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Annexin A5 prevents post-interventional accelerated atherosclerosis development in a dose-dependent fashion in mice

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ABSTRACT

Background: Activated cells in atherosclerotic lesions expose phosphatidylserine (PS) on their surface. Annexin A5 (AnxA5) binds to PS and is used for imaging atherosclerotic lesions. Recently, AnxA5 was shown to inhibit vascular inflammatory processes after vein grafting. Here, we report a therapeutic role for AnxA5 in post-interventional vascular remodeling in a mouse model mimicking percutaneous coronary intervention (PCI).

Methods and results: Associations between the rs4833229 (OR = 1.29 (CI 95%), $p_{allelic} = 0.011$) and rs6830321 (OR = 1.35 (CI 95%), $p_{allelic} = 0.003$) SNPs in the AnxA5 gene and increased restenosis-risk in patients undergoing PCI were found in the GENDER study. To evaluate AnxA5 effects on post-interventional vascular remodeling and accelerated atherosclerosis development in vivo, hypercholesterolemic ApoE$^{-/-}$ mice underwent femoral arterial cuff placement to induce intimal thickening. Dose-dependent effects were investigated after 3 days (effects on inflammation and leukocyte recruitment) or 14 days (effects on remodeling) after cuff placement. Systemically administered AnxA5 in doses of 0.1, 0.3 and 1.0 mg/kg compared to vehicle reduced early leukocyte and macrophage adherence up to 48.3% ($p = 0.001$) and diminished atherosclerosis development by 71.2% ($p = 0.012$) with a reduction in macrophage/foam cell presence. Moreover, it reduced the expression of the endoplasmic reticulum stress marker GRP78/BiP, indicating lower inflammatory activity of the cells present.

Conclusions: AnxA5 SNPs could serve as markers for restenosis after PCI and AnxA5 therapeutically prevents vascular remodeling in a dose-dependent fashion, together indicating clinical potential for AnxA5 against post-interventional remodeling.

1. Introduction

Post-interventional vascular remodeling and accelerated atherosclerosis development are important complications of revascularization strategies and limit treatment success rate [1]. These features are elicited by endothelial and atherosclerotic plaque injury, triggering inflammatory activation and leukocyte recruitment to the injured arterial segment. These cells are the driving factors behind smooth muscle cell (SMC) proliferation and extracellular matrix deposition leading to intimal hyperplasia. Subendothelial retention and oxidation of low-density lipoprotein (LDL) cholesterol is central to the initial lesion formation in both native atherosclerosis and restenosis development [2,3]. Recently, it was postulated that endoplasmic reticulum (ER) stress, leading to the unfolded protein response (UPR), is involved in the regulation of inflammation in activated vascular cells and the link between UPR and arterial inflammation is emerging as an important factor in (accelerated) atherosclerosis development [4–7].

AnxA5 is a member of the annexin family of proteins that calcium-dependently bind to negatively charged phospholipid surfaces and was originally discovered as an anticoagulant and antithrombotic protein [8–11] and has been shown to inhibit the prothrombinase complex [12] and to down-regulate the surface expression of tissue factor [13]. It is now known to have anti-inflammatory and anti-atherosclerotic properties [14,15] and to regulate interferon γ signalling [16]. Viable cells express phosphatidylserine (PS) on their inner cellular membrane leaflet. When

