Novel pharmaeutical interventions in experimental atherosclerosis and myocardial infarction

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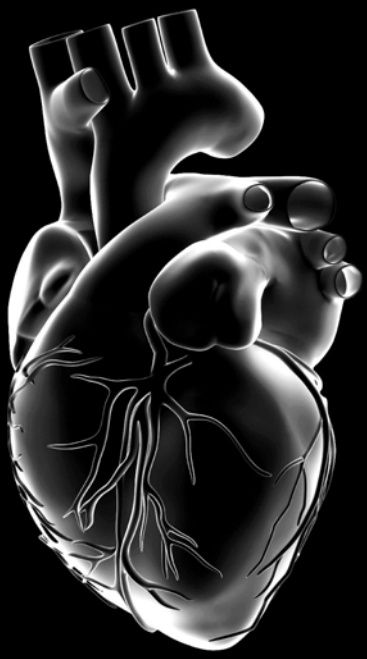

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The thromboxane prostanoid receptor antagonist S18886 (terutroban), combined with dietary cholesterol-lowering, blocks the progression of atherosclerosis in APOE*3Leiden mice

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Abstract

Objective To evaluate the effect of Thromboxane Prostanoid (TP) receptor inhibition by increasing doses of the antagonist S18886 (terutroban) in combination with dietary cholesterol-lowering on existing atherosclerotic lesions in APOE*3Leiden transgenic mice.

Methods and Results APOE*3Leiden mice were fed an atherogenic diet resulting in plasma cholesterol levels of 27.6 mM to develop atherosclerosis. At 10 weeks, 15 mice were sacrificed to assess atherosclerosis development. Then cholesterol in the diet was reduced to reach plasma cholesterol levels of 5-6 mM and the remaining mice were treated with: vehicle (control), S18886 (10, 30 or 60 mg/kg bw/d) or ADP-receptor antagonist clopidogrel (3.8 mg/kg bw/d). After 12 weeks all groups were sacrificed and atherosclerosis was measured.Cholesterol-lowering alone decreased levels of inflammation markers SAA (-95%), MCP-1 (-70%) and E-selectin (-36%;all $p<0.001$), lessened the number of lesions (-20%;$p<0.05$) and improved the lesion stability. The latter was revealed by a reduced macrophage content (-95% $p<0.001$) and by a 7-fold increase of smooth muscle cells and collagen areas as compared to baseline. Supplementary treatment with S18886 reduced additionally and dose-dependently the lesion area (up to -55%), the progression of lesion severity (up to -43%) and the monocyte adherence (up to -70%) when compared to control (all $P<0.01$). Clopidogrel did not add to the effect of cholesterol-lowering on atheroclerosis development.

Conclusion In APOE*3Leiden mice dietary cholesterol-lowering decreased the systemic and vascular inflammatory status and improved lesion stability. S18886 demonstrated additional atheroprotective effects indicated by a dose-dependently reduced lesion size and severity and endothelial monocyte adherence.

Introduction

Atherothrombosis is a multifactorial disease responsible for most cardiovascular events. The reduction of high plasma cholesterol by nutritional and/or pharmaceutical intervention is the first choice approach for treatment in preventing atherosclerosis development. However, different studies have shown that substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of LDL cholesterol[1]. More recently, it has been evidenced that circulating platelets, when activated, are not only involved in thrombus formation leading to clinical complications, but are also able to activate vascular cells. Accumulating evidence has shown that they are involved in the initiation and the progression of atherosclerosis[2], and inhibition of platelet adhesion reduces leukocyte infiltration and atherosclerosis in hypercholesterolemic mice[3]. Therefore, platelet inhibition not only leads to a significant decrease in cardiovascular events but could also result in a slower progression of atherosclerosis.

The most widely prescribed anti-platelet treatment is aspirin, which clinical efficacy is based on inhibition of the platelet cyclo-oxygenase-1 (COX-1), thus inhibiting the generation of platelet thromboxane A_2 (TxA_2,), which binds to the thromboxane-prostanoid endoperoxide (TP) receptor[4] and triggers platelet aggregation. TP receptors are widely expressed by vascular cells (smooth muscle cells, endothelial cells) and by circulating leucocytes, and their activation leads to vasoconstriction, inflammation and cell proliferation, phenomena widely involved in atherosclerosis initiation and progression. Aspirin treatment has clearly shown a beneficial effect in the secondary prevention of cardiovascular diseases (CVD), but is less accepted for the primary prevention. This is mainly caused by the increased incidence of bleeding and the very modest non-significant CVD risk reduction in individuals at low risk[5-7]. Additionally, aspirin has controversial effects on vasoconstriction, endothelial dysfunction or vascular wall proliferation[8,9] known to be of major importance in the atherosclerotic process.

