

This item is the archived peer-reviewed author-version of:

Spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques via induction of autophagy

Reference:

Michiels Cédéric, Kurdi Ammar, Timmermans Jean-Pierre, De Meyer Guido, Martinet Wim.- Spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques via induction of autophagy
Atherosclerosis - ISSN 0021-9150 - 251(2016), p. 319-327
Full text (Publishers DOI): <http://dx.doi.org/doi:10.1016/j.atherosclerosis.2016.07.899>

Spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques via induction of autophagy

Cédéric F. Michiels,¹ Ammar Kurdi,¹ Jean-Pierre Timmermans,²

Guido R.Y. De Meyer,¹ Wim Martinet¹

¹Laboratory of Physiopharmacology, University of Antwerp, Antwerp, Belgium

²Laboratory of Cell Biology and Histology, University of Antwerp, Antwerp, Belgium

Keywords: atherosclerosis - autophagy – spermidine – cholesterol efflux – necrosis

Number of Figures/tables: 7

Corresponding author: Prof. dr. Wim Martinet

Laboratory of Physiopharmacology, University of Antwerp, Universiteitsplein 1, B-2610

Antwerp, Belgium; Tel. +32 3 265 26 79; Fax. +32 3 265 25 67; E-mail:

wim.martinet@uantwerpen.be

ABSTRACT

Background and aims: Spermidine is an endogenous biological polyamine that exhibits broad longevity-extending activities via the induction of autophagy. Because basal autophagy is atheroprotective during early atherosclerosis but dysfunctional in advanced plaques, the aim of the present study was to assess the potential beneficial effects of autophagy induction by spermidine on atherosclerotic plaque progression and composition.

Methods: Apolipoprotein E-deficient ($ApoE^{-/-}$) mice were fed a Western-type diet for 20 weeks with or without 5 mM spermidine in the drinking water.

Results: (Immuno-)histochemical analysis of plaques in the aortic root, proximal ascending aorta and brachiocephalic artery showed that spermidine changed neither the size of the plaque nor its cellular composition. However, spermidine treatment significantly reduced necrotic core formation ($6.6\pm 0.5\%$ vs. $3.7\pm 0.5\%$ in aortic root, $P=0.0008$) and lipid accumulation inside the plaque ($27\pm 3\%$ vs. $17\pm 1\%$ oil red O positivity in thoracic aorta, $P=0.017$). *In vitro* experiments showed that macrophages, unlike vascular smooth muscle cells (VSMCs), are relatively insensitive to autophagy induction by spermidine. Along these lines, spermidine triggered cholesterol efflux in autophagy-competent VSMCs ($5.7\pm 1.2\%$ vs. $8.7\pm 0.2\%$, $P=0.0118$), but not in autophagy-deficient $Atg7^{F/F} SM22\alpha-Cre^{+}$ VSMCs or macrophages. Analogous to the experiments *in vitro*, spermidine affected neither necrosis nor lipid load in plaques of $Atg7^{F/F} SM22\alpha-Cre^{+} ApoE^{-/-}$ mice.

Conclusions: Spermidine inhibits lipid accumulation and necrotic core formation through stimulation of cholesterol efflux, albeit without changing plaque size or cellular composition. These effects, which are driven by autophagy in VSMCs, support the general idea that autophagy induction is potentially useful to prevent vascular disease.

1. Introduction

Atherosclerosis is one of the leading underlying causes of death and morbidity in the Western world [1, 2]. It occurs as a chronic inflammatory disease of the vascular system, and progressively leads to the formation of atherosclerotic plaques in the vessel wall. Plaques that are formed in an early stage contain very few macrophages and have a small necrotic core that is separated from the lumen of the blood vessel by a thick fibrous cap consisting of vascular smooth muscle cells (VSMCs) and collagen fibers. However, stable plaques can progress to more unstable lesions [3], which are characterized by a thin fibrous cap with few VSMCs, numerous macrophage-derived foam cells and a large necrotic core. Eventually, unstable plaques can rupture and cause severe complications such as acute myocardial infarction and stroke. Fortunately, our insight into the progression of the disease has expanded in recent years, making atherogenesis a process that is adaptable instead of being unavoidable. Indeed, a healthy diet, physical exercise and lipid-lowering drugs (such as statins) are known to improve atherosclerosis [1, 2]. However, despite these preventive measures, the morbidity and mortality remains considerable, which increases the need for new and more adequate therapies.

Recent evidence suggests that autophagy plays an important role in atherosclerosis [4, 5]. During autophagy, unwanted intracellular content is sequestered in double membranous structures, better known as autophagosomes, and degraded after fusion with lysosomal vesicles [6, 7]. In this way, autophagy functions as a protective mechanism in atherosclerosis and safeguards plaque cells from various adverse stimuli such as reactive oxygen species, oxidized LDL, hypoxia and endoplasmic reticulum stress [4, 8, 9]. However, autophagy is an age-dependent process that declines over time. Decreased autophagic levels have been associated with the development of several age-related diseases including vascular disease [10-12].

Conversely, stimulation of the autophagic process appears to be a promising therapeutic strategy to stabilize atherosclerotic plaques and/or slow down atherogenesis [4, 5, 13].

Among the agents that are known to induce autophagy, the polyamine spermidine may represent the most attractive compound for therapeutic use, for multiple reasons. First, spermidine is a naturally occurring polyamine, present in all mammalian cells, suggesting that serious adverse effects after exogenous supply are unlikely to occur. Second, a polyamine-rich diet is known to reduce cardiovascular morbidity and mortality [14, 15], probably by boosting the autophagic process during aging, although other mechanisms (e.g. modulation of inflammation and lipid metabolism) are also feasible [16]. Third, spermidine is known to increase the lifespan of multiple species, from yeast to rodents [17, 18], promotes resistance against stress [19], and retards the manifestation of several age-related disorders including arterial aging [20], colon cancer [21] and neurodegeneration [22, 23]. These findings led us to investigate the hypothesis that spermidine, through its autophagy-stimulating effect, has atheroprotective properties.