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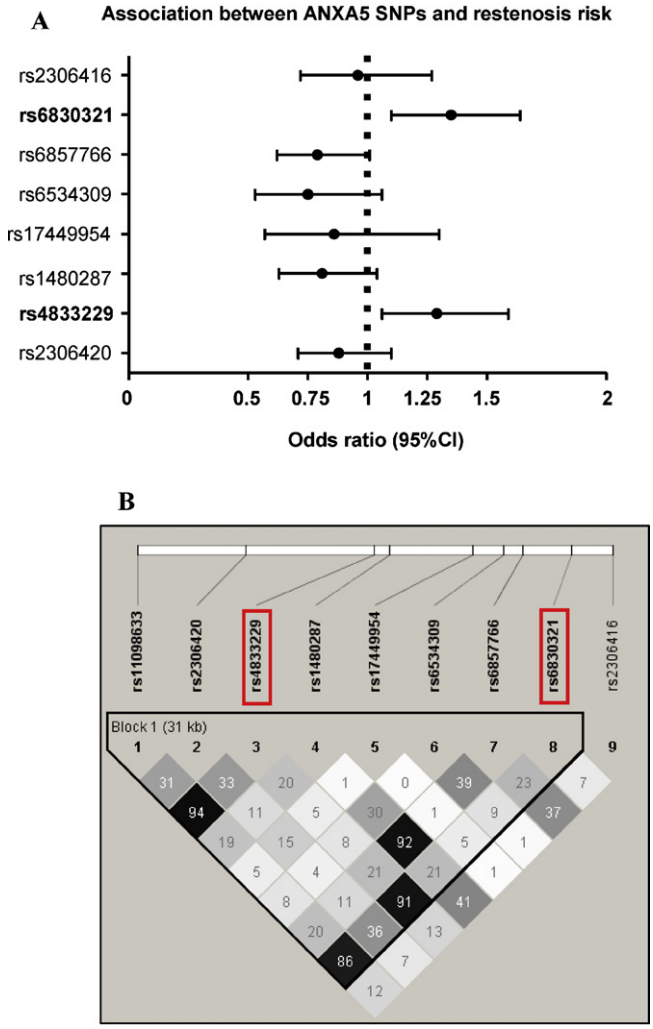

Fig. 1. AnxA5 is a genetic risk marker for clinical restenosis after PCI. Association results for the allelic test for eight SNPs in the ANXA5 gene (A). LD plot shows that rs4833229 and rs6830321 SNPs are in high LD ($r^2 = 0.91$) (B).

PS is externalized, it serves as an 'eat-me' signal. Annexin A5 (AnxA5) binds reversibly, specifically and with high affinity to PS [15]. PS becomes externalized during apoptosis, which makes AnxA5 a powerful tool to detect apoptosis (and atherosclerosis) both in vitro and in vivo [17]. PS is expressed in native atherosclerosis and after revascularisation procedures, and circulating AnxA5 binds with high affinity to these cells, and is therefore present in high concentrations in atherosclerotic plaques and injured vascular segments. PS externalization is normally thought to be associated to apoptosis, but can also be externalized in a controlled and reversible way in non-apoptotic cells [18,19].

Plasma levels of AnxA5 are inversely related to the severity of coronary stenosis and are indicative of the extent of atherosclerotic plaques [20], but are also elevated in subjects with left ventricular hypertrophy and following myocardial infarction [21,22]. It was recently shown that systemically administered AnxA5 can prevent vein graft disease and vascular inflammation [23] and that the dimer of annexin A5, diannexin, can protect against renal ischemia–reperfusion injury and inflammatory cell infiltration into transplanted islet grafts [24,25]. Patients with hypercholesterolemia and previous coronary heart disease (CHD) undergoing PCI for atherosclerosis are most at risk for inflammatory-driven post-interventional restenosis development. The risk for development of restenosis may partially be determined by genetic factors. It has been shown that genetic variations in genes encoding inflammatory factors (SNPs) can predict the risk for restenosis after percutaneous coronary intervention (PCI) [26]. The effects of genetic variation in the AnxA5 gene on clinical restenosis after PCI or cardiovascular disease progression have thus far not been elucidated.

In the present study we investigated the association between AnxA5 SNPs and restenosis-risk in patients undergoing PCI, followed by in vivo evaluation of the therapeutic effectiveness of AnxA5 in a humanized mouse model for post-interventional vascular remodeling using ApoE3*Leiden mice. Our findings point to a potential diagnostic and therapeutic clinical role for AnxA5 against post-PCI vascular remodeling.

2. Materials and methods

Association between single nucleotide polymorphisms (SNPs) in the AnxA5 gene, extracted from the GENDER genome wide association study (GWAS) dataset [27] and restenosis-risk following PCI was investigated.

We performed in vivo intervention studies in which hypercholesterolemic ApoE$^{-/-}$ mice on a Western-type diet were subjected to femoral artery cuff placement to induce vascular injury and remodeling [28]. Cuff placement leads to a localized vascular inflammation, which in turn produces concentric intimal lesions that can affect vessel patency. The lesions consist of SMCs, connective tissue and infiltrated leukocytes such as macrophages/foam cells and are strongly inflammation-dependent [29]. In these vascular segments, inflammatory cell adhesion, infiltration, intimal thickening and lesion composition were assessed using histology, morphometry and immunohistochemistry (IHC), as described previously [29]. Treatment with vehicle, 0.1, 0.3 and 1.0 mg/kg AnxA5 was given to operated ApoE$^{-/-}$ mice. A three day protocol was used to evaluate effects on leukocyte recruitment, and a 14 day protocol to evaluate effects on vascular remodeling. All materials and methods are described in detail in Supplemental material.