Platelet inhibition can also be achieved by blocking TP receptors with specific antagonists which have advantages over aspirin. These compounds not only block the effects of TxA_2, but also inhibit the binding of non specific TP receptor ligands, mostly isoprostanes and HETEs, formed in response to oxidative stress and able to exert deleterious effects[10,11]. Furthermore, in contrast to aspirin, TP receptor antagonists do not affect the synthesis of prostacyclin (PGI_2) by the endothelial cells, which is a endogenous antiplatelet and vasodilatory prostanoid[10].

S18886 (terutroban) is a selective TP receptor antagonist developed for the secondary prevention of atherothrombotic complications in patients[12,13]. It has been shown to be a well-tolerated and powerful antiplatelet agent, able to inhibit

thrombus formation more efficiently than aspirin [14,15]. Previous studies in animal models have shown that S18886 displays anti-inflammatory properties combined with a decreased expression of adhesion molecules and a reduced recruitment of monocytes/macrophages within the arterial wall, resulting in the inhibition of atherogenesis [16].

A third approach to inhibit platelet activation is antagonizing the adenosine diphosphate (ADP) receptor on the platelet with compounds like clopidogrel, which, like aspirin, is a generally applied treatment in the clinic. Clopidogrel has been shown to be at least as effective as aspirin in preventing ischemic stroke, myocardial infarction and vascular death in patients suffering from CVD[17].

The purpose of this study was to evaluate the effects of inhibition of TP receptors by incremental doses of S18886 on existing atherosclerotic lesions in combination with dietary cholesterol intake lowering. For this study APOE*3Leiden transgenic mice were used, which is a well-established model for hyperlipidemia and atherosclerosis[18,19]. These mice become hyperlipidemic upon dietary cholesterol and respond to various hypolipidemic drugs in a similar way as humans[20] As a control for anti-platelet therapy an additional group of mice was treated with the ADP receptor antagonist clopidogrel.

Methods

Mice

Eighty-three female heterozygous APOE*3Leiden transgenic mice (16 to 18 weeks of age), characterized for human apoE[18], were used. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were bred by TNO.

Diets

To induce atherosclerosis development, all animals received a semi-synthetic western type diet (WD)[18] supplemented with 0.75% (w/w) cholesterol and 0.05% (w/w) cholate for ten weeks. Thereafter (on t=0 weeks) on the basis of age, body weight and plasma cholesterol and triglyceride levels, the mice were matched into five groups (n=13-14): The control group, fed WD + 0.025% (w/w) cholesterol only and four treatment groups, fed the same diet as the control group supplemented with either clopidogrel (3.8 mg/kg bw/d) or S18886 (10, 30 or 60 mg/kg bw/d) for 12 weeks (compounds provided by Institut de Recherches Internationales Servier, France). To determine the amount of atherosclerosis at the start of treatment 15

mice were sacrificed after matching at t=0 weeks and were considered the reference for baseline levels. The animals received food and water *ad libitum*.

Lipid and lipoprotein analysis and plasma SAA, MCP-1, E-selectin and VCAM-1
After a 4-hour fasting period, EDTA plasma was collected (Sarstedt, Germany). Total plasma cholesterol and triglyceride levels were measured (Roche Diagnostics, No-1489437 and No-1488872). Lipoprotein profiles were obtained by FPLC. Serum amyloid A (SAA) (Biosource International, Belgium), MCP-1, E-selectin and VCAM-1 (all R&D systems Inc, Minneapolis, USA) were determined by ELISA[19,21].

