

Evaluation of anti-atherogenic properties of ezetimibe using ^3H -labeled LDL and $^{99\text{m}}\text{Tc}$ -cAbVCAM1-5 SPECT imaging in ApoE $^{-/-}$ mice fed a paigen diet

Author names and affiliations.

Laurent S. Dumas^{1,2,3*}, François Briand^{4*}, Romain Clerc^{1,2,4}, Emmanuel Brousseau⁴, Christopher Montemagno^{1,2}, Mitra Ahmadi^{1,2}, Sandrine Bacot^{1,2}, Audrey Soubies^{1,2}, Pascale Perret^{1,2}, Laurent M. Riou^{1,2}, Nick Devoogdt⁵, Tony Lahoutte⁵, Gilles Barone-Rochette^{1,2,6}, Daniel Fagret^{1,2,6}, Catherine Ghezzi^{1,2}, Thierry Sulpice⁴ and Alexis Broisat^{1,2}

¹Inserm U1039 Radiopharmaceutiques Biocliniques, Grenoble, France ;

²UGA, Université Grenoble Alpes, Grenoble, France

³Advanced Accelerator Applications, Saint-Genis-Pouilly, France

⁴Physiogenex, 516 rue Pierre et Marie Curie, 31670 Labège, France ;

⁵ICMI, Vrije Universiteit Brussel, Brussels, Belgium

⁶CHRU Grenoble, Hôpital Michallon, 38000 Grenoble, France

*Authors contributed equally

First author:

Laurent S Dumas

Laboratoire "Radiopharmaceutiques Biocliniques"

INSERM UMR S 1039

Faculté de Médecine de Grenoble

Domaine de la Merci

38700 La Tronche

Fax # +33 4 76 63 71 42

Telephone # +33 4 76 63 71 02

Email address: laurent.dumas.ujf@gmail.com

Corresponding author:

Alexis Broisat

Laboratoire "Radiopharmaceutiques Biocliniques"

INSERM UMR S 1039

Faculté de Médecine de Grenoble

Domaine de la Merci

38700 La Tronche

Fax # +33 4 76 63 71 42

Telephone # +33 4 76 63 71 02

Email address: Alexis.broisat@inserm.fr

Total word counts: 4468

Abstract word counts: 330

Number of figure: 4

Keywords:

Atherosclerosis, Ezetimibe, LDL-cholesterol catabolism, VCAM-1, $^{99\text{m}}\text{Tc}$ -cAbVCAM1-5

Short running title:

Anti-atherogenic properties of ezetimibe

ABSTRACT

Background: Intestinal cholesterol absorption inhibitor ezetimibe added to statin therapy has recently demonstrated clinical benefits in the IMProved Reduction of Outcomes: Vytorin Efficacy International Trial trial by further reducing Low Density Lipoprotein-Cholesterol (LDL-C) levels than statin therapy alone. Here we investigated the mechanisms by which inhibition of intestinal cholesterol absorption could contribute to the clinically observed cardiovascular events reduction by evaluating its effect on inflammatory plaque development in apolipoprotein E^{-/-} (ApoE^{-/-}) mice. **Methods:** ApoE^{-/-} mice were fed a Paigen diet (1.25% cholesterol, 0.5% cholic acid and 15% fat) without or with ezetimibe (7 mg/kg/day) for 6 weeks. A first set of mice (n=15) was used to test whether ezetimibe promotes LDL derived cholesterol fecal excretion by injecting intravenously ³H-cholesteryl oleate labeled human LDL and a second set (n=20) was used to evaluate the expression of the inflammatory marker Vascular Cell Adhesion Molecule-1 (VCAM-1) in atherosclerotic plaques using the imaging agent ^{99m}Tc-cAbVCAM1-5. A third set of mice (n=21) was used to compare VCAM-1 expression and ^{99m}Tc-cAbVCAM1-5 uptake in various tissues. **Results:** Mice treated with ezetimibe showed a 173% higher LDL-cholesteryl ester plasma disappearance rate ($p<0.001$ vs. control) after ³H-cholesteryl oleate labeled LDL injection. At time 96 hours after injection, hepatic fraction of ³H-tracer was 61% lower in mice treated with ezetimibe ($p<0.001$). Meanwhile, LDL-derived ³H-cholesterol excretion in the feces was 107% higher ($p<0.001$). The anti-atherogenic effect of ezetimibe monitored by ^{99m}Tc-cAbVCAM1-5 Single Photon Emission Computed Tomography (SPECT) imaging showed a 49% reduction in aortic tracer uptake (0.95 ± 0.04 vs. 1.87 ± 0.11 %ID/cm³, $P<0.01$).

In addition to hypercholesterolemia, the pro-inflammatory Paigen diet significantly increased VCAM-1 expression with respect to the control group in various tissues including the aorta and this expression was highly correlated with ^{99m}Tc-cAbVCAM1-5 uptake ($r = 0.75$; $P<0.05$). **Conclusion:** Inhibition of intestinal cholesterol absorption with ezetimibe promotes anti-atherosclerotic effects through increased

LDL-C catabolism and LDL-derived cholesterol fecal excretion, and reduces inflamed atherosclerotic plaques. These mechanisms may contribute to the benefits of adding ezetimibe to a statin therapy.

INTRODUCTION

Cardiovascular diseases are mainly caused by coronary artery disease and more specifically by the rupture of a vulnerable atherosclerotic plaque (1). In order to prevent cardiovascular events, lowering LDL-C level with statins is the most widely used therapy (2). Nevertheless, some patients treated with statins are not reaching the adequate levels of LDL-C or experienced adverse effects (3), calling for alternative LDL lowering therapies.

Ezetimibe is a dietary and biliary intestinal cholesterol absorption inhibitor which selectively inhibits the Niemann Pick C1 like 1 receptor without affecting intestinal absorption of triglycerides (4). Davis and coll. demonstrated that ezetimibe decreased plasma cholesterol levels by 61% and reduced the development of atherosclerotic lesions in ApoE^{-/-} mice (5). Ezetimibe is prescribed as a second line therapy (6) and its effectiveness for reducing LDL-C levels has been demonstrated in several clinical studies(7,8). Moreover, a beneficial 22% reduction in LDL-C levels has been reported when ezetimibe was given together with a statin, as compared to statin alone (9). Results from the clinical trial IMPROVED Reduction of Outcomes: Vytorin Efficacy International Trial that started in 2005, showed that reducing LDL-C levels below the recommended target value by adding ezetimibe to simvastatin treatment granted additional benefit by significantly reducing cardiovascular events, as compared to patients taking simvastatin alone in subjects with acute coronary syndrome (10). Interestingly, ezetimibe in combination with statin significantly decreased the levels of c-reactive protein and might have other metabolic effects than simply lowering LDL-C levels (11,12).