3. Results

3.1. Annexin A5 SNP as risk marker for clinical restenosis

AnxA5 plasma levels are linked to the severity of coronary stenosis and AnxA5 is a marker of cardiovascular disease progress. These data indicate a potential role of AnxA5 in (post-interventional) accelerated atherosclerosis development. Therefore we investigated the association between AnxA5 SNPs and restenosis risk in patients undergoing PCI enrolled in the GENDER study, composed of 866 patients (295 cases that developed restenosis following PCI and 571 controls that did not develop restenosis). Clinical outcome was linked to genetic data obtained through a genome-wide association analysis.

The allelic association test identified two SNPs, rs4833229 and rs6830321, which are significantly associated with restenosis risk after PCI (Fig. 1A). Both SNPs increased the risk for restenosis (rs4833229, odds ratio (OR) = 1.29 (95% confidence interval (CI) 1.06–1.58), $p_{\text{allelic}} = 0.011$ and rs6830321, OR = 1.35 (95% CI 1.10–1.64), $p_{\text{allelic}} = 0.003$), even after adjustment for clinical risk factors, such as total occlusion, diabetes, smoking and residual stenosis (Table 1). The minor allele frequencies for cases and controls from the GENDER population are 0.481 and 0.418 for rs4833229 and 0.510 and 0.436 for rs6830321 respectively, indicating they are present in a large proportion of the population. The AnxA5 gene linkage disequilibrium (LD) plot shows that rs4833229 and rs6830321 are in high LD ($r^2 = 0.91$, Fig. 1B). Haplotype analysis in the gene showed similar association results with restenosis

Table 1
Association between restenosis risk and SNPs in the ANXA5 gene.

SNP	Base position	Minor/major allele	MAF cases/controls	OR (95% CI)	*p* Value
rs2306420	122810925	T/C	0.283/0.309	0.88 (0.71–1.10)	0.2622
rs4833229	**122820114**	**A/G**	**0.481/0.418**	**1.29 (1.06–1.58)**	**0.0114**
rs1480287	122821231	A/G	0.481/0.215	0.81 (0.63–1.04)	0.0954
rs17449954	122827178	C/T	0.181/0.067	0.86 (0.57–1.30)	0.4705
rs6534309	122829379	C/T	0.058/0.108	0.75 (0.53–1.06)	0.1040
rs6857766	122830735	A/G	0.083/0.230	0.79 (0.62–1.01)	0.0636
rs6830321	**122834205**	**T/C**	**0.510/0.436**	**1.35 (1.10–1.64)**	**0.0034**
rs2306416	122837138	CT	0.139/0.145	0.96 (0.72–1.27)	0.7564

Allelic association results for 8 SNPs included in the annexin A5 gene in the GENDER study. Positions are based on hg18 build. *Abbreviations*: Chr, chromosome; MAF, minor allele frequency. ORs are computed for the minor allele from the two by two allele contingency table. Significant association was observed for SNPs rs4833229 and rs6830321 and restenosis-risk with SNPs displaying high linkage.

as found in the single SNP analysis (haplotype ACAGTTGTT, frequency: 0.427, OR = 1.275, $p = 0.018$). These data link AnxA5 SNPs to restenosis-risk after PCI and suggest that AnxA5 genotype functions as risk marker for restenosis. We therefore further explored AnxA5's therapeutic potential using an in vivo model for restenosis and intimal hyperplasia.

3.2. Annexin A5 dose-dependently prevents leukocyte recruitment after vascular injury

Effects of AnxA5 on leukocyte recruitment to injured arterial segments was investigated in the femoral artery cuff model in ApoE$^{-/-}$ mice receiving daily vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 through IP injection. Total plasma cholesterol was not affected by annexin A5 treatment (Supplementary Table I). Three days after cuff placement there is inflammation in the cuffed arteries, with leukocytes both adherent to the endothelial surface and with cells that have migrated into the media layer (Fig. 2A). Staining of arterial lesions at this time point revealed that 0.1, 0.3 and 1.0 mg/kg/d AnxA5-treated animals displayed a reduced percentage of endothelial leukocyte adhesion by 26.7% ($p = 0.014$), 34.9% ($p = 0.010$) and 48.3% ($p = 0.001$) respectively (Fig. 2B). For monocytes/macrophages, this percentage was reduced by 40.0% ($p = 0.029$), 66.9% ($p = 0.001$), and 45.0% ($p = 0.037$) respectively (Fig. 2C).