Histological assessment of atherosclerosis
After the 10-week induction and the 12-week treatment period the mice were sacrificed the hearts were dissected, formalin-fixed and embedded in paraffin. Per mouse, 4 serial aortic root sections (5µm, 50µm spaced) were used for quantification and qualification of the atherosclerotic lesions after staining with hematoxylin-phloxin-saffron (HPS). For determination of severity of atherosclerosis, the lesions were classified into 5 categories as described before[19,21]: I) early fatty streak, II) regular fatty streak, III) mild lesion, IV) moderate lesion, V) severe lesion. Per mouse the percentages of all lesions found in the respective categories were calculated. The total lesion area was calculated per cross-section. Collagen content of the lesion after Sirius Red staining, macrophage area after immunostaining with anti-mouse Mac-3 (BD Pharmingen, the Netherlands) and smooth muscle cells (SMCs) area after immunostaining with mouse anti-human actin (DAKO, Denmark), cross reacting with mouse actin, were quantified morphometrically. Also the number of monocytes adhering to the endothelium was counted after immunostaining with AIA31240 (Accurate Chemical and Scientific, New York, USA). All analyses were performed by the same operator, who was blinded for experimental group allocation.

Statistical analysis
Significance of differences was calculated by using parametric t-test for comparison of baseline levels at the start of the study with the control group. ANOVA tests, followed by Dunnett test were applied for comparison of the intervention treatment groups with the control group. A parametric Pearson's correlation was calculated for dose dependency. The Extreme Studentized Deviate method was used to identify and exclude outliers. $P<0.05$ was considered significant. All data are presented as mean ± SD.

Results

Plasma lipid levels

During the first 10 weeks of study, when the mice were fed the high cholesterol diet prior to intervention, average plasma cholesterol and triglyceride levels were 27.6 ± 7.3 mM and 2.3 ± 0.6 mM, respectively. As aimed for, these values decreased significantly to 5.3 ± 1.0 mM and 1.5 ± 0.3 mM ($p<0.001$) when the dietary cholesterol intake was reduced during the 12-week intervention period. The reduction was confined to the apoB-containing lipoproteins (data not shown). The total cholesterol exposure (plasma cholesterol level x duration of study) did not differ between all treatment groups and the control group. **Figure 1** represents the plasma cholesterol levels.

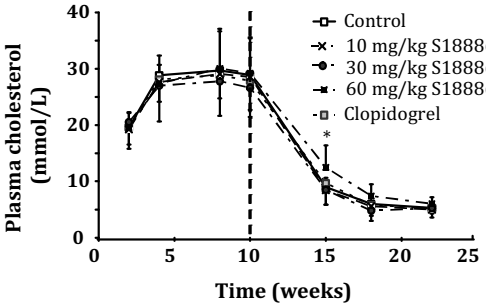

Figure 1 Mean plasma cholesterol levels over time. APOE*3Leiden mice were fed an atherogenic diet for 10 weeks to induce atherosclerosis development. Thereafter dietary cholesterol was lowered and supplementary intervention was started. *P=0.014; 60 mg/kg S18886 vs control.

	SAA (μg/mL)	**MCP-1** (pg/mL)	**E-Selectin** (μg/mL)	**VCAM-1** (μg/mL)
Baseline	13.3± 7.3	81 ± 34	188 ± 26	2.5 ± 0.3
Control	2.5 ± 0.8$^{\lozenge}$	24 ± 7$^{\lozenge}$	120 ± 10$^{\lozenge}$	2.3 ± 0.2
S18886 (10 mg/kg)	2.6 ± 1.0	26 ± 12	124 ± 16	2.5 ± 0.5
S18886 (30 mg/kg)	2.7 ± 0.7	21 ± 5	133 ±15	2.4 ± 0.4
S18886 (60 mg/kg)	3.4 ± 1.7	19 ± 8	127 ± 15	2.6 ± 0.4
Clopidogrel	3.0 ± 1.1	14 ± 5*	115 ± 19	2.5 ± 0.4

Table 1. Plasma levels of systemic inflammation marker Serum Amyoid A (SAA) and vascular inflammation markers MCP-1, E-selectin and VCAM-1 at baseline (t=0) and after 12 weeks of intervention. Cholesterol-lowering with or without additional treatment resulted in a significant decrease of SAA, MCP-1 and E-selectin levels. $^{\lozenge}P<0.001$ compared to baseline, *P<0.001 compared to control group.

