Chapter 5

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.

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 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.

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 the supplemental material.

Results

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), pallelic=0.011 and rs6830321, OR=1.35 (95% CI 1.10-1.64), pallelic=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 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.

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

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

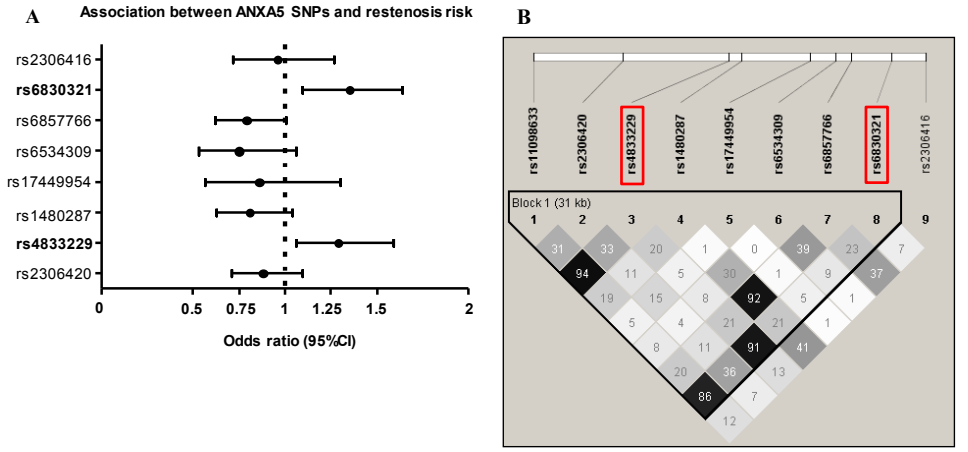


Figure 1. AnxA5 is a genetic risk marker for clinical restenosis after PCI. Association results for the allelic test for eight SNPs in the ANXA5 gene (A). LD plot shows that rs4833229 and rs6830321 SNPs are in high LD (r^2 = 0.91) (B).

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.