2. Materials and methods

2.1. Mice

Male apolipoprotein E-deficient ($ApoE^{-/-}$) mice (6-8 weeks old) were fed a Western type diet (WD, TD88137, Harlan Teklad) containing 0.2% cholesterol, 21% total fat and 34% sucrose for 20 weeks. Meanwhile, spermidine was added to the drinking water at 5 mM final concentration. Spermidine in the drinking water was replaced every 2-3 days and freshly prepared from 1 M aqueous spermidine stock solutions. Stock solutions were adjusted to pH 7.4 with HCl and stored light protected at -20°C for no longer than one month. Control $ApoE^{-/-}$ mice were given plain drinking water. The animals were housed in groups of 6 per cage in a temperature-controlled room with a 12-hour light/dark cycle and with *ad libitum* access to water and food. At the end of the experiment, mice were sacrificed by an overdose of sodium pentobarbital (250 mg/kg, i.p.) and retro-orbital blood samples were taken for total cholesterol analysis by commercially available kits (Randox). Blood lipid profiles were obtained by separating the different lipoproteins (HDL, LDL and VLDL) by fast protein liquid chromatography. The aortic root, proximal ascending aorta and brachiocephalic artery were fixed in 4% formaldehyde (pH 7.4) for 24 hours and then paraffin-embedded. Serial sections (5 μm) were prepared for histological analysis.

For an acute treatment of established plaques, male $ApoE^{-/-}$ mice (6-8 weeks old) were fed WD for 20 weeks. In the last 4 weeks of WD, spermidine was added to the drinking water as described above. Control $ApoE^{-/-}$ mice were given plain drinking water. In some experiments, GFP-LC3 transgenic mice (strain GFP-LC3#53, RIKEN BioResource Center) [24] containing a rat LC3-eGFP fusion under control of the chicken beta-actin promoter were used to detect spermidine-induced autophagy and were treated with 5 mM spermidine or plain water for 1

month. Four hours before sacrifice, GFP-LC3 mice were injected intraperitoneally with 100 mg/kg chloroquine (Sigma-Aldrich) dissolved in saline to prevent autophagy-mediated degradation of LC3B.

To investigate the significance of autophagy during spermidine treatment, we used mice homozygous for the *Atg7^{Flox}* allele (*Atg7^{F/F}* mice), crossbred with *SM22 α -Cre⁺* or *LysM-Cre⁺* mice to obtain VSMC or macrophage-specific *Atg7* knockout mice, respectively [25, 26]. For atherosclerosis studies, *Atg7^{F/F} SM22 α -Cre⁺* and *Atg7^{+/+} SM22 α -Cre⁺* mice on an *ApoE^{-/-}* background [26] were fed a WD and treated with spermidine as described above. All mouse experiments were approved by the Ethical Committee of the University of Antwerp.

2.2. Histological analysis

The plaque area (defined as the region between the lumen/intima interface and the internal elastic lamina) was measured on hematoxylin & eosin-stained sections. The acellular/anuclear area in the plaque, better known as the necrotic core, was quantified in three 50 μm -spaced sections. A 3000 μm^2 threshold was implemented in order to avoid counting of regions that likely do not represent substantial areas of necrosis. For the detection of apoptosis, a terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) technique was used [27]. Alternatively, apoptotic cells were stained by immunohistochemistry using anti-cleaved caspase-3 (9661; Cell Signaling Technology). The cellular composition of the plaques was analyzed by immunohistochemistry using anti- α -smooth muscle cell actin (A2547; Sigma-Aldrich) and anti-Mac-3 (553322; Pharmingen). After primary antibody incubation, specimens were incubated with species appropriate horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories), followed by 60 minutes of reactive ABC (Vector Laboratories).

Immunocomplexes were detected with 3,3'-diaminobenzidine or 3-amino-9-ethyl-carbazole (Vector Laboratories). Collagen content was determined on Sirius red-stained slides. All plaque components were expressed as percentage positivity of total plaque area. Images were analyzed with a color image analysis system (Image Pro Plus 4.1, Media Cybernetics Inc.). To investigate lipid accumulation inside the plaque, an oil red O staining was performed on Neg-50-embedded aortic tissue material. The surface area occupied by atherosclerotic plaques was quantified by *en face* staining of entire thoracic aortas with oil red O for 24 hours. Autophagosome accumulation in GFP-LC3 mice was studied by staining paraffin-embedded tissue samples with rabbit anti-LC3B (3868, clone D11; Cell Signaling Technology) as previously described [28, 29].

2.3. Isolation of vascular smooth muscle cells and bone marrow-derived macrophages

VSMCs were isolated from mouse aorta as previously described [30]. Briefly, after excision and removal of adherent fatty tissue, the aorta was cut open starting from the diaphragm up to the aortic arch and incubated as a whole in a 100 μ M calcium solution supplemented with 1.5 mg/ml papain (Sigma-Aldrich) and 0.5 mg/ml dithiothreitol (Roche Diagnostics) for 30 minutes at 37°C while being aerated by 95% O₂/5% CO₂. The entire aorta was transferred to a 100 μ M calcium solution containing 2 mg/ml collagenase type II (315 IU/mg, Worthington) and incubated for 30 minutes under the same conditions. Subsequently, the aorta was flushed in 0 Ca²⁺ solution to obtain single VSMCs. After centrifugation, VSMCs were resuspended in 1:1 DMEM/F-12 medium (Gibco) containing 20% heat-inactivated fetal bovine serum (Sigma-Aldrich) and supplemented with 100 U/ml penicillin-100 μ g/ml streptomycin (Life Technologies) and 20 U/ml polymyxin B sulfate (Fagron). Cells were allowed to attach and grew in culture plates at 37°C in 95% O₂/5% CO₂.

Bone marrow-derived macrophages (BMDMs) were harvested by flushing bone marrow from the hind limbs of mice with heparinized (10 IU/ml) RPMI 1640 medium containing antibiotics. After filtration and washing, cells were cultured for 7 days in full RPMI 1640 medium supplemented with 15% L-cell conditioned medium (containing monocyte colony stimulating factor (M-CSF)) at 37°C in 95% O₂/5% CO₂ until 80-90% confluency was reached.

2.4. Transmission electron microscopy

Transmission electron microscopy was performed as described [29]. Briefly, cells were fixed in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 hours at 4°C, then rinsed three times in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5% sucrose and post-fixed in 1% OsO₄ solution for 1 hour. After dehydration in an ethanol gradient (70% ethanol (20 minutes), 96% ethanol (20 minutes)), 100% ethanol (2 x 20 minutes)), samples were embedded in EMbed 812 (EMS). Ultrathin sections were stained with 2% uranyl acetate for 15 minutes and Reynolds solution pH 12.4 for 10 minutes. Sections were examined with a FEI Tecnai microscope at 120 kV.