Cholesterol-lowering reduces inflammation markers

Inflammation plays a major role in the development of atherosclerosis and is described to be influenced by lipid lowering[22-24]. Therefore we investigated the effect of dietary cholesterol-lowering and the additional treatments on plasma levels of the liver-derived plasma inflammation marker serum amyloid A (SAA), which reflects the overall systemic inflammatory state, and VCAM-1, MCP-1 and E-selectin, as markers for endothelial activation. Cholesterol-lowering from 27.6 to 5.3 mM by itself had clear anti-inflammatory effects as demonstrated by decreased plasma levels of inflammation markers SAA (from 13.3 ± 7.3 to 2.5 ± 0.8 μg/mL; $p<0.001$), MCP-1 (from 81 ± 34 to 24 ± 7 pg/mL; $p<0.0001$) and E-selectin (from 188 ± 26 to 120 ± 10 μg/mL; $p<0.0001$) (**table 1)**. Additional treatment with S18886 did not add to this effect, while clopidogrel significantly decreased MCP-1 levels (14 ± 5 pg/mL; $P<0.001$). Plasma VCAM-1 levels were not affected by cholesterol-lowering or supplementary treatments.

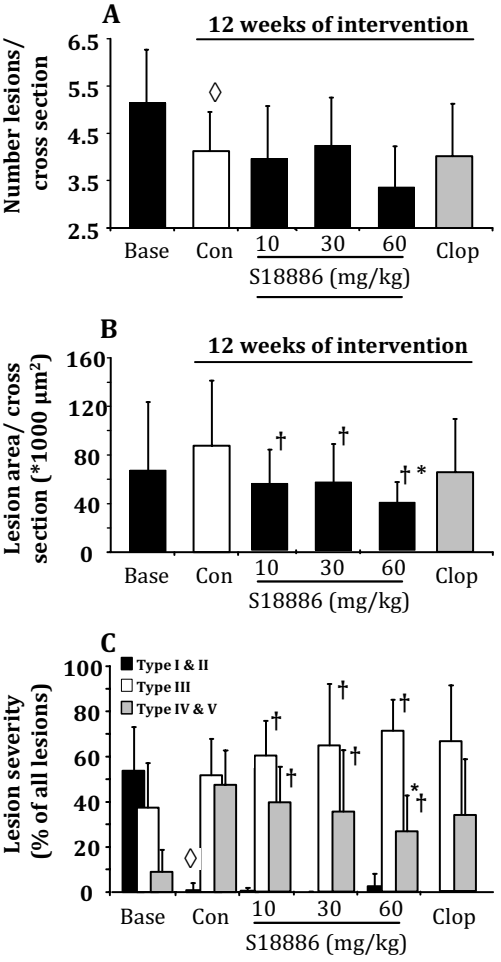

Figure 2. Effect of S18886 and clopidogrel (Clop) on the number of lesions per cross sections (A), the lesion area per cross section (B, † P_{trend} =0.006) and the lesion severity (C). Per lesion type the percentage of all lesions was calculated. ◊p<0.05 compared to baseline (Base) and *P<0.0001 compared to control (Con). † P_{trend} =0.011 type III and P_{trend} =0.007 type IV & V (compared to control).

Treatment with S18886 in combination with cholesterol-lowering inhibits progression of pre-existing atherosclerosis

Ten weeks of high cholesterol diet resulted in the development of a moderate amount of atherosclerosis in the aortic root, as measured in the mice sacrificed at t=0 weeks (baseline: 5.1 ± 1.1 lesions and 67.6 ± 56.0*1000 μm² lesion area per cross section; **figure 2A and 2B**). The

lesions were mainly foam cell rich lesions**.** As presented in **figure 2C,** 54 ± 19 % of the lesions were fatty streaks, consisting of only foam cells (type I and II), 37 ± 20 % were mild lesions, made up of foam cells covered by a fibrous cap (type III) and the remaining 9 ± 10% were severe lesions, which are infiltrated into the media and contain necrosis and cholesterol clefts (type IV and V). Despite a significant reduction in the number of lesions per cross section (from 5.1 ± 1.1 to 4.1 ± 0.8; $p<0.05$), lowering of plasma cholesterol alone during 12 weeks failed to inhibit the progression of atherosclerosis, as reflected by the progression of lesion area (87.5 ± 53.8 *1000 μm^2 in the control group vs 67.6 ± 56.0*1000 μm^2 at baseline, NS). Furthermore, a major shift in the distribution of lesion types as compared to baseline was observed. Almost all fatty streaks (type I and type II) had disappeared (1% ± 3 %; $p<0.0001$) and both mild type III lesions (52 ± 16 %; $p<0.05$) and severe type IV-V lesions (47 ± 16 %; $p<0.0001$) were present, reflecting a progression of atherosclerosis severity.