In the present study, we aimed to investigate the mechanisms by which ezetimibe may reduce cardiovascular events. We investigated if blocking intestinal cholesterol absorption may promote the fecal excretion of atherogenic cholesterol deriving from LDL particles. The effects of ezetimibe on LDL-C metabolism and LDL-derived cholesterol fecal excretion was therefore evaluated with ³H-cholesteryl

oleate-LDL kinetics, while its effect on inflammatory plaque development was evaluated using ^{99m}Tc -cAbVCAM1-5 imaging in ApoE $^{-/-}$ mice fed a Paigen diet. ^{99m}Tc -cAbVCAM1-5 is a Single Photon Emission Computed Tomography (SPECT) radiotracer directed against the inflammatory marker Vascular Cell Adhesion Molecule 1 (VCAM-1) currently under clinical translation (13,14). This molecular imaging agent belongs to a new class of radiotracer derived from Single Domain Antibodies (sdAb) or VHH, which are composed of the single variable domain of camelidae immunoglobulins. Aortic atherosclerotic lesions have been successfully detecting in ApoE $^{-/-}$ mice fed a western diet using ^{99m}Tc -cAbVCAM1-5 imaging, and the sensitivity of this tool has been demonstrating using statin therapy has a gold standard (13,14). This molecular imaging agent can therefore be employed for the preclinical non-invasive evaluation of anti-atherosclerotic therapies.

MATERIALS AND METHODS

Animals and Diet

All experimental procedures were in accordance with institutional guidelines and approved by the Animal Care and Use committees of Midi-Pyrénées and Grenoble University. Fifty-six (56) 7-weeks-old female mice were used. ApoE $^{-/-}$ mice (n=50) were fed a pro-atherogenic Paigen diet (1.25% cholesterol, 0.5% cholic acid and 15% fat) supplemented (Paigen + EZE) or not (Paigen) with ezetimibe (0.005% in diet, equivalent to 7 mg/kg/day) for 6 weeks, while the C57Bl6/J control mice (n=6) remained on chow diet. Body weight and food consumption were monitored weekly.

The effect of ezetimibe on LDL-C metabolism and excretion was evaluated using *in vivo* ^3H -cholesteryl oleate-LDL kinetics (n=15), and the effect of ezetimibe on atherosclerosis development and inflammation was investigated using *in vivo* SPECT imaging and *post mortem* analysis (n=41).

Evaluation of the Effect of Ezetimibe on LDL-C Metabolism and LDL-derived cholesterol fecal excretion

LDL particles were isolated from human plasma by ultracentrifugation ($1.019 < d < 1.063$), extensively dialyzed and then labeled with ^3H -cholesteryl oleate in the presence of lipoprotein-deficient serum isolated from human plasma, as described previously (15). After isolation of labeled LDL by ultracentrifugation and extensive dialysis, mice (Paigen group, $n=7$ and Paigen + EZE group, $n=8$) were weighed and individually caged for feces collection, and injected intravenously with ^3H -cholesteryl oleate LDL (~3-4 million dpm/mouse).

Blood was collected from the tail tip at 5 minutes and then 1, 3, 6, 24 and 48 hours after intravenous injection to isolate plasma (5 μl) and count ^3H -radioactivity. The plasma ^3H -tracer decay curve was then used to calculate LDL-cholesteryl ester fractional catabolic rate (i.e. the fraction of tracer irreversibly removed from the plasma per unit of time) using the SAAMII program, as described previously (16).

Mice were kept in individual cages for up to 96 hours after tracer injection to collect feces and to measure fecal ^3H -cholesterol and ^3H -bile acids following chemical extraction as described previously (17). Mice were then euthanized and exsanguinated. The liver was harvested and weighted. A ~50 mg liver sample was used to measure ^3H -radioactivity in the whole liver as well as in cholesterol and bile acids fraction. Hepatic and fecal total cholesterol and bile acid masses were also measured from the same samples using colorimetric kits as described previously and tracer recovery was expressed as a percent of injected dose (18).

Evaluation of the Effect of Ezetimibe on Atherosclerosis

In vivo SPECT imaging of inflamed atherosclerotic lesions was performed using $^{99\text{m}}\text{Tc}$ -cAbVCAM1-5 in ApoE^{-/-} mice (Paigen group, $n=10$ and Paigen + EZE group, $n=10$). Moreover, VCAM-1 protein expression was further investigated by Enzyme-Linked ImmunoSorbent Assay (ELISA) in ApoE^{-/-} mice fed

a Paigen diet (Paigen group, n=9 and Paigen + EZE group, n=6) as well as in control C57Bl/6J mice fed a chow diet (control, n=6).

In vivo SPECT imaging. Radiolabeling of cAbVCAM1-5 was performed as previously described using the tricarbonyl method (13). SPECT/CT acquisition was performed 2 hours after the intravenous injection of 47.2 ± 10.7 MBq of ^{99m}Tc -cAbVCAM1-5, and the aortic uptake expressed as a percentage of injected dose per cm^3 (%ID/ cm^3) (13). *Ex vivo* evaluation of biodistribution was then performed and expressed as a percentage of injected dose per gram (%ID/g). Then, frozen sections of aortic root were obtained for histology, VCAM-1 immunostaining and ^{99m}Tc cAbVCAM1-5 autoradiography. Plaque surface was expressed in mm^2 and VCAM-1 intensity level was graded as low (1), moderate (2) or strong (3) by two blinded observers. ^{99m}Tc cAbVCAM1-5 uptake in autoradiographic images was expressed either as a concentration (%ID/g) or as the total uptake per slice (%ID/slice) as previously described (14).

VCAM-1 ELISA. Three (3) hours after injection of ^{99m}Tc -cAbVCAM1-5, mice were euthanized by CO_2 inhalation. Tissues of interest were rapidly harvested, rinsed in NaCl 0.9% and weighed. ^{99m}Tc -cAbVCAM1-5 uptake was measured by gamma well counting (Wizard², PerkinElmer). After tissue grinding with a Potter-Elvehjem in ice-cold RPPA buffer, homogenates were centrifuged at 10,000 g for 10 min at 4°C. Supernatants were extracted and the quantification of VCAM-1 protein levels was performed using a commercial kit (sVCAM-1/CD-106, R&D Systems).

