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To cite this version:

Thomas Gautier, Valérie Deckert, Virginie Aires, Naïg Le Guern, Lil Proukhnitzky, et al.. Human apolipoprotein C1 transgenesis reduces atherogenesis in hypercholesterolemic rabbits. Atherosclerosis, 2021, 320, pp.10-18. 10.1016/j.atherosclerosis.2021.01.011 . hal-03130754

HAL Id: hal-03130754

https://hal.science/hal-03130754v1

Submitted on 3 Feb 2021

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Atherosclerosis 320 (2021) 10–18

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Human apolipoprotein C1 transgenesis reduces atherogenesis in hypercholesterolemic rabbits

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ARTICLE INFO

Keywords:
Apolipoprotein C1
CETP
Atherosclerosis
HDL
Transgenic
Rabbit

ABSTRACT

Background and aims: Apolipoprotein (apo) C1 is a 6.6 kDa protein associated with HDL and VLDL. ApoC1 alters triglyceride clearance, and it also favors cholesterol accumulation in HDL, especially by inhibiting CETP in human plasma. Apart from studies in mice, which lack CETP, the impact of apoC1 on atherosclerosis in animal models expressing CETP, like in humans, is not known. This study aimed at determining the net effect of human apoC1 on atherosclerosis in rabbits, a species with naturally high CETP activity but with endogenous apoC1 without CETP inhibitory potential.
Methods: Rabbits expressing a human apoC1 transgene (*HuApoC1Tg*) were generated and displayed significant amounts of human apoC1 in plasma.
Results: After cholesterol feeding, atherosclerosis lesions were significantly less extensive (−22%, $p < 0.05$) and HDL displayed a reduced ability to serve as CETP substrates (−25%, $p < 0.05$) in *HuApoC1Tg* rabbits than in WT littermates. It was associated with rises in plasma HDL cholesterol level and PON-1 activity, and a decrease in the plasma level of the lipid oxidation markers 12(S)-HODE and 8(S)HETE. In chow-fed animals, the level of HDL-cholesterol was also significantly higher in *HuApoC1Tg* than in WT animals (0.83 ± 0.11 *versus* 0.73 ± 0.11 mmol/L, respectively, $p < 0.05$), and it was associated with significantly lower CETP activity (cholesteryl ester transfer rate, −10%, $p < 0.05$; specific CETP activity, −14%, $p < 0.05$).
Conclusions: Constitutive expression of fully functional human apoC1 in transgenic rabbit attenuates atherosclerosis. It was found to relate, at least in part, to the inhibition of plasma CETP activity and to alterations in plasma HDL.

1. Introduction

Human apolipoprotein (apo) C1 is the smallest plasma apolipoprotein, with 57 amino-acid residues and a molecular weight of 6.6 kDa [1]. ApoC1 contains only two alpha helices [2] and is thought to be similar to the common ancestor of the apo A/C/E family [3]. Despite its very simple structure, the actual role of apoC1 in lipoprotein metabolism and related diseases is not clearly established although several functions have been proposed. In healthy subjects, apoC1 is mostly associated with high-density lipoproteins (HDL, 80%) and to a lesser extent with very low-density lipoproteins (VLDL, 20%) [4,5]. Therefore, most research has focused on the possible involvement of apoC1 in the metabolism of these two lipoprotein classes.

Regarding HDL metabolism, apoC1 was shown to inhibit phospholipase A_2 [6], hepatic lipase [7] and SR-BI-mediated HDL uptake [8], and to activate cholesterol efflux from lipid-laden macrophages [9] as

Abbreviations: Apo, apolipoprotein; HDL, high-density lipoprotein; LDL, low density lipoprotein; VLDL, very low-density lipoprotein; TRL, triglyceride-rich lipoproteins; WT, wild-type; *HuApoC1Tg*, human apoC1 transgenic; PCR, polymerase chain reaction; RT, reverse transcription.

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https://doi.org/10.1016/j.atherosclerosis.2021.01.011
Received 6 July 2020; Received in revised form 3 December 2020; Accepted 8 January 2021
Available online 12 January 2021
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well as lecithin:cholesterol acyltransferase activity [10]. In addition, apoC1 is a potent inhibitor of cholesteryl ester transfer protein (CETP) *in vitro* [11] and in transgenic mice expressing human CETP [12]. Accordingly, plasma apoC1 levels are inversely correlated with CETP activity in healthy humans [13]. Taken together, these data suggest that apoC1 might be atheroprotective. In contrast, the effects of apoC1 on VLDL metabolism would rather suggest a pro-atherogenic effect. Indeed, *in vitro* studies showed that apoC1 can inhibit lipoprotein lipase and hepatic lipase activity [14,15] as well as VLDL uptake [16]. Studies in transgenic mice overexpressing apoC1 showed a hypertriglyceridemic effect of apoC1 *in vivo* [17], but hypertriglyceridemia could also be observed in some apoC1-deficient mouse models [18]. In humans, larger amounts of apoC1 were present in VLDL from hypertriglyceridemic subjects [4]. Finally, and in addition to its role in lipoprotein metabolism, it was shown in mice that apoC1 can bind to endotoxins and alter the inflammatory response with either protective or deleterious effects [19] that might affect the onset of atherosclerosis.

Mouse studies showed that apoC1 expression increases atherosclerosis in a hyperlipidemic [9] or in an inflammatory context [20]. However, these results were obtained in mice, a species devoid of active CETP [21]. Studies about the relationship between apoC1 and atherosclerosis in humans are scarce, and were conducted only in small populations. On the one hand, elevated plasma apoC1 was associated with increased thickness of the vessel wall in men with metabolic syndrome and high C-reactive protein, but not in counterparts with low C-reactive protein [22]. On the other hand, high apoC1 levels were associated with a lower hazard ratio for cardiovascular death in the elderly [23]. In fact, previous human studies were only observational and were performed in very specific pathophysiological contexts. Therefore, conclusions should be drawn with caution, and whether apoC1 may directly influence the cardiovascular risk remains unclear. More recently, genome-wide association studies showed that apoC1 single nucleotide polymorphisms can be associated with HDL-C and CHD risk [24] or IMT [25], but interpretation is difficult due to the strong linkage disequilibrium with the *apoE* gene [24,26,27].