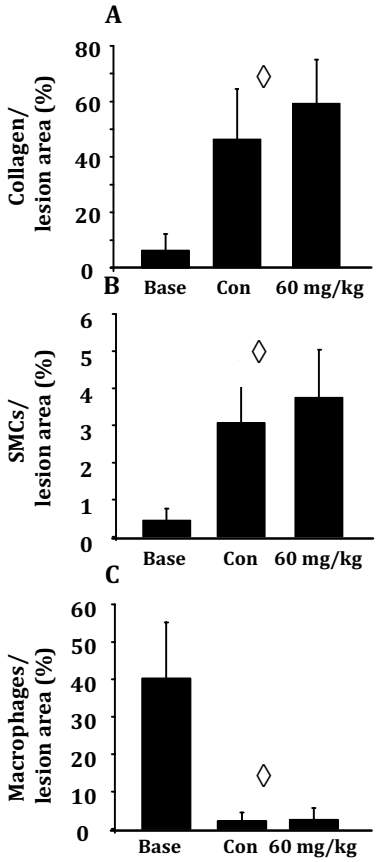

Figure 3 Effect of dietary cholesterol lowering and S18886 on lesion composition. The collagen content of the lesions as percentage of lesion area (A). The SMC area as percentage of total lesion area (B). The macrophage area as percentage of total lesion area per group (C). ◊P<0.001 compared to baseline. Base = baseline, Con = control.

Additional treatment with increasing doses of S18886 showed a dose-dependent reduction of the amount of atherosclerosis as compared to the control group ($p=0.006$ for Pearson's correlation coefficient) as reflected by the decreased lesion area. Whereas the doses of 10 and 30 mg/kg bw/d tended to reduce the lesion area by 37 % and 36 % respectively (NS), the highest dose of 60 mg/kg bw/d resulted in a significant decrease of 55 % ($p=0.001$). Moreover, the progression of the lesions towards more severe lesions as observed in the control group was partly prevented with increasing doses of S18886 ($p=0.011$ and 0.007 for Pearson's correlation coefficient for the increase in type III lesions and the decrease in type IV-V lesions, respectively). The highest dose of 60 mg/kg bw/d S18886 displayed 42% less severe type IV-V lesions as compared to the control group (27 ± 16 % vs 47 ± 16 %). Treatment with

clopidogrel failed to reach a statistically significant effect, either in lesion size, number or severity, when compared to control group.

Cholesterol-lowering stabilizes lesions

To assess whether platelet inhibition with S18886 together with cholesterol-lowering had additional effects next to the reduction of lesion size and severity, the lesion composition was analyzed. To this end the amounts of collagen, SMCs (both assumed to stabilize lesions) and macrophages (considered as an instable component) of the lesions were measured (**figure 3A-C)**. At baseline only 6.3 ± 5.9% of the total lesion area was filled with collagen, and SMCs were nearly absent with a presence of 0.5 ± 0.3% of the total lesion area. The major component of these lesions was macrophage foam cells (40.3 ± 14.9% of the total lesion area). Dietary cholesterol-lowering increased both the collagen and SMC content of the lesions about a 7-fold (46.4 ± 18.0%, $p<0.001$ and 3.1 ± 1.7%, $p<0.005$ respectively), and concomitantly dramatically decreased the amount of macrophages by 95% ($p<0.001$), resulting in more stable lesions. Supplementary treatment with S18886 did not add to this improved lesion composition induced by dietary cholesterol-lowering.

S18886 combined with cholesterol-lowering reduces monocyte adhesion

The onset of lesion development is considered to be the adhesion of monocytes to the activated endothelium followed by infiltration into the media and maturation to macrophages and foam cells. Feeding a high cholesterol diet resulted in 4.4 ± 2.9 adhering monocytes per cross section at baseline (**figure 4)**. Cholesterol-lowering did not prevent monocyte adhesion (3.7 ± 2.8 monocytes per cross section). As compared to the control, treatment with S18886 also reduced the adherence of monocytes to the endothelium in a dose-dependent manner ($p=0.007$ for Pearson's correlation coefficient) with only 1.1 ± 0.9 adhering monocytes per cross section in animals receiving the highest dose of S18886 ($p<0.05$). Treatment with clopidogrel tended to decrease the number to 1.7 ± 2.0 monocytes per cross section, but not significantly.