Western Blot analysis. Evaluation of direct anti-inflammatory effect of ezetimibe on VCAM-1 expression in human THP1 monocyte differentiated into macrophages using with phorbol 12-mirystate 13-acetate and stimulated with tumor necrosis factor- α was performed by western blot analysis as detailed in supplemental material and method.

Statistical Analysis

All results are presented as mean \pm s.e.m. Student t-test were used to compare data sets with equal variance, and Mann-Whitney U for unpaired data sets with unequal variance. Significance of linear correlation was assessed with Pearson's test. Multiple comparison statistical test was used to compare data between 3 groups (Paigen, Paigen + EZE, control). The significance level was set to $P < 0.05$.

RESULTS

In Vivo ^3H -Cholesteryl Oleate-LDL Kinetics

Plasma cholesterol levels and body weight. After 6 weeks on diet, ezetimibe treatment resulted in a significant reduction in plasma total cholesterol levels (Paigen 29.0 ± 3.1 ; Paigen + ezetimibe 14.6 ± 2.3 mmol/L, $P < 0.01$ vs. Paigen). Moreover, body weight was significantly higher in ezetimibe treated group than in control group (21.0 ± 0.2 vs 18.4 ± 0.5 respectively, $p < 0.01$) (supplemental Table 1).

After injection of ^3H -cholesteryl oleate labeled human LDL, mice treated with ezetimibe showed a higher ^3H -tracer disappearance rate (Fig. 1A), resulting in a 173% higher LDL-cholesteryl ester catabolism (Fig. 1B, $P < 0.001$ vs. Paigen). At time 96 hours after radiolabeled LDL injection, ^3H -tracer hepatic recovery in the whole liver (Fig. 1C) was reduced by 61% with ezetimibe ($P < 0.001$). Accordingly, ^3H -tracer hepatic recovery in cholesterol and bile acids fraction were significantly reduced by 55 and 52%, respectively, in mice treated with ezetimibe. Meanwhile, LDL-derived ^3H -tracer excretion in the feces (Fig. 1D) was increased by 107% in the fecal cholesterol fraction ($P < 0.001$). Similar trends were observed for hepatic cholesterol levels (Fig. 1E) and fecal cholesterol mass excretion (Fig. 1F), with a 75% reduction and 99% increase with ezetimibe, respectively (both $P < 0.001$).

Effect on Plaque Inflammation

In vivo SPECT/CT imaging. VCAM-1 expression was evaluated using ^{99m}Tc -cAbVCAM1-5 *in vivo* imaging in order to investigate the effect of ezetimibe on atherosclerosis-related inflammation (Fig. 2; Supplemental Fig. 1). Paigen diet induced rapid atherosclerotic plaque formation in the aortic root of ApoE^{-/-} mice ($0.33 \pm 0.03 \text{ mm}^2$), which was significantly reduced by 73% by ezetimibe ($0.11 \pm 0.03 \text{ mm}^2$, $P < 0.0001$) (Fig. 2A). As demonstrated by hematoxylin erythrosine saffron quantification, this was attributed to a significant decrease in core thickness, and therefore Intima:Media ratio, whereas cap thickness remained unchanged (Supplemental Fig. 2). Moreover, as determined by immunohistochemistry, VCAM-1 density in atherosclerotic lesions was significantly reduced in ezetimibe treated mice (1.56 ± 0.23 vs 2.4 ± 0.18 in Paigen Group, $P < 0.01$) (Fig. 2B). Accordingly, ^{99m}Tc -cAbVCAM1-5 aortic uptake was significantly reduced in ezetimibe treated mice as determined by *ex vivo* autoradiography (Fig. 2C). Indeed, ^{99m}Tc -cAbVCAM1-5 density was 39% lower in the ezetimibe treated group than in the untreated group (2.7 ± 0.29 vs $4.4 \pm 0.38 \text{ \%ID/g}$, respectively, $P < 0.01$). Overall, the ezetimibe-induced decreases in VCAM-1 expression and burden of atherosclerotic lesions resulted in a profound, 81% reduction in total ^{99m}Tc -cAbVCAM1-5 aortic uptake. This effect was successfully monitored non-invasively by SPECT/CT imaging (Fig. 2D). Indeed, ^{99m}Tc -cAbVCAM1-5 uptake in the ascending aorta was visible, and was decreased by 49% when Paigen diet was supplemented with ezetimibe (0.95 ± 0.04 vs $1.87 \pm 0.11 \text{ \%DI/cm}^3$ in Paigen group, $P < 0.001$).

Effect of ezetimibe on VCAM-1 protein expression. VCAM-1 protein expression was further investigated in various tissues by ELISA (Fig. 3). Low levels of VCAM-1 were found in the aortas of control mice fed a chow diet, and no difference in VCAM-1 expression was found in lesion-free abdominal aortas among experimental groups. The Paigen diet induced a significant, 11-fold increase in the ascending aorta and aortic arch of ApoE^{-/-} mice. This effect was significantly inhibited by ezetimibe (Fig. 3A). Indeed,

VCAM-1 expression in the ascending aorta was reduced by 47% in the ezetimibe-treated group in comparison to the Paigen group (2.40 ± 0.60 vs 4.54 ± 0.37 ng/mg, $P < 0.05$) (Fig. 3A). ^{99m}Tc -cAbVCAM1-5 aortic uptake was correlated with this VCAM-1 expression pattern. Indeed, ^{99m}Tc -cAbVCAM1-5 uptake significantly increased in atherosclerotic lesions of Paigen-fed mice, and ezetimibe significantly reduced this effect by approximately 50% (3.97 ± 0.31 vs 1.98 ± 0.5 %ID/g in treated group, $p < 0.01$) (Fig. 3B). Consequently, ^{99m}Tc -cAbVCAM1-5 uptake strongly correlated with VCAM-1 expression in the aorta ($R = 0.76$, $P < 0.0001$) (Supplemental Fig. 3).

Interestingly, Paigen diet also induced an increase in VCAM-1 expression in all other investigated tissues, which reached statistical significance in the liver, spleen, thymus and heart. This effect was significantly reduced by ezetimibe in the liver, thymus, heart and muscle (Fig. 4A). As observed in the aorta, the ^{99m}Tc cAbVCAM1-5 biodistribution profile was correlated with that of VCAM-1 expression. Indeed, Paigen diet induced an increase in ^{99m}Tc -cAbVCAM1-5 uptake in all investigated tissues, which reached statistical significance in the liver, spleen and lymph nodes. This effect was significantly inhibited by ezetimibe in the thymus, heart and muscle (Fig. 4B).