Rabbits are highly susceptible to atherosclerosis when fed a cholesterol-rich diet [28]. Interestingly, they have naturally high CETP activity [29], a feature that might be due, at least in part, to the fact that endogenous rabbit apoC1, unlike human and mouse apoC1, lacks any CETP inhibitory potential [30]. In the present study, we investigated the effect of human apoC1 on plasma lipids, lipoprotein profiles, and eventually atherosclerosis in a new line of transgenic rabbits expressing human apoC1 (*HuApoC1Tg* rabbits).

2. Materials and methods

2.1. Human ApoC1 gene construct

The *HuApoC1* construct encompassed the human *ApoC1* cDNA from the site of initiation of translation (ATG) to the STOP codon and its expression was driven by the promoter of the gene of the human elongation factor *EF1 alpha*. Complete description of the construct is available in Supplemental data.

2.2. Transgenic rabbits

*HuApoC1*Tg rabbits were generated as described in Supplemental data.

*HuApoC1*Tg and New-Zealand WT rabbits were fed a standard chow (2.4% fat; 16.4% starch; 40.6% total fiber; energy, 2340 kCal/kg) and had free access to water and food. They were housed in standard, specific pathogen-free conditions. Blood samplings were performed after an overnight fast. Blood was drawn with a 25G needle at the marginal ear vein under local anesthesia (cutaneous application of lidocaine 2.5%/prilocaine 2.5%; EMLA cream, Astra-Zeneca). For the atherosclerosis study, the rabbits were fed a 1% cholesterol-enriched diet (HC diet, based on chow diet enriched with cholesterol) for 8 weeks. At the end of the 8-week diet, euthanasia was performed by electrical stunning of the rabbits immediately followed by complete bleeding by section of carotid artery. Both males and females were used with the following ratios: wild-type, 11 M/11F; HuApoC1Tg, 13 M/11F.

2.3. Immunodetection of human apoC1

Mass levels of human apoC1 in total plasma or lipoprotein fractions were determined by a specific immunoassay with specific anti-human apoC1 antibodies. Briefly, total plasma from rabbits, or lipoprotein fractions isolated by FPLC were diluted 1:10 to 1:100 in phosphate-buffered saline (PBS), and applied onto nitrocellulose membranes (Whatman, Dassel, Germany) with a Slot Blot system (Hoefer Scientific, San Francisco, CA). The resulting blots were blocked for 1 h in 5% low-fat dried milk in PBS containing 0.1% Tween and then washed with PBS/Tween. Human apoC1 was revealed by successive incubations with anti-human apoC1 antibodies (ACRIS, Herford, Germany) and horseradish peroxidase-coupled anti-goat secondary antibodies (Dako, Glostrup, Denmark). Blots were finally developed by using an ECL kit (GE Healthcare), acquired with a ChemiDoc system (BioRad) and signal intensities were analyzed with the ImageLab software (BioRad). The apoC1 mass level in each sample was estimated by comparison with a calibration curve that was obtained with serial dilutions of a human plasma standard blotted together with the samples. Plasma from wild-type rabbits displayed no signal.

2.4. Measurement of CETP activity

CETP activity was determined in microplates by a fluorescent method using commercially available donor liposomes enriched with nitrobenz-oxadiazole (NBD)-labelled cholesteryl esters (CETP activity assay kit, Sigma Aldrich). For the measurement of plasma CETP activity, the incubation media contained 5 μl of donor liposomes and 10 μl of total plasma as a source of endogenous CETP and lipoprotein acceptors. To measure the activity in the presence of isolated HDL, 5 μl of donor liposomes and 1 μg of partially purified CETP [11] were incubated in the presence of native HDL acceptors (final amount, 5 nmol cholesterol) [31]. HDL ($1.063 < d < 1.21$) were isolated by sequential ultracentrifugation in a TLA100 rotor (TLX ultracentrifuge, Beckman, Palo Alto, CA). Briefly, density was adjusted at 1.063 by adding KBr solution to total plasma and apoB containing lipoproteins (supernatant) were discarded after a 3-h spin. The density of infranatant was subsequently adjusted to 1.21 with NaBr solution and HDL (supernatant) were obtained after a 5.5-h spin. Fluorescence readings were performed on a Victor^2 multilabel counter (PerkinElmer) over a 3-h period at 37 °C. The CETP-mediated transfer of NBD-cholesteryl esters from self-quenched donors to acceptor lipoproteins was monitored by the increase in fluorescence intensity (excitation, 465 nm; emission, 535 nm), and results were expressed in fluorescence arbitrary units after deduction of blank values that were obtained with control mixtures without CETP.

2.5. Fast protein liquid chromatography (FPLC) analysis

Two hundred microliters of pooled rabbit plasma ($n = 11–24$) were injected into a Superose 6 HR 10/30 column which was connected to an FPLC (fast protein liquid chomatography) system (GE Healthcare) [31]. Lipoproteins were eluted at a constant flow rate of 0.3 ml/min with TBS (50 mM; pH 7.4) containing 0.074% EDTA and 0.02% sodium azide. Total cholesterol in isolated 0.3 mL fractions was determined with an enzymatic kit (Cholesterol FS, Cat#11300, DiaSys, Holzheim, Germany). TRL (triglyceride-rich lipoproteins lipoproteins) are contained in fractions 5–14, LDLs (low-density lipoproteins) are contained in fractions 15–25 and HDLs are contained in fractions 26–45.

2.6. Plasma lipid analysis

Plasma total cholesterol (Cat#98181813, ThermoFisher), free cholesterol (Cat#11360, Diasys), triglycerides (Cat#981786, ThermoFisher), phospholipids (Cat#296-63801, Wako) and fatty acids (Cat#434-91795/436-91995) were assayed with enzymatic kits on an Indiko Chemistry System (Thermo Fisher). Triglyceride-rich lipoproteins (chylomicrons, VLDL, d < 1.006, 2 h spin), IDL and LDL (1.006 < d < 1.063, 3 h spin) and HDL (1.063 < d < 1.21, 5.5 h spin) were isolated by sequential ultracentrifugation in a TLA100 rotor (TLX ultracentrifuge, Beckman, Palo Alto, CA) [32]. Densities were adjusted by addition of KBr and NaBr solutions at each step. Cholesterol content in isolated lipoprotein fractions was determined with an enzymatic kit (Cholesterol FS, Cat#11300, DiaSys).