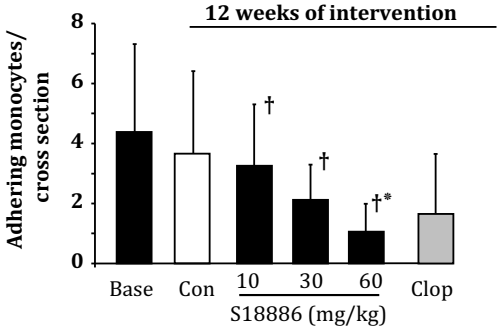

Figure 4. Effect of S18886 and clopidogrel (Clop) on the number of monocytes adhering to the endothelium per cross-section. *P<0.05 compared to control. †P_{trend} =0.007 (compared to control). Base = baseline, Con = control.

Discussion

This study shows that in a relevant model of advanced atherosclerosis in APOE*3Leiden mice the TP receptor antagonist S18886 together with cholesterol lowering in the diet reduced additionally and dose-dependently the atherosclerotic lesion area, the progression of lesion severity and the amount of adhering monocytes as compared to control.

In patients with increased risk for cardiovascular diseases, cholesterol-lowering by treatment with mainly statins is the first choice approach to reduce or prevent the progression of atherosclerosis or to even regress it. In the present study, dietary cholesterol was reduced and this resulted in lowering of plasma cholesterol to human-like levels of 5-6 mM within 4-8 weeks. Positive effects of cholesterol-lowering on atherogenesis were reflected by a strong decrease in systemic and vascular inflammation markers (SAA, MCP-1 and E-selectin) and by a reduction in lesion number (-20%). A decrease in macrophage content (-95%) and a 7-fold increase in SMC and collagen content of the lesions indicated a shift toward more stable lesions. Nevertheless, despite the strong decrease of plasma cholesterol levels in the intervention phase, the development of atherosclerosis continued. Albeit that a decreased number of lesions was observed, lesions kept on growing as demonstrated by an increase in lesion size (NS) and, more strikingly, by a massive progression from mild towards severe advanced lesions (when comparing control group to baseline levels). These observations revealed that in the present model, dietary cholesterol-lowering was unable to fully inhibit atherosclerosis progression and are in accordance with previous studies in APOE*3Leiden mice in which dietary cholesterol reduction was less efficient than drug-induced reduction by hypolipidemics[19,21].

A second line of defence in treatment of clinical atherosclerosis complications is platelet inhibition. Although not all physiological functions of platelets are fully clear yet, there is accumulating evidence that they go beyond aggregation and actively participate in atherosclerotic processes[25,26]. It has been described that platelets can oxidize LDL particles and stimulate their accumulation in monocytes and even stimulate CD34+ cells to differentiate into foam cells[27,28]. Moreover, activated platelets have been reported to increase the adhesive properties of monocytes[29] via the expression of adhesion molecules and the release of thromboxane A_2, a prostanoid that acts as a pro-inflammatory and chemotactic agent. This contention is supported by the observation of atheroprotection in

animals in which platelet inhibition was achieved[16,30,31] and of aggravating atherosclerosis after platelet activation[32] .

In the present study, platelet inhibition by S18886 was in part responsible for the decreased progression of atherosclerosis and reduced vascular inflammation. Treatment of the APOE*3Leiden mice with incremental doses of the selective TP-receptor antagonist S18886 resulted in dose-dependent beneficial effects on important parameters of the development of atherosclerosis. S18886 inhibited lesion progression, as represented by a reduction in lesion size (up to -55%) and lesion severity with a reduced progression towards more complicated lesions. This observation is of critical importance as complicated lesions are more prone to trigger clinical events after rupture. The adhesion of monocytes to the endothelium, a primary step in the formation of atherosclerotic lesions, was dose-dependently decreased by S18886 (up to -70%) as compared to the control group.