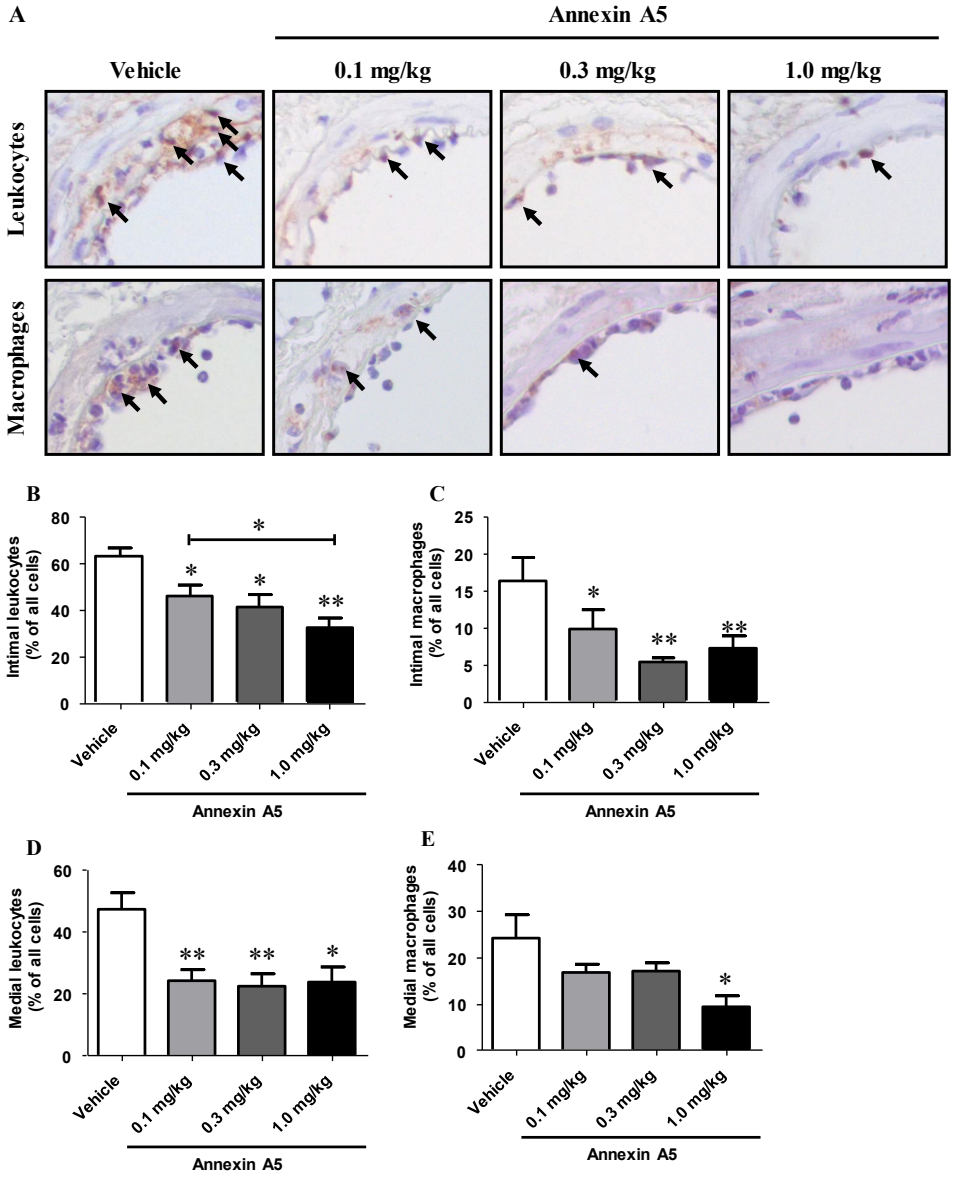
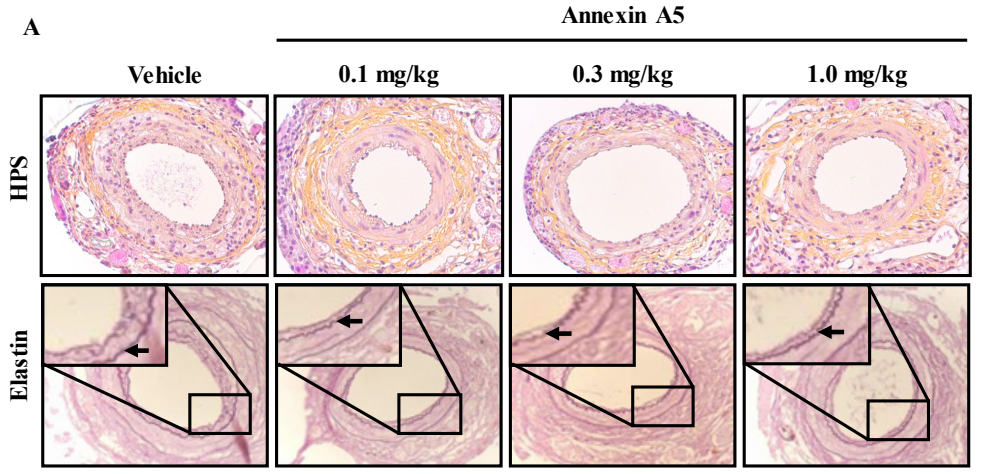


Figure 2. Annexin A5 dose-dependently prevents leukocyte recruitment after vascular injury. Representative cross-sections of cuffed-femoral arteries of ApoE$^{-/-}$ mice treated with vehicle or 0.1, 0.3 or 1.0 mg/kg/d AnxA5 (leukocyte and macrophage staining, magnification 80x, arrows indicate positive 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.

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, B).



