

## RESEARCH PAPER

# Rosuvastatin does not affect human apolipoprotein A-I expression in genetically modified mice: a clue to the disputed effect of statins on HDL

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### Keywords

human apolipoprotein A-I; rosuvastatin; HDL; transgenic mice; cholesterol

### Received

23 March 2010

### Revised

17 March 2011

### Accepted

29 March 2011

## BACKGROUND AND PURPOSE

Besides a significant reduction of low-density lipoprotein (LDL) cholesterol, statins moderately increase high-density lipoprotein (HDL) levels. *In vitro* studies have indicated that this effect may be the result of an increased expression of apolipoprotein (apo)A-I, the main protein component of HDL. The aim of the present study was to investigate *in vivo* the effect of rosuvastatin on apoA-I expression and secretion in a transgenic mouse model for human apoA-I.

## EXPERIMENTAL APPROACH

Human apoA-I transgenic mice were treated for 28 days with 5, 10 or 20 mg·kg<sup>-1</sup>·day<sup>-1</sup> of rosuvastatin, the most effective statin in raising HDL levels. Possible changes of apoA-I expression by treatment were investigated by quantitative real-time RT-PCR on RNA extracted from mouse livers. The human apoA-I secretion rate was determined in primary hepatocytes isolated from transgenic mice from each group after treatment.

## KEY RESULTS

Rosuvastatin treatment with 5 and 10 mg·kg<sup>-1</sup>·day<sup>-1</sup> did not affect apoA-I plasma levels, whereas a significant decrease was observed in mice treated with 20 mg·kg<sup>-1</sup>·day<sup>-1</sup> of rosuvastatin (−16%, *P* < 0.01). Neither relative hepatic mRNA concentrations of apoA-I nor apoA-I secretion rates from primary hepatocytes were influenced by rosuvastatin treatment at each tested dose.

## CONCLUSIONS AND IMPLICATIONS

In human apoA-I transgenic mice, rosuvastatin treatment does not increase either apoA-I transcription and hepatic secretion, or apoA-I plasma levels. These results support the hypothesis that other mechanisms may account for the observed HDL increase induced by statin therapy in humans.

## Abbreviations

apo, apolipoprotein; AU, arbitrary units; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; PLTP, phospholipid transfer protein; VLDL, very-low-density lipoprotein

## Introduction

Epidemiological studies have established that low plasma high-density lipoprotein (HDL) cholesterol levels are a major risk factor for cardiovascular disease (Barter and Rye, 2006). It is well established that HDL-cholesterol (HDL-C) is a strong and independent inverse predictor of coronary artery disease even in patients with normal levels of low-density lipoprotein cholesterol (LDL-C) (Gordon and Rifkind, 1989).

Statin treatment is recommended as a first-line drug therapy because of its well-established efficacy in decreasing cardiovascular events in both primary and secondary prevention (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). The main mechanism of action of statins is the reduction of cholesterol biosynthesis by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, resulting in up-regulation of hepatic LDL receptor activity and consequently marked reduction of LDL cholesterolaemia (Brown and Goldstein, 1986). In addition to LDL-cholesterol (LDL-C), plasma triglycerides are lowered by statins, possibly as a consequence of diminished very-low-density lipoprotein (VLDL) production and induction of receptor-mediated remnant clearance (Vega and Grundy, 1998). Furthermore, statin treatment moderately increases plasma HDL-C concentration in a range between 4% and 10% (McTaggart and Jones, 2008). Although modest, this increase may, however, provide important protection from cardiovascular disease, because it has been estimated that cardiovascular risk decreases by 2–3% for every 10 mg·L<sup>-1</sup> increase in HDL-C levels (Gordon and Rifkind, 1989).

The mechanisms behind the effect of statins on HDL have not been yet clarified. In hepatic cells, inhibition of HMG-CoA reductase by statins has been shown to increase peroxisome proliferator activated-receptor  $\alpha$  (PPAR $\alpha$ ) activity and, as found with the fibrates, to elevate the hepatic synthesis of apolipoprotein (apo)A-I, the main protein component of HDL, with consequent increase of HDL particle formation (Martin *et al.*, 2001). A second explanation relates to the metabolic interaction between HDL and triglyceride-rich atherogenic lipoproteins mediated by the cholesteryl ester transfer protein (CETP). The VLDL reduction by statins would reduce the CETP-mediated cholesteryl ester transfer from HDL to VLDL, thus increasing HDL-C levels (Guerin *et al.*, 2000; de Haan *et al.*, 2008).