2.7. Native polyacrylamide gradient gel electrophoresis

HDL from pooled rabbit plasma (n = 22–24) were isolated by sequential ultracentrifugation as described above and applied onto a 15–250 g/l polyacrylamide gradient gel (LaraSpiral, Couternon, France). Total lipoproteins were separated by ultracentrifugation at 100000 rpm for 5.5h as the d < 1.21 g/ml plasma in a TLA100 rotor in a TLX ultracentrifuge (Beckman). Lipoproteins were then applied on to a 15–250 g/l polyacrylamide gradient gel (Spiragel 1.5–25.0; Spiral, Couternon, France) in a 90 mM Tris, 80 mM boric acid, 3 mM EDTA running buffer. Electrophoresis was run at 4 °C for 15 h at 200 V [33]. HDL proteins were subsequently stained with Coomassie (Instant Blue, Expedeon, Cambridge, UK). Images were acquired with a ChemiDoc system (BioRad), HDL distribution profiles were analyzed with the ImageLab software (BioRad) and compared with protein globular standards that were subjected to electrophoresis together with the samples (HMW kit, GE Healthcare).

2.8. Quantification and analysis of atherosclerosis

Areas of atherosclerotic lesions in rabbits fed a high-cholesterol diet for 8 weeks were quantified by *en-face* analysis [32]. The aortic arch and the thoracic aorta were opened longitudinally and fixed with 4% paraformaldehyde (PFA) in PBS. Fixed aortas were subsequently pinned onto a black wax surface and stained with 0.3% Oil-Red-O (Sigma) in 60% isopropanol for 15 min at room temperature, washed with 60% isopropanol for 10 min and finally rinsed with water before being stored in 4% PFA in PBS before imaging. The area of atherosclerotic plaques was quantified by color thresholding with the Photoshop CS6 software (Adobe Systems, San José, CA). For histological analyses, serial sections of aorta were stained with Masson's Trichrome or immunostained with specific antibody (Dako Ram-11 antibody for macrophages).

2.9. Statistical analyses

All statistical analyses were performed using GraphPad Prism 6.0 software. The significance of differences between groups was determined by Student's t-test after checking the equality of variances. Differences were considered significant for p values below 0.05. Correlation analyses were performed using Pearson's test.

3. Results

3.1. Generation of transgenic rabbits expressing human apoC1

Sixty-five rabbits were born after the transfer of 620 embryos injected with the construct (Supplemental Fig. IA) into 25 recipient does. Seven transgenic founders were identified and one transgenic line harboring 3 copies of the transgene was successfully established. After puberty, all rabbits were viable, did not show any overt abnormality and had a body weight similar to that of WT animals (2.11 ± 0.19 kg *versus* 2.11 ± 0.15 kg, respectively, NS).

By using RT-PCR (Supplemental Fig. IB), an amplified fragment of expected size (1076 base pairs) and sequence encompassing the full-length human *ApoC1* mRNA was found, supporting the production of a full-length apoC1 protein in HuApoC1Tg rabbits.

The expression of the endogenous rabbit *apoC1* gene was similar in transgenic and wild-type rabbit liver (Fig. 1A), *i.e.* the main source of apoC1 in mammals [27]. Due to the presence of the human *eF1-α* gene promoter in the construct, human *ApoC1* transgene expression could be detected in all organs tested, with the highest mRNA levels in heart and pancreas and the lowest levels in adipose tissue and spleen (Fig. 1B).

Since apoC1 is a secreted protein, we investigated plasma levels and distribution of human apoC1 in transgenic rabbits. Human apoC1 could be detected in plasma of chow-fed animals, with an average concentration of 4.59 mg per liter (Supplemental Table I). Most human apoC1 co-localized with HDL, with smaller amounts present in the low-density lipoprotein (LDL) as well as in the lipoprotein-free fraction (Fig. 1C). HDL were the prominent cholesterol carriers in both transgenic and wild-type animals (Fig. 1D).

3.2. Effect of human apoC1 expression on lipid profile and atherosclerosis in rabbits fed a high cholesterol diet

In order to determine the impact of human apoC1 on atherosclerosis in our model, WT and *HuApoC1Tg* rabbits were fed a high-cholesterol diet for eight weeks. Cholesterol feeding led to a dramatic increase in plasma cholesterol levels, which reached 32.35 ± 5.14 and 31.76 ± 6.98 mmol/L in WT and *HuApoC1Tg* rabbits, respectively (Supplemental Table II). In both genotypes, it was mostly due to cholesterol accumulation in the apoB-containing lipoproteins (Supplemental Table II). Plasma level of human apoC1 was 62.02 ± 19.71 mg/L in *HuApoC1Tg* rabbits (Supplemental Table II). It located predominantly in the most prominent apoB-containing lipoproteins, but substantial amounts were also found in HDL (Fig. 2A).