2.5. Cholesterol efflux assay

BMDMs and VSMCs were loaded with a combination of cholesterol (20 mg/ml in 100% absolute ethanol) and ³[H]-cholesterol (1 µCi/ml) in serum-free DMEM (glucose, HEPES, glutamine, 10% fatty acid free BSA and antibiotics). Before addition to the cells, radioactivity was measured by liquid scintillation counting. The same procedure was followed 24 hours later to calculate the incorporation percentage. Cells were then washed with PBS and fresh medium was added to allow equilibration. After 1 hour, cells were washed with PBS and treated for 24

hours with 10 μ M spermidine in the presence of the acceptor human apoA-I (10 μ g/ml). The radioactivity of the supernatant and cell fraction (lysed in 0.1 M NaOH for 30 minutes at room temperature) was measured and cholesterol efflux was calculated by the following formula: cholesterol efflux (%) = $(\text{dpm}_{\text{supernatant}}) / (\text{dpm}_{\text{supernatant}} + \text{dpm}_{\text{cell fraction}})$. The efflux to apoA-I was calculated by subtracting effluxes of the wells without apoA-I from those containing apoA-I.

2.6. Western blot analysis

Cells were lysed with Laemmli sample buffer (Bio-Rad Laboratories). Cell lysates were heat-denatured for 5 minutes in boiling water and loaded on a 4-12% SDS-polyacrylamide gel. After gel electrophoresis, proteins were transferred to an Immobilon-P Transfer membrane (Millipore) according to standard procedures. Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk (Bio-Rad) for 1 hour. After blocking, membranes were probed overnight at 4°C with primary antibodies in antibody dilution buffer (Tris-buffered saline/0.05% Tween 20 containing 1% nonfat dry milk), followed by a 1 hour incubation with peroxidase-conjugated secondary antibodies (Dako) at room temperature. Antibody detection was accomplished with SuperSignal West Pico or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) using a Lumi-Imager (Roche Diagnostics). The following primary antibodies were used: rabbit anti-LC3B (3868, clone D11; Cell Signaling Technology), rabbit anti-GFP (ab6556; Abcam), rabbit anti-SQSTM1/p62 (P0067; Sigma-Aldrich), mouse anti-EP300 (ab3164, clone NM11; Abcam) and mouse anti- β -actin (A5441, clone AC-15; Sigma-Aldrich).

2.7. *Statistical analysis*

Results are expressed as mean \pm SEM. All analyses were performed using SPSS software (version 23.0). Statistical tests are specified in the figure legends. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Spermidine induces autophagy in vivo, but does not affect the size or cellular composition of atherosclerotic plaques

ApoE^{-/-} mice were fed a Western-type diet (WD) for 20 weeks. Meanwhile, mice were treated with spermidine via the drinking water. Western blots of aortic plaques revealed an increased LC3-II/LC3-I ratio and decreased levels of SQSTM1/p62 after spermidine treatment, indicative of autophagy induction (Fig. 1A). To confirm autophagy induction, GFP-LC3 mice were treated similarly with spermidine for one month. Western blot analysis of endothelium-denuded aortic segments of spermidine-treated GFP-LC3 mice showed increased levels of cleaved GFP (Fig. 1B). Because the expression levels of GFP-LC3 in VSMCs are too low for immunohistochemical analysis [29], stimulation of autophagosome formation by spermidine was evaluated by immunostaining LC3B in liver tissue. One month of spermidine treatment clearly increased the formation of LC3B positive dots in the liver as compared to control treated GFP-LC3 mice (Fig. 1C). In *ApoE*^{-/-} mice that were fed a WD, spermidine affected neither total blood cholesterol levels (spermidine vs control: 461±47 vs 434±36 mg/dl) nor plasma lipoprotein profiles as analyzed by FPLC (supplemental Fig. 1). Analysis of hematoxylin & eosin-stained cross-sections demonstrated that the overall plaque size was not changed after 20 weeks of spermidine treatment (Table 1). Mac3- and α -SMC actin immunostains were performed to quantify the number of macrophages and VSMCs in the plaque, respectively. Both Mac3- and α -SMC actin-positive areas in the plaque were not significantly different when compared to control treated animals (Table 1). Analogous with the chronic (20 weeks) treatment of developing plaques, acute treatment (4 weeks) of established plaques changed neither plaque size nor its composition (Table 2).

3.2. Spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques

Even though the size and cellular composition of the plaques did not change after spermidine treatment, *en face* oil red O-stained aortas demonstrated that spermidine significantly reduced lipid distribution over the entire thoracic aorta after 20 weeks of treatment, particularly in the descending part of the thoracic aorta (Fig. 2A). Moreover, oil red O stained cross-sections of aortic segments showed that lipid accumulation inside the plaque of spermidine-treated *ApoE*^{-/-} mice was significantly reduced (Fig. 2B). In addition, plaques of spermidine-treated mice showed less plaque necrosis in comparison with plaques of control animals, while plaque apoptosis was unchanged (Table 1). Unlike chronic treatment of developing plaques, acute treatment of established plaques did not change the necrotic core (Table 2).

3.3. Spermidine induces autophagy in cultured VSMCs, but not in macrophages

Because VSMCs and macrophages are the most prominent cell types inside atherosclerotic plaques, both cell types were isolated from wild type C57BL/6 mice and treated with increasing concentrations of spermidine *in vitro*. Western blot analysis revealed typical features of autophagy induction in VSMCs, such as reduced SQSTM1/p62 levels and accumulation of LC3-II, but not in macrophages (Fig. 3A). In line with these findings, formation of autophagosomes as demonstrated by TEM analysis was only evident in spermidine-treated VSMCs (Fig. 3B). Given that the acetyltransferase EP300 is a major target of spermidine treatment [31], expression of EP300 was further analyzed. Western blot results showed that EP300 was present at much higher

levels in macrophages vs VSMCs (Fig. 3C). EP300 expression did not significantly change during spermidine treatment.

