 10^5 - 8.74 \times 10^5$, $p=0.007$; **Fig. 4 A, C**). In contrast, we found no difference in the numbers of T lymphocytes, B lymphocytes or neutrophils in the spleen of the different mice groups (*Supplementary Table S3*). There was no significant difference in the plasma concentrations of the following pro-inflammatory cytokines in control and nanoliposome-administered mice: MCP-1, TNF- α , IL-12p70, IFN- γ , IL-10 and IL-6 (*Supplementary Table S4*).

Nanoliposome administration limited hepatic steatosis and increased hepatic expression of genes involved in RCT

Since the liver is the major site of cholesterol excretion (Dietschy et al., 1993), we analyzed the effect of administering the nanoliposomes on the morphology of the liver by ORO staining (**Fig 5. A**). Control mice showed obvious vacuolization and accumulation of lipid droplets in the hepatocytes. At the completion of the experiment the total liver weight was significantly greater in mice receiving vehicle control compared to those administered nanoliposomes ($p=0.031$), however there was no difference in the hepatic TC levels between the two groups ($p=0.420$; **Table 1**). The number of vacuoles and nuclear size of hepatocytes appeared to be less in the livers of mice receiving nanoliposomes. The median percentage area of lipid deposits was significantly less in mice receiving nanoliposomes (16.09%, IQR 13.71-22.21) than controls (35.43%, IQR 31.47-38.87, $p=0.007$; **Fig. 5. B**). We assessed the effect of nanoliposome administration on the hepatic expression of genes involved in the RCT pathway. Mice receiving nanoliposomes had significantly greater relative *Abc-a1* expression than controls (**Fig 5. C**). Mice receiving nanoliposomes also had significantly higher relative expression of other ABC family members such as *Abc-g1* ($p=0.007$), *Abc-g5* ($p=0.031$) and *Abc-g8* ($p=0.015$) than controls. Furthermore, there was higher expression of *Lxr- α* ($p=0.015$) and *Scarb-1* ($p=0.015$) within the liver of mice receiving nanoliposomes than controls. At the end of the experiment, mice receiving nanoliposomes had significantly higher median plasma LCAT activity in comparison to controls (67014 RFU, IQR 52356 - 135511, control; 189246 RFU, IQR 135393 - 203835, nanoliposome, $p=0.031$; **Fig. 6. A**). Plasma lipid profiles were assessed following 3 months of a HFC diet (i.e. immediately prior to the start of administration of the interventions) and after 4 weeks of nanoliposome or vehicle control administration. Plasma TC levels were similar in the 2 groups prior to and 4 weeks after starting the interventions (**Table 2**). Of note, compared to the saline controls a lower median plasma LDL-C concentration was observed in mice receiving nanoliposomes but this was only of borderline significance (1119 mg/dL, IQR 761.50-1728, control; 457.70 mg/dL, IQR 415.40-807.70, nanoliposome, $p=0.055$; **Table 2**).

Administration of nanoliposome promoted increased aortic expression of *Abc-a1* and *Abc-g1*

Gene expression analyses demonstrated that in mice receiving nanoliposomes aortic *Abc-a1* and *Abc-g1* expressions were significantly higher than controls (**Fig 6. B**). Nanoliposome administration did not impact on aortic *Scarb-1* expression.

Discussion

The main finding from this study was that administration of nanoliposomes limited atherosclerosis severity within a mouse model. The finding was similar for two different methods of assessing atherosclerosis severity. These results suggest that the nanoliposomes may have potential as an anti-atherosclerosis treatment. Most atherosclerosis-associated

events in human have been linked to atherosclerotic plaque rupture (Schwartz et al., 2007). Plaques with thin and inflamed caps are thought to be prone to rupture (Bentzon et al., 2014; van der Wal and Becker, 1999). Mice receiving nanoliposomes had reduced macrophage staining and increased collagen deposition within the fibrous cap of atherosclerotic plaque within the BCA compared to controls. The findings suggest that administration of nanoliposomes may have a plaque stabilising effect with potentially important implications for reducing atherosclerosis-related events. The mechanisms by which nanoliposomes induced these changes in plaque morphology are not currently clear and require further investigation.