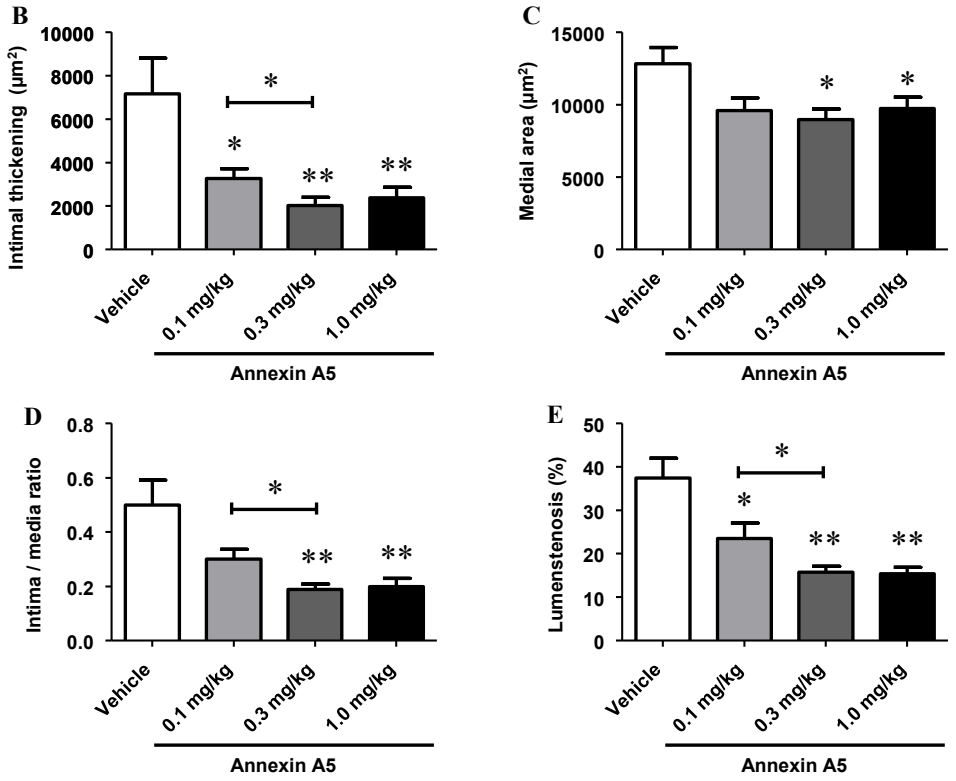


Figure 3. Annexin A5 reduces accelerated atherosclerosis development in a dose-dependent fashion. Representative cross-sections of cuffed arteries of ApoE$^{-/-}$ mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 (A) after 14d (HPS and Weigert's elastin staining, magnification 40x, arrows indicate 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. * $p<0.05$, ** $p<0.01$.

IHC showed profound intravascular macrophages/foam cell areas, which co-localized with AnxA5 (supplementary fig IIA, 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 up-regulation 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).

