J. Cell. Mol. Med. Vol 13, No 9B, 2009 pp. 3226-3235

Up-regulation of reverse cholesterol transport key players and rescue from global inflammation by ApoA-I$_{Milano}$

Giovanni Cimmino [a, #], Borja Ibanez [a, c, #], Gemma Vilahur [b], Walter S. Speidl [a], Valentin Fuster [a], Lina Badimon [b], Juan J. Badimon [a, *]

[a] Cardiovascular Biology Research Laboratory, Cardiovascular Institute, Mount Sinai School of Medicine, New York, NY, USA
[b] Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau, UAB, Barcelona, Spain
[c] Current Address: Cardiology Department, Fundación Jiménez Díaz-Capio, Madrid, Spain

Received: July 9, 2008; Accepted: November 10, 2008

Abstract

Recombinant-ApoA-I$_M$ (rApoA-I$_M$) administration has been shown to regress and stabilize atherosclerotic plaques. However, the mechanisms responsible for these beneficial effects are not fully understood. The aims of the present study were to define whether the benefits of rApoA-I$_M$ treatment were mediated *via* an enhanced reverse cholesterol transport (RCT) and/or anti-inflammation-related mechanisms. Advanced aortic lesions were induced in New Zealand White rabbits (n = 16). Animals were randomized to placebo or rApoA-I$_M$ (rApoA-I$_M$/phospholipids; ETC-216), two infusions 4 days apart. Four days after last dose, aortas and livers were processed for cholesterol content, expression of RCT-related receptors (ATP-binding cassette A-1 [ABCA-1] and scavenger receptor BI [SR-BI]), and inflammation-related markers (inducible nitric oxide synthase [iNOS] and capase-3). Oxidative stress was assessed in the vessel wall and in plasma. rApoA-I$_M$ administration resulted in a significant reduction in the hepatic and aortic cholesterol content without differences in plasma levels. This effect was associated with an up-regulation of vessel wall ABCA-1, as well as a hepatic and arterial-wall SR-BI up-regulation. Systemic and atherosclerotic-plaque inflammation markers were significantly reduced by the rApoA-I$_M$ administration, as demonstrated by a reduction in circulating oxidative stress markers and prostaglandin F1-α levels, and the down-regulation of the iNOS and caspase 3 in the aortic lesions. rApoA-IM up-regulated the ABCA-1 and SR-BI levels to a greater extent than the wild-type form of apoA-I in *in vitro* studies done with lipid-rich macrophages. Our data suggest that rApoA-I$_M$ administration enhances RCT and induces a 'rescue' from the global inflammatory status associated with atherosclerotic disease. The Milano form of apoA-I seems to be more efficient in RCT than the apoA-I wild-type.

Keywords: atherosclerosis • lipoproteins • ApoA-I • reverse cholesterol transport • inflammation

Introduction

The recruitment of inflammatory cells in the arterial wall is triggered by the deposition and oxidation of lipid material in the arterial wall. This is a defensive mechanism that by self-perpetuation becomes injurious leading to the development and progression of atherosclerotic lesions [1]. The accumulation of lipid material is the result of an imbalance between the influx and efflux of cholesterol within the arterial wall. High levels of plasma low-density lipoprotein (LDL)-cholesterol are considered the major mechanism responsible for the influx and accumulation of cholesterol in the arterial wall, while high-density lipoprotein (HDL)-cholesterol seems responsible for its efflux [2–4]. The mechanism by which cholesterol is removed from extra-hepatic organs and delivered to the liver for its catabolism and excretion is called reverse cholesterol transport (RCT).

Epidemiological evidence has associated high levels of HDL-cholesterol/apoA-I with protection against atherosclerotic disease, but the ultimate mechanism(s) responsible for the beneficial effect is not well established. Recently, research efforts have focused on ApoA-I$_{Milano}$, a naturally occurring mutation of apoA-I with potent anti-atherogenic properties [5]. Benefits associated with the administration of the recombinant form of ApoA-I$_{Milano}$ (rApoA-I$_M$) have been described in several experimental models of atherosclerosis [6–8] and even in human beings [9]. We have recently reported that acute administration of rApoA-I$_M$ induces a rapid regression of advanced atherosclerotic lesions in a rabbit model of atherosclerosis [10]. The observed rapid plaque regression was

[#]These authors contributed equally.
*Correspondence to: Juan BADIMON,
Ph.D., F.A.C.C., Mount Sinai School of Medicine.
One Gustave L. Levy Place, Box 1030. New York NY 10029, USA.
Tel.: +212-241-8484
Fax: +212-426-6962
E-mail: juan.badimon@mssm.edu

doi:10.1111/j.1582-4934.2008.00614.x

associated with molecular changes suggesting a transition from a vulnerable to a more stable plaque phenotype [10]. The mechanism by which rApoA-I_M infusion results in a high efficient cholesterol removal from the vessel wall and plaque stabilization remains to be fully elucidated.

The aim of the present work was to improve the knowledge on the mechanisms of action responsible for the observed acute benefits following the administration of rApoA-I_M. For such purpose, we have assessed the effect of rApoA-I_M on the cholesterol removal, RCT-related receptors, inflammation and oxidative stress in a rabbit model of atherosclerosis and in a murine foam cell line.

Methods

In vivo study

Study design

Abdominal aortic atherosclerotic plaques were induced in rabbits ($n = 16$) by a combination of 9-month 0.2% cholesterol-rich diet plus two aortic denudations as previously described [11, 12]. At the end of the atherosclerosis induction period, animals were randomized to receive two intravenous (i.v.) injections, 4 days apart, of recombinant ApoA-I_{Milano} (rApoA-I_M)-phospholipids ($n = 8$) or equal volume of placebo (saline, $n = 8$) [10]. Four days after the last dose, animals were killed and the aortas and livers were harvested and processed for lipid content measurement, atherosclerotic plaque oxidative stress, as well as for gene and protein expression of markers involved in RCT and inflammation. Plasma from animals receiving rApoA-I_M was collected immediately before the first dose and at sacrifice for determination of systemic cholesterol levels, systemic lipid peroxidation products and prostaglandin F1-α (PGF1-α) levels. The study protocol was approved by an institutional animal research committee. All animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals'. All experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, 1996.

Experimental procedures

The atherosclerosis induction protocol has been described in detail elsewhere [11, 13]. Briefly, male New Zealand White rabbits (age 5 months) were fed with 0.2% cholesterol atherogenic diet (Research Diets, Inc., NJ, USA) for 9 months. Two aortic balloon endothelial denudations were performed 1 and 12 weeks after atherogenic diet initiation. Before undergoing any procedure, animals were fully anesthetized by intramuscular injections of ketamine (30 mg/kg) and xylazine (2.2 mg/kg).