No significant difference was found in total plasma cholesterol, triglyceride, phospholipid and fatty acid levels between WT and *HuApoC1Tg* rabbits fed the high cholesterol diet (Supplemental Table II), and cholesterol content and distribution in apoB-containing lipoproteins were similar in both groups (Supplemental Table II, Fig. 2B). Importantly, HDL-cholesterol levels were higher in *HuApoC1Tg* rabbits than in WT rabbits (1.08 ± 0.51 *versus* 0.83 ± 0.34 mmol/L, respectively, $p < 0.05$) (Supplemental Table II). In addition, as determined by native gradient gel electrophoresis, the higher amounts of HDL appeared to rely mostly on large-sized subfractions (Fig. 2C). More in details, by studying pooled samples from rabbits on a HC diet, there was a 20.4% increase in the relative amounts of HDL with a diameter above 14 nm (9.9% in HuApoC1Tg *versus* 8.2% in WT rabbits) together with a 7.2% decrease in the relative amounts of HDL with a diameter below 10 nm (34.8% in *HuApoC1Tg versus* 37.5% in WT rabbits). Although plasma MCP-1 levels (Fig. 2D), CRP levels (Fig. 2E) and efflux capacities of HDL (Fig. 2F) were similar in both groups, PON-1 activity was significantly increased in *HuApoC1Tg* rabbits compared to WT rabbits (46 ± 10 *versus* 30 ± 13 U/liter, respectively, $p < 0.05$) (Fig. 2 G) and correlated positively with HDL-cholesterol levels (r-0.393, p = 0.024, Pearson test, Supplemental Table IV). This was accompanied by a trend to a global decrease in plasma levels of the lipid oxidation markers HODEs and HETEs in *HuApoC1Tg* rabbits compared with WT rabbits (see Supplemental Table III). More specifically, a significant 16% decreases 12(S)-HODE (71.1 ± 4.4 *versus* 84.0 ± 10.0 in *HuApoC1Tg* and WT rabbits, respectively, $p = 0.0328$) and a significant 9% decrease in 8(S)-HETE (68.9.1 ± 7.4 *versus* 76.2 ± 7.8 in *HuApoC1Tg* and WT rabbits, respectively, $p = 0.0297$) could be observed. Interestingly, total HODE levels correlated positively with cholesteryl ester transfer activity (r = 0.397, $p = 0.014$) and, together with total HETE levels, with specific CETP activity (r = 0.346, $p = 0.033$ and r = 0.363, $p = 0.025$, respectively, Pearson test; see Supplemental Table IV).

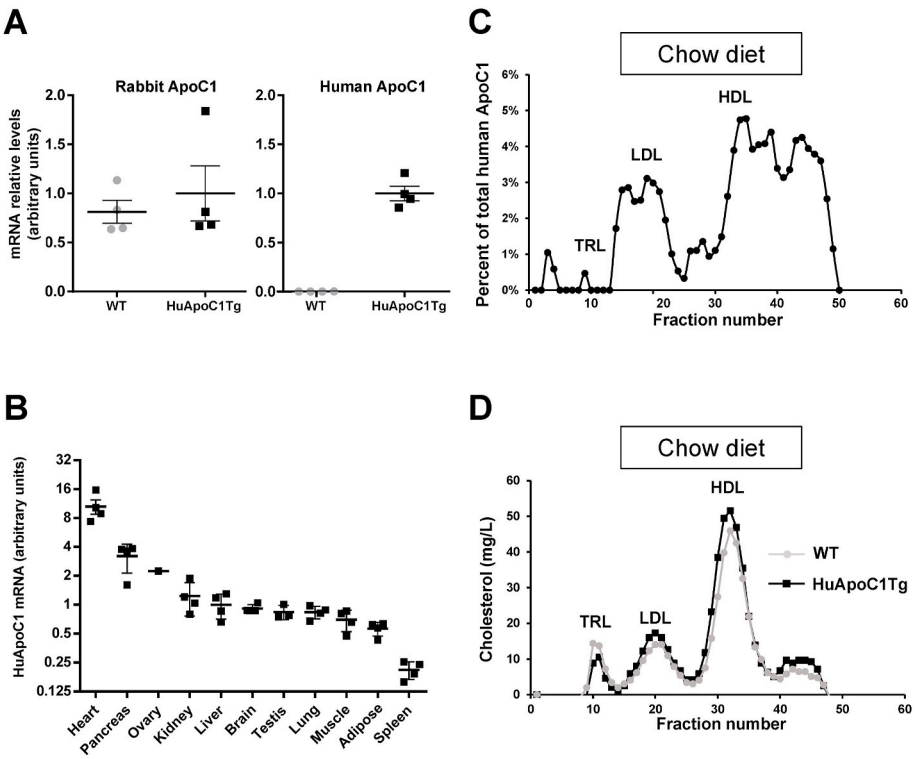

Fig. 1. ApoC1 expression and distribution in plasma of chow-fed rabbits.
(A) Quantification of rabbit (left panel) and human *apoC1* mRNA (right panel) obtained from livers of WT (grey circles) and *HuApoC1Tg* rabbits (black squares). Mean mRNA levels in *HuApoC1Tg* rabbits were set to 1. Means ± S.E.M. (n = 4 animals). N.S., not significant, Mann-Whitney test. (B) Human *apoC1* mRNA levels were compared in organs of *HuApoC1Tg* rabbits. Liver mRNA mean levels were set to 1. Means ± S.E.M. (n = 4). (C and D) Plasma from fasting WT (n = 14) and *HuApoC1Tg* rabbits (n = 11) was subjected to FPLC. (C) Human apoC1 levels in FPLC fractions obtained from *HuApoC1Tg* rabbit plasma were determined by immunoassay and expressed as a percentage of human apoC1 levels found in total plasma. (D) Cholesterol levels in FPLC fractions obtained from WT (grey line) and *HuApoC1Tg* rabbits (black line).

After Oil Red O staining, descending aortas were analyzed for the extent of atherosclerotic lesions (Fig. 3A). Strikingly, *en-face* imaging of aortas from *HuApoC1Tg* rabbits showed a 21.9% reduction in atherosclerotic lesions as compared to those in WT rabbits ($p = 0.019$). Hematoxylin-eosin staining revealed significant and similar thickening of arterial intima in both WT and *HuApoC1Tg* rabbits (Fig. 3B and C). Masson's Trichrome staining (Fig. 3D and E) showed similar collagen enrichment in plaques from WT and HuApoC1Tg rabbits (25.7 ± 7.4% *versus* 23.6 ± 11.3%, respectively, $p = 0.5427$, NS). Immunohistochemistry on histological slices revealed similar macrophage infiltration with 33.0 + 3.7% and 33.5 + 5.0% of RAM-11 staining in the plaques of WT and *HuApoC1Tg* rabbits, respectively (p = 0.7027, NS) (Fig. 3F and G).

3.3. Effect of human apoC1 expression on CETP activity in transgenic rabbits

Plasma cholesteryl ester transfer activity was first assessed by using donor liposomes containing fluorescently labelled cholesteryl esters (Table 1).