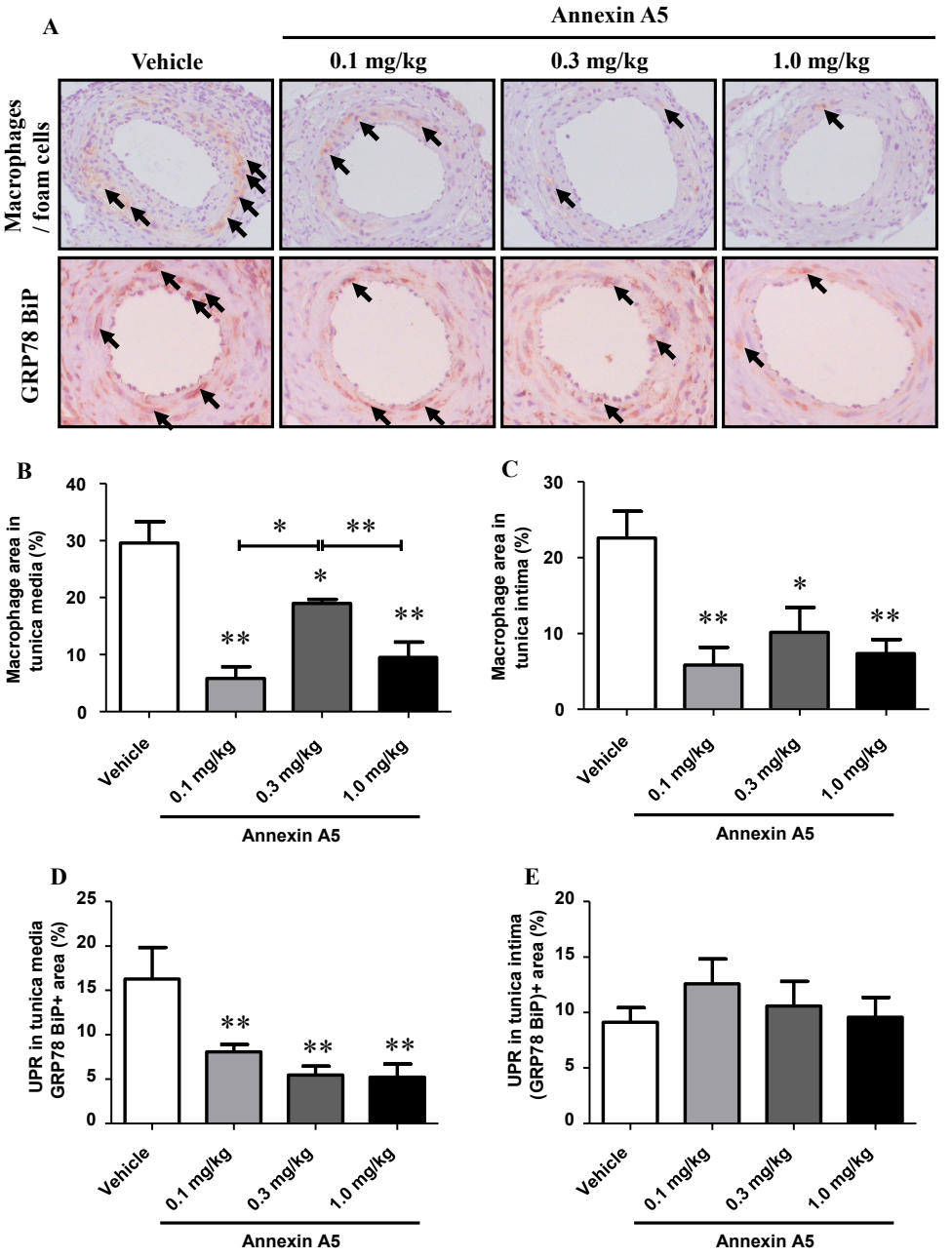


Figure 4. Annexin A5 leads to a less-inflammatory phenotype with reduced intravascular signs of ER-stress. Representative cross-sections of cuffed arteries of ApoE$^{-/-}$ mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 (A) after 14d (macrophages and GRP78 BiP staining, magnification 40x, arrows 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.

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 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.

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Online supplements

Materials and Methods

GENDER project

The GENetic DEterminants of Restenosis (GENDER) study was designed to investigate the association between genetic polymorphisms and clinical restenosis[1]. In brief, it is a large multicenter prospective follow-up study conducted during 1999-2001 and comprised of patients treated successfully by percutaneous coronary intervention (PCI) for an acute coronary syndrome. Clinical restenosis was established during a nine-month follow-up period for death, myocardial infarction and target vessel revascularization (TVR), which occurred in 9.8% of all patients. Eight Single Nucleotide Polymorphisms (SNPs) included in the AnxA5 gene were extracted from the GENDER genome wide association study (GWAS) dataset[2] composed of 866 patients (295 cases that developed restenosis following PCI and 571 controls that did not develop restenosis after PCI). The GWAS was conducted using Illumina Human 610-Quad Beadchips (Illumina) and the infinium II assay, following the manufacturer's instructions. After genotyping, samples and genetic markers were subjected to a stringent quality control protocol, described in detail elsewhere[2]. The open source software PLINK[3] was used to perform genetic association analysis. All p values were corrected for multiple testing. For linkage disequilibrium (LD) analyses in terms of r^2 and haplotype block delineation, we used Haploview software[4].

Mice

All experiments were approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center (LUMC). $ApoE^{-/-}$ mice, purchased from the Jackson Laboratory (Bar Harbor) on a C57BL/6J background were used for these studies. All animals were 10-12 weeks at the start of a dietary run-in period before surgery. $ApoE^{-/-}$ mice were fed a Western-type diet containing 0.15% cholesterol (Lantmännen Lantbruk, diet R638). The diet was given three weeks prior to surgery and was continued throughout the entire experiment. All animals received food and water ad libitum during the experiment.