At the end of the 9-month atherosclerotic induction period, animals were randomized to receive two i.v. infusions (75 mg/kg) of rApoA-I_M-palmitoyl-2-oleoyl phosphatidylcholine (ETC-216, Pfizer. From now on rApoA-I_M) or equal volume of placebo, as previously reported [10]. ETC-216 was supplied as frozen vials containing approximately 15 mg/ml by Pfizer Pharmaceuticals.

Four days after the last dose of rApoA-I_M or placebo, animals were killed with an overdose of pentobarbital (Sleepaway®, Fort Dodge. 75 mg/kg). Abdominal aortas and livers were excised with sterile tools, washed in autoclaved phosphate-buffered saline (PBS), and frozen in liquid nitrogen and stored at –80 °C for further analysis.

Plasma samples were stored frozen at −80°C. The determination of lipid peroxidation products after placebo or rApoA-I_M treatments, as well as PGF1-α levels before and after rApoA-I_M were done as previously described [14–16].

Aortic and hepatic tissue cholesterol content quantification

Aortic tissue cholesterol was determined always in the proximal half of the descending aorta harvested using a previously validated assay [17]. Briefly, atherosclerotic wall and hepatic tissues were grounded to a fine powder with a ceramic pestle in liquid nitrogen. Ten milligrams of powder were measured and homogenized, and the lipid content was extracted by treatment with chloroform-Triton X-100 followed by centrifugation. The lower phase was collected and vacuum dried. The extracted lipids were dissolved in cholesterol reaction buffer by vortexing extensively for 5 min. and measured *via* a cholesterol assay kit (Biovision®) utilizing absorbance spectrophotometry according to manufacturer.

Western blot analysis

Aortic and hepatic protein levels of markers involved in RCT (ATP binding cassette A-1 [ABCA-1] and scavenger receptor BI [SR-BI]), as well as of inducible nitric oxide synthase (iNOS) and caspase-3 were analysed by Western blot analysis. Aortic and liver segments were excised, removed of surrounding adherent and connective tissue, and snap-frozen in liquid nitrogen. Thereafter, the frozen liver and aortas were pulverized, homogenized in lysis buffer (50 mmol/l Tris-HCl, 1 mmol/l ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.1 mg/ml PMSF, 1 μg/ml aprotinin, 10 μg/ml leupetin, pH 7.4), briefly sonicated, and the protein concentration was measured with the bicinchoninic acid method (BCA, Pierce Biotechnology; Rockford, IL, USA) using bovine serum albumin (BSA) as a standard. Samples (50 μg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were placed in Tris-buffered saline, 0.05% Tween 20 (TBST) supplemented with PBS containing 3% BSA for 2 hrs at room temperature and then incubated with iNOS (R&D system Inc., Minneapolis, MN, USA), caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), SR-BI (Novus Biological, Inc., Littleton, CO, USA) or ABCA-1 (GenScript, Piscataway, NJ, USA) overnight at 4°C.

The blots were washed three times with TBS-Tween, and the membranes were incubated with horseradish peroxidase-conjugated antibodies for 1 hr at room temperature and washed again as described previously. Membrane-bound secondary antibodies were detected using the ECL™ chemiluminescence system (Amersham Bioscience, Pierce Biotechnology). Changes in expression were measured by densitometry (ImageJ) and β-actin (Santa Cruz Biotechnology) was used for protein loading control.

Real-time PCR mRNA analysis

RNA was isolated from 30 mg frozen aortic or hepatic tissue with the RNeasy-mini kit (Qiagen™) according to the manufacturer's guidelines. Concentration of RNA was determined by measuring absorbance at 260 nm (A_{260}). The A_{260}/A_{280} ratio of the samples ranged from 1.8 to 2.2.

Real-time PCR assays were carried out using SYBR green PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA) on a GeneAmp 5700 Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. Intron spanning primers (TibMolBiol) were designed using Primer Express software (Applied Biosystems) and are presented in Table S1. Results were normalized to GADPH (housekeeping gene) expression.

In vitro study

Study design

The *in vitro* study was designed to better recognize the effect of rApoA-I_M on receptors involved in RCT in cholesterol-loaded macrophages (foam cells). In order to understand whether the effect of rApoA-I_M on these receptors is shared with other forms of apoA-I or by contrast are uniquely inherent to the Milano form, two different types of apoA-I were tested (apoA-$I_{wild\text{-}type}$ [apoA-I_{wt}] and lipid-free rApoA-I_M. Culture media free of any of these agents was used as control.

Foam cells were generated by adding oxidized LDL (oxLDL) to the macrophages' culture media. After 72 hrs of oxLDL exposure, the media was replaced by oxLDL-free culture media containing apoA-I_{wt} (pure protein bought from CalBiochem®), lipid-free rApoA-I_M and or regular media free of any of these agents.

Macrophage's cholesterol content, as well as protein expression of receptors implicated in RCT (ABCA-1 and SR-BI) was determined after each treatment at the different time-points expressed in the results section. In addition, a protein expression time course of macrophage's ABCA-1 and SR-BI was performed throughout the 72 hrs oxLDL exposure.

Cell culture

Murine macrophages (J774A.1 cell line, ATCC, Rockville, MD) were cultured in T25 flasks with RPMI 1640 medium containing 10% foetal calf serum, 50 μg/ml each of penicillin and streptomycin, and 2 mM glutamine. Experiments were performed when cells were ≈90% confluent.

Isolation of LDL and preparation of oxLDL

LDL (density = 1.019–1.063 g/ml) was isolated from human plasma by sequential ultracentrifugation. For oxidation, LDL was diluted to 0.1 mg protein/ml with EDTA-free PBS and incubated with $CuSO_4$ (10 μmol/l) for 18 hrs at 37°C. At the end of incubation, 0.1 mmol/l EDTA was added to prevent further oxidation and the oxLDL was concentrated to 1 mg/ml. This procedure resulted in extensive LDL oxidation and the resulting preparations are referred to in the text as oxLDL.

Foam cell formation and exposure to treatments

J774 macrophages were seeded (1×10^6 /ml) in six-well plates and then incubated with oxLDL (100 μg/ml) for 72 hrs. At the end of incubation, medium was removed and fresh medium containing lipid-free rApoA-I_M (10 μg/ml) or apoA-I_{wt} (10 μg/ml), respectively, was added for 24 hrs. Untreated J774 cells (not exposed to oxLDL) were used as control.