It was thus of interest to evaluate, *in vivo*, the possible impact on HDL of statin treatment in a naturally CETP-deficient animal model, the mouse, transgenic for human apoA-I expression (Rubin *et al.*, 1991). This transgenic model has previously been shown to respond to PPAR $\alpha$  activators, such as fibrates (Berthou *et al.*, 1996). Human apoA-I transgenic mice were treated for 28 days with three different doses of rosuvastatin. Among statins, rosuvastatin appears to be the most effective in increasing HDL plasma concentrations (McTaggart and Jones, 2008) and, unlike other statins, maintains a relatively constant effect across doses (Jones *et al.*, 2003). The results of the present study indicate that, in human apoA-I transgenic mice, rosuvastatin did not affect human apoA-I expression, at each tested dose.

## Methods

### *Animals and treatments*

All animal care and experimental procedures were in accordance with institutional guidelines that are in compliance with national (D.L. no. 116, G.U. Suppl. 40, 18 February 1992, Circolare no. 8, G.U. July 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, 12 December 1987; Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 1996). The study was performed on forty male 20- to 25-week-old C57BL/6 transgenic mice expressing human apoA-I in the absence of murine apoA-I (hA-I/A-IKO). This was achieved by multiple crosses between human apoA-I transgenic mice (hA-I) (Rubin *et al.*, 1991), kindly provided by Dr Edward M. Rubin, and mice lacking murine apoA-I (A-IKO) due to gene targeting (Williamson *et al.*, 1992), kindly provided by Dr Nobuyo Maeda. The transgenic model expresses human apoA-I through the microinjection of an 11 Kb genomic fragment of the human apoA-I gene; this fragment contains the PPAR $\alpha$  response element known to confer fibrate responsiveness to the human apoA-I gene (Berthou *et al.*, 1996) and potentially implicated in the HDL increase by statin treatment (Martin *et al.*, 2001). Transgenic mice were randomly divided into four groups of ten mice each and fed a standard diet for rodents (control group) for 28 days or the same standard diet containing 25, 50 or 100 mg·kg<sup>-1</sup> of rosuvastatin (AstraZeneca, UK).

### *Lipid/Lipoprotein analyses*

Before and at the end of treatment, after an overnight fast, blood was collected, under isoflurane anaesthesia, from the retro-orbital plexus into tubes containing 0.1% (w/v) EDTA and centrifuged in a microcentrifuge for 10 min at 5900× *g* at 4°C.

Plasma total cholesterol and triglycerides were measured by enzymatic methods (ABX Diagnostics, Montpellier, France). HDL-C levels were measured after precipitation of apoB-containing lipoproteins with PEG 8000 (20% w/v) in 0.2 M glycine, pH 10 (Schultz *et al.*, 1992). Human apoA-I concentrations were determined by immunoturbidimetric assays, using a sheep antiserum specific for human apoA-I (LTA, Milan, Italy).

To analyse plasma lipoprotein distribution, plasma was pooled for each group and applied onto a Superose 6 10/30 column (Pharmacia) for fast protein liquid chromatography (FPLC) analysis (van Gent and van Tol, 1990). On the collected fractions, total cholesterol and triglycerides were measured by enzymatic methods.

To determine HDL particle size distribution, total lipoproteins ( $d < 1.215$  g·mL<sup>-1</sup>) were isolated by salt gradient ultracentrifugation (Havel *et al.*, 1955). An aliquot of pooled plasmas from each group was adjusted to a density of 1.215 g·mL<sup>-1</sup> with solid KBr and centrifuged for 6 h at 4°C at 541 000× *g* in a Beckman TL100 ultracentrifuge equipped with a Beckman TL100.3 rotor. HDL particle size distribution was determined by non-denaturing polyacrylamide gradient gel electrophoresis (GGE), essentially as described by Nichols *et al.* (Nichols *et al.*, 1986). Aliquots (20  $\mu$ L) of the total lipo-

protein fraction were loaded onto a non-denaturing 4–30% polyacrylamide gradient gel and separated by electrophoresis for 25 h at 125 V at 4°C. The proteins were stained with Coomassie R-250 and HDL particle size was determined by computer-assisted scanning densitometry, as previously described (Nichols *et al.*, 1986).

### Phospholipid transfer protein (PLTP) activity

PLTP activity was measured in mouse plasma before and after treatment, as previously described (Klein *et al.*, 2006), using a commercially available fluorescence activity assay (Roar Biomedical Inc., New York, NY, USA) according to the manufacturer's instructions. This fluorimetric assay measures the transfer (unquenching) of fluorescent phosphatidylcholine from donor to acceptor synthetic liposomes.