3.4. Spermidine attenuates lipid accumulation in VSMCs by stimulating cholesterol efflux in an autophagy-dependent manner

To evaluate whether autophagy mediates the spermidine-induced downgrading of lipid content in atherosclerotic plaques, VSMCs and macrophages were isolated from $Atg7^{F/F} SM22\alpha-Cre^+$ and $Atg7^{F/F} LysM-Cre^+$ mice, respectively, lacking the essential autophagy gene $Atg7$ specifically in VSMCs and macrophages, respectively. Lack of ATG7 expression in isolated $Atg7^{F/F} SM22\alpha-Cre^+$ and $Atg7^{F/F} LysM-Cre^+$ cells is associated with several hallmarks of impaired autophagy such as SQSTM1/p62 accumulation and absence of LC3-II formation, as previously described [25, 26]. Spermidine significantly increased cholesterol efflux in $^3[H]$ -cholesterol-loaded, autophagy-competent $Atg7^{+/+} SM22\alpha-Cre^+$ VSMCs (Fig. 4A), while the same process was severely attenuated in autophagy-deficient $Atg7^{F/F} SM22\alpha-Cre^+$ VSMCs, both in spermidine-treated and untreated conditions (Fig. 4A). Spermidine did not facilitate cholesterol clearance in macrophages, neither in $Atg7^{+/+} LysM-Cre^+$ nor in $Atg7^{F/F} LysM-Cre^+$ cells (Fig. 4B).

3.5. Spermidine reduces lipid accumulation and necrotic core formation in atherosclerotic plaques by stimulating autophagy in VSMCs

To investigate whether the beneficial effects of spermidine on atherosclerotic plaques were directly related to stimulation of autophagy in VSMCs, $Atg7^{+/+} SM22\alpha-Cre^+$ and $Atg7^{F/F} SM22\alpha-Cre^+$ mice on an $ApoE^{-/-}$ background were fed a WD and treated with spermidine or plain drinking water for 14 weeks. Spermidine reduced the necrotic core (Fig. 5A) and lipid load (Fig.

5B, supplemental Fig. 2) in plaques from autophagy-competent *Atg7*^{+/+} *SM22 α -Cre*⁺ *ApoE*^{-/-} mice, but these effects were completely absent in spermidine-treated *Atg7*^{F/F} *SM22 α -Cre*⁺ *ApoE*^{-/-} mice containing autophagy-deficient VSMCs (Fig. 5A, B, supplemental Fig. 2).

4. Discussion

Growing evidence indicates that autophagy in atherosclerotic plaques is a cytoprotective mechanism against many pathophysiological stimuli, including oxidative stress, hypoxia and metabolic stress [4, 5, 8]. However, recent findings suggest that autophagy in advanced plaques is impaired. Levels of the autophagy substrate SQSTM1/p62, for example, dramatically increase in mouse atherosclerotic aortas with increasing age and plaque burden [32]. Moreover, mRNA and protein levels of LC3B decrease approximately 5-fold in symptomatic versus asymptomatic human carotid plaques [33]. Although the mechanisms of impaired autophagy in advanced plaques are unclear, it has been proposed that several factors such as ceroid deposition, production of high amounts of nitric oxide and aging could be responsible, as they are all known to inhibit the autophagic process [4]. The goal of the present study was to investigate potential beneficial effects of autophagy induction on atherosclerotic plaques in mice by administering the autophagy-enhancing agent spermidine. Previous findings have shown that spermidine restores the expression of autophagy markers in arteries of old mice [20]. Moreover, spermidine reverses large elastic artery stiffness, improves NO-mediated endothelial function and reduces oxidative stress [20, 34], suggesting that spermidine counteracts arterial aging. In line with these findings, data from the present study indicate that spermidine inhibits lipid accumulation and necrotic core formation through stimulation of cholesterol efflux inside the plaque, though without changing plaque size and cellular composition. Because a reduction in lipid load and necrotic core formation by spermidine was only obvious in plaques from autophagy-competent mice, but not in spermidine-treated *Atg7^{F/F} SM22 α -Cre⁺ ApoE^{-/-}* mice containing an autophagy defect in VSMCs (lack of the essential autophagy gene *Atg7*), it is plausible to assume that these effects are completely driven by stimulating the autophagic process particularly in VSMCs. Indeed, *in*

vitro studies showed that spermidine induces autophagy and cholesterol efflux to apoA-I in VSMCs, but neither initiation of autophagy nor enhanced cholesterol efflux was observed in macrophages. Importantly, our data do not suggest that macrophages are insensitive to autophagy stimulation. Macrophages induce autophagy in response to a number of pathophysiological stimuli such as IFN γ [35] or 7-ketocholesterol [36], and unlike VSMCs, show extensive autophagosome formation after mTOR inhibition [37], indicating that the autophagy stimulus is a critical factor for initiation of autophagy. Spermidine-mediated cholesterol efflux was autophagy-dependent since VSMCs from *Atg7^{F/F} SM22 α -Cre⁺* mice did not reveal enhanced cholesterol efflux after spermidine treatment. Moreover, basal cholesterol efflux was clearly impaired in autophagy-deficient cells, both in VSMCs and macrophages lacking ATG7, indicating that even basal levels of autophagy play an important role in cholesterol metabolism.

Several studies confirm that autophagy is strongly involved in managing intracellular lipids [38-40]. Indeed, autophagy is induced under lipid-loading conditions, and mediates the delivery of lipid droplets to lysosomes [39]. Lysosomal acid lipases hydrolyze cholesteryl esters to generate free cholesterol for ATP-binding cassette transporter (ABCA1) dependent cholesterol efflux. It is, however, surprising that spermidine induced neither autophagy nor autophagy-mediated cholesterol efflux in macrophages. A large body of evidence indicates that spermidine acts as an autophagy inducer by virtue of its capacity to inhibit acetyltransferases [17, 18]. Particularly noteworthy is the capacity of spermidine to inhibit EP300 [31], an acetyltransferase that directly acetylates and inhibits several core proteins of the autophagy machinery such as ATG5, ATG7, ATG8 and ATG12 [41]. Given that a knockdown of EP300 stimulates autophagy, EP300-driven deacetylation of autophagy proteins seems sufficient to initiate autophagosome formation. Interestingly, depletion of acetyl-coenzyme A under starvation conditions inhibits

EP300 [42, 43], which may explain at least partly why nutrient-deprived mammalian cells induce autophagy. In the present study, we demonstrate that macrophages, unlike VSMCs, contain high levels of EP300 protein. This finding suggests that spermidine may not be able to fully suppress EP300 activity in this cell type so that the autophagic machinery remains unaffected.