In addition, a dose–response time-dependent experiment using escalating doses of both forms of apoA-I protein was performed.

Cholesterol content assays

The lipid content (10^6 cells) was extracted by treatment with chloroform-Triton X-100 followed by centrifugation. The lower phase was collected and vacuum dried. The extracted lipids were dissolved in cholesterol reaction buffer by vortexing extensively for 5 min. and measured *via* a cholesterol assay kit (Biovision®) utilizing absorbance spectrophotometry.

Delipidation of ETC-216 was done according to previously reported protocols [18, 19]. SDS-PAGE and Coomassie staining after ETC-216 delipidation revealed a single band in the apoA-I region (Fig. S1).

Western blot analysis

ABCA-1 and SR-BI protein levels were measured at selected time-points. Macrophages were washed twice with cold PBS and then scraped and lysed in Cytobuster (Novogen Inc., New Canaan, CT, USA) according to the manufacturer's instructions. Equal amount of total proteins were loaded on SDS PAGE Mini-gel and then transferred onto nylon-enhanced nitrocellulose membrane. Membranes were blocked with a solution of PBS containing 3% BSA for 2 hrs and then incubated with rabbit polyclonal anti-ABCA-1 and -SR-BI (Novus Biological, Inc.) overnight at 4°C followed by washing (3 × 10 min.) with 0.1%Tween/PBS (PBS-T) buffer. The blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for another 1 hr at room temperature. After washing three times (10 min. each) with PBS-T, the membrane was incubated for 5 min. in a mixture of equal volumes of Western blot chemiluminescence reagents 1 and 2. The membrane was then exposed to film before development.

Statistical analysis

Continuous variables are expressed as mean ± S.E. of the mean. For statistical comparisons, Wilcoxon and Mann-Whitney tests were applied appropriately. All experiments were done three times, and the mean of the results were taken for statistical comparisons. A value of $P < 0.05$ (two-tailed) was considered statistically significant. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Effect of rApoA-I_{Milano} on circulating cholesterol levels

There were no significant differences in plasma levels of total cholesterol between animals receiving rApoA-I_M or placebo either before or after treatment. Total cholesterol levels were similar in both groups before the treatment (1025 ± 89 mg/dl *versus* 1028 ± 243 mg/dl in the rApoA-I_M and placebo groups, respectively). Four days after the last dose of treatments , total cholesterol levels were similar in both groups: 1118 ± 137 mg/dl in rApoA-I_M *versus* 954 ± 143 mg/dl in placebo group). The effect of the administration of rApoA-I_M on circulating levels of apoA-I_M has been previously reported [10].

Total HDL levels were similar in both groups at the end of atherosclerotic induction: 104 ± 7 in rApoa-I_M *versus* 97 ± 12 mg/dl

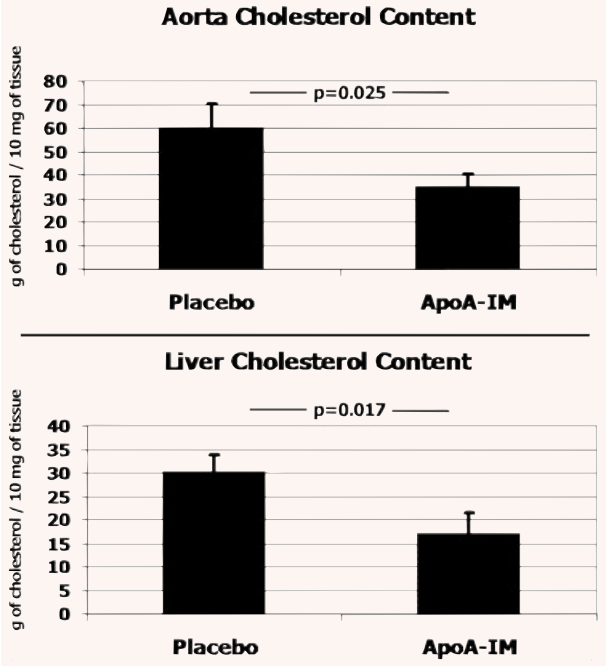

Fig. 1 Mean ± S.E.M. tissue cholesterol content in the aortic arterial wall (top) and liver (bottom panel) from animals receiving rApoA-I_{Milano} (rApoA-I_M) or placebo. The *Y*-axis represents μg of cholesterol per 10 mg of tissue.

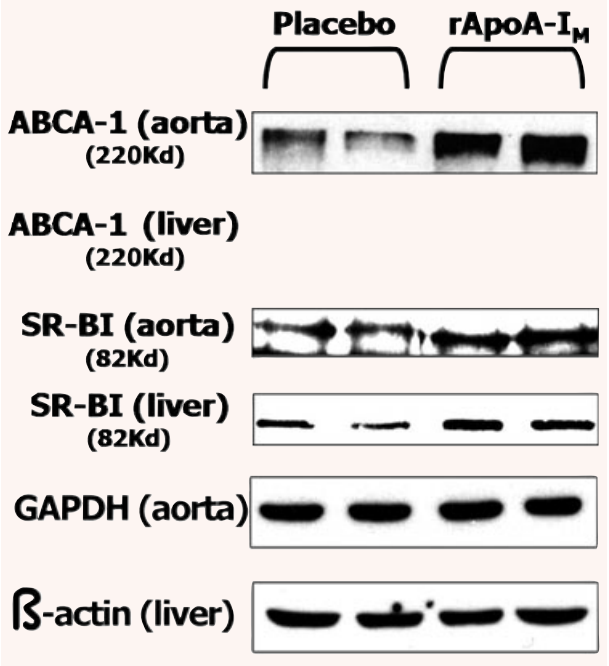

Fig. 2 Protein expression by Western blot of aortic and hepatic receptors involved in reverse cholesterol transport from animals receiving rApoA-I_M or placebo. ABCA-1: ATP binding cassette A-1, SR-BI: scavenger receptor type B class I.

in placebo, P = NS). At the time of killing, total HDL was significantly higher in rApoA-I_M animals than in placebo (138 ± 22 in rApoA-I_M *versus* 89 ± 15 in placebo, $P = 0.012$).

Effect of rApoA-I_{Milano} on the aortic vessel wall

The effects of the administration of rApoA-I_M (a 5% of plaque regression and changes in lesion composition) have been previously reported [10]. Aortas of rApoA-I_M-treated animals had significantly lower lipid content than those of placebo animals (Fig. 1, upper panel). Reduction on both size and lipid content in the vessel wall with unchanged plasma lipid levels strongly suggests an enhanced net cholesterol removal from aortic lesions. The enhanced RCT is also supported by the significant ($P < 0.001$) increase in ABCA-1 protein levels and up-regulation of SRB1 at the gene and protein levels in the aortic wall as compared to placebo (Figs 2 and 3).