In animals on chow diet, the cholesteryl ester transfer rate was significantly lower in *HuApoC1Tg* rabbits than in WT rabbits (466 ± 53 *versus* 516 ± 72 AU/min, respectively, $p < 0.05$). Plasma CETP mass concentration as measured by ELISA did not differ between the two groups (1.30 ± 0.39 and 1.19 ± 0.13 mg/L, respectively, NS). As a consequence, human apoC1 expression resulted in a significant, 14% decrease in specific CETP activity in *HuApoC1Tg* rabbits compared with WT rabbits on chow diet (37.5 ± 7.5 *versus* 43.7 ± 6.1 AU/min/ng, respectively, $p < 0.05$). HDL-cholesterol levels in *HuApoC1Tg* rabbits were higher than those in WT rabbits (0.83 ± 0.11 *versus* 0.73 ± 0.11 mmol/L, respectively, $p < 0.05$, Supplemental Table I and Fig. 1D).

After 8 weeks on a high-cholesterol diet, the cholesteryl ester transfer rate in total plasma was significantly increased in both WT and *HuApoC1Tg* rabbits compared to what was observed on a chow diet (+62% and +72%, respectively, $p < 0.0001$ in both cases) to reach similar levels in both genotypes (836 ± 157 AU/min in wild type and 803 ± 152 AU/min in *HuApoC1Tg*, N.S.). The plasma CETP mass concentration was also increased in response to the high cholesterol diet (+55% in WT and +36% in *HuApoC1Tg*, $p < 0.0001$ and $p = 0.0013$, respectively) and was not different between the two genotypes. Specific CETP activity in total plasma was similar in hypercholesterolemic WT and *HuApoC1Tg* rabbits (Table 1).

Human apoC1 is known to be a potent CETP inhibitor when associated with HDL, and it was reported in earlier studies that adding apoC1 to isolated HDL is able to reduce CETP-lipoprotein interactions. Thus, we tested the ability of isolated HDL from hypercholesterolemic rabbits to serve as substrates for CETP. In the presence of constant amounts of HDL and purified human CETP, cholesteryl ester transfer occurred at a significantly slower rate with HDL from *HuApoC1Tg* rabbits than with HDL from WT rabbits (Fig. 4A), with an overall 25% decrease in CETP activity in HDL containing human apoC1 ($p = 0.0023$; Fig. 4B). Importantly, CETP activity in the presence of purified HDL correlated strongly and positively with the extent of atherosclerotic lesions and negatively with plasma levels of human apoC1 in rabbits on a high-cholesterol diet ($r = 0.3764$, $p = 0.0099$ and $r = -0.321$, p=0.030, respectively, Pearson test, see Supplemental Table IV).

4. Discussion

The present study reports the generation of a new line of transgenic rabbits expressing human apoC1. Levels of human apoC1 in plasma ranged from 10% (chow diet) to 100% (high cholesterol diet) of the levels that are normally measured in healthy human plasma [30]. Despite the ubiquitous expression pattern of the transgene, which significantly differs from what is observed in mice and primates [27], human apoC1 was a secreted protein mainly associated with circulating lipoproteins. In a similar way to what is observed in humans, in transgenic rabbits most human apoC1 bound to HDL in the normolipidemic state, and it also accumulated in large amounts in the prominent apoB-containing lipoproteins in the hypercholesterolemic state [34], leading to a net increase in total human apoC1 plasma levels. It has been suggested that this typical distribution pattern relies on both the high