It is generally accepted that cholesterol mainly accumulates in atherosclerotic lesions due to the scavenging function of macrophages [44]. In this respect, spermidine may not have a significant effect on lipid accumulation and foam cell formation in plaques as it does not stimulate cholesterol efflux in macrophages. However, recent evidence suggests a much larger role for intimal VSMCs in foam cell formation than previously assumed [45]. First, ABCA1 expression is significantly reduced in late-stage intimal VSMCs and provides a likely reason for the contribution of VSMCs to total foam cell formation, and as a repository for much of the excess cholesterol in the arterial wall. Second, the expression of macrophage markers by intimal SMCs suggests that many of the intimal macrophages previously assumed to be of monocyte origin are in fact VSMCs.

Apart from autophagy induction, it is plausible that other mechanisms of action are involved. Indeed, Carmona-Gutiérrez et al. [46] showed that the anti-necrotic function of yeast pro-cathepsin D is spermidine-dependent, but autophagy-independent. Spermidine is a well-known anti-oxidant capable of preventing oxidative damage of lipids and DNA through direct scavenging of reactive oxygen species (ROS), especially singlet oxygen and hydroxyl radicals [47, 48]. Because plaque formation is associated with the production of high levels of ROS mediating oxidative damage and necrotic cell death [49], ROS scavenging by spermidine might help to resolve oxidative damage in atherosclerotic plaques, thereby preventing necrotic core formation via the inhibition of ROS-induced primary necrosis. However, it should be noted that

antiatherogenic effects triggered by dietary antioxidant ingestion are often disappointing [50]. Probably dietary antioxidants are only effective in high oxidative stress conditions and/or after depletion of natural antioxidant defense systems. Moreover, spermidine is also capable of reducing Fe^{3+} to Fe^{2+} and may act as a pro-oxidant rather than an anti-oxidant in the presence of free iron ions and H_2O_2 [51]. Accordingly, there is no hard evidence that spermidine inhibits plaque necrosis *in vivo* via anti-oxidant activity.

It should be noted that the present study contains some limitations. First, even though we included an acute treatment of established plaques, we could not observe any effect, possibly due to the short duration (4 weeks) of the treatment. A longer regimen is required to fully assess the curative effects of spermidine on established plaques. Second, plaque rupture is a typical feature of advanced human plaques, but rarely observed in $\text{ApoE}^{-/-}$ mice. Accordingly, it remains unclear whether spermidine can prevent the life-threatening complications related to plaque rupture such as myocardial infarctions, stroke or even sudden death. Administration of spermidine in recently developed mouse models (e.g. $\text{ApoE}^{-/-}\text{Fbn1}^{\text{C1039+/-}}$ mice)[52] that form advanced rupture-prone plaques may help to solve this issue. Third, spermidine was administered in this study to male mice only. Nonetheless, gender-related differences in polyamine oxidase activity have been reported [53], suggesting that male and females might react differently to spermidine treatment. Finally, it is worthwhile to mention that $\text{Atg7}^{\text{F/F}}\text{SM22a-Cre}^+\text{ApoE}^{-/-}$ mice (used in the present study to investigate the significance of autophagy in VSMCs during spermidine treatment) could not be fed a Western-type diet for 20 weeks (similar to experiments with regular $\text{ApoE}^{-/-}$ mice) because $\text{Atg7}^{\text{F/F}}\text{SM22a-Cre}^+\text{ApoE}^{-/-}$ mice develop heart failure and sudden death at later age due to partial autophagy deficiency in the heart. This complication forced us to perform the

experiment at an earlier stage (14 weeks), a time point that provides plaques that are quite similar in size and composition.

In conclusion, spermidine is a promising anti-atherosclerotic compound that promotes cholesterol efflux in VSMCs and prevents necrosis, albeit without changing the size or cellular composition of the plaques. Given that these effects are autophagy-dependent, we provide novel evidence that (moderate) induction of autophagy is an attractive strategy for stabilization of atherosclerotic plaques and the prevention of cardiovascular disease.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Financial support

This study was funded by the University of Antwerp (BOF) and the Fund for Scientific Research (FWO)-Flanders (projects G.0074.12N and G.0443.12N). Cédéric F. Michiels is a fellow of the Agency for Innovation by Science and Technology (IWT). The Tecnai G2 Spirit BioTwin TEM was purchased with support of the Hercules Foundation.

Author contributions

Study conception and design : Michiels, De Meyer, Martinet

Acquisition of data: Michiels, Kurdi, Timmermans

Analysis and interpretation: all authors

Drafting of manuscript: all authors

Acknowledgments

The authors acknowledge Dr Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science, Japan) and Dr Noboru Mizushima (Tokyo Medical and Dental University, Japan) for providing *Atg7^{F/F}* and GFP-LC3 mice, respectively, and Rita Van den Bossche, Hermine Fret, Anne-Elise Van Hoydonck, Lieve Svensson and Dominique De Rijck for excellent technical support.

References

1. Weber C, Noels H (2011) Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med* 17:1410-1422.
2. Libby P (2013) Mechanisms of acute coronary syndromes and their implications for therapy. *N Engl J Med* 368:2004-2013.
3. Sakakura K, Nakano M, Otsuka F, Ladich E, Kolodgie FD, Virmani R (2013) Pathophysiology of atherosclerosis plaque progression. *Heart Lung Circ* 22:399-411.
4. De Meyer GRY, Grootaert MOJ, Michiels CF, Kurdi A, Schrijvers DM, Martinet W (2015) Autophagy in vascular disease. *Circ Res* 116:468-479.
5. Vindis C (2015) Autophagy: an emerging therapeutic target in vascular diseases. *Br J Pharmacol* 172:2167-2178.
6. Mizushima N, Komatsu M (2011) Autophagy: renovation of cells and tissues. *Cell* 147:728-741.
7. Feng Y, He D, Yao Z, Klionsky DJ (2014) The machinery of macroautophagy. *Cell Res* 24:24-41.
8. Martinet W, De Meyer GRY (2009) Autophagy in atherosclerosis: a cell survival and death phenomenon with therapeutic potential. *Circ Res* 104:304-317.
9. Nussenzweig SC, Verma S, Finkel T (2015) The Role of Autophagy in Vascular Biology. *Circ Res* 116:480-488.