The effects of rApoA-I_M on iNOS, caspase-3 and lipid peroxidation products, as markers of inflammation, were evaluated. The group receiving rApoA-I_M showed a significant down-regulation of iNOS and caspase-3 mRNA and protein levels as compared with the placebo group, Fig. 4. Atherosclerotic plaque oxidative stress was significantly lower in the rApoA-I_M than the placebo group (9.1 ± 1.7 *versus* 17.2 ± 2.3 μM malondialdehyde [MDA], respectively, $P = 0.032$).

Effect of rApoA-I_{Milano} on the liver

The effects of rApoA-I_M administration on the hepatic accumulation of cholesterol and liver expression of ABCA-1 and SR-BI were also assessed.

Recombinant-ApoA-I_M dramatically reduced the hepatic cholesterol content of the livers *versus* those receiving placebo, Fig. 1, lower panel).

A significant gene and protein SR-BI, and ABCA-1 protein up-regulation was observed in the livers of rApoA-I_M-treated animals *versus* the placebo group (Figs 2 and 3).

Effect of rApoA-I_{Milano} on oxidative stress and circulating isoprostanes

Circulating lipid peroxidation products at sacrifice were significantly lower in rApoA-I_M-treated animals (72.7 ± 5.9 *versus* 102.9 ± 7.8 μM MDA in rApoA-I_M *versus* placebo, respectively, $P = 0.016$). Circulating levels of PGF1-α were significantly

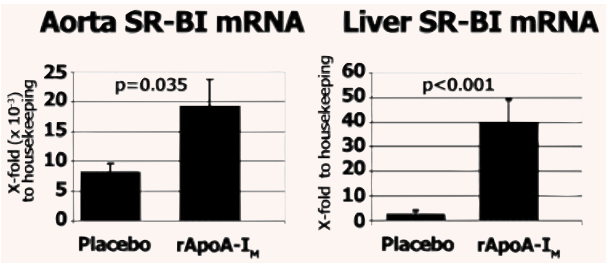

Fig. 3 SR-BI gene expression in aorta (vessel wall) and liver wall. *Y*-axis in left panel corresponds to amount of RNA $\times 10^{-3}$ (expressed as mean $\pm$ S.E.M. of X-fold to housekeeping gene). *Y*-axis in the right panel corresponds to amount of RNA (expressed as mean $\pm$ S.E.M. of X-fold to housekeeping gene).

reduced from baseline (pre-Rx) by the rApoA-I_M treatment (166.7 $\pm$ 36.9 pg/ml *versus* 102.8 $\pm$ 27.4, $P = 0.02$).

Comparative study of Milano and wild-type ApoA-I isoforms on macrophage cholesterol content

Murine macrophages (J774A.1 cell line) were exposed for 72 hrs to oxLDL. This process resulted in a significant increase in their cholesterol content (3.15 $\pm$ 0.1 *versus* 7.9 $\pm$ 0.2 $\mu g \times 10^{-6}$ cells before and 72 hrs after oxLDL exposure, respectively, $P < 0.001$).

Lipid-rich macrophages were treated with wild-type human apoA-I (apoA-I_{wt}) or delipidated apoA-I_M. Both treatments resulted in a significant reduction in the cellular cholesterol content *versus* pre-treatment (3.4 $\pm$ 0.2 and 2.8 $\pm$ 0.0 $\mu g \times 10^{-6}$ cells, respectively, $P < 0.001$). Interestingly, the incubation of macrophages with apoA-I_M resulted in a significantly lower content of intracellular cholesterol as compared with those treated with apoA-I_{wt} ($P < 0.05$) – see Fig. 5. These results strongly suggest an enhanced cholesterol removal from macrophages by the *Milano* form as compared with the wild-type apoA-I.

In order to better delineate whether apoA-I_M was more effective than native apoA-I, in cholesterol removal from macrophages, four different concentrations of each type of apoA-I (2.5, 5, 10 and 20 μg/ml) were tested. The cholesterol content of macrophages was determined at 12 and 24 hrs. As can be seen in Fig. S2, all four concentrations of apoA-I_M were significantly more efficient at 12 hrs as compared to apoA-I_{wt}. Interestingly, at 24 hrs, only the two highest concentrations of apoA-I_M were more efficient than apoA-I_{wt}. This results suggest that apoA-I_M is not only more efficient, but also faster that apoA-I_{wt} in the removal of cholesterol from foam cells. There was no incremental effect of the 20 μg/ml concentration neither for apoA-I_M nor for apoA-I_{wt}. As can be seen in Fig. S2, the effect of apoA-I_M at all concentrations were not different between 12 and 24 hrs, while the effect of apoA-I_{wt} was in all superior at 24 hrs than at 12 hrs.

Effect of oxLDL on macrophages'ABCA-1 and SR-BI density

ABCA-1 and SR-BI density was assessed at baseline (before), and 24, 48 and 72 hrs after oxLDL exposure. The accumulation of cholesterol by the exposure of the murine macrophages to ox-LDL resulted in an early up-regulation of ABC1, followed by a significant ($P < 0.03$) down-regulation at 48 and 72 *versus* pre-oxLDL exposure values. Following oxLDL exposure, there was a gradual decrease in SR-BI density. See Fig. S3.

Effect of different forms of apoA-I on macrophages' ABCA-1 and SR-BI density

Foam cells were generated by culture of macrophages with oxidized LDL. After 72 hrs of exposure to oxLDL, there was a dramatic ABCA-1 and SR-BI protein down-regulation, as noted above (Fig. S3). Treatment with both forms of apoA-I induced a significant up-regulation of both key receptors involved in RCT (ABCA-1 and SR-BI). Interestingly, macrophages' ABCA-1 and SR-BI density after treatment with the Milano form of apoA-I was significantly higher than after treatment with apoA-I_{wt}. See Fig. 6.