### Quantitative real-time PCR

At the end of the dietary treatment, five hA-I/A-IKO mice from each group were killed and total RNA was isolated from mouse livers using the NucleoSpin RNA extraction kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. One µg of DNase-treated total RNA was reverse-transcribed using TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA, USA). PCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on an iCycler Optical System (Bio-Rad Laboratories, Hercules, CA, USA). The selected primers used for amplification of human apoA-I cDNA were AGCTTGCTGAAGGTGGAGGT (in exon 4) and ATCGAGTGAAGGACCTGGC (in exon 3). The primers amplify a 154-bp product. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. All of the procedures and calculation of the results were carried out according to the manufacturer's recommendations.

### Hepatic human apoA-I secretion rate

The apolipoprotein secretion rate was determined in primary hepatocytes isolated from three hA-I/A-IKO mice of each group at the end of the dietary treatment. Animals were fasted for 5 h and anesthetized with 5% sodium pentobarbital, and the hepatocytes were prepared with slight modification of a previously described method (Hayek *et al.*, 1993). Cell viability was assessed by Trypan blue staining, and  $5 \times 10^5$  live cells were plated on 35 mm plates. The culture medium (Williams' E medium; Sigma, St. Louis, MO, USA) was changed after 4 h of incubation at 37°C. The next day, to assess the human apolipoprotein secretion rate, cells were washed once with phosphate-buffered saline, pre-incubated for 1 h in leucine-free Dulbecco's modified Eagle's medium without serum, and then incubated for 30, 60, 90, 120 min with 1 mL of the same medium containing  $200 \mu\text{Ci}\cdot\text{mL}^{-1}$  [ $^3\text{H}$ ]leucine (PerkinElmer Life Sciences). The medium was centrifuged at  $12\,000\times g$  at 4°C for 5 min to remove cell debris. Radiolabelled human apolipoproteins were quantitatively isolated from cell lysates by immunoprecipitation using a rabbit polyclonal anti-human apoA-I antibody (DAKO, Glostrup, Denmark). The immunoprecipitate was further purified by SDS-PAGE under reducing conditions. A band corresponding to human apoA-I was excised from the gel; the label was extracted with Solvable (Packard Instru-

ments Co., Inc., Meriden, CT, USA) and counted (Azrolan *et al.*, 1995). The results were normalized to cellular protein content of each plate determined by the Bradford method (Bradford, 1976). The data presented are the means of triplicate measurements and are representative of three independent experiments.

### Cell treatments

Primary hepatocytes were prepared from hA-I/A-IKO mice as described above (see *Hepatic human apoA-I secretion rate*). Cells ( $5 \times 10^5$  per dish) were plated on 35 mm plates and the culture medium (Williams' E medium; Sigma) was changed after 4 h of incubation at 37°C. The next day, cells were washed once with phosphate-buffered saline and incubated for a total of 48 h with various concentrations of statins in Williams' medium containing 2.5% serum. Medium supplemented with the drugs was renewed after 24 h incubation. At the end of treatment, cells were harvested and total RNA was isolated as described above (see *Quantitative real-time PCR*). Human apoA-I cDNA was quantified by real-time PCR on an Applied Biosystems 7000 sequence detector as described. The housekeeping gene cyclophilin was used for normalization.

Fenofibrate and simvastatin were purchased from Sigma (St. Louis, MO, USA), cerivastatin from Toronto Research Chemicals Inc (North York, Ontario, Canada), whereas pitavastatin was kindly provided by Kowa Company Ltd. (Tokyo, Japan).

### Statistical analysis

Data are expressed as mean  $\pm$  SD. Group differences were tested for statistical significance by analysis of variance for repeated measurements with one grouping factor or one-way ANOVA, followed by a Bonferroni's *post hoc* test. A value of  $P < 0.05$  was considered statistically significant.