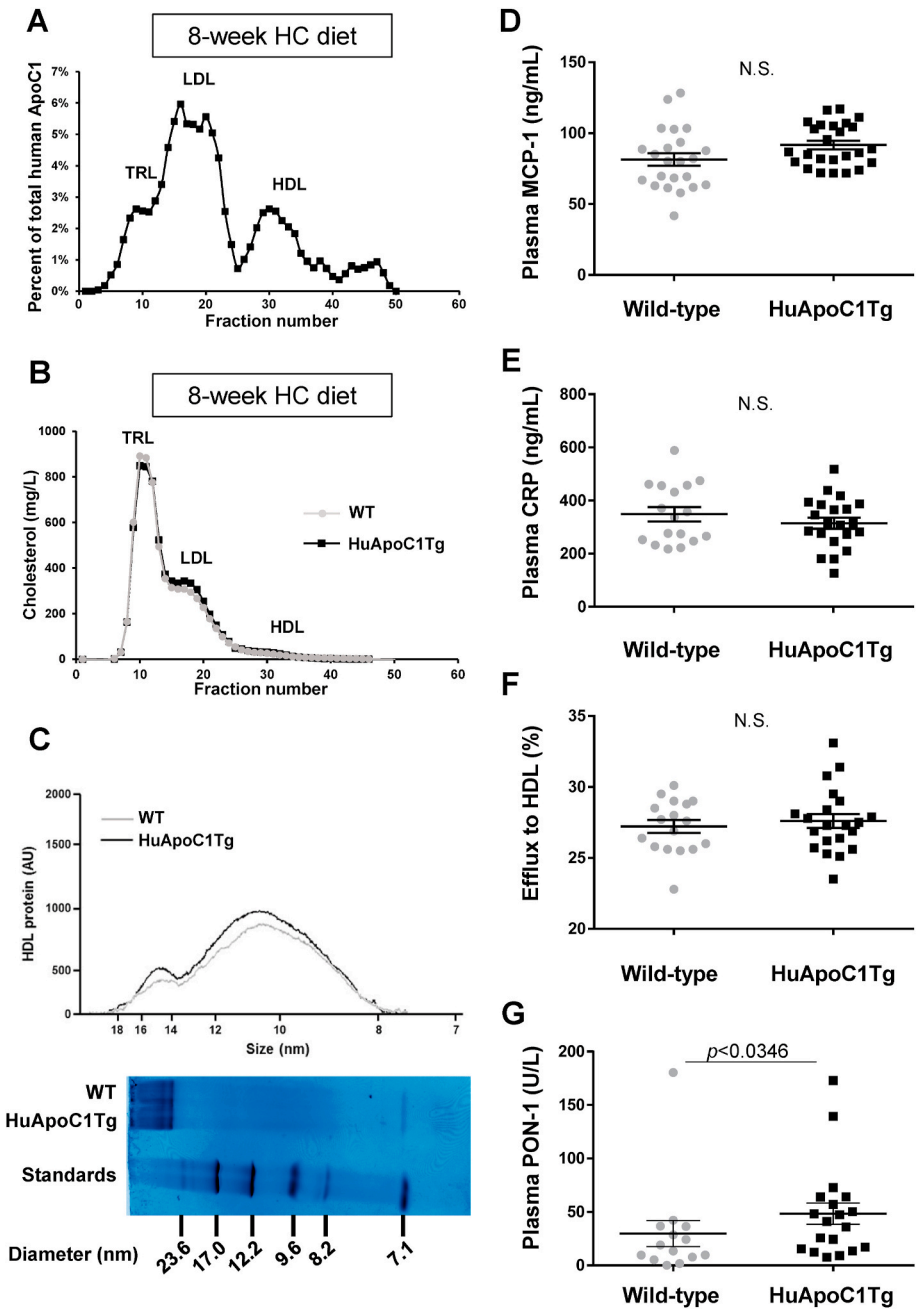

Fig. 2. Plasma distribution of human apoC1, inflammatory and lipoprotein parameters in rabbits fed a high-cholesterol diet.
(A and B) Pooled plasmas from fasting WT (n = 22) and *HuApoC1Tg* rabbits (n = 24) were subjected to FPLC. (A) Human apoC1 levels in FPLC fractions from *HuApoC1Tg* rabbits were determined by immunoassay and expressed as percentage of total plasma human apoC1. (B) Cholesterol levels in FPLC fractions from WT (grey line) and *HuApoC1Tg* rabbits (black line). (C) Lipoproteins from WT (grey line) and *HuApoC1Tg* rabbits (black line) were isolated from pooled plasmas by ultracentrifugation and subjected to native gradient gel electrophoresis prior to Coomassie staining. HDL size distribution profiles were then analyzed. Plasma MCP-1 (D) and CRP (E) levels were determined with specific ELISA kits. (F) Cholesterol efflux from THP-1 macrophages in the presence of HDL isolated from wild-type or *HuApoC1Tg* rabbits was performed as described in Materials and methods. (G) Plasma paraoxonase-1 (PON-1) activity was measured with a fluorescent kit. Means ± S.E.M. in (D–G). Significance of difference between groups was determined by Student's *t*-test. N.S., not significant.

exchangeability of apoC1 and its affinity for individual lipoprotein fractions, and not on the cell type it originates from [4].

Here, and in the hypercholesterolemic state, human apoC1 expression led to fewer atherosclerotic lesions in the transgenic rabbit model than in WT littermates. This brings the first direct evidence of an atheroprotective effect of human apoC1 at physiological levels in a species, which, like humans and unlike mice, spontaneously develops atherosclerosis when fed a cholesterol-rich diet, expresses active CETP, and has substantial amounts of apoB-containing lipoproteins when fed a high-cholesterol diet [35]. The underlying causes of the reduced atherosclerosis in the presence of human apoC1 do not seem to relate to the modulation of inflammation or macrophage recruitment since MCP-1 and CRP levels were similar in *HuApoC1Tg* and WT rabbits. These results differ from what was observed in naturally CETP-deficient mice, where human apoC1 had a pro-inflammatory effect in endotoxemia and sepsis models [19] when overexpressed at levels substantially higher than in the present rabbit study [17].

Regarding the influence of human apoC1 on plasma lipid metabolism, the anti-atherogenic effect was in all likelihood not related to an effect on apoB-containing lipoproteins. First, *HuApoC1* expression in our model had no impact on plasma triglyceride levels and distribution, whether the rabbits were in a hyperlipidemic state or not, despite substantial amounts of human apoC1 associated with TRL in cholesterol-fed rabbits. As supported by earlier studies [17,33], *HuApoC1* expression more likely triggers hypertriglyceridemia in the mouse, than in the rabbit model. Although previous studies reported an effect of apoC1 on lipase activities and TRL clearance *in vitro* and in transgenic mouse models [14–17], the lack of triglyceride elevation might rely on different causes. On the one hand, the lack of effect of human apoC1 in our model can be due to very active LPL in rabbits [36,37] which, again and in this respect, cannot compare with mice. On the other hand, and contrarily to previously developed transgenic mice, our *HuApoC1Tg* rabbits do not