10. Sridhar S, Botbol Y, Macian F, Cuervo AM (2012) Autophagy and disease: always two sides to a problem. *J Pathol* 226:255-273.
11. Choi AM, Ryter SW, Levine B (2013) Autophagy in human health and disease. *N Engl J Med* 368:651-662.
12. Jiang P, Mizushima N (2014) Autophagy and human diseases. *Cell Res* 24:69-79.
13. Schrijvers DM, De Meyer GRY, Martinet W (2011) Autophagy in atherosclerosis: a potential drug target for plaque stabilization. *Arterioscler Thromb Vasc Biol* 31:2787-2791.
14. Soda K (2010) Polyamine intake, dietary pattern, and cardiovascular disease. *Med Hypotheses* 75:299-301.
15. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pinto X, Basora J, Munoz MA, Sorli JV, Martinez JA, Martinez-Gonzalez MA (2013) Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med* 368:1279-1290.
16. Minois N (2014) Molecular basis of the 'anti-aging' effect of spermidine and other natural polyamines - a mini-review. *Gerontology* 60:319-326.
17. Eisenberg T, Knauer H, Schauer A, Buttner S, Ruckstuhl C, Carmona-Gutierrez D, Ring J, Schroeder S, Magnes C, Antonacci L, Fussi H, Deszcz L, Hartl R, Schraml E, Criollo A, Megalou E, Weiskopf D, Laun P, Heeren G, Breitenbach M, Grubeck-Loebenstien B, Herker E, Fahrenkrog B, Frohlich KU, Sinner F, Tavernarakis N, Minois N, Kroemer G,

- Madeo F (2009) Induction of autophagy by spermidine promotes longevity. *Nat Cell Biol* 11:1305-1314.
18. Madeo F, Eisenberg T, Buttner S, Ruckenstuhl C, Kroemer G (2010) Spermidine: a novel autophagy inducer and longevity elixir. *Autophagy* 6:160-162.
 19. Minois N, Carmona-Gutierrez D, Bauer MA, Rockenfeller P, Eisenberg T, Brandhorst S, Sigrist SJ, Kroemer G, Madeo F (2012) Spermidine promotes stress resistance in *Drosophila melanogaster* through autophagy-dependent and -independent pathways. *Cell Death Dis* 3:e401.
 20. LaRocca TJ, Gioscia-Ryan RA, Hearon CM, Jr., Seals DR (2013) The autophagy enhancer spermidine reverses arterial aging. *Mech Ageing Dev* 134:314-320.
 21. Soda K, Kano Y, Chiba F, Koizumi K, Miyaki Y (2013) Increased polyamine intake inhibits age-associated alteration in global DNA methylation and 1,2-dimethylhydrazine-induced tumorigenesis. *PLoS One* 8:e64357.
 22. Velloso NA, Dalmolin GD, Gomes GM, Rubin MA, Canas PM, Cunha RA, Mello CF (2009) Spermine improves recognition memory deficit in a rodent model of Huntington's disease. *Neurobiol Learn Mem* 92:574-580.
 23. Buttner S, Broeskamp F, Sommer C, Markaki M, Habernig L, Alavian-Ghavanini A, Carmona-Gutierrez D, Eisenberg T, Michael E, Kroemer G, Tavernarakis N, Sigrist SJ, Madeo F (2014) Spermidine protects against alpha-synuclein neurotoxicity. *Cell Cycle* 13:3903-3908.

24. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y (2004) In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell* 15:1101-1111.
25. Michiels CF, Fransen P, De Munck DG, De Meyer GRY, Martinet W (2015) Defective autophagy in vascular smooth muscle cells alters contractility and calcium homeostasis in mice. *Am J Physiol Heart Circ Physiol* 308:H557-H567.
26. Grootaert MOJ, da Costa Martins PA, Bitsch N, Pintelon I, De Meyer GRY, Martinet W, Schrijvers DM (2015) Defective autophagy in vascular smooth muscle cells accelerates senescence and promotes neointima formation and atherogenesis. *Autophagy* 11:2014-2032.
27. Schrijvers DM, Martinet W, De Meyer GRY, Andries L, Herman AG, Kockx MM (2004) Flow cytometric evaluation of a model for phagocytosis of cells undergoing apoptosis. *J Immunol Methods* 287:101-108.
28. Martinet W, Schrijvers DM, Timmermans J-P, Bult H, De Meyer GRY (2013) Immunohistochemical analysis of macroautophagy: recommendations and limitations. *Autophagy* 9:386-402.
29. Martinet W, Timmermans J-P, De Meyer GRY (2014) Methods to assess autophagy in situ: transmission electron microscopy versus immunohistochemistry. *Methods Enzymol* 543:89-114.
30. Jackson WF, Huebner JM, Rusch NJ (1997) Enzymatic isolation and characterization of single vascular smooth muscle cells from cremasteric arterioles. *Microcirculation* 4:35-50.

31. Pietrocola F, Lachkar S, Enot DP, Niso-Santano M, Bravo-San Pedro JM, Sica V, Izzo V, Maiuri MC, Madeo F, Marino G, Kroemer G (2015) Spermidine induces autophagy by inhibiting the acetyltransferase EP300. *Cell Death Differ* 22:509-516.
32. Razani B, Feng C, Coleman T, Emanuel R, Wen H, Hwang S, Ting JP, Virgin HW, Kastan MB, Semenkovich CF (2012) Autophagy Links Inflammasomes to Atherosclerotic Progression. *Cell Metab* 15:534-544.
33. Swaminathan B, Goikuria H, Vega R, Rodriguez-Antiguedad A, Lopez MA, Freijo MM, Vandebroek K, Alloza I (2014) Autophagic marker MAP1LC3B expression levels are associated with carotid atherosclerosis symptomatology. *PLoS One* 9:e115176.
34. Fetterman JL, Holbrook M, Flint N, Feng B, Bretomicronn-Romero R, Linder EA, Berk BD, Duess MA, Farb MG, Gokce N, Shirihai OS, Hamburg NM, Vita JA (2016) Restoration of autophagy in endothelial cells from patients with diabetes mellitus improves nitric oxide signaling. *Atherosclerosis* 247:207-217.
35. Matsuzawa T, Kim BH, Shenoy AR, Kamitani S, Miyake M, Macmicking JD (2012) IFN-gamma Elicits Macrophage Autophagy via the p38 MAPK Signaling Pathway. *J Immunol* 189:813-818.
36. Liao X, Sluimer JC, Wang Y, Subramanian M, Brown K, Pattison JS, Robbins J, Martinez J, Tabas I (2012) Macrophage Autophagy Plays a Protective Role in Advanced Atherosclerosis. *Cell Metab* 15:545-553.