Discussion

We have previously reported the acute benefits associated with the administration of rApoA-I_{Milano} to an experimental model of advanced atherosclerosis [10]. We are now describing the potential mechanisms responsible for the observed beneficial effects. The main results of the present work are: (1) Two administrations of rApoA-I_M in atherosclerotic rabbits resulted in a significant reduction of aortic and hepatic cholesterol content without affecting plasma cholesterol levels. (2) These observations were associated with a significant up-regulation of several of key players involved in the RCT (ABCA-1 and SR-BI) both at the vascular and hepatic level. (3) The administration of rApoA-I_M resulted in a significant reduction of inflammatory markers and oxidative stress both at vascular (atherosclerotic lesion) and systemic (circulating) levels, suggesting a *rescue* of the global pro-inflammatory state associated with atherosclerotic disease. (4) Finally, *in vitro* experiments on oxLDL-loaded macrophages suggested a more potent cholesterol removal activity of the ApoA-I_{Milano} than the wild-type form of apoA-I.

Atherosclerosis is a diffuse disease characterized by the deposition/accumulation of cholesterol and inflammatory cells within the arterial wall. Free cholesterol is considered a highly toxic substance that contributes to the perpetuation of the inflammatory process [1]. Removal of cholesterol from the vessel wall may, therefore, have an impact not only on the size and composition of the atherosclerotic lesions, but also on its inflammatory status.

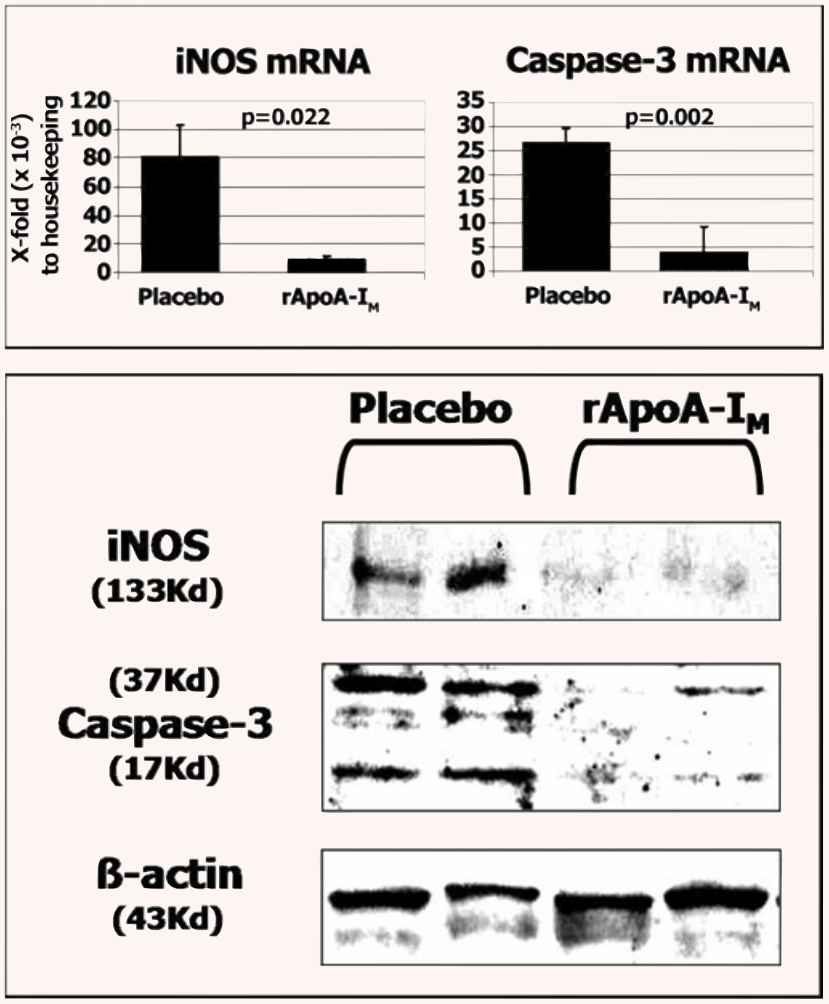

Fig. 4 Inducible nitric oxide synthase (iNOS) and caspase-3 gene expression (amount of RNA $\times 10^{-3}$ [expressed as mean $\pm$ S.E.M. of X-fold to housekeeping gene]) by real-time PCR is shown in top panel. Bottom panel illustrates protein expression of same markers. Note that rApoA-I_M induced a significant down-regulation of iNOS and of both forms of caspase-3 (pre-active [37 kD band], and cleaved [17 kD band]).

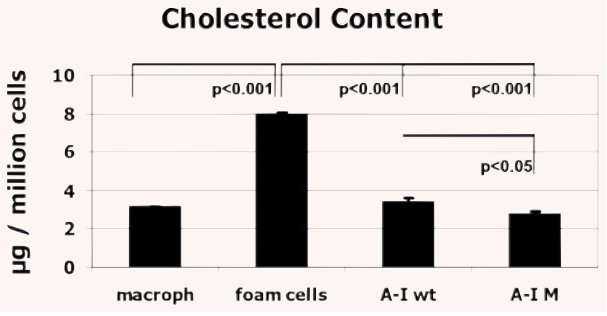

Fig. 5 *In vitro* cholesterol content (mean $\pm$ S.E.M. of $\mu g \times 10^{-6}$ cells) from macrophages (macroph, pre-exposure to ox-LDL), foam cells (after 72 hrs exposure to oxLDL), and after 24 hrs treatment of foam cells with apoA-I wild-type (A-I_{wt}), and ApoA-I_{Milano} (A-I_M). Bars represent mean values. $n = 4$ per time-point/treatment.

The effects of rApoA-I_M on the regression of previously established atherosclerotic lesions have been reported by different groups not only in experimental models but also in human beings [6–10, 20]. In the present study we are describing a significant reduction in aortic wall and hepatic cholesterol content and the up-regulation of ABCA-1 and SR-BI. These observations strongly suggest that the described benefits of rApoA-I_M on plaque regression could, at least in part, be mediated *via* an enhanced RCT.