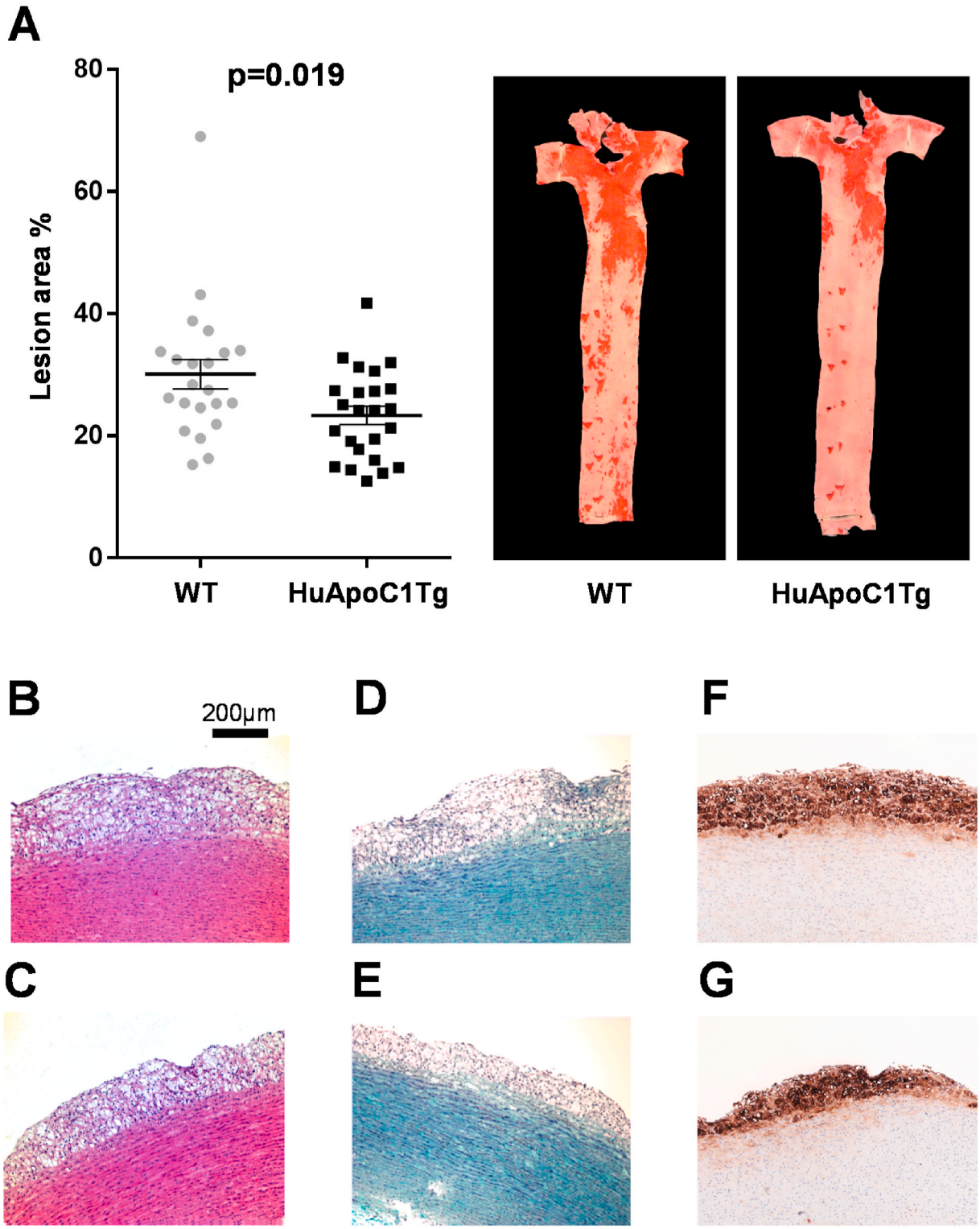

Fig. 3. Aortic lesions in aorta from WT and *HuApoC1Tg* rabbits fed a high-cholesterol diet.
WT (n = 22, grey circles) and *HuApoC1Tg* rabbits (n = 24, black squares) were fed a high-cholesterol diet for 8 weeks. (A) Aorta was stained with Oil Red O and results were expressed as % of area covered with lesions. Means ± S.E.M. Significance of difference between groups was determined by Student's *t*-test. (B–G) Immunohistological analysis of atherosclerotic lesions in wild-type (B, D, F) and *HuApoC1Tg* rabbits (C, E, G) fed a high cholesterol diet. Hematoxylin-eosin staining (B and C), Masson's Trichrome staining (D and E) and immunostaining for macrophages (RAM-11; F and G) were performed on section of arteries. Black bar, 200-µm scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
CETP activity in rabbits on a chow diet or a high cholesterol diet.

	WT chow n = 14	*HuApoC1Tg* chow n = 11	WT HC n = 22	*HuApoC1Tg* HC n = 24
CE transfer rate (AU/min)	516 ± 72	466 ± 53[a]	836 ± 157[b]	803 ± 152[e]
CETP mass (mg/l)	1.19 ± 0.13	1.30 ± 0.39	1.85 ± 0.49[c]	1.77 ± 0.38[f]
Specific activity (AU/min/ng)	43.7 ± 6.1	37.5 ± 7.5[d]	47.5 ± 14.3	47.1 ± 12.9[g]

Cholesteryl ester (CE) transfer rate and CETP mass levels in plasma were measured in WT (n = 22) and *HuApoC1Tg* rabbits (n = 24) on a chow or a high-cholesterol (HC) diet. Specific CETP activity is the ratio between the CE transfer rate and amounts of CETP protein. Means are shown ±S.D. [a, b, c, d]Significantly different from WT chow, $p = 0.0305$, $p < 0.0001$, $p < 0.0001$ and $p = 0.0327$, respectively. [e, f, g]Significantly different from *HuApoC1Tg* chow, $p < 0.0001$, $p = 0.0013$ and $p = 0.0227$, respectively, Student's *t*-test.

represent an overexpression model since observed circulating human apoC1 levels remained moderate and did not exceed the levels observed in healthy humans. In comparison, human apoC1 levels in transgenic mouse models of overexpression were 2- to 4.5-fold higher than in healthy humans [17,38]. The lack of a triglyceride effect in our model might be due to lower apoC1 levels in our rabbits than in apoC1 overexpressing mice. A possible role of endogenous CETP in blunting the hypertriglyceridemic effect of apoC1 can be ruled out since human apoC1 expression could still induce a significant rise in triglyceride levels in a previous study using transgenic mice expressing CETP [33]. Finally, *HuApoC1* expression did not modify LDL cholesterol levels in the present rabbit studies. Again, it may relate to the level of human apoC1, which was kept moderate in the present study.

Unlike apoB-containing lipoproteins, HDL levels were significantly higher in *HuApoC1Tg* rabbits than in their WT littermates, both with the normal chow and high-cholesterol diets. The consistent rise in HDL level might account for the atheroprotective effect of human apoC1 expression in our model. Accordingly, *HuApoC1Tg* rabbits displayed a significant rise in HDL size and in activity of PON-1, an HDL-bound antiatherogenic enzyme involved in the neutralization of oxidized lipids [39]. It might account, at least in part, for the significant decrease in some HODE and HETE species, i.e. oxidized lipid derivatives which can be considered as a hallmark of a reduced oxidative stress in *HuApoC1Tg* rabbits. Finally, the correlation between the extent of atherosclerotic plaques and the ability of isolated HDL to serve as substrates for CETP also supports the HDL-mediated effect of apoC1 on atherosclerosis.

The present data suggest that the HDL-raising effect of *HuApoC1* expression most likely relates to the inhibition of CETP activity. Indeed, we measured significant reductions in specific CETP activity as well as in cholesteryl ester transfer rates in total plasma of *HuApoC1Tg* rabbits on a chow diet and with a moderate, but long-lasting CETP inhibitory potential. Consistent with earlier studies [11], human apoC1 was again shown to behave as a potent CETP inhibitor, and even moderate levels of human apoC1 in rabbit plasma are sufficient to significantly inhibit CETP activity. This comes in support of earlier human studies, which reported that even small variations in plasma apoC1 level could impact CETP activity and the plasma lipoprotein profile in healthy individuals and patients with diabetes [40] and dyslipidemia [13]. Plasma HDL isolated from *HuApoC1Tg* rabbits fed a high-cholesterol diet were poorer substrates for CETP when compared with HDL from WT animals and we could observe a significant, negative correlation between apoC1 levels and the ability of isolated HDL to serve as substrates for CETP. However, specific CETP activity and cholesteryl ester transfer in hyperlipidemic plasma were unchanged on a high-cholesterol diet, probably due to the drastic accumulation of triglyceride-rich lipoproteins (i.e. a main driving force for the CETP-mediated reaction in cholesteryl ester transfer assays [13,41,42]). Thus, the inhibition of CETP activity by apoC1 resulted in increased HDL levels and in the production of larger HDL in *HuApoC1Tg* rabbits in the present study, with consistent observations in animals fed normal chow or a high-cholesterol diet.