To our knowledge this is the first study that demonstrates that S18886 has additional atheroprotective effects on top of cholesterol-lowering. In different studies in apoE$^{-/-}$ mice[16], apobec1/LDLR DKO mice[33] and rabbits[34], it was shown that S18886 reduced the progression of atherosclerosis in a prevention design, in which the animals were treated during progression of atherosclerosis. Regarding the effect of S18886 on pre-existing atherosclerotic lesions, which is of more therapeutic relevance, inconsistent data were found. Without concomitant cholesterol lowering S18886 showed no beneficial effect in apobec1/LDLR DKO mice[33]. In rabbits the compound induced regression of pre-existing lesions as observed by MRI analysis in the absence of cholesterol-lowering[35] and showed no effect when cholesterol levels were lowered concomitantly[34]. In agreement with our data, S18886 was also shown to increase the stability of lesions as reflected by a decreased content in pro-inflammatory macrophages and lytic enzymes such as MMP[34,35].

Limited and ambiguous data on the effects of the platelet inhibitor clopidogrel on progression of atherosclerosis in animal models have been reported. In rabbits the compound showed atheroprotective and anti-inflammatory effects[31] at a similar dose as used in the present study, whereas it did not hamper atherosclerosis development in apoE deficient mice[36] at a higher dose. In accordance with the latter report, no atheroprotective effects of clopidogrel in combination with cholesterol-lowering on pre-existent atherosclerosis was observed.

Other positive effects of platelet inhibition on atherosclerosis were recently reported with the novel dual TP-receptor antagonist and thromboxane synthase inhibitor BM-573, by hampering the progression of pre-exisiting lesions in LDLr$^{-/-}$

mice[37]. No effect on inflammation markers was described in the latter study. In contrast, anti-inflammatory properties of S18886 were reported *in vitro* as well as *in vivo* in mice and rabbits[16,34]. It is possible that these anti-inflammatory effects of S18886 on plasma parameters are not visible in the present study, since they may be overshadowed by the strong effect of dietary cholesterol-lowering. However, S18886 demonstrated clear anti-inflammatory activity as evidenced by a marked reduction in monocyte adhesion, which is considered as a functional marker of activation of the endothelium *in vivo*. In conclusion, the present and reported data provide evidence for an anti-inflammatory capacity of S18886, ultimately resulting in inhibition of lesion progression.

In humans, the anti-platelet drug aspirin is widely accepted to reduce the risk of CVD. However, the major adverse side effect bleeding and the large prevalence of aspirin resistance (5-45%) are drawbacks of this drug[7,38]. In the large CAPRIE trial[17] clopidogrel was shown to be at least as effective as aspirin in preventing ischemic stroke, myocardial infarction and vascular death. Combining the two in the MATCH[39] and CHARISMA[40] study did, however, not significantly decrease cardiovascular events and may even increase major bleedings. Besides their proven benefit in patients with CVD, the variety in response to these anti-platelet compounds is considerable, this might be called 'resistance', possibly reflecting the unravelled complexity of mechanisms affected by these treatments[41]. Analysis of urine samples of aspirin treated patients of the HOPE study demonstrated that thromboxane B_2 levels were predictive for the risk of myocardial infarction and vascular death, providing evidence that inhibition of thromboxane production or activity might be protective for cardiovascular death[42]. This hypothesis was confirmed in the DAVID study[43], in which picotamide, a dual inhibitor of thromboxane A_2 synthase and receptor, was shown to be more effective in reducing all cause mortality in diabetic patients as compared to aspirin. Previously picotamide was shown to inhibit the progression of plaque growth in the carotid artery in diabetic patients[44]. These data suggest that antagonizing the TP-receptor may indeed have clinical benefits.

In conclusion, this study demonstrates that the TP-receptor antagonist S18886 combined with dietary cholesterol-lowering prevents the progression of established atherosclerosis lesions towards more advanced lesions. The atheroprotective effect of S18886 is suggested to stem from the combination of platelet inhibition and its additional effects resulting from TP antagonism in vascular and inflammatory cells, as reflected by a dose-dependent reduction in adhesion of activated monocytes. Data from ongoing clinical trials will indicate

whether treatment with the TP receptor antagonist S18886 in humans is also successful in the secondary prevention of atherosclerosis and its complications.

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Disclosures

L.C. is an employee of I.R.I.S., Courbevoie, France.

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