The percentage leukocyte infiltration into the media was reduced by all AnxA5 treatments by 49.4% ($p = 0.008$), 53.3% ($p = 0.006$) and 49.9% ($p = 0.011$) respectively (Fig. 2D). The percentage medial macrophages was reduced by 61.2% ($p = 0.025$) by 1.0 mg/kg AnxA5, the other dosages did not significantly affect monocyte/macrophage extravasation (Fig. 2E). Together, these data indicate an important role for AnxA5 in low dosages in the prevention of leukocyte recruitment to injured arterial segments.

3.3. Annexin A5 dose-dependently prevents accelerated atherosclerosis development

The inflammation caused by cuff placement leads to an inflammation driven intimal hyperplasia. Therapeutic effectiveness of AnxA5 on (neo-)intima development was evaluated 14 days after cuff placement. Annexin A5 treatment did not affect plasma total cholesterol concentration (Supplementary Table I). Accelerated atherosclerotic lesion development was measured on sections stained with HPS and Weigert's elastin (Fig. 3A). Vehicle-treated animals developed intimal thickening, resulting in luminal stenosis. Quantitative analysis displayed reduced intimal thickening (expressed as μm^2 per cross-section) after 0.1, 0.3 and 1.0 mg/kg AnxA5-treatment by 54.6% ($p = 0.041$), 71.2% ($p = 0.012$) and 66.9% ($p = 0.009$) respectively (Fig. 3B). Intimal thickening was 38.1% more reduced ($p = 0.031$) by 0.3 compared to 0.1 mg/kg AnxA5.

AnxA5 (0.3 and 1.0 mg/kg) also decreased the absolute medial surface area (μm^2) by 30.1% ($p = 0.012$) and 24.1% ($p = 0.025$, Fig. 3C) and intima/media ratio by 62.3% ($p = 0.004$) and 60.3% ($p = 0.007$, Fig. 3D), although the lowest dose was ineffective. Furthermore, luminal stenosis (%) was reduced by 58.0% ($p = 0.001$) and 58.8% ($p = 0.0004$, Fig. 3E), identifying a potent role for AnxA5 in the control of inflammatory post-interventional vascular remodeling. Compared to 0.1 mg/kg, 0.3 mg/kg AnxA5 had increased protective effects on both the intima/media ratio (by 38.5%, $p = 0.016$) and luminal stenosis percentage (by 33.2%, $p = 0.042$). The total vessel wall diameter and luminal areas were both similar in all AnxA5 dosages, except for 1.0 mg/kg, which displayed 27.1% ($p = 0.043$) reduced total vessel area (Supplementary Fig. IA and B).

IHC showed profound intravascular macrophages/foam cell areas, which co-localized with AnxA5 (Supplementary Fig. IIA and B) staining at both 3d and 14d after surgery. AnxA5 in all dosages strongly reduced the accumulation of the percentage of macrophages/foam cell area (Fig. 4A) in the tunica media (Fig. 4B, $p = 0.0002$, $p = 0.028$ and $p = 0.0005$ respectively) and in the tunica intima (Fig. 4C, $p = 0.002$, $p = 0.011$ and $p = 0.002$ respectively) after 14d. The 78 kDa glucose regulated protein/BiP (GRP78) is an ER protein and associates permanently with mutant or defective incorrectly folded proteins, preventing their export from the ER lumen. ER stress including upregulation of GRP78 is present in unstable atherosclerotic lesions. We investigated if annexin A5 affected GRP78 expression in cuffed femoral arteries. AnxA5 in all dosages strongly reduced GRP78 BiP expressing cells in the media (Fig. 4D) by 50.2% ($p = 0.006$), 66.3% ($p = 0.0006$) and 68.0% ($p = 0.004$) respectively, but not in the intima (Fig. 4E).