37. Verheye S, Martinet W, Kockx MM, Knaapen MW, Salu K, Timmermans JP, Ellis JT, Kilpatrick DL, De Meyer GRY (2007) Selective clearance of macrophages in atherosclerotic plaques by autophagy. *J Am Coll Cardiol* 49:706-715.
38. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ (2009) Autophagy regulates lipid metabolism. *Nature* 458:1131-1135.
39. Ouimet M, Franklin V, Mak E, Liao X, Tabas I, Marcel YL (2011) Autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase. *Cell Metab* 13:655-667.
40. Ouimet M, Marcel YL (2012) Regulation of lipid droplet cholesterol efflux from macrophage foam cells. *Arterioscler Thromb Vasc Biol* 32:575-581.
41. Lee IH, Finkel T (2009) Regulation of autophagy by the p300 acetyltransferase. *J Biol Chem* 284:6322-6328.
42. Eisenberg T, Schroeder S, Andryushkova A, Pendl T, Kuttner V, Bhukel A, Marino G, Pietrocola F, Harger A, Zimmermann A, Moustafa T, Sprenger A, Jany E, Buttner S, Carmona-Gutierrez D, Ruckenstuhl C, Ring J, Reichelt W, Schimmel K, Leeb T, Moser C, Schatz S, Kamolz LP, Magnes C, Sinner F, Sedej S, Frohlich KU, Juhasz G, Pieber TR, Dengjel J, Sigrist SJ, Kroemer G, Madeo F (2014) Nucleocytosolic depletion of the energy metabolite acetyl-coenzyme a stimulates autophagy and prolongs lifespan. *Cell Metab* 19:431-444.
43. Marino G, Pietrocola F, Eisenberg T, Kong Y, Malik SA, Andryushkova A, Schroeder S, Pendl T, Harger A, Niso-Santano M, Zamzami N, Scoazec M, Durand S, Enot DP,

- Fernandez AF, Martins I, Kepp O, Senovilla L, Bauvy C, Morselli E, Vacchelli E, Bennetzen M, Magnes C, Sinner F, Pieber T, Lopez-Otin C, Maiuri MC, Codogno P, Andersen JS, Hill JA, Madeo F, Kroemer G (2014) Regulation of autophagy by cytosolic acetyl-coenzyme A. *Mol Cell* 53:710-725.
44. Kruth HS (2001) Macrophage foam cells and atherosclerosis. *Front Biosci* 6:D429-D455.
45. Allahverdian S, Chehroudi AC, McManus BM, Abraham T, Francis GA (2014) Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation* 129:1551-1559.
46. Carmona-Gutierrez D, Bauer MA, Ring J, Knauer H, Eisenberg T, Buttner S, Ruckenstuhl C, Reisenbichler A, Magnes C, Rechberger GN, Birner-Gruenberger R, Jungwirth H, Frohlich KU, Sinner F, Kroemer G, Madeo F (2011) The propeptide of yeast cathepsin D inhibits programmed necrosis. *Cell Death Dis* 2:e161.
47. Ha HC, Sirisoma NS, Kuppusamy P, Zweier JL, Woster PM, Casero RA, Jr. (1998) The natural polyamine spermine functions directly as a free radical scavenger. *Proc Natl Acad Sci U S A* 95:11140-11145.
48. Rider JE, Hacker A, Mackintosh CA, Pegg AE, Woster PM, Casero RA, Jr. (2007) Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. *Amino Acids* 33:231-240.
49. Martinet W, Schrijvers DM, De Meyer GRY (2011) Necrotic cell death in atherosclerosis. *Basic Res Cardiol* 106:749-760.

50. Cherubini A, Vigna GB, Zuliani G, Ruggiero C, Senin U, Fellin R (2005) Role of antioxidants in atherosclerosis: epidemiological and clinical update. *Curr Pharm Des* 11:2017-2032.
51. Mozdzan M, Szemraj J, Rysz J, Stolarek RA, Nowak D (2006) Anti-oxidant activity of spermine and spermidine re-evaluated with oxidizing systems involving iron and copper ions. *Int J Biochem Cell Biol* 38:69-81.
52. Van der Donckt C, Van Herck JL, Schrijvers DM, Vanhoutte G, Verhoye M, Blockx I, Van Der Linden A, Bauters D, Lijnen HR, Sluimer JC, Roth L, Van Hove CE, Franssen P, Knaapen MW, Hervent A-S, De Keulenaer GW, Bult H, Martinet W, Herman AG, De Meyer GRY (2015) Elastin fragmentation in atherosclerotic mice leads to intra-plaque neovascularization, plaque rupture, myocardial infarction, stroke, and sudden death. *Eur Heart J* 36:1049-1058.
53. Ferioli ME, Pinotti O, Pirona L (1999) Gender-related differences in polyamine oxidase activity in rat tissues. *Amino Acids* 17:139-148.

Figures and tables

Fig. 1. Spermidine induces autophagy in vivo. (A) *ApoE*^{-/-} mice were fed a Western-type diet for 20 weeks with or without spermidine in the drinking water. The LC3-II/LC3-I ratio and SQSTM1/p62 expression levels were examined in atherosclerotic aortic segments via western blotting. ****p*<0.001 vs control (Student's unpaired t-test, n=5). (B-C) Spermidine was administered to GFP-LC3 transgenic mice via the drinking water for one month. Cleavage of GFP-LC3 was analyzed via western blotting in endothelium-denuded aortic segments of untreated (C) or spermidine-treated (SP) GFP-LC3 mice (B). LC3 was also analyzed via immunohistochemistry in liver of spermidine-treated or control animals (C). Scale bar=20 μm. The LC3 positive area was quantified. ****p*<0.001 vs control (Student's unpaired t-test, n=6).

Fig. 2. Spermidine reduces lipid accumulation in atherosclerotic plaques. *ApoE*^{-/-} mice were fed a Western-type diet for 20 weeks with or without spermidine in the drinking water. (A) En face oil red O staining of the entire thoracic aorta. **p*<0.05 versus control (Student's unpaired t-test, n=6). Scale bar=2 mm. (B) Intraplaque oil red O staining of aortic segments. ****p*<0.001 versus control (Student's unpaired t-test, n=12). Scale bar=200 μm.