Previous studies suggest that the spleen represents an important reservoir of monocytes in mice prone to atherosclerosis and that these cells contribute directly to atherosclerosis progression (Robbins et al., 2012; Swirski et al., 2009). Mature monocytes reside in the sub-capsular red pulp of the spleen, where they are rapidly deployed in response to inflammatory signals. To examine the effect of administration of anionic nanoliposomes on splenic leukocyte homeostasis, we performed flow cytometry of freshly isolated spleen cells. In accordance with previous reports, a HFC diet promoted a high number of monocytes within the spleen (Robbins et al., 2012). The numbers of CD11b⁺ Ly6G^{hi} monocytes were significantly lower within the spleen of mice receiving nanoliposomes compared to controls. The findings suggested that the liposomal formulation limited differentiation of monocytes into M1 macrophages, which secretes pro-inflammatory cytokines (Meng et al., 2014). In contrast, we found no significant difference in circulating concentrations of pro-inflammatory cytokines in mice receiving nanoliposomes or vehicle control.

A recent study suggested that phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by stimulating expansion of polyreactive IgM producing B1 lymphocytes (Hosseini et al., 2015). The study suggested that the athero-protective effect of the liposome was due to expansion of B1 cells in the spleen and increased IgM production by the B1 cells. In the present study, a direct effect of liposomes on splenic B1 cells could not be detected as we only assessed the total splenic B-cell number. Nonetheless, it could be possible that liposome administration attenuated atherosclerosis development through effects on immune cells.

LDLR is a key receptor for the endocytosis of circulating LDL-C and a dysfunctional LDLR promotes premature atherosclerosis. The liver is considered to be an important contributor to systemic inflammation in *Ldlr*^{-/-} mice since these animals show elevated hepatic inflammation compared to wild-type mice (Luyendyk and Guo, 2011). Previous findings also suggest that hepatic LDLR functions as a "gatekeeper" targeting dense ApoB100-containing lipoproteins for degradation (Larsson et al., 2004; Twisk et al., 2000). We observed significant reduction in hepatic fat accumulation in mice receiving nanoliposomes. The liver has previously been implicated in controlling systemic inflammation in situations of high cholesterol intake [51]. To gain insight into the apparent enhancement of lipoprotein clearance in mice receiving liposomes, we analysed the expression of the genes involved in RCT. Nanoliposome administration led to upregulation of *Abc-a1*, *Abc-g1*, *Abc-g5*, *Abc-g8*, *Lxr-α* and *Scarb-1* within the liver suggesting that nanoliposomes enhanced the RCT pathway by promoting expression of the ABC transporters.

LCAT promotes esterification of cholesterol and promotes formation and maturation of HDL-C. However, recent evidence suggests that the anti-atherogenic effect of LCAT correlates mainly with its plasma ApoB-lowering ability rather than the HDL-C raising

ability (Rousset et al., 2009). In the current study, we found that nanoliposome administration significantly increased plasma LCAT activity. High LCAT activity has been shown to promote RCT since excess cholesterol is removed from the cells by HDL-C and delivered to the liver for excretion (Rousset et al., 2009). Adenovirus-mediated transient expression of LCAT has been reported to decrease ApoB-lipoprotein due to increased catabolism in an experimental model (Amar et al., 2009). Similarly, adenovirus-mediated LCAT gene transfer has been reported to decrease oxidative stress and atherosclerosis in LDLR-Leptin double-mutant mice (Mertens et al., 2003). It could be possible that, similar to the situation of adenovirus mediated LCAT overexpression, the increased LCAT activity that we observed in the mice administered nanoliposomes might have promoted ApoB-lipoprotein clearance.

Nanoliposome administration not only promoted genes responsible for RCT in the liver, but also promoted upregulation of *Abc-a1* and *Abc-g1* within the aorta. *Abc-a1* and *Abc-g1* are the principal targets of LXR that promotes macrophage cholesterol efflux and initiates RCT. Activation of LXR- α stimulates direct intestinal excretion *in vivo* (van der Veen et al., 2009). Mice deficient in *Abc-a1*, *Abc-g1* or *Lxr-a* have marked cholesterol accumulation within macrophage (Alberti et al., 2001; Kennedy et al., 2005; McNeish et al., 2000). LXR agonists promote increased ABC-A1 and ABC-G1 expression in macrophages, resulting in increased cholesterol efflux (Venkateswaran et al., 2000). LXR activation promotes increased expression of ABC-G5, ABC-G8 (Repa and Mangelsdorf, 2002) and cholesterol 7- α -hydroxylase (CYP7A1) (Repa et al., 2002) within the liver, resulting in increased sterol secretion into bile. LXR activation also promotes increased ABC-G5 and ABC-G8 expression in the small intestine resulting in increased cholesterol excretion into the feces (Chiang et al., 2001; Repa et al., 2002). Overall, our findings suggest that the ability of nanoliposomes to upregulate genes involved in the RCT pathway both within the liver and aorta promoted cholesterol clearance. This is consistent with previously reported effects of anionic phospholipids in stimulating the expression of ABC-A1 and apo-A1, and inhibiting cholesteryl ester transfer protein (Burgess et al., 2003; Masson et al., 1996; Sparks et al., 1998).