4. Discussion

This study demonstrates an important therapeutic role for AnxA5 in post-interventional intimal hyperplasia and accelerated atherosclerosis development. Association between AnxA5 SNPs and increased restenosis-risk in patients undergoing PCI was found. Systemic AnxA5 was effective in preventing intimal thickening and could dose-dependently reduce leukocyte and macrophage recruitment to injured arterial segments in ApoE$^{-/-}$ mice in 0.3 and 0.1 mg/kg dosages. Finally, we demonstrate that sustained therapy reduces accelerated atherosclerosis with fewer infiltrated macrophages/foam cells and UPR-expressing cells in the injured arterial wall. Together, these date indicate high diagnostic and therapeutic potential for AnxA5 against post-PCI vascular remodeling.

Association between AnxA5 SNPs and restenosis development were investigated using a large study population that underwent PCI, the GENDER population. It has already been shown in this material that mutations in several genes associated with inflammation were associated to restenosis development (24). Our results demonstrate that SNPs rs4833229 and rs6830321 show significant association with increased risk for clinical restenosis (OR 1.29 and

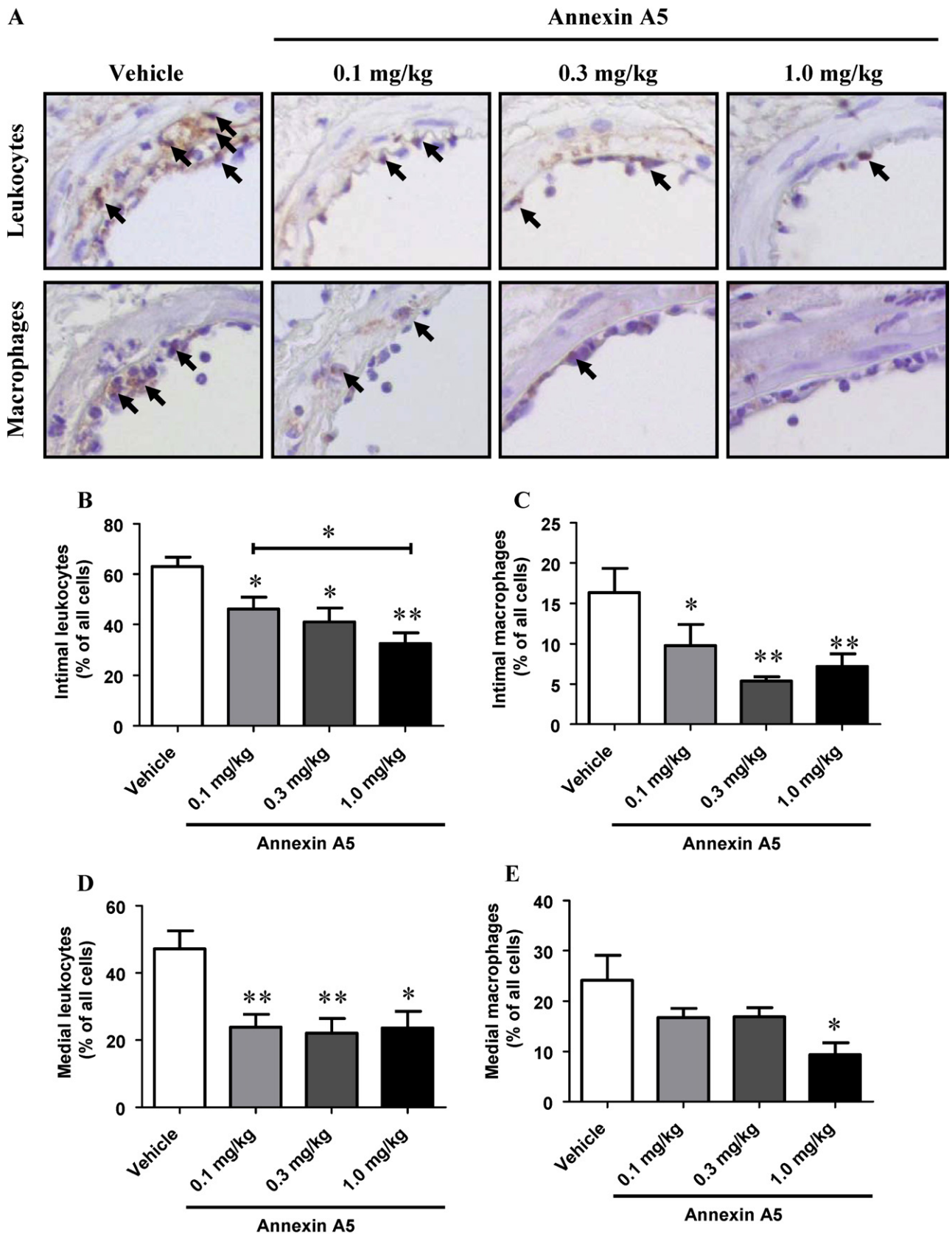

Fig. 2. Annexin A5 dose-dependently prevents leukocyte recruitment after vascular injury. Representative cross-sections of cuffed-femoral arteries of ApoE$^{-/-}$ mice treated with vehicle or 0.1, 0.3 or 1.0 mg/kg/d AnxA5 (leukocyte and macrophage staining, magnification 80×, arrows indicate positive staining) after 3d (A). Quantification of intimal adhering leukocytes (B) and macrophages (C) as percentage of all cells within the internal elastic lamina and medial infiltrated leukocytes (D) and macrophages (E) (%). Results indicated as mean ± SEM, $n = 10$. $^{*}p < 0.05$, $^{**}p < 0.01$.