The present findings extend the previously described vasculoprotective effects of recombinant apoA-I_M administration, providing insight into the mechanism of action. The effects of rApoA-I_M administration on the removal of cholesterol from the established lesions is well documented [7, 8, 21]. We are now reporting that the lesion regression is accompanied by a reduced cholesterol accumulation in the liver. These observations combined with the up-regulation of the major players involved in the efflux of cholesterol, strongly suggest that rApoA-I_M enhances the RCT. However, since we have measured neither the bile acid synthesis nor faecal excretion of cholesterol, we cannot be completely rule out that a

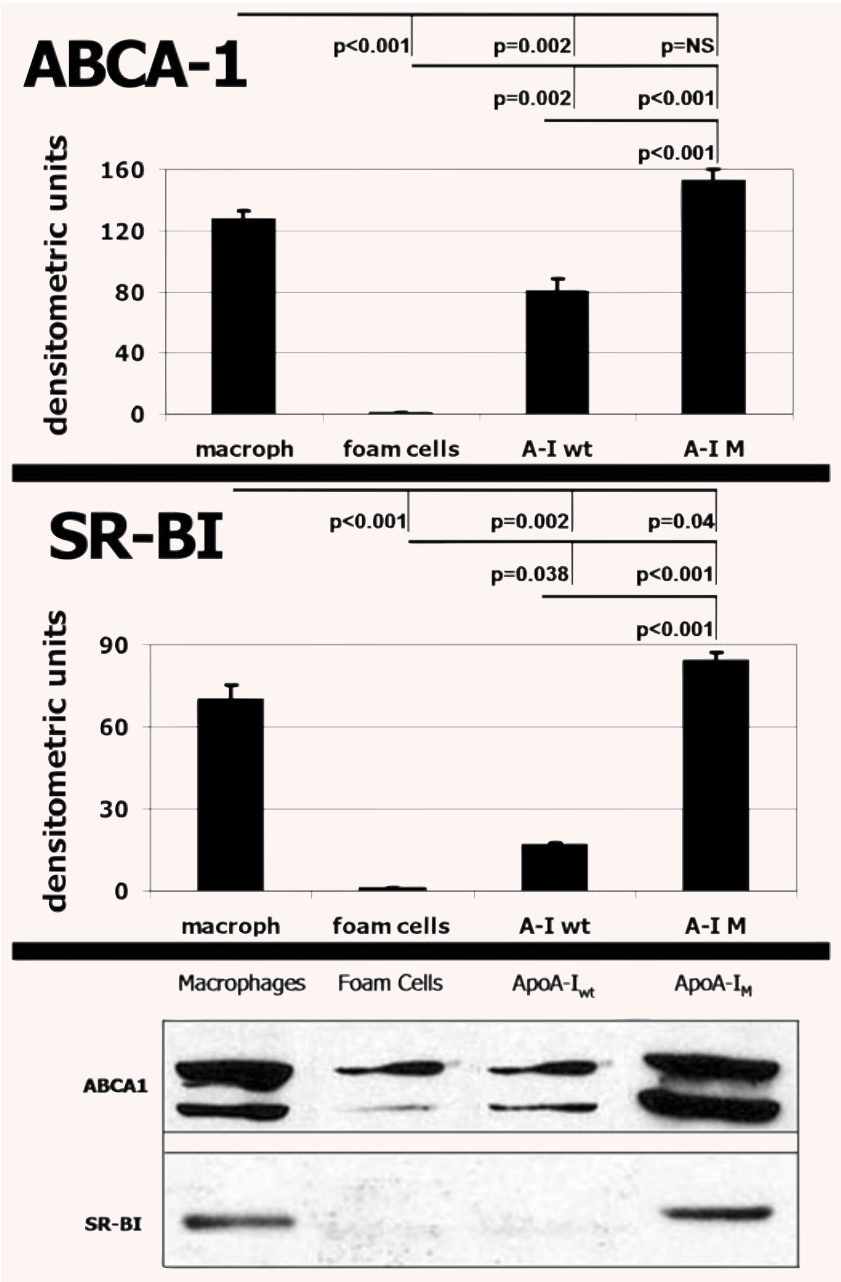

Fig. 6 *In vitro* ABCA-1 and SR-BI protein expression (by Western blot) in macrophages (pre-oxLDL exposure), foam cells (72 hrs after oxLDL exposure), and after 24 treatment of foam cells with different forms of apoA-I (human apoA-I wild-type (A-I_{wt}) and delipidated ApoA-I_{Milano} (A-I_M), see text). Data are expressed as mean ± S.E.M. of arbitrary densitometric units. Bottom panel illustrates representative Western blot images from this set of *in vitro* experiments. $n = 4$ per time-point/treatment.

mobilization of cholesterol to different areas from the vessel wall and the liver, something very unlikely, took place. The gross post-mortem evaluation of the animals at sacrifice did not show any extraneous deposition of cholesterol. Another plausible explanation for the observed hepatic cholesterol reduction is that the decrease in hepatic cholesterol levels reflect the transport of cholesterol to nascent HDL particles *via* ABCA-1, particularly given the up-regulation of ABCA-1 in the liver. Previous studies have shown that hepatic ABCA-1 plays an important role in the formation of HDL [22].

Prior reports showing a 35% increase in faecal excretion of cholesterol in hypercholesterolaemic patients after the infusion of rApo A-I [23] seems to be indirectly corroborated by our observations showing less cholesterol accumulation in liver and aorta without affecting the systemic levels.

To understand the mechanism of action responsible of the enhanced RCT observed, we analysed the effect of rApoA-I_M administration on the major players involved in this process (ABCA-1 and SR-BI) both *in vivo* and *in vitro* conditions. The *in vitro* set of experiments were specifically designed to

understand whether, and eventually how, the *Milano* form of apoA-I exerts stronger vasculoprotective properties than the wild-type form of apoA-I. We have showed that rApoA-I_M infusion induces a significant up-regulation of ABCA-1 and SR-BI levels both in the atherosclerotic plaque and in the liver. The incubation of oxLDL-loaded-macrophages (foam cells) with apoA-I_M confirmed the significant up-regulation of ABCA-1 and SR-BI observed *in vivo*. In addition, the experiments on oxLDL-loaded macrophages, suggest a more efficient cholesterol removal activity with the Milano isoform as compared with the wild-type apoA-I. Whether the up-regulation of SR-BI observed in oxLDL-loaded-macrophages in our *in vitro* set of experiments plays a role in the observed cholesterol removal from the aortas in animals is not addressed by the present work. While previous *in vitro* experiments have clearly shown that macrophages SR-BI mediate cholesterol efflux to HDL [24], a recent study has challenged the involvement of macrophages SR-BI in RCT *in vivo* [25].

Whether the *Milano* form of apoA-I is more effective than the wild-type in cholesterol removal has been the matter of heated discussion. Some reports claim that both forms are equally effective in their cholesterol removal and athero-protective activities [26, 27], while others have shown that ApoA-I_{Milano} is not only more effective in cholesterol removal [28, 29], but also exert increased anti-atherogenic properties *in vivo*. The latter has been corroborated by a recent study showing that over-expression of apoA-I_M had a stronger anti-atherogenic effect than over-expression of wild-type apoA-I on murine atherosclerosis [20]. Our observations support the concept that the *Milano* form of apoA-I is more effective in the removal of cholesterol from macrophages/foam cells than the wild-type form. Potential reasons for the different results might be due to the different techniques used for macrophage loading (ox-LDL *versus* acetylated LDL by Weibel *et al*. [26]). Finally, we have observed that apoA-I_M is faster than apoA-I_{wt} in achieving its maximum effect. In fact, there no differences in macrophages' cholesterol content after 12 or 24 hrs exposure to apoA-I_M. This suggests that, beyond being more efficient in cholesterol removal, the Milano form of apoA-I seems to be also faster. Whether these observations are responsible of the incremental beneficial effects of apoA-I_M *in vivo* is still unknown.