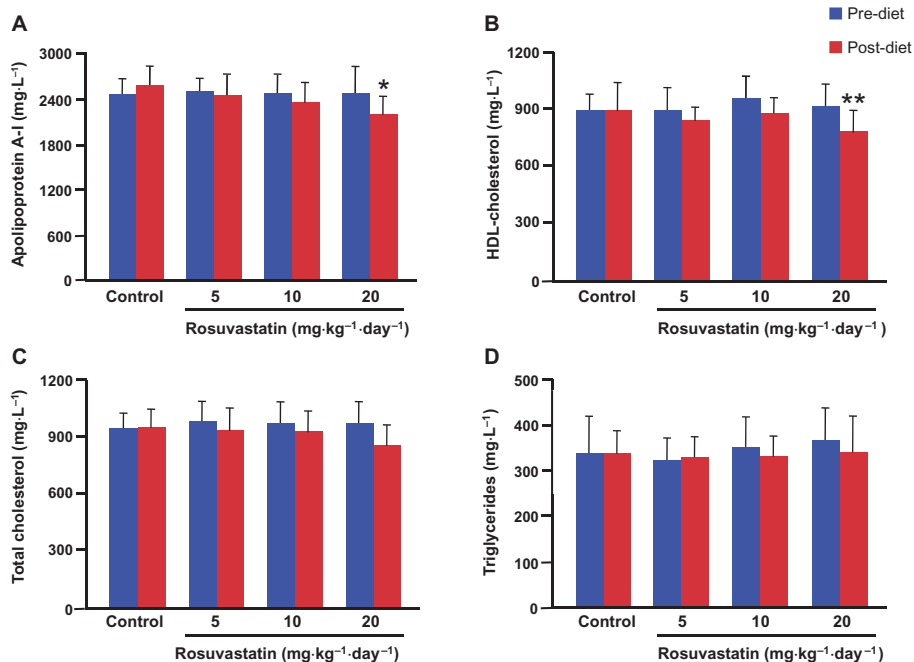
## Results

### Treatments

Daily food intake was monitored during the study and no differences were observed among groups (data not shown). Based on food consumption, it was calculated that the three groups of mice fed the rosuvastatin-containing diets took, approximately, 5, 10 or  $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  of rosuvastatin.

### Lipid/apolipoprotein analyses

To evaluate the influence of rosuvastatin treatment on lipid/apolipoprotein plasma levels, blood samples were taken from each mouse before and after treatment. In controls, as expected, apoA-I and lipid plasma levels remained unchanged over the 28 day period (Figure 1). Treatment with 5 and  $10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  of rosuvastatin did not affect apoA-I plasma levels, whereas a significant decrease was observed in mice treated with the highest dose of rosuvastatin (Figure 1A). Rosuvastatin treatment did not significantly modify HDL-C levels in mice treated with the two lower doses, whereas the highest rosuvastatin dose caused a significant reduction in HDL-C (Figure 1B). Unlike humans, the main cholesterol-carrying lipoprotein in the mouse is HDL



**Figure 1**

Effect of rosuvastatin treatment on human apolipoprotein A-I (A), HDL-cholesterol (B), total cholesterol (C) and triglyceride (D) plasma levels. Data are expressed as mean  $\pm$  SD. \* $P < 0.01$ , \*\* $P < 0.005$  significantly different from pretreatment. HDL, high-density lipoprotein.

and this feature is even more pronounced in mice overexpressing human apoA-I (Rubin *et al.*, 1991), that is, in the transgenic model used in this study. Variations in total cholesterol with drug treatment reflected therefore the trend observed for HDL-C levels, although no significant variations were observed in each treated group (Figure 1C). Rosuvastatin treatment did not induce any significant change in triglyceride levels at each tested dose (Figure 1D).

Plasma collected before and at the end of treatment was analysed by FPLC to assess possible variations in cholesterol and triglyceride distribution among lipoprotein fractions (Figure 2). FPLC confirmed that over 80% of plasma cholesterol was mostly carried in HDL particles. No variations were detected in the control group before and after the 28 days treatment (Figure 2A); the drug treatment caused a slight decrease of the HDL-C content that was more pronounced with the highest dose (Figure 2B–D). Moreover, rosuvastatin appeared to shift the HDL peak towards larger particles. No changes in the other lipoprotein fractions were observed in each treated group. The triglyceride content in the lipoprotein fractions was not modified by the pharmacological treatment, possibly because of the extremely low triglyceride starting concentration and the almost negligible VLDL levels of this mouse model (data not shown).

Changes in the HDL particle size induced by treatment were assessed by non-denaturing GGE of total lipoproteins and subsequent densitometric analysis (Figure 3). As previously reported (Rubin *et al.*, 1991; Chiesa *et al.*, 1998), human apoA-I transgenic mice are characterized by the presence of two major HDL subpopulations with diameters of about 9.8 and 8.5 nm and a shoulder corresponding to larger HDL particles (10.3 nm in diameter). Whereas no variations were

detected in the control group (Figure 3A), rosuvastatin treatment caused a redistribution of HDL subpopulations (Figure 3B–D). With increasing doses of rosuvastatin, a relative decrease in the amount of smaller particles and an increase in that of larger particles was observed.