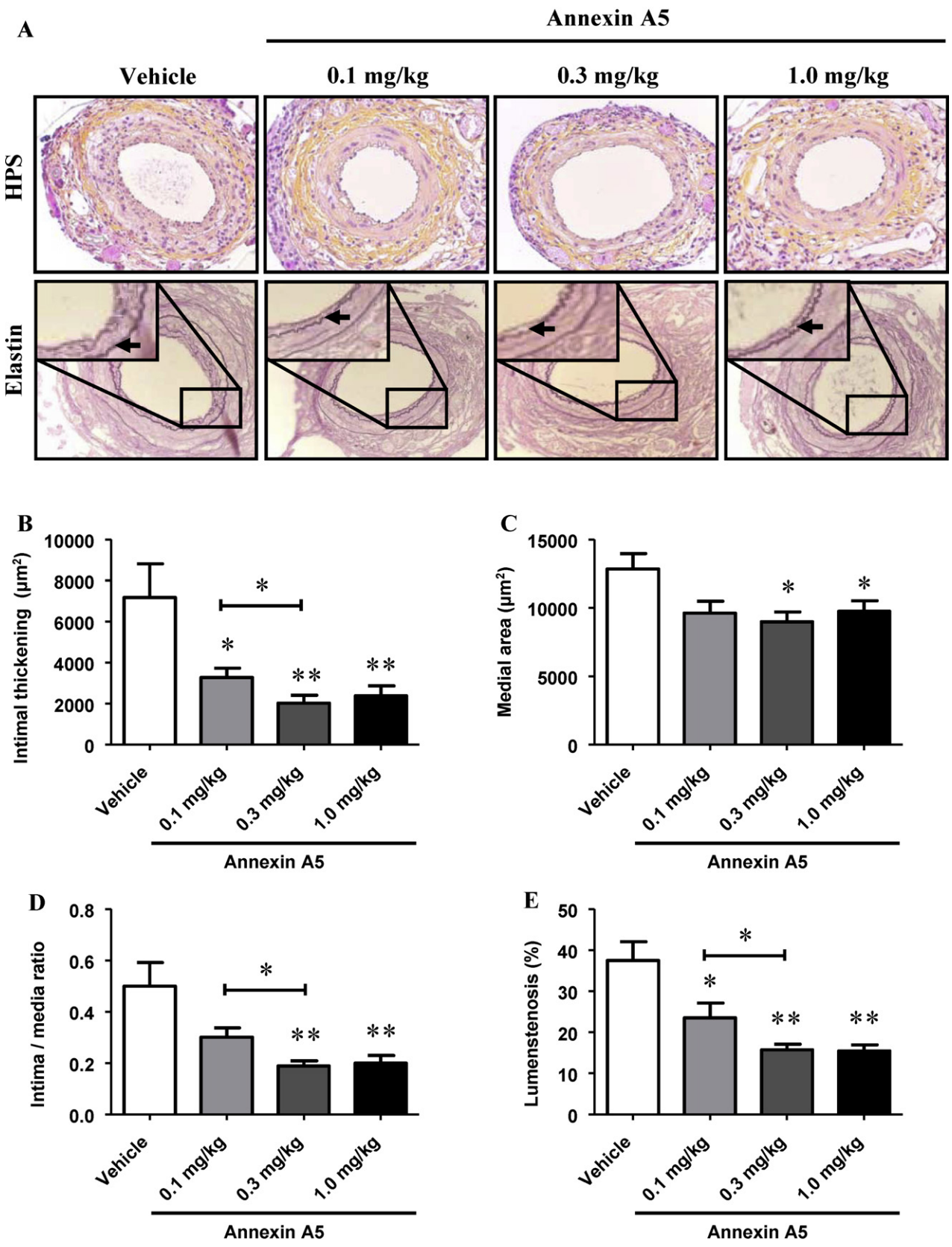

Fig. 3. Annexin A5 reduces accelerated atherosclerosis development in a dose-dependent fashion. Representative cross-sections of cuffed arteries of ApoE$^{-/-}$ mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 (A) after 14d (HPS and Weigert's elastin staining, magnification 40×, arrows indicate internal elastic laminae). Quantification of intimal thickening (μm^2) (B), medial area (μm^2) (C), intima/media ratio (D) and luminal stenosis (%) (E). Results indicated as mean ± SEM, $n = 10$. $^*p < 0.05$, $^{**}p < 0.01$.