Moreover, as determined in vitro on human THP1 cells by western-blot analysis, VCAM-1 expression was observed on differentiated and stimulated macrophages, but not on monocytes. Interestingly, Ezetimibe reduced VCAM-1 expression by 18% (Supplemental Fig. 4).

DISCUSSION

Ezetimibe is a potent lipid lowering drug that targets NCP1L1 in enterocytes, which in turn results in intestinal cholesterol absorption inhibition. Consequently, not only does ezetimibe inhibit the exogenous dietary cholesterol absorption but also the reabsorption of biliary cholesterol. Therefore,

whereas statins act on endogenous cholesterol synthesis, ezetimibe lowers circulating cholesterol by inhibiting exogenous cholesterol absorption and endogenous cholesterol reabsorption.

In the present study, the effect of ezetimibe was evaluated for the first time *in vivo* in ApoE^{-/-} mice fed a Paigen diet. The Paigen diet is a hyperlipidemic diet containing a combination of cholesterol and cholic acid. Cholic acid is one of the two major bile acids which potentiates cholesterol influx (19). Therefore, ApoE^{-/-} mice fed a Paigen diet exhibit pronounced hypercholesterolemia resulting in the rapid development of atherosclerotic lesions within only 6 weeks. The efficacy of ezetimibe was investigated *in vivo* by evaluating the metabolism of LDL-C and the excretion of LDL-derived cholesterol in feces through ³H-cholesteryl oleate LDL kinetics. LDL-C catabolism and LDL-derived cholesterol fecal excretion were found to be significantly increased in ezetimibe treated mice. Our data therefore demonstrate that intestinal cholesterol absorption inhibition with ezetimibe promotes the elimination of pro-atherogenic LDL-C, thereby potentially preventing its accumulation into atherosclerotic plaques. The benefits of the 50% reduction of the Paigen diet-induced hypercholesterolemia were quantified *in vivo* using ^{99m}Tc-cAbVCAM1-5 SPECT imaging. This radiolabeled nanobody, which is currently undergoing clinical translation, is targeting the inflammatory marker VCAM-1 and has previously been validated as a sensitive and reproducible tool for the imaging of inflammation in mouse atherosclerotic lesions (14). In the present study, atherosclerotic lesions were readily observable in the aortic root and ascending aorta on SPECT images. As demonstrated by Contrast-CT based image quantifications, ezetimibe induced a 2-fold reduction in ^{99m}Tc-cAbVCAM1-5 uptake. Such a result is in accordance with the profound and significant 73% decrease in plaque area that was observed from the aortic root histological sections. Meanwhile, it has been previously reported that ^{99m}Tc-cAbVCAM1-5 SPECT quantification in mouse atherosclerotic lesion reflects not only plaque volume but also the level of VCAM-1 expression within the lesion (14). Interestingly, the results from the present study indicated that ^{99m}Tc-cAbVCAM1-5 uptake in atherosclerotic lesions was reduced in ezetimibe-treated mice when tracer uptake was corrected to

plaque volume as demonstrated by *ex vivo* autoradiography. This result suggests that, independently of the reduction in plaque size, ezetimibe induced a decrease in VCAM-1 expression as well. The reduction in VCAM-1 expression might be attributed either to a decrease in cholesterolemia and the associated lipotoxicity, or to a direct anti-inflammatory effect of the ezetimibe as suggested by recent studies. Indeed, Munoz-Pacheco and coll. showed that ezetimibe decreased the expression of adhesion molecules (ICAM-1, CD11a, and CD11b) in monocyte differentiated cells line THP1 into macrophage-like cells, and modified the expression of microRNA-155 (miR-155) involved in the development of atherosclerosis (20). Moreover, Cerda and coll. demonstrated that ezetimibe reduced the expression of miR-221, a proatherogenic microRNA, on tumor necrosis factor α -stimulated human umbilical vein endothelial cells (21). These results are in accordance with that obtained in the present study by western blot analysis on THP1 macrophages. This could explain part of the reduction of VCAM-1 expression in aortic sinus and aortic arch of ApoE^{-/-} mice. Furthermore, the fact that ezetimibe acts on monocyte/macrophages could explain part of the reduction of vcam-1 expression in lymphoid organs such as the spleen.

The effect of ezetimibe on VCAM-1 expression was further evaluated by ELISA. The results confirmed the 2-to 3-fold decrease in VCAM-1 protein density induced by ezetimibe in the ascending aorta and aortic arch lesions. Moreover, the amount of VCAM-1, expressed by atherosclerotic lesions was found to be significantly correlated to ^{99m}Tc-cAbVCAM1-5 uptake as quantified *ex vivo* by gamma well counting, thereby confirming the sensitivity of this imaging agent. Interestingly, the Paigen diet was found to induce a significant increase in VCAM-1 expression in most other investigated organs, which reached statistical significance in the liver, spleen, thymus and heart, and this effect was inhibited by ezetimibe in most of them. This result is in accordance with previous studies demonstrating the pro-inflammatory effect of the Paigen diet in addition to its pro-atherosclerotic effect. Indeed, Vergnes and

coll. found that a Paigen diet induced hepatic inflammation, and that this effect could be attributed to the combination of cholesterol and cholic acid since the removal of one of these two compounds from the diet resulted in significant changes in the pattern of pro-inflammatory genes expression (22). In the present study, we further demonstrated that the Paigen diet induced inflammation in other organs & tissues including the aorta, as demonstrated by the level of VCAM-1 expression, and that this effect was significantly reduced by ezetimibe. Moreover, the pattern of ^{99m}Tc -cAbVCAM1-5 biodistribution was in accordance with VCAM-1 expression in all investigated organs, suggesting that this imaging agent might be a suitable tool for the non-invasive imaging of chronic inflammation in other relevant pathophysiological settings.

LIMITATIONS

The relative contribution of endothelial cells and intraplaque cells expressing VCAM-1 to ^{99m}Tc -cAbVCAM1-5 uptake within the lesion still needs to be determined. Indeed, image quantification and thereby inflammatory score will depend on targeted cells, and an imaging agent targeting endothelial and intraplaque cells will not provide the same information as an imaging agent limited to endothelial cells, such as anti-VCAM-1 microbubbles. Moreover, identifying the targeted cells would allow a better understanding of the effect of a therapy on a specific cell type.