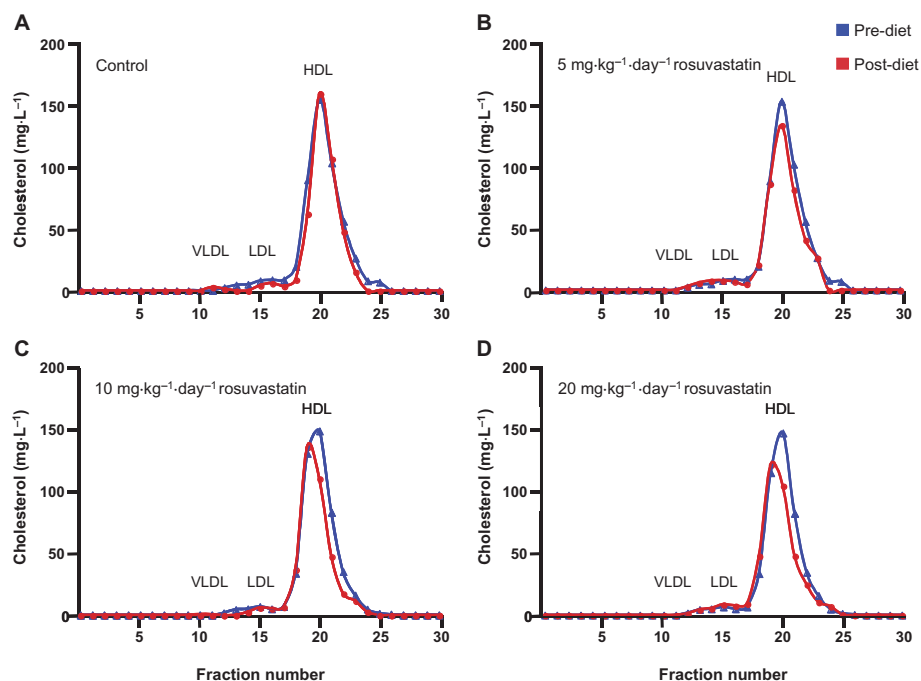
### Phospholipid transfer activity

To try to explain the redistribution of HDL particle size towards larger particles by rosuvastatin treatment, PLTP activity was measured in mouse plasma and results are shown in Figure 4. PLTP activity was significantly decreased by rosuvastatin treatment at each tested dose. Moreover, the reduction was proportional to the rosuvastatin dose used.

### Hepatic human apoA-I expression and secretion

To evaluate a possible influence of rosuvastatin on human apoA-I expression, at the end of the pharmacological treatment, mice were killed and hepatic mRNA concentrations of human apoA-I were measured by real-time RT-PCR. As reported in Figure 5, the relative mRNA concentration of apoA-I was not affected by rosuvastatin treatment at any tested dose.

To assess the impact of rosuvastatin treatment on apoA-I secretion, the human apoA-I secretion rate was determined using primary hepatocytes obtained from a subset of transgenic mice from each group. As shown in Figure 6, secretion of human apoA-I was linear for all the groups; no differences were observed in the secretion rates between controls and animals treated with 5 and 10 mg·kg<sup>-1</sup>·day<sup>-1</sup> of rosuvastatin ( $P > 0.05$ ). Although not significant, apoA-I secretion rate in



**Figure 2**

Fast protein liquid chromatography (FPLC) profile of plasma cholesterol before and after rosuvastatin treatment at each tested dose. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

the group treated with the highest dose of rosuvastatin tended to be lower than that of control animals.

### *Human apoA-I expression in primary hA-I/A-IKO mouse hepatocytes*

To investigate if the results obtained with rosuvastatin *in vivo* could be translated to other statins, primary hepatocytes from hA-I/A-IKO mice were incubated with rosuvastatin (5, 10, 20  $\mu$ M), cerivastatin (0.25, 0.5, 1  $\mu$ M), pitavastatin (2.5, 5, 10  $\mu$ M) and simvastatin (2.5, 5, 10  $\mu$ M). These concentration ranges have been previously demonstrated to be effective in increasing apoA-I expression in HepG2 cells (Martin *et al.*, 2001; Maejima *et al.*, 2004; Qin *et al.*, 2008). Whereas fenofibrate increased human apoA-I expression, no variations were observed for each statin, at every concentration tested (Figure 7).