This study has several limitations. The study involved a small sample size in each group since multiple intravenous injections of the nanoliposomal formulations were necessary over a period of 4 weeks. We did not monitor the intravenous delivery of nanoliposomes over the course of administration since a previous study using the same liposome formulation showed that a reduction in LDL-C was achieved as quickly as 1 hr after liposome injection. Furthermore, we did not look at the effect of nanoliposomes on circulating cells. Cell phenotyping was only performed on spleen cells. Another limitation of the current study is that we did not employ classical methods of assessing the RCT pathway. Instead, we assessed the gene expression patterns of the most crucial mediators of RCT in both the liver and the aorta. The mechanism by which the nanoliposomal formulation increased LCAT enzymatic activity was not investigated and requires further study.

It is known that anionic liposomes can enhance LDL-C removal from the circulation via direct coalescence and electrostatic interaction with apoB-100. Moreover, a selective uptake of anionic liposomes by vulnerable atherosclerotic plaques of apolipoprotein E deficient mice (*ApoE*^{-/-}) has been reported (Zhaorigetu et al., 2014). The *Ldlr*^{-/-} mouse model is considered an extreme model of dyslipidemia by comparison to human dyslipidemia. Atherosclerosis in these mice develops within a very short time period by comparison to humans. The exaggerated kinetics of atherosclerosis formation might conceal the full potential of the nanoliposomal formulations. Further research in other atherosclerotic models, such as

apolipoprotein E deficient mice, is needed to identify the exact therapeutic potential of the liposomal formulation. Even though mouse models are the most commonly used in pre-clinical research, they also have a number of limitations. Hence, the inherent limitations of currently available rodent dyslipidemic models must also be kept in mind when interpreting and extrapolating these data. Finally, the primary goal of this study was to test the hypothesis that this novel therapeutic approach might increase the cholesterol clearance and reduce atherosclerotic plaque burden within the aorta in a mouse model and full understanding of the mechanisms by which this was achieved were beyond the scope of this study.

Conclusions

In conclusion, this study suggests that administration of anionic nanoliposomes limits the severity of atherosclerosis within a mouse model, with concurrent hepatic and aortic upregulation of genes important in the RCT pathway. The research suggests the potential of this approach for treating human atherosclerosis given that these nanoliposomal formulations have been suggested to be safe for human administration (Hua and Wu, 2013).

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Competing Financial Interests

Amirhossein Sahebkar and Mahmoud R. Jaafari have a patent on the use of liposomes for treating dyslipidemia (US20150147383).

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Author contributions

The authors contributed to the manuscript as follows: S.M.K., J.M., A.S., and J.G. designed the study; S.M.K. and J.G. wrote the manuscript; R.J.J. performed flow cytometry analysis; S.M.K. performed immunohistochemistry and gene expression analysis; J.V.M. contributed to the statistical analysis; J.L., performed biochemical analysis; A.S., M.R.J., M.H., developed the nanoliposome; D.L., assisted in animal experiments. All authors contributed to the preparation of the manuscript.

Figure legends

Figure 1. The effect of nanoliposomes on the development of atherosclerotic lesions in *Ldlr*^{-/-} mice maintained on a HFC diet for 4 months. (A) At necropsy, the aortic arch was dissected and fixed overnight and stained with Sudan IV. Representative *en face* preparations of aortic arch segments from each group. (B) Quantification of the digital images of *en face* preparations. All data presented as median and interquartile range (n= 5/group). ** $p= 0.007$. (C) Representative hematoxylin and eosin images of sections of BCA from each group showing atherosclerotic plaques. Scale bar 100 μ m. Black asterisks highlight the lumens of the vessels. Note that the lumen is narrowed. The region shown is the advanced fibrous plaque showing a large necrotic core with a fibrous cap. (D) The panel shows magnified regions of the BCA section shown in C. Scale bar 50 μ m. Abbreviations: HFC, high fat and cholesterol; BCA, brachiocephalic arteries.