The atheroprotective effect of human apoC1 in our transgenic model might rely either on the quantity or on the functional properties of lipoproteins. Interestingly, two clinical studies using proteomics strategies reported that the relative amounts of apoC1 were reduced in HDL from patients with coronary artery disease [43,44]. In addition, apoC1-poor HDL were shown to display markers of protein oxidation [44] and a positive correlation between apoC1 content ant antioxidant activity in HDL was reported in healthy humans [45]. Although these observational studies were not designed to determine whether apoC1 depletion is a cause, a consequence or a surrogate marker of CAD and increased lipoprotein oxidation, they come in support of our observation that oxidized lipid derivatives accumulate to a lesser extent when human apoC1 is expressed in hypercholesterolemic *HuApoC1Tg* rabbits.

Finally, as a limitation, it must be pointed out that our study was conducted in one single transgenic line. Although our results were obtained among more than 20 individuals of each genotype, one single

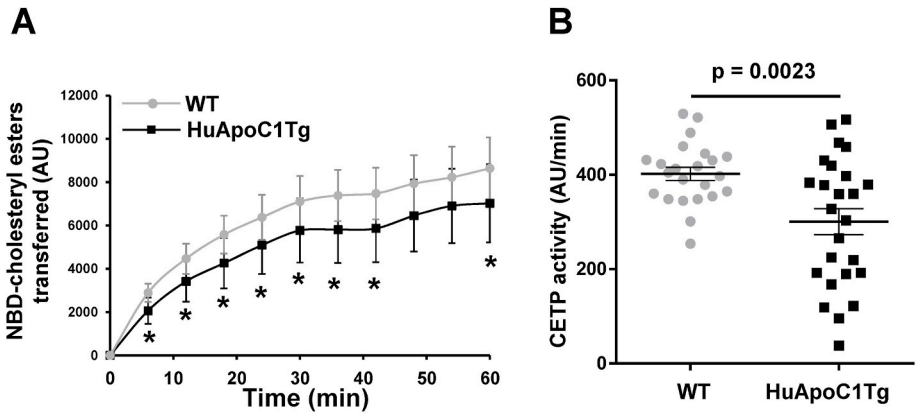

Fig. 4. HDL from WT and *HuApoC1Tg* rabbits as substrates for CETP.
HDL isolated from rabbits on a high cholesterol diet were incubated in the presence of purified CETP and donor liposomes containing fluorescently labelled cholesteryl esters. (A) Time-course of cholesteryl ester transfer with HDL from WT (grey circles) or *HuApoC1Tg* (black squares) rabbits. Each point is the mean ± S.D. of 22 (WT) or 25 (*HuApoC1Tg*) rabbits. *Significantly different from WT, $p = 0.0112$, 0.0077, 0.0043, 0.0066, 0.0095, 0.0040, 0.0059 and 0.0479 for 6, 12, 18, 24, 30, 36, 42 and 60 min, respectively, Student's *t*-test. (B) CETP activity in the presence of HDL from WT (grey bar) or *HuApoC1Tg* (black bars) was calculated from the initial transfer rates. Results are shown with means ± S.E.M. of 22 (WT) or 24 (*HuApoC1Tg*) rabbits. $p = 0.0023$, Student's *t*-test.

transgenic line did not allow to assess a gene dose effect on lipid profile and atherosclerosis extent.

Taken together, our data show that human apoC1 expression in rabbits leads to a reduction in the extent of atherosclerosis. Concomitantly, it was associated with the inhibition of CETP activity together with increases in cholesterol, PON-1 and HDL size, and a decrease in lipid oxidation. The protective effect of apoC1 against atherosclerosis will deserve further attention and clinical relevance in humans remains to be established.

Financial support

This work was supported by grants from the University of Bourgogne Franche-Comté; the Institut National de la Santé et de la Recherche Médicale (INSERM); the Fondation de France [Project Cardio 2013 R13057MM-RAF13002MMA to TG]; ANR grant ATHEROLIP; the European Union program FEDER; and by a French Government grant managed by the French National Research Agency under the program 'Investissements d'Avenir' [reference ANR-11-LABX-0021 (Lipstic Labex)].

CRediT authorship contribution statement

Thomas Gautier: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft. **Valérie Deckert:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Virginie Aires:** Investigation, Data curation, Methodology. **Naig Le Guern:** Investigation, Data curation, Formal analysis, Methodology. **Lil Proukhnitzky:** Investigation, Formal analysis. **Danish Patoli:** Investigation, Formal analysis. **Stéphanie Lemaire:** Investigation, Formal analysis. **Guillaume Maquart:** Investigation, Formal analysis. **Amandine Bataille:** Investigation, Formal analysis. **Marion Xolin:** Investigation. **Charlène Magnani:** Investigation, Formal analysis. **David Masson:** Conceptualization, Writing - review & editing. **Erwana Harscoët:** Investigation, Methodology, Data curation, Formal analysis. **Bruno Da Silva:** Investigation, Methodology, Data curation, Formal analysis. **Louis-Marie Houdebine:** Conceptualization, Writing - review & editing. **Geneviève Jolivet:** Conceptualization, Methodology, Data curation, Funding acquisition, Writing - review & editing. **Laurent Lagrost:** Conceptualization, Funding acquisition, Methodology, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful for the care given to animals at the facility by the team of UCEA (INRA, Jouy-en-Josas, France). We thank Karim Helimi (MSc, UMR1231, Dijon) for technical help and Philip Bastable for English editing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2021.01.011.

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