CONCLUSION

In conclusion, we demonstrated the anti-atherogenic effect of ezetimibe on atherosclerotic plaque progression in the *in vivo* experimental model of ApoE^{-/-} mice fed a Paigen diet. Increased LDL cholesterol catabolism and LDL-derived cholesterol fecal excretion, as well as reduced inflammation in atherosclerotic plaques as demonstrated using ^{99m}Tc -cAbVCAM1-5 imaging, contributed to those effects.

DISCLOSURE

This work was partly funded by France Life Imaging, grant “ANR-11-INBS-0006” as well as by the Agence Nationale pour la Recherche (ANR) and the Direction Générale de l’Offre de Soins (DGOS), grant “ANR-13-PRTS-0019”.

Tony Lahoutte is consultant for Camel-IDS NV, Member of ‘comité stratégique’ Institute for RadioElements (IRE). Nick Devoogdt is consultant for Camel-IDS NV.

ACKNOWLEDGMENTS

We thank Dimitri Tissot, Laurie Arnaud and Hadjer Guetarni, students from Université Grenoble Alpes, for their help on data collection. We thank Stéphane Tanguy for assistance with ELISA.

REFERENCES

1. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* 2000;20:1262-1275.
2. Taylor F, Huffman MD, Macedo AF, et al. Statins for the primary prevention of cardiovascular disease. *Cochrane Database Syst Rev.* 2013;1:CD004816
3. Armitage J. The safety of statins in clinical practice. *Lancet Lond Engl.* 2007;370:1781-1790.
4. Van Heek M, France CF, Compton DS, et al. In vivo metabolism-based discovery of a potent cholesterol absorption inhibitor, SCH58235, in the rat and rhesus monkey through the identification of the active metabolites of SCH48461. *J Pharmacol Exp Ther.* 1997;283:157-163.
5. Davis HR, Compton DS, Hoos L, Tetzloff G. Ezetimibe, a potent cholesterol absorption inhibitor, inhibits the development of atherosclerosis in ApoE knockout mice. *Arterioscler Thromb Vasc Biol.* 2001;21:2032-2038.
6. Kondo Y, Hamai J, Nezu U, et al. Second-line treatments for dyslipidemia in patients at risk of cardiovascular disease. *Endocr J.* 2014;61:343-351.
7. Dujovne CA, Bays H, Davidson MH, et al. Reduction of LDL cholesterol in patients with primary hypercholesterolemia by SCH 48461: results of a multicenter dose-ranging study. *J Clin Pharmacol.* 2001;41:70-78.
8. Dujovne CA, Suresh R, McCrary Sisk C, et al. Safety and efficacy of ezetimibe monotherapy in 1624 primary hypercholesterolaemic patients for up to 2 years. *Int J Clin Pract.* 2008;62:1332-1336.
9. Ballantyne CM, Houri J, Notarbartolo A, et al. Effect of ezetimibe coadministered with atorvastatin in 628 patients with primary hypercholesterolemia: a prospective, randomized, double-blind trial. *Circulation.* 2003;107:2409-2415.
10. Cannon CP, Blazing MA, Giugliano RP, et al. Ezetimibe Added to statin therapy after acute coronary syndromes. *N Engl J Med.* 2015;372:2387-2397.
11. Sager PT, Capece R, Lipka L, et al. Effects of ezetimibe coadministered with simvastatin on C-reactive protein in a large cohort of hypercholesterolemic patients. *Atherosclerosis.* 2005;179:361-367.
12. Kastelein JJP, Akdim F, Stroes ESG, et al. Simvastatin with or without ezetimibe in familial hypercholesterolemia. *N Engl J Med.* 2008;358:1431-1443.
13. Broisat A, Hernot S, Toczek J, et al. Nanobodies targeting mouse/human VCAM1 for the nuclear imaging of atherosclerotic lesions. *Circ Res.* 2012;110:927-937.
14. Broisat A, Toczek J, Dumas LS, et al. 99mTc-cAbVCAM1-5 imaging is a sensitive and reproducible tool for the detection of inflamed atherosclerotic lesions in mice. *J Nucl Med.* 2014;55:1678-1684.

15. Briand F, Tréguier M, André A, et al. Liver X receptor activation promotes macrophage-to-feces reverse cholesterol transport in a dyslipidemic hamster model. *J Lipid Res.* 2010;51:763-770.
16. Briand F, Thieblemont Q, Muzotte E, Sulpice T. Upregulating reverse cholesterol transport with cholesteryl ester transfer protein inhibition requires combination with the LDL-lowering drug berberine in dyslipidemic hamsters. *Arterioscler Thromb Vasc Biol.* 2013;33:13-23.
17. Briand F, Tréguier M, André A, et al. Liver X receptor activation promotes macrophage-to-feces reverse cholesterol transport in a dyslipidemic hamster model. *J Lipid Res.* 2010;51:763-770.
18. Briand F, Thieblemont Q, André A, Ouguerram K, Sulpice T. CETP inhibitor torcetrapib promotes reverse cholesterol transport in obese insulin-resistant CETP-ApoB100 transgenic mice. *Clin Transl Sci.* 2011;4:414-420.
19. Nishina PM, Verstuyft J, Paigen B. Synthetic low and high fat diets for the study of atherosclerosis in the mouse. *J Lipid Res.* 1990;31:859-869.
20. Muñoz-Pacheco P, Ortega-Hernández A, Miana M et al. Ezetimibe inhibits PMA-induced monocyte/macrophage differentiation by altering microRNA expression: a novel anti-atherosclerotic mechanism. *Pharmacol Res.* 2012;66:536-543.
21. Cerda A, Fajardo CM, Basso RG, Hirata MH, Hirata RDC. Role of microRNAs 221/222 on statin induced nitric oxide release in human endothelial cells. *Arq Bras Cardiol.* 2015;104:195-201.
22. Vergnes L, Phan J, Strauss M, Tafuri S, Reue K. Cholesterol and cholate components of an atherogenic diet induce distinct stages of hepatic inflammatory gene expression. *J Biol Chem.* 2003;278:42774-42784.