Femoral artery cuff mouse model

Mice were subjected to arterial femoral arterial cuff placement to induce intimal thickening and accelerated atherosclerosis development, as described previously[5-7]. In brief, animals were anesthetized before surgery with a combination of intraperitoneally (IP)-injected Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen). The right femoral artery was isolated and sheathed with a rigid non-constrictive polyethylene cuff (Portex, 0.40mm inner diameter, 0.80mm outer diameter and an approximate length of 2.0mm).
Animals received vehicle (0.9% sterile NaCl) or AnxA5 (Athera Biotechnologies AB) through IP injection. Three and 14 days after cuff placement, mice were anesthetized as before and euthanized. At sacrifice, blood was drawn in EDTA collection tubes (Sarstedt B.V.) and centrifuged at 6000 r.p.m. for 10 min at 4°C to obtain plasma, which was stored at -20°C. Next, the thorax was opened and mild pressure-

perfusion (100mm Hg) with phosphate-buffered saline for 5 min by cardiac puncture in the left ventricle. After perfusion, the cuffed femoral artery was harvested, fixed in 3.7% formaldehyde in water (w/v) and paraffin-embedded. Serial cross-sections (5 µm thick) were made from the entire length of the artery for analysis.

Biochemical analysis
Total plasma cholesterol (Roche Diagnostics, kit 1489437) concentration was measured enzymatically before randomization at surgery.

Quantification of cuffed femoral artery lesions
Immunohistochemical (IHC) staining was performed using positive and negative tissue-specific controls as indicated by the antibody manufacturer. Samples were stained with hematoxylin-phloxine-saffron (HPS) and specific vessel wall composition was visualized for elastin (Weigert's elastin staining) and with antibodies against GRP78 BiP (1:200, Abcam, to identify cells displaying signs of UPR), CD45 for leukocytes (1:200, Pharmingen), MAC3 for monocytes/macrophages/foam cells (1:200, BD Biosciences) and anti-annexin V for injected protein accumulation (1:100, BioVision). Using image analysis software (Leica Qwin), total cross-sectional medial area was measured between the external and internal elastic laminae; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina, as was GRP78 BiP+ surface area. The luminal stenosis is expressed as the percentage of surface area (μm^2) within the internal elastic lamina (comprised of the luminal and neointimal areas) that is taken up by neointimal tissue (in μm^2). The number of leukocytes and monocytes/macrophages attached to the endothelium, within the neointimal tissue or infiltrated in the medial layer of the femoral arteries was quantified and is displayed as a percentage of the total number of present cells. All quantification in this study was performed on six equally spaced (150 µm distance) serial stained perpendicular cross-sections throughout the entire length of the vessel and was performed by blinded observers.

Statistical analysis
All data are presented as mean± standard error of the mean (SEM). Association between clinical outcome and individual SNPs was tested using an allelic association test. Groups were compared using a Mann-Whitney sum test for non-parametric data. Total plasma cholesterol concentrations in time were compared using a Wilcoxon matched pairs test. All statistical analyses were performed with SPSS 17.0 software for Windows or using Prism software. P-values <0.05 were regarded as statistically significant and are indicated with an asterisk (*).

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Supplemental figures

Group	Total plasma cholesterol (mmol/L)	
	Surgery	Sacrifice
Early time point (3d)		
Vehicle	19.5±1.3	25.0±0.8
1.0 mg/kg annexin A5	15.4±0.4	19.8±1.6
0.3 mg/kg annexin A5	16.6±0.6	20.0±1.7
0.1 mg/kg annexin A5	19.6±1.8	22.8±2.2
Late time point (14d)		
Vehicle	23.7±2.7	21.1±2.4
1.0 mg/kg annexin A5	18.0±2.7	17.1±2.7
0.3 mg/kg annexin A5	19.9±4.1	19.2±3.3
0.1 mg/kg annexin A5	19.3±1.6	19.8±1.6

Table I. Plasma cholesterol in mice undergoing annexin A5 dose-response investigations.
Plasma total cholesterol (mmol/L) of ApoE$^{-/-}$ mice receiving vehicle or annexin A5 (1.0, 0.3 or 0.1 mg/kg/d) trough IP injection, measured at surgery or at sacrifice (day 3 or 14). No significant differences were observed (mean±SEM, n=10).