Our results on ABCA-I up-regulation confirm the recently reported that sera from apoA-I_M carriers promotes an ABCA-1-driven cholesterol efflux from macrophages to a greater extent than that observed in sera from matched controls [29]. Conversely, to the best of our knowledge, the SR-BI up-regulation observed by us has never been reported after treatment with apoA-I_M. Whether the up-regulation of ABCA-1 and SR-BI is the main feature responsible of the enhanced RCT and lipid excretion observed cannot be assured at this point but our results suggest that it certainly plays an important role in the beneficial effects resulting from rApoA-I_M administration in atherosclerosis.

It is well known that local and systemic inflammation is a major player in the vulnerability of atherosclerotic plaques. We are showing that apoA-I_M administration resulted in a rescue of the global inflammatory status of the atherosclerotic animals. At the systemic level, we have shown a reduced oxidative stress (less lipid peroxidation products) compared with the placebo animals. In addition, we found that circulating levels of PGF1-α (a biomarker of inflammation) [30] significantly decreased following rApoA-I_M treatment. These two different assays combined with our previous observation of a reduced MCP-1 levels in the lesions of the animals receiving rApo A-I_M [10], suggest a systemic anti-inflammatory effect associated with rApoA-I_M administration.

Furthermore, we have shown that treatment with rApoA-I_M induced a significant vessel wall iNOS and caspase-3 down-regulation. These molecular changes suggest a profound modulation of the lesions phenotype expanding our previous observations in this regard [10]. Inducible NOS has been shown to be involved in LDL-cholesterol oxidation [31], therefore the beneficial effects of rApoA-I_M administration may not only be restricted in time to the acute phase, but also it may change the fate of the atherosclerotic lesion, blocking the vicious cycle of LDL-cholesterol oxidation and atherosclerosis progression. The significant reduction in local lipid peroxidation products supports this hypothesis. Recombinant apoA-I_M seems to decrease apoptosis in the atherosclerotic lesions, as noted by the significant reduction in constitutive and cleaved forms of caspase-3. Previous reports associated apoptotic macrophages as the source for plaque tissue factor [32]. In this regard, it has been shown that short-term treatment with apoA-I_M reduces the tissue factor content within the atherosclerotic lesions, and this effect may be mediated by the significant reduction in caspase-3 expression observed in the current work.

Taken together our *in vivo* and *in vitro* studies, we are reporting an enhanced cholesterol removal and an anti-inflammatory activity associated with the rApoA-I_M infusion. Whether the enhanced removal of cholesterol is responsible for the anti-inflammatory effect of the apoA-I_M or other pleiotropic or non-lipid mediated mechanisms are responsible for these observations remain to be established.

Limitations

The major limitation of the current study is that we have not tested *in vivo* the effect of native apoA-I. Although it would have been interesting, the authors lacked the expertise to assemble native apoA-I with palmitoyl-2-oleoyl phosphatidylcholine (at the same ratio as rApoA-I_M is assembled with the phospholipids in ETC-216). For this reason we tested *in vitro* the effect of the pure proteins (native and Milano apoA-I) after the delipidation of the latter.

In addition, it has been suggested that phospholipidic micelles may have by itself some impact in cholesterol removal from macrophages. Although Chiesa *et al*. showed that phospholipids

alone had no effect on plaque volume while rApoA-I_M-phospholipids significantly regressed atherosclerotic lesions [33], it cannot completely be ruled out that the phospholipids contained in ETC-216 may have an impact, if any, on the beneficial *in vivo* effects here reported.

Acknowledgements

The drug tested in this work was provided by Pfizer Research & Development. B.I. is recipient of a grant by the Working Group on Ischemic Heart Disease of the Spanish Society of Cardiology. G.V. is recipient of a Juan de la Cierva grant.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Coomassie staining gel run in non-reducing conditions (left). Recombinant apoA-I_{Milano} is a protein dimmer with a molecular weight of ≈56 kD. When the gel is run in reducing conditions (right), delipidated apoA-I_{Milano} appears as a single band in the 28-kD position. Human wild-type apoA-I migrates to the 28-kD position.

Fig. S2. Mean ± S.E.M. of *in vitro* cholesterol content (mean of $\mu g\ x10^{-6}$ cells) from macrophages (macroph, pre-exposure to ox-LDL), foam cells (after 72 hrs exposure to oxLDL), and after 12- and 24-hr treatment of foam cells with apoA-I wild-type (A-I_{wt}), and apoA-I_{Milano} (A-I_M) at four different concentrations. $N = 4$ per time-point/concentration. $^{*}P$-value <0.05 for the comparison between apoA-I_M and apoA-I_{wt}. $^{\#}P$-value <0.05 between 12 and 24 hrs.

Fig. S3. Time course macrophages' ABCA-1 and SR-BI protein levels during exposure to oxLDL. Data are expressed as mean ± S.E.M. of arbitrary densitometric units. See text.