## Discussion

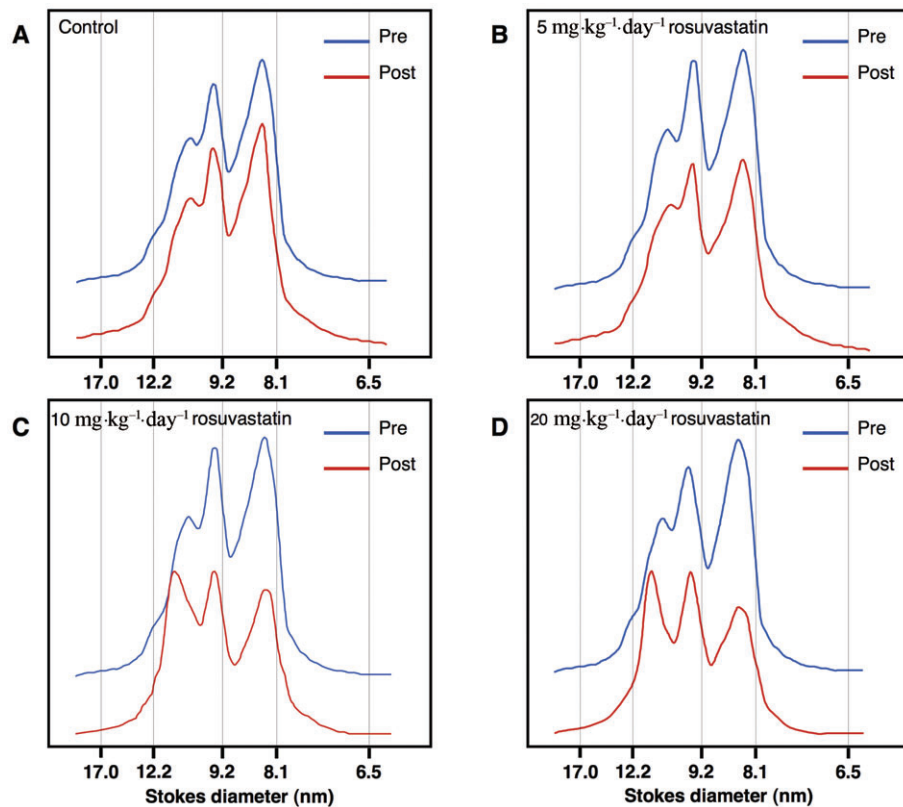
The development of statins has been a major advance in cardiovascular medicine, mainly because of the great efficacy of these molecules in lowering plasma LDL-C levels (Jones *et al.*, 2003). However, in view of the strong inverse relationship between HDL levels and cardiovascular risk, it is important to assess the manner and degree of statin effects on these lipoproteins. Placebo-controlled trials have demonstrated that statin treatment moderately, but constantly, increases HDL-C levels, whereas it affects apoA-I levels to a lesser degree and not always significantly (Davidson *et al.*, 1997; 2002a,b;

Bays *et al.*, 2004). Consistent with these observations, is the reporting of the formation of larger, cholesterol-rich HDL particles by statins (Asztalos and Schaefer, 2003).

The molecular basis for the effect of statin treatment on HDL levels has received limited attention and it has been investigated mostly *in vitro*. The present study had the objective of evaluating, *in vivo*, the effect of rosuvastatin on apoA-I expression and HDL-C levels in a mouse model expressing human apoA-I.

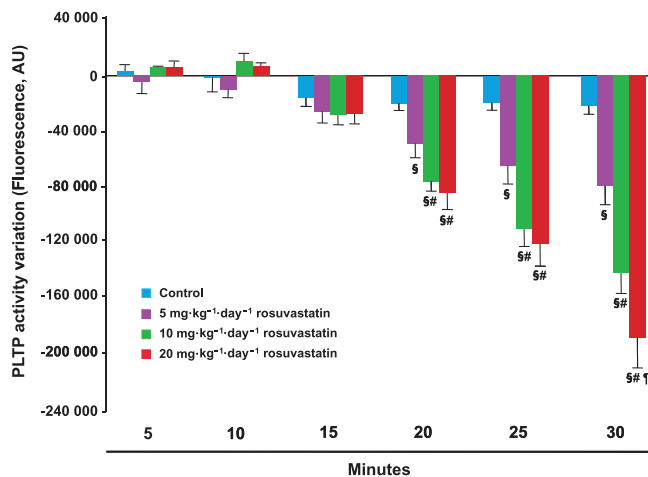
Previous studies on HepG2 cells had indicated that an elevation of apoA-I mRNA levels was involved in the observed HDL increase by statin treatment (Martin *et al.*, 2001). The authors identified a statin response element in the human apoA-I promoter that coincided with a PPAR $\alpha$  response element known to confer fibrate responsiveness to the human apoA-I gene. To test this hypothesis *in vivo*, in the present study a transgenic model was chosen, expressing human apoA-I through the microinjection of an 11 Kb genomic fragment of the human apoA-I gene containing the PPAR $\alpha$  response element (Rubin *et al.*, 1991). Indeed, in this same transgenic model, fibrate treatment had been associated with a strong increase in hepatic human apoA-I mRNA and an elevation of human apoA-I and HDL-C plasma concentrations (Berthou *et al.*, 1996). Interestingly, fibrates had an opposite effect on mouse apoA-I expression, which was significantly down-regulated by the pharmacological treatment (Berthou *et al.*, 1996). Thus, to avoid any confounding effect on murine apoA-I, the animal model used in the present study expressed human apoA-I in the absence of mouse apoA-I through crosses between human apoA-I transgenic mice and knock-out animals for murine apoA-I.