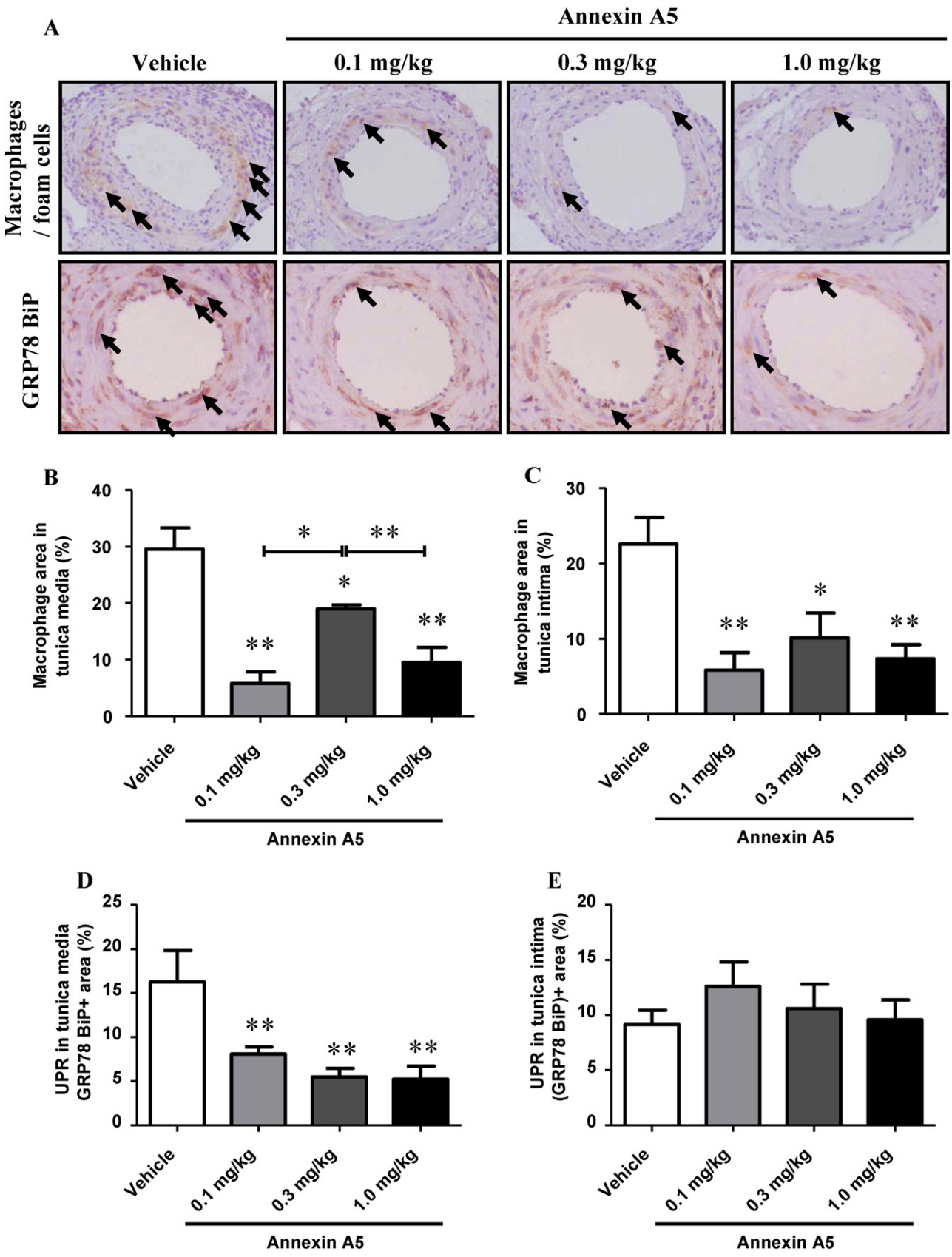

Fig. 4. Annexin A5 leads to a less-inflammatory phenotype with reduced intravascular signs of ER-stress. Representative cross-sections of cuffed arteries of ApoE$^{-/-}$ mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 (A) after 14d (macrophages and GRP78 BiP staining, magnification 40×, arrows indicate positive staining) and quantification of medial (B) and intimal (C) macrophage/foam cell area (%) and medial (D) and intimal (E) GRP78 BiP expression (%). Results indicated as mean ± SEM, $n = 10$. $^{*}p < 0.05$, $^{**}p < 0.01$.

1.35, Fig. 1A). This genetic variance in addition to plasma levels [19] would allow for excellent stratification of patients that are most at risk for restenosis development, enabling individual tailor-made treatment strategy. Additionally, our results support the notion that genetic programming of not only pro-inflammatory mediators, but also the endogenous anti-inflammatory system exerts a significant role in post-interventional remodeling.

In this study, a perivascular cuff-mediated arterial injury model was applied, which allows for quick and reproducible lesion formation with continuous blood flow in a patent vessel segment, although the perivascular approach rather differs from clinical endovascular injury through balloon inflation and stent deployment during PCI. This perivascular approach could affect the amount of exposure of subendothelial thrombogenic material and thrombosis, which are important targets for AnxA5.

Therapeutic effects were shown to most likely result from local AnxA5 binding to activated cells in the injured vascular segment. Local AnxA5 can reduce adherence of platelets leukocytes and eventually prevent their inflammatory activation, with reduced signs of ER-stress and the UPR within these cells. We found reduced GRP78/BiP expression in the tunica media (Fig. 4D) but not in the intima (Fig. 4E) Prolonged intracellular cholesterol storage leads to increased ER stress in cells, which is more likely to occur in foam cells than in early monocyte/macrophages. In this study, such cells should predominantly be found among cells that have migrated towards the tunica media, which in turn may explain the difference between GRP78/BiP expression between the media and intima layers.