Figure 1:

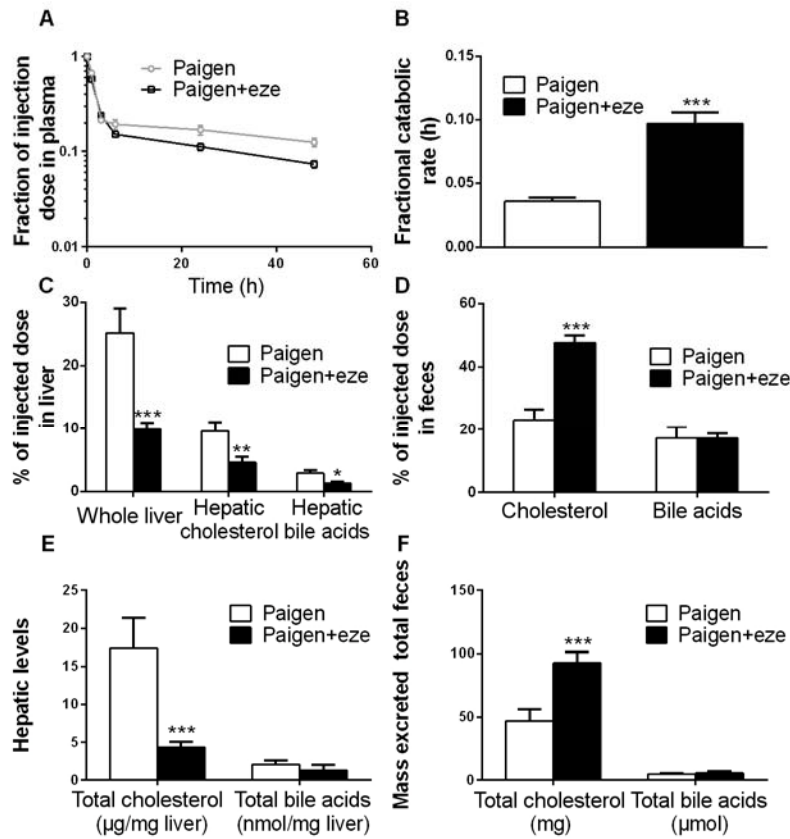


Figure 1: Effect of ezetimibe on LDL-C metabolism and excretion using *in vivo* ³H-cholesteryl. ³H-tracer plasma disappearance (A), LDL-cholesteryl ester fractional catabolic rate (B). Hepatic (C) and fecal (D) ³H-tracer tracer recovery after ³H-cholesteryl oleate labeled human LDL intravenous injection, hepatic (E) and fecal (F) total cholesterol and bile acids mass in ApoE^{-/-} mice. * *P*<0.05; ** *P*<0.01; *** *P*<0.001 vs Paigen group.

Figure 2 :

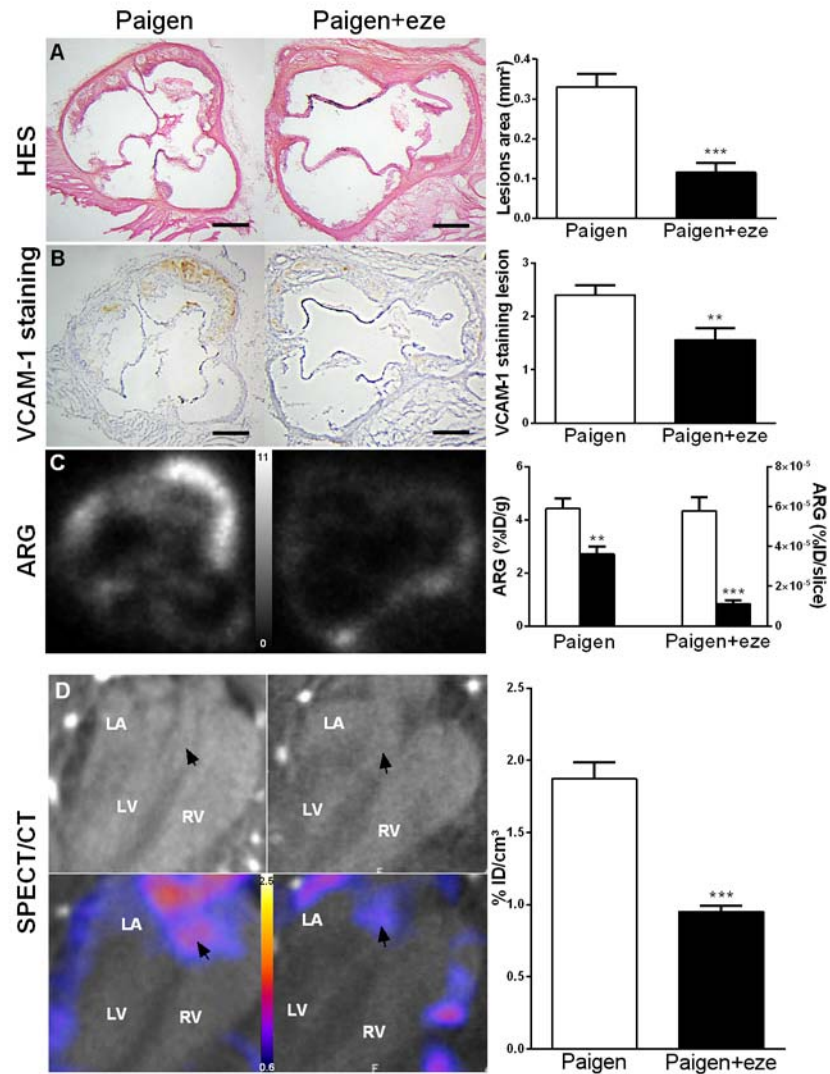


Figure 2: Effect of ezetimibe on plaque inflammation. Paigen diet induced aortic lesions and ezetimibe significantly reduced plaque volume and VCAM-1 expression as determined by histology (A) and immunohistochemistry (B). As shown on autoradiographic images, ^{99m}Tc -cAbVCAM1-5 aortic uptake was significantly reduced in ezetimibe treated mice either when expressed as a concentration or a total uptake per slice (C). *In vivo* quantification of ^{99m}Tc -cAbVCAM1-5 aortic uptake was in accordance with *ex vivo* data and the effect of ezetimibe was successfully monitored as shown on representative CT and SPECT/CT coronal views selected at the level of the aortic roots (arrow) (D). ** $P < 0.01$; *** $P < 0.001$ vs Paigen. Scale bar = 200 μm (A, B). Autoradiography scale: 0-11%DI/g; SPECT scale: 0.8-2.6% ID/cm³ (D). HES: Hematoxylin Erythrosine Saffron; ARG: Autoradiography; LA: Left Atrium; LV: Left Ventricle; RV: Right Ventricle

Figure 3:

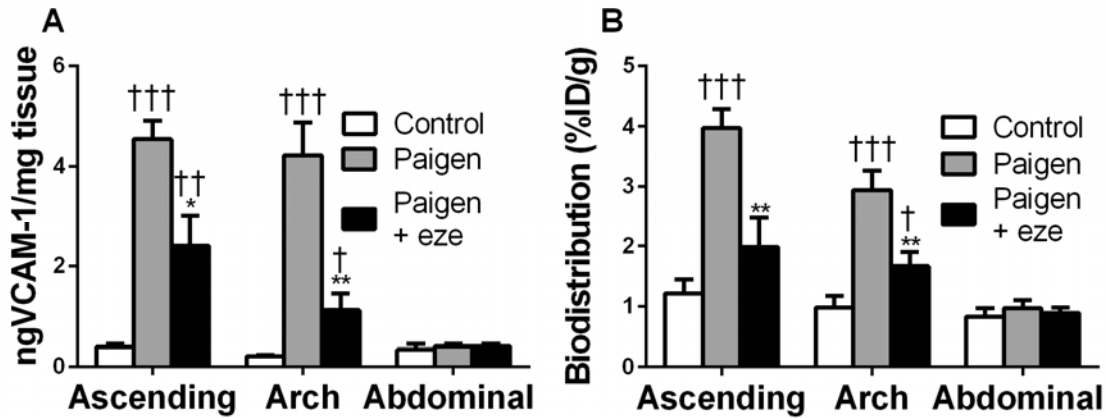


Figure 3: Anti-inflammatory effect of ezetimibe on aortic atherosclerotic lesion. ^{99m}Tc -cAbVCAM1-5 uptake was reduced in Paigen+eze group and was well correlated with VCAM-1 expression in aorta. VCAM-1 expression was evaluated on ascending aorta, aortic arch and abdominal aorta by ELISA and expressed as ngVCAM-1/mg tissue (A). ^{99m}Tc -cAbVCAM1-5 uptake was determined by gamma well counting and expressed as %ID/g (B). Significant ** $P < 0.01$ vs Paigen; † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$ vs control.

Figure 4:

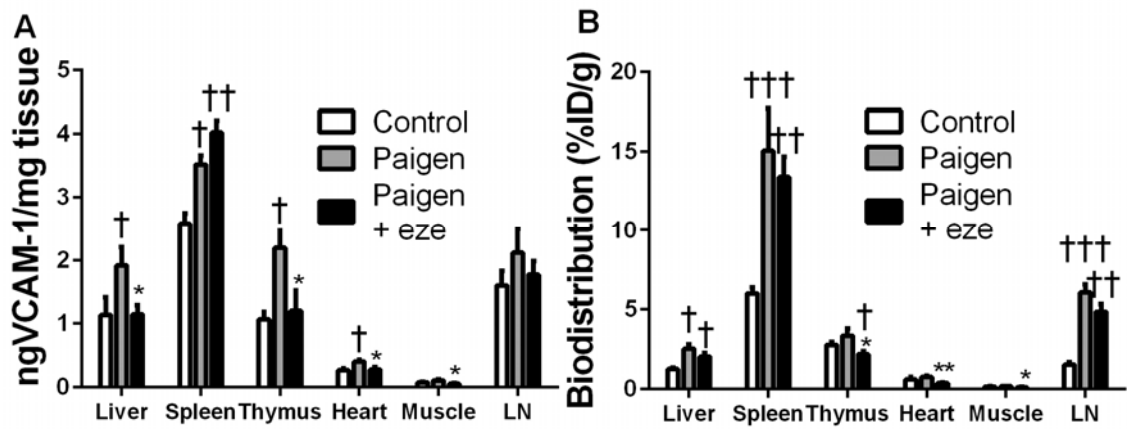


Figure 4: Effect of ezetimibe on VCAM-1 expression in the liver, spleen, thymus, heart, muscle and Lymph Node. VCAM-1 expression was evaluated by ELISA assay and expressed as ngVCAM-1/mg tissue (A). ^{99m}Tc -cAbVCAM1-5 uptake was determined by gamma well counting and expressed as %ID/g (B). Significant: * $P < 0.05$; ** $P < 0.01$ vs Paigen. † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$ vs control. LN: Lymph Node.

Supplementary data:

Supplemental Material and Methods

Cells culture and treatment:

Human THP-1 cells line were culture in Roswell Park Institute Medium 1640 (Sigma-Aldrich) containing 10% of heat inactivated fetal bovine serum, supplemented with 2mmol/l glutamine and 1% penicillin/streptomycin at 37°C in an atmosphere of 5 % CO₂. A total of 1 x 10⁶ cells/well were suspended in 6 well plates and differentiated into macrophages during 24h with phorbol 12-myristate 13-acetate at 5ng/ml in the presence or absence of ezetimibe (100nM). Then, cells were rinsed with RPMI 1640 and rested during 24h. Differentiated THP-1 macrophages were stimulated with Tnf-α at 50 ng/ml during 24h with or without ezetimibe (100nM).

Western blot:

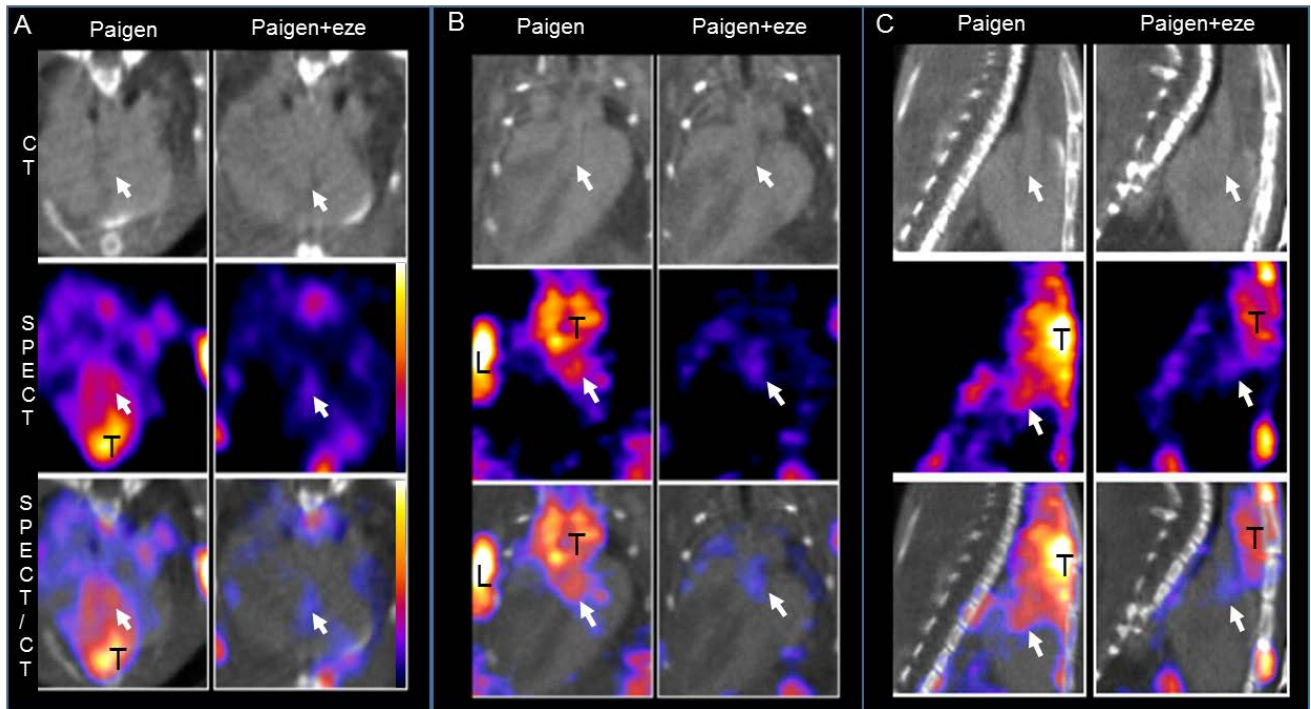
VCAM-1 expression was analyzed by western blot in THP-1 cells treated or not with ezetimibe. After treatment, cells were rinsed with PBS and lysed with ice cold RIPA buffer at -80°C. Cells lysates were centrifuges at 10 000g for 10 min at 4°C and supernatant was collected. Protein quantification was assayed by BCA method and 30 µg protein samples were loaded into SDS-polyacrylamide gel (7.5/%) and then transferred on nitrocellulose membrane. Rabbit anti VCAM-1 antibody was employed as the primary antibody (abcam, ab13047, 1/1000) 4°C overnight. Goat anti-rabbit was employed as the secondary antibody at 1/2000 (Horseshoe peroxidase-labeled goat anti-rabbit IgG; Dako) for 1h at room temperature. The protein signal, observed at 110kda, was quantified using ImageJ software and normalized to the expression of α-actin.

Supplemental Table 1:

Parameter	Diet	Paigen group	Paigen+eze group	vs Paigen group
Total plasma cholesterol (mmol/L)	Pre	7.1±0.4	7.04±0.8	-50% $P < 0.01$
	Post	29.0±3.1	14.6±2.3**	
Body weight (g)	Pre	19.1±0.4	18.9±0.9	+14% $P < 0.001$
	Post	18.4±0.5	21.0±0.2***	

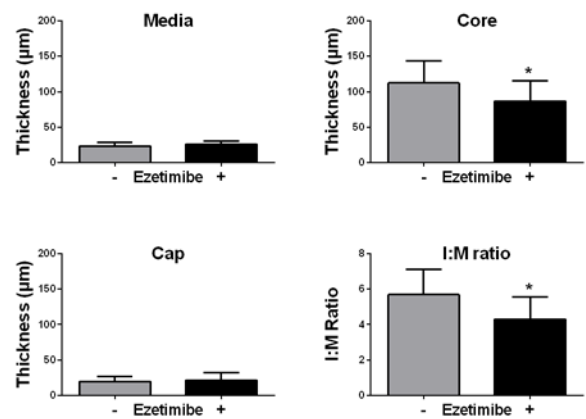
Supplemental Table 1: Effect of diets on total plasma cholesterol levels and body weight. ** $P < 0.01$; *** $P < 0.001$ vs Paigen group.

Supplemental figure 1:



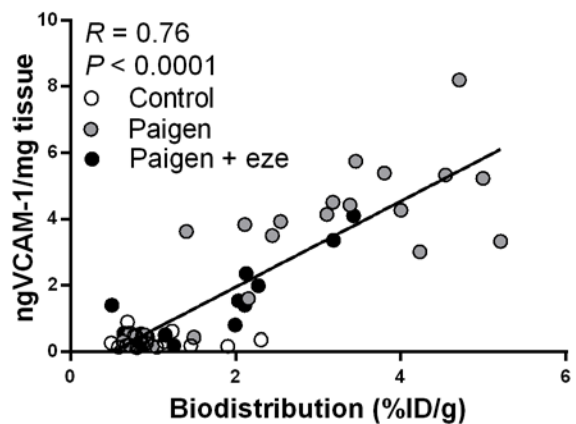
Supplemental figure 1: In vivo SPECT/CT imaging. Transverse (A), coronal (B) and sagittal (C) views centered on ascending aorta (white arrow) of ApoE^{-/-} mice fed a Paigen diet supplemented or not with ezetimibe. Retro-orbital injection of hexabrix allowed us to identify ascending aorta on CT. Scale was 0.8% to 2.6% ID/g. T= Thymus, L=Lymph Node.

Supplemental figure 2:



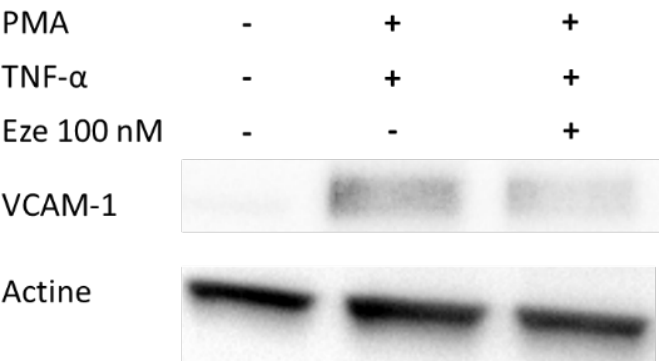
Supplemental figure 2: Quantification of cap, media and core thickness as well as Intima:Media (I:M) ratio in the cross sectional area of aortic sinus of ApoE^{-/-} mice treated or not with ezetimibe. * $P < 0.05$ vs Paigen group.

Supplemental figure 3:



Supplemental figure 3: Pearson correlation between VCAM-1 expression and ^{99m}Tc-cAbVCAM1-5 uptake in ascending aorta, aortic arch and abdominal aorta.

Supplemental figure 4:



Supplemental figure 4: Direct anti-inflammatory effect of ezetimibe on VCAM-1 expression in THP-1 macrophages cells. THP-1 cells, treated or not with 100nM of ezetimibe, were induced into macrophages with PMA for 24h and then stimulated with TNF- α for 24h. Detection of VCAM-1 expression and α -actin was performed by western blot analysis.