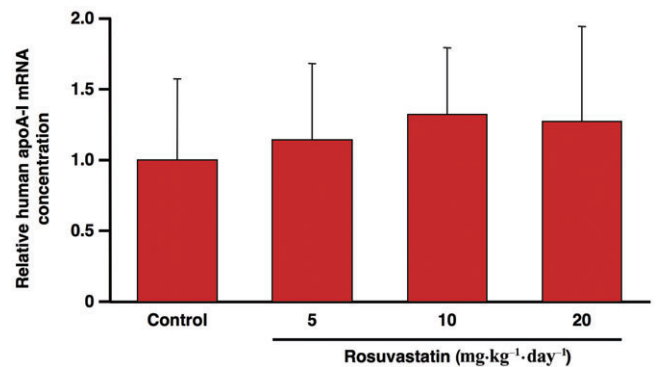
**Figure 3**

Non-denaturing 4–30% gradient gel electrophoresis of mouse plasma lipoproteins ( $d < 1.215 \text{ g}\cdot\text{mL}^{-1}$ ) before and after rosuvastatin treatment. Proteins were stained with Coomassie R-250 and HDL particle size was determined by computer-assisted scanning densitometry.



**Figure 4**

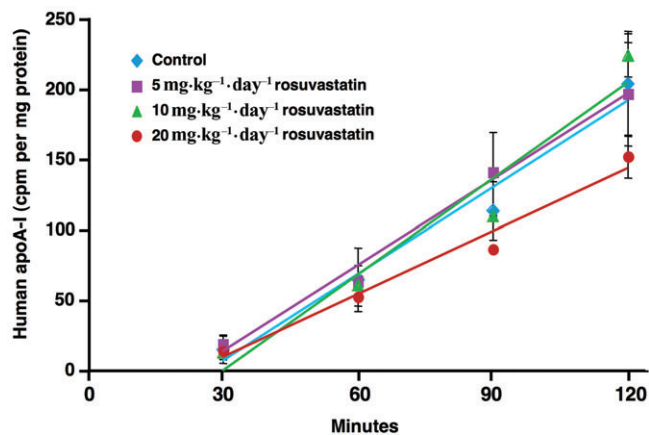
Mean change in phospholipid transfer protein (PLTP) activity measured in mouse plasma after rosuvastatin treatment. Data are expressed as mean  $\pm$  SD. AU = arbitrary units; § $P < 0.05$  significantly different from control; # $P < 0.05$  versus rosuvastatin at  $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ; ¶ $P < 0.05$  significantly different from rosuvastatin at  $10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ .



**Figure 5**

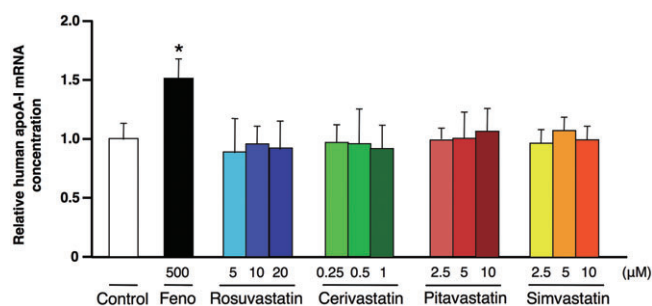
Relative mRNA concentrations of human apolipoprotein A-I in the liver of control mice or mice treated with three different rosuvastatin doses. Values are normalized to the reference gene for GAPDH and are expressed relative to the levels in control mice set as 1. Each bar represents mean values  $\pm$  SD.

In the present study, biochemical evaluations on whole plasma and FPLC fractions did not show any increase of either apoA-I or HDL-C levels with rosuvastatin treatment at each tested dose. Specifically, no variations were observed with 5 and  $10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  of rosuvastatin, whereas the



**Figure 6**

*In vitro* analysis of human apolipoprotein A-I (apoA-I) secretion from primary hepatocytes obtained from control mice or mice treated with 5, 10, 20 mg·kg<sup>-1</sup>·day<sup>-1</sup> of rosuvastatin. Data are representative of three separate experiments and each point is the mean ± SD of triplicate measurements.



**Figure 7**

Relative mRNA concentrations of human apolipoprotein A-I in primary hepatocytes isolated from hA-I/A-IKO mice. Hepatocytes were treated for 48 h with fenofibrate (Feno, 500 μM), rosuvastatin (5, 10, 20 μM), cerivastatin (0.25, 0.5, 1 μM), pitavastatin (2.5, 5, 10 μM), and simvastatin (2.5, 5, 10 μM). Values are normalized to the reference gene for cyclophilin and are expressed relative to the levels in control (untreated) set as 1. Data are representative of three separate experiments and each bar represents the mean ± SD of triplicate measurements. \**P* < 0.05 significantly different from control.