Figure 2. Nanoliposome administration attenuated BCA atheroma severity in *Ldlr*^{-/-} mice maintained on a HFC diet for 4 months. (A) Representative Elastin van Giessen (EVG) stained BCA sections. Black asterisks highlight the lumens of the vessels. Scale bar 100 μ m. The lower panels (B) show magnified images of the BCA section shown in A. Scale bar 50 μ m. (C) Quantification graphs of atherosclerosis plaque area measured from EVG stained BCA sections. Plaque area was the measured area of plaque enclosed by the internal elastic laminae (IEL) (D) Quantification graphs of intima/ media ratio measured from EVG stained BCA sections. Intima/media ratio was obtained by dividing the area inside the IEL by the medial area [obtained by subtracting the area inside the IEL from area inside the external elastic lamina (EEL)]. All data presented as median and interquartile range (n= 5/group). * $p= 0.030$; ** $p= 0.007$. Abbreviations: HFC, high fat and cholesterol; BCA, brachiocephalic arteries.

Figure 3. Nanoliposome administration improved markers of plaque stability in advanced atherosclerotic lesions in *Ldlr*^{-/-} mice. (A) Representative images of BCA sections of collagen deposition by picrosirius red staining and subsequent polarization microscopy. White asterisks highlight the lumen of the vessels. Scale bar 100 μ m. (B) Quantification graphs of % picrosirius stained plaque area viewed as birefringence under polarized light. All data presented as median and interquartile range (n=5/group). * $p=0.031$. Abbreviations: HFC, high fat and cholesterol; BCA, brachiocephalic arteries.

Figure 4. Analysis of the number and phenotype of spleen cells from *Ldlr*^{-/-} mice administered saline or nanoliposomes. (A) i. Representative flow-cytometric plots showing CD11b⁺Ly6G^{hi}Ly6B.2^{hi} cells from the spleen of *Ldlr*^{-/-} mice maintained on HFC-diet for 4 months. Spleen cells were isolated and stained with fluorophore-conjugated antibodies. Samples were processed on a FACS Calibur flow cytometer and analysed using FlowJo software. The numbers represent cells stained. ii. Quantification graph depicting the proportions of CD11b⁺Ly6G^{hi}Ly6B.2^{hi} cells from the spleens of HFC-fed *Ldlr*^{-/-} mice from control and nanoliposome administered mice. (B) Nanoliposomes attenuated the number of macrophages within the BCA. Representative immunohistochemical images showing MOMA-2 staining. Black asterisks highlight the lumen of the vessels. Scale bar 50 μ m. (C) Quantification graph showing absolute numbers of CD11b⁺Ly6G^{hi}Ly6B.2^{hi} cells from the spleens of HFC-fed *Ldlr*^{-/-} mice from control and nanoliposome administered mice. Data are presented as median and interquartile range. ** $p=0.007$, as analysed by Mann Whitney U test. (D) Quantification graphs of % macrophage stained area of BCA sections. Data presented as median and interquartile range (n=5/group). * $p=0.015$. Abbreviations: HFC, high fat and cholesterol.

Figure 5. Nanoliposome administration attenuated lipid deposition and upregulated reverse cholesterol transport pathway genes in the liver of HFC-fed *Ldlr*^{-/-} mice. (A) The effects of nanoliposome administration on the hepatic accumulation of cholesterol was assessed by Oil Red O (ORO) staining. A representative ORO stained section showing reduced vacuoles and nuclear size in mice receiving nanoliposomes. Scale bar 50 μ m. (B) Quantitation graph showing percentage of ORO stained area in the liver sections. Data presented as median and interquartile range (n= 5/group) as analyzed by Mann Whitney U test. ** $p=0.007$. (C). Effect of liposome administration on the hepatic gene expression. Nanoliposome administration significantly increased the hepatic gene expression of *Abc-a1*, *Abc-g1*, *Lxr- α* and *Scrab1*. Values are median and interquartile range (n= 5/group) as analyzed by Mann Whitney U test. Abbreviations: HFC, high fat and cholesterol.