Fig. 3. Spermidine induces autophagy in vascular smooth muscle cells (VSMCs), but not in macrophages (MΦ). Mouse aortic VSMCs and bone-marrow derived MΦ were isolated and treated *in vitro* with 0-10 μM spermidine (SP) for 24 hours. (A) Western blot analysis of the autophagy-related proteins p62 and LC3. β-actin served as a loading control. Expression of p62 (vs β-actin) and LC3-II/LC3-I ratio were quantified. **p*<0.05, ***p*<0.01 versus 0 μM (One-way ANOVA, followed by Dunnett test, n=3). (B) Transmission electron microscopy of cells after

treatment with vehicle (control) or 10 μ M spermidine for 24 hours. Scale bar=2 μ m. The number of autophagic vesicles (AVs, arrows) per cell was quantified. *** p <0.001 versus control (Mann-Whitney U test, n=40 cells from two independent experiments). (C) Western blot analysis of acetyltransferase EP300.

Fig. 4. Spermidine promotes cholesterol efflux in VSMCs via autophagy. ~~VSMCs (A) and macrophages (B) were isolated from $Atg7^{F/F}$ - $SM22\alpha$ - Cre^+ (+/+) and $Atg7^{F/F}$ - $LysM$ - Cre^+ (F/F) mice, lacking the essential autophagy gene $Atg7$ specifically in VSMCs and macrophages, respectively.~~ VSMCs (A) were isolated from $Atg7^{+/+}$ $SM22\alpha$ - Cre^+ (+/+) and $Atg7^{F/F}$ $SM22\alpha$ - Cre^+ (F/F) mice. Likewise, macrophages (B) were isolated from $Atg7^{+/+}$ $LysM$ - Cre^+ (+/+) and $Atg7^{F/F}$ $LysM$ - Cre^+ (F/F) mice. Cells were loaded with 3 [H]-cholesterol and then treated for 24 hours with 10 μ M spermidine (SPERM) in the presence of the acceptor apoA-I. Efflux of cholesterol to apoA-I was measured via scintillation counting. * p <0.05 versus control; $^{##}$ p <0.01, $^{###}$ p <0.001 versus +/+ (One-way ANOVA followed by Bonferroni test, n=4).

Fig. 5. Spermidine reduces necrosis and lipid accumulation in atherosclerotic plaques by stimulating autophagy in VSMCs. $Atg7^{F/F}$ $SM22\alpha$ - Cre^+ $ApoE^{-/-}$ (F/F) mice with an autophagy defect (deletion of $Atg7$) specifically in VSMCs as well as $Atg7^{+/+}$ $SM22\alpha$ - Cre^+ $ApoE^{-/-}$ (+/+) control mice were fed a Western-type diet and treated with spermidine or plain drinking water for 14 weeks. Plaque necrosis (A) and lipid content (B) in frozen sections of the aortic root, proximal ascending aorta and brachiocephalic artery were determined via histology. * p <0.05, ** p <0.01 versus control (Student's unpaired t-test, n=6).

Table 1. Characteristics of atherosclerotic plaques in the aortic root, proximal ascending aorta and brachiocephalic artery of *ApoE*^{-/-} mice after 20 weeks on Western-type diet with or without spermidine in the drinking water

	Aortic root		Proximal ascending aorta		Brachiocephalic artery	
	Control	Spermidine	Control	Spermidine	Control	Spermidine
Plaque size (x 10³ μm²)	123 ± 12	129 ± 11	216 ± 29	219 ± 24	195 ± 13	209 ± 14
Macrophages (%)	2.3 ± 0.5	3.3 ± 0.6	6.0 ± 1.3	5.4 ± 1.2	7.7 ± 0.9	8.7 ± 1.2
Smooth muscle cells (%)	2.6 ± 0.5	2.3 ± 0.2	8.3 ± 1.5	6.2 ± 1.3	3.8 ± 0.5	5.3 ± 0.5
Collagen (%)	31 ± 5	28 ± 5	43 ± 4	41 ± 4	56 ± 4	56 ± 6
Apoptosis						
TUNEL (%)	0.4 ± 0.5	0.4 ± 0.4	0.1 ± 0.1	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1
Cleaved caspase 3 (%)	1.2 ± 0.1	1.3 ± 0.3	1.7 ± 0.3	2.0 ± 0.4	1.1 ± 0.4	2.2 ± 0.7
Necrosis (%)	6.6 ± 0.5	3.7 ± 0.5***	8.8 ± 1.2	5.7 ± 0.9*	13 ± 1	9.0 ± 1.3*

^a **p*<0.05, ****p*<0.001 versus control (Student's unpaired t-test, n=11-12)

Table 2. Characteristics of atherosclerotic plaques in the aortic root, proximal ascending aorta and brachiocephalic artery of *ApoE*^{-/-} mice after 20 weeks on Western-type diet (WD). Spermidine was added to the drinking water in the last four weeks of WD.

	Aortic root		Proximal ascending aorta		Brachiocephalic artery	
	Control	Spermidine	Control	Spermidine	Control	Spermidine
Plaque size (x 10³ μm²)	164 ± 16	174 ± 19	255 ± 30	301 ± 26	243 ± 13	246 ± 26
Macrophages (%)	1.8 ± 0.5	2.0 ± 0.3	3.7 ± 1.0	3.6 ± 0.4	2.5 ± 0.8	2.9 ± 0.5
Smooth muscle cells (%)	1.7 ± 0.5	2.1 ± 0.4	4.0 ± 1.0	5.6 ± 0.6	1.8 ± 0.6	1.9 ± 0.3
Collagen (%)	26 ± 1.8	27 ± 1.9	62 ± 3.3	65 ± 3.1	54 ± 1.9	51 ± 1.9
Apoptosis (%)						
TUNEL (%)	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Cleaved caspase-3 (%)	1.5 ± 0.3	1.4 ± 0.4	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.5	1.9 ± 1.1
Necrosis (%)	7.9 ± 1.2	7.1 ± 1.0	10 ± 1.5	8.2 ± 1.2	6.8 ± 1.1	6.5 ± 1.6

^a Statistically significant differences were not present (Student t-test, n=10-12 per group).