20 mg·kg<sup>-1</sup>·day<sup>-1</sup> dose significantly decreased apoA-I and HDL-C. Hepatic apoA-I expression, evaluated by quantitative real-time PCR, was not modified by the pharmacological treatment. Similar results were obtained *in vitro*, by incubating primary hA-I/A-IKO hepatocytes with different doses of rosuvastatin. Moreover, *in vitro* assessment of the effect of other statins on apoA-I expression showed no variation of human apoA-I mRNA levels. These results are in contrast with previous investigations, all performed *in vitro*, in human hepatoma cells (Martin *et al.*, 2001; Maejima *et al.*, 2004; Qin *et al.*, 2008). Although possible limitations of the experimental model used may account for the results obtained, it should be noted that this animal model is indeed responsive to

PPARα agonists as also shown by other investigators (Berthou *et al.*, 1996; Rotllan *et al.*, 2011; Srivastava *et al.*, 2011). Altogether, our results suggest that transcriptional activation of apoA-I may not be the major mechanism explaining the HDL increase triggered by statin treatment.

In order to assess whether rosuvastatin treatment could post-transcriptionally influence human hepatic apoA-I production, apoA-I secretion was measured on primary hepatocytes isolated from the different treatment groups. No differences were observed among groups, except for a lower, although not significant, secretion rate in the mice treated with the highest rosuvastatin dose. These results do not support the hypothesis that rosuvastatin treatment may increase HDL levels by directly affecting human apoA-I production. Clinical studies generally show a trend towards increase of apoA-I levels (Brown *et al.*, 2002; Davidson *et al.*, 2002a; Capuzzi *et al.*, 2003; Hunninghake *et al.*, 2004), although there is no real evidence for increased apoA-I production in subjects receiving rosuvastatin treatment. Indeed, investigations on the effect of rosuvastatin on the kinetics of apoA-I in patients affected by metabolic syndrome (Ooi *et al.*, 2008) or type 2 diabetes (Verges *et al.*, 2009) showed no variations or even a reduction in apoA-I production rate versus placebo.

The unexpected result obtained with the highest dose, that is, a reduction of HDL-C and human apoA-I after rosuvastatin treatment, could possibly be a consequence of hepatic cholesterol depletion due to the inhibition of HMG-CoA reductase, that may reduce hepatic HDL production (Brewer *et al.*, 2004). This possibility is partially supported by the slight reduction of apoA-I secretion observed for primary hepatocytes of mice treated with the highest rosuvastatin dose. Alternatively, the increase of LDL receptor activity by statin treatment, by enhancement of the catabolism of apoE-containing lipoproteins, could accelerate the catabolism of apoE-containing HDL particles and thus reduce plasma HDL levels. However, Western blot analysis on plasma from controls and rosuvastatin treated mice showed that apoE levels were not modified by the treatment (data not shown).

Finally, GGE analysis of HDL particles showed, in rosuvastatin treated mice, a relative increase of larger particles and a relative decrease of smaller HDL particles. This variation in HDL size was modest with the lower rosuvastatin dose, but increased progressively at higher doses. The appearance of larger HDL particles after statin treatment has been frequently described in clinical studies (Asztalos and Schaefer, 2003; Ikewaki *et al.*, 2009) and it is generally considered a consequence of a reduced CETP-mediated exchange of triglycerides and cholesteryl esters between HDL and VLDL particles (Guerin *et al.*, 2000). In the present study, although mice lack CETP, a redistribution of HDL particles, towards larger subpopulations, was also observed. These results may imply that other mechanisms could be involved in the inter-conversion of HDL particles occurring after statin treatment. A recent study reported that statins increased HDL size by lowering PLTP activity, thus decreasing pre-β-HDL formation (Dallinga-Thie *et al.*, 2009). Indeed, in the present study, a reduced PLTP activity by rosuvastatin treatment was observed.

In summary, in human apoA-I transgenic mice lacking CETP, rosuvastatin treatment did not increase apoA-I tran-

scription and hepatic secretion, or apoA-I plasma levels. These results indirectly support the hypothesis that the effect of statins on HDL levels observed in humans can be mostly attributed to reduced CETP activity.

## Acknowledgements

This study was supported by AstraZeneca, Italy. We thank Dr Lorenzo Arnaboldi, Professor Maurizio Crestani and coworkers for helpful discussion.

## Conflict of interest

The authors state no conflict of interest.

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