Figure 6. Nanoliposome administration upregulated expression of genes involved in reverse cholesterol transport within the aorta and promoted Lecithin acyl transferase (LCAT) activity in the plasma of HFC-fed *Ldlr*^{-/-} mice. (A) LCAT promotes the transfer of excess cell-associated cholesterol from peripheral tissues to the liver to be excreted. LCAT activity in the plasma of HFC-fed *Ldlr*^{-/-} mice was measured using a fluorogenic assay at an excitation wavelength of 342 nm and an emission wavelength of 400nm. Values are median, interquartile range (n= 5/group) and are expressed as relative fluorescence units (RFU). (B) Effects of nanoliposome administration on mRNA expression within the aorta of HFC-fed *Ldlr*^{-/-} mice. Nanoliposome administration significantly increased the gene expression of *Abc-a1* and *Abc-g1*. Values are median and interquartile range (n= 5/group) as analyzed by Mann Whitney U test. Abbreviations: HFC, high fat and cholesterol.

Tables

Table 1. Total body weight of high fat and cholesterol (HFC)-fed *Ldlr*^{-/-} mice administered saline or nanoliposomes at baseline, 3 months after diet commenced and 4 weeks after the liposome or control intervention started.

Table 2. Plasma total cholesterol, low density lipoprotein, high density lipoprotein and triglyceride levels in high fat and cholesterol (HFC)-fed *Ldlr*^{-/-} mice administered saline or nanoliposomes.

Table 1. Total body weight of high fat and cholesterol (HFC)-fed *Ldlr*^{-/-} mice administered saline or nanoliposomes at baseline, 3 months after diet commenced and 4 weeks after the liposome or control intervention started.

	Saline control	Nanoliposome	<i>p</i> <i>value</i>
Baseline Body weight (g)	29 (28 - 30)	29 (28.50-31.50)	0.761
Body weight 3 months HFC (g)	38 (35.50-42.50)	37 (35.50-42.50)	0.904
Final Body weight (g)	45 (43.50-45.50)	34 (31.50-42)	0.055
Change in Body weight following intervention (%)	18.42 (5 - 26.86)	10.53 (-18 - 9.34)	0.354
Liver weight (g)	2.85 (2.15 - 3.16)	1.22 (1.03 - 2.26)	0.031
Hepatic TC (μmol/g tissue)	33.20 (20.26 – 82.63)	22.80 (7.66 – 55.65)	0.420

Values are presented as median (interquartile range). * p-values denote comparisons between groups by Mann-Whitney U test. Abbreviations: HFC, high fat high cholesterol diet; N, number of mice per group; TC, Total cholesterol.

Table 2. Plasma total cholesterol, low density lipoprotein, high density lipoprotein and triglyceride levels in high fat and cholesterol (HFC)-fed *Ldlr*^{-/-} mice administered saline or nanoliposomes.

	Saline			Nanoliposome			After intervention (Saline vs Nanoliposome)
	Before intervention	After intervention	<i>p</i> - <i>value</i> *	Before intervention	After intervention	<i>p</i> - <i>value</i> *	<i>p</i> - <i>value</i> ¶

Total cholesterol (mg/dL)	1405.0 (582.7-878.2)	2008.0 (1708.0-2383.0)	0.313	1474.0 (962.3-1837.0)	1500.0** (1421.0-2117.0)	0.625	0.190
HDL-C (mg/dL)	68.1 (56.9-116.2)	74.9 (61.1-180.6)	>0.999	84.1 (72.7-173.3)	117.0 (103.3-134.3)	>0.999	0.412
LDL-C (mg/dL)	738.5 (398.1-378.8)	1119.0 (761.5-1728.0)	0.063	746.2 (232.7-957.7)	457.7 (415.4-807.7)	0.813	0.055
Triglycerides (mg/dL)	207.1 (146.2-253.3)	165.1 (93.6-262.2)	0.625	172.2 (136.5-278.5)	50.8 (39.2-125.9)	0.063	0.095

Data are expressed as median (interquartile range).

* p-values calculated using the Wilcoxon matched-pairs signed rank test.

** Data from one mouse had to be excluded as values were 10-fold higher than expected suggesting technical failure of the test.

¶ p-values calculated using the Mann-Whitney U test.

Abbreviations: HDL-C, high density lipoprotein cholesterol; HFC, high fat high cholesterol diet; LDL-C, low density lipoprotein cholesterol; TG, triglyceride.

Figure 1 .

Figure 2 .

Figure 3 .

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Figure 4 .

Figure 5 .

Figure 6 .

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