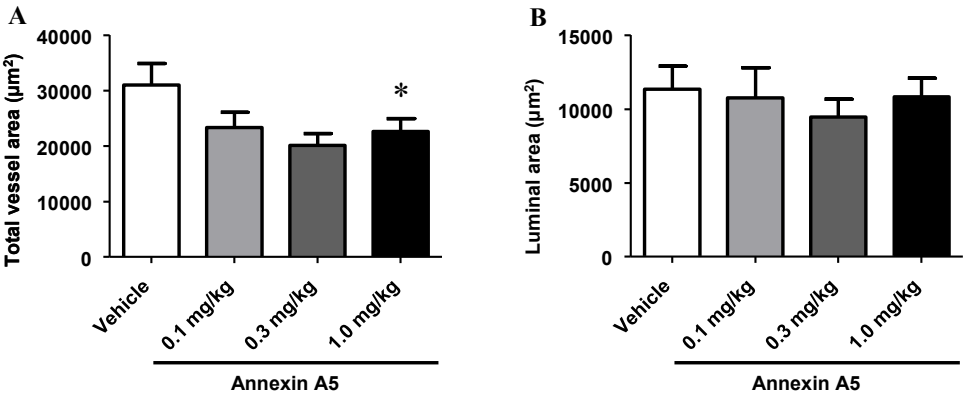

Figure I. Annexin A5 reduces accelerated atherosclerosis development in a dose-dependent fashion. Quantification of total vessel area (µm^2) (A) and luminal area (µm^2) (B) in ApoE$^{-/-}$ mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 after 14d. Results indicated as mean±SEM, n=10. * p<0.05, ** p<0.01, n.s. not significant.

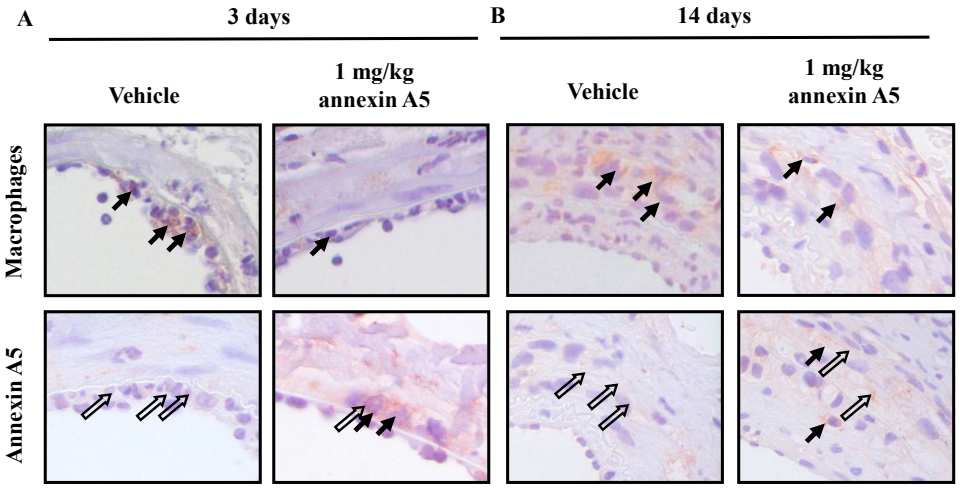

Figure II. Co-localization of injected AnxA5 at macrophage (3d) and macrophage/foam cell (14d) areas after 3d (A) and 14d (B). Representative cross-sections of cuffed arteries of ApoE$^{-/-}$ mice receiving vehicle or 1.0 mg/kg AnxA5 (MAC3 and annexin A5 staining, magnification 80x, closed arrows indicate positive macrophage and AnxA5 staining, open arrows indicate projected macrophages in consecutive slides (5 µm distance) in annexin A5 stained femoral artery cross-sections to indicate co-localization).