The fact that clearance of AnxA5 is much slower from the arterial wall than from plasma [30] and accumulates in the injured vascular wall after systemic injection [23], supports the hypothesis that AnxA5 could act anti-inflammatory in levels lower than originally investigated (<1.0 mg/kg). Current results confirm this, with AnxA5 already effective in reducing leukocyte (Fig. 2B) and macrophage (Fig. 2C) recruitment and intimal thickening (Fig. 3B) in dosages 3–10 times lower than previously investigated. This would favour clinical application, where undesired side-effects can be kept to a minimum.

In conclusion, this study shows that systemic AnxA5 treatment strongly influences post-interventional accelerated atherosclerosis development and can dose-dependently prevent vascular remodeling. AnxA5 has previously been successfully applied to diagnose atherosclerotic patients non-invasively [19]. These results therefore may have important clinical implications. Immune-mediated interventions directed towards therapeutically controlling the leukocyte recruitment and vascular remodeling process could strongly benefit from systemic AnxA5, which could be applied in an early phase following revascularization or bypass grafting to prevent accelerated atherosclerosis development. AnxA5 SNPs could function as biomarkers in the assessment of restenosis risk in patients undergoing PCI, improving patient screening. Together, these data indicate high clinical potential for AnxA5 against post-interventional remodeling.

Disclosures

K. Pettersson is an employee of Athera Biotechnologies, Stockholm, Sweden. None of the other authors has any disclosure to report.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2012.01.037.

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