Table S1. Primers sequences for quantitative real-time PCR

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1582-4934.2008.00614.x
(This link will take you to the article abstract).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. **Ibanez B, Vilahur G, Badimon J.** Plaque progression and regression in atherothrombosis. *J Thromb Haemost*. 2007; 5: 292–9.
2. **Viles-Gonzalez JF, Fuster V, Corti R, *et al.*** Emerging importance of HDL cholesterol in developing high-risk coronary plaques in acute coronary syndromes. *Curr Opin Cardiol*. 2003; 18: 286–94.
3. **Badimon JJ, Badimon L, Fuster V.** Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *J Clin Invest*. 1990; 85: 1234–41.
4. **Badimon JJ, Badimon L, Galvez A, *et al.*** High density lipoprotein plasma fractions inhibit aortic fatty streaks in cholesterol-fed rabbits. *Lab Invest*. 1989; 60: 455–61.
5. **Franceschini G, Sirtori CR, Capurso A 2nd, *et al.*** A-IMilano apoprotein. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. *J Clin Invest*. 1980; 66: 892–900.
6. **Ameli S, Hultgardh-Nilsson A, Cercek B, *et al.*** Recombinant apolipoprotein A-I Milano reduces intimal thickening after balloon injury in hypercholesterolemic rabbits. *Circulation*. 1994; 90: 1935–41.
7. **Shah PK, Nilsson J, Kaul S, *et al.*** Effects of recombinant apolipoprotein A-I(Milano) on aortic atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 1998; 97: 780–5.
8. **Kaul S, Coin B, Hedayiti A, *et al.*** Rapid reversal of endothelial dysfunction in hypercholesterolemic apolipoprotein E-null mice by recombinant apolipoprotein A-I(Milano)-phospholipid complex. *J Am Coll Cardiol*. 2004; 44: 1311–9.
9. **Nissen SE, Tsunoda T, Tuzcu EM, *et al.*** Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *Jama*. 2003; 290: 2292–300.
10. **Ibanez B, Vilahur G, Cimmino G, *et al.*** Rapid change in plaque size, composition and molecular footprint following recombinant ApoA-I_{Milano} (ETC-216) administration. Magnetic Resonance Imaging Study in an Experimental Model of Atherosclerosis. *J Am Coll Cardiol*. 2008; 51: 1104–9.
11. **Corti R, Osende J, Hutter R, *et al.*** Fenofibrate induces plaque regression in hypercholesterolemic atherosclerotic rabbits: *in vivo* demonstration by high-resolution MRI. *Atherosclerosis*. 2007; 190: 106–13.
12. **Corti R, Osende JI, Fallon JT, *et al.*** The selective peroxisomal proliferator-activated receptor-gamma agonist has an additive effect on plaque regression in combination with simvastatin in experimental atherosclerosis: *in vivo* study by high-resolution magnetic resonance imaging. *J Am Coll Cardiol*. 2004; 43: 464–73.
13. **Helft G, Worthley SG, Fuster V, *et al.*** Atherosclerotic aortic component quantification by noninvasive magnetic resonance imaging: an *in vivo* study in rabbits. *J Am Coll Cardiol*. 2001; 37: 1149–54.
14. **Yagi K.** Simple assay for the level of total lipid peroxides in serum or plasma. *Methods Mol Biol*. 1998; 108: 101–6.
15. **Dawn-Linsley M, Ekinci FJ, Ortiz D, *et al.*** Monitoring thiobarbituric acid-reactive substances (TBARs) as an assay for oxidative damage in neuronal cultures and central nervous system. *J Neurosci Methods*. 2005; 141: 219–22.

16. **Frolich JC.** Measurement of icosanoids. Report of the Group for Standardization of Methods in Icosanoid Research. *Prostaglandins*. 1984; 27: 349–68.
17. **Folch J, Lees M, Sloane Stanley GH.** A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957; 226: 497–509.
18. **Miller JP, Mao SJ, Patsch JR, *et al.*** The measurement of apolipoprotein A-I in human plasma by electroimmunoassay. *J Lipid Res*. 1980; 21: 775–80.
19. **Badimon JJ, Kottke BA, Chen TC, *et al.*** Quantification and immunolocalization of apolipoprotein E in experimental atherosclerosis. *Atherosclerosis*. 1986; 61: 57–66.
20. **Wang L, Sharifi BG, Pan T, *et al.*** Bone marrow transplantation shows superior atheroprotective effects of gene therapy with apolipoprotein A-I Milano compared with wild-type apolipoprotein A-I in hyperlipidemic mice. *J Am Coll Cardiol*. 2006; 48: 1459–68.
21. **Parolini C, Marchesi M, Lorenzon P, *et al.*** Dose-related effects of repeated ETC-216 (recombinant apolipoprotein A-Imilano/1-palmitoyl-2-oleoyl phosphatidylcholine complexes) administrations on rabbit lipid-rich soft plaques *in vivo* assessment by intravascular ultrasound and magnetic resonance imaging *J Am Coll Cardiol*. 2008; 11: 1098–103.
22. **Timmins JM, Lee JY, Boudyguina E, *et al.*** Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J Clin Invest*. 2005; 115: 1333–42.
23. **Eriksson M, Carlson LA, Miettinen TA, *et al.*** Stimulation of fecal steroid excretion after infusion of recombinant proapolipoprotein A-I. Potential reverse cholesterol transport in humans. *Circulation*. 1999; 100: 594–8.
24. **Yancey PG, de la Llera-Moya M, Swarnakar S, *et al.*** High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *J Biol Chem*. 2000; 275: 36596–604.
25. **Wang X, Collins HL, Ranalletta M, *et al.*** Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport *in vivo*. *J Clin Invest*. 2007; 117: 2216–24.
26. **Weibel GL, Alexander ET, Joshi MR, *et al.*** Wild-type ApoA-I and the Milano variant have similar abilities to stimulate cellular lipid mobilization and efflux. *Arterioscler Thromb Vasc Biol*. 2007; 27: 2022–9.
27. **Lebherz C, Sanmiguel J, Wilson JM, *et al.*** Gene transfer of wild-type apoA-I and apoA-I Milano reduce atherosclerosis to a similar extent. *Cardiovasc Diabetol*. 2007; 6: 15.
28. **Franceschini G, Calabresi L, Chiesa G, *et al.*** Increased cholesterol efflux potential of sera from ApoA-IMilano carriers and transgenic mice. *Arterioscler Thromb Vasc Biol*. 1999; 19: 1257–62.
29. **Favari E, Gomaraschi M, Zanotti I, *et al.*** A unique protease-sensitive high density lipoprotein particle containing the apolipoprotein A-I(Milano) dimer effectively promotes ATP-binding Cassette A1-mediated cell cholesterol efflux. *J Biol Chem*. 2007; 282: 5125–32.
30. **McAdam BF, Catella-Lawson F, Mardini IA, *et al.*** Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci USA*. 1999; 96: 272–7.
31. **Miyoshi T, Li Y, Shih DM, *et al.*** Deficiency of inducible NO synthase reduces advanced but not early atherosclerosis in apolipoprotein E-deficient mice. *Life Sci*. 2006; 79: 525–31.
32. **Hutter R, Valdiviezo C, Sauter BV, *et al.*** Caspase-3 and tissue factor expression in lipid-rich plaque macrophages: evidence for apoptosis as link between inflammation and atherothrombosis. *Circulation*. 2004; 109: 2001–8.
33. **Chiesa G, Monteggia E, Marchesi M, *et al.*** Recombinant apolipoprotein A-I(Milano) infusion into rabbit carotid artery rapidly removes lipid from fatty streaks. *Circ Res*. 2002; 90: 974–80.