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Annexin A5 haplotypes in familial hypercholesterolemia: Lack of association with carotid intima-media thickness and cardiovascular disease risk

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ABSTRACT

Objective: Annexin A5 (ANXA5) has been suggested to possess antiatherogenic properties. We investigated whether *ANXA5* genetic variations and plasma ANXA5 levels were associated with carotid atherosclerosis and contributed to cardiovascular disease (CVD) risk in patients with familial hypercholesterolemia (FH). **Methods**: We sequenced the promoter region and exon 2 of *ANXA5* in 284 FH patients from the ASAP (Atorvastatin versus Simvastatin on Atherosclerosis Progression) trial. Common haplotypes (H) were constructed based on seven single nucleotide polymorphisms (SNPs). We studied whether plasma ANXA5 levels or *ANXA5* haplotypes were associated with the extent of atherosclerosis (evaluated by carotid intima-media thickness (IMT). The association between *ANXA5* haplotypes and the risk for CVD events was investigated in 1730 FH patients from the GIRaFH (Genetic Identification of Risk factors in Familial Hypercholesterolemia) study. **Results**: In ASAP, individuals carrying the *ANXA5* haplotype H2 exhibited lower plasma ANXA5 levels, whereas H4 carriers had increased levels of circulating ANXA5 compared to non-carriers. Plasma ANXA5 levels were not associated with carotid IMT. None of the four *ANXA5* haplotypes correlated with the age-related IMT progression (ASAP study) or contributed to CVD risk (GIRaFH cohort). **Conclusions**: Both *ANXA5* haplotypes and plasma ANXA5 levels were not associated with carotid IMT progression or CVD risk in FH patients.

1. Introduction

Annexin A5 (ANXA5), a calcium-dependent phospholipid-binding protein, is highly expressed by endothelial cells [1] and present in atherosclerotic plaques, especially at sites with high prothrombotic potential [2,3]. ANXA5 is considered to have antithrombotic capacities, by virtue of its scavenging effect on anionic phospholipids and downregulation of the surface expression of tissue factor [4–7]. Since atherosclerotic vessel walls contain apoptotic and activated cells that expose negatively charged phospholipids [8,9], ANXA5 may form an antithrombotic shield on these cell surfaces, thereby restricting their tendency to induce thrombus formation. Apart from its antithrombotic property, ANXA5 has also been shown to exert potent anti-inflammatory activities, which are attributed to downregulation of interferon-gamma (IFN-γ)-mediated inflammatory cellular responses [10]. In addition, ANXA5 inhibits phospholipase A2 activity, an enzyme essential for the generation of pro-inflammatory mediators [11,12]. Concomitantly, it has been shown that ANXA5 binds to oxidized low-density lipoprotein (oxLDL) thereby preventing the ox-LDL induced procoagulant and pro-inflammatory effects [13]. In proatherogenic *ApoE*$^{-/-}$ mouse models, it has been demonstrated that ANXA5 administration reduces local inflammation and vascular remodeling as well as improves vascular function, confirming the notion that ANXA5 has antiatherogenic effects [14,15].

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Additionally to the biochemical properties of ANXA5, attention is paid to a number of single nucleotide polymorphisms (SNPs) in *ANXA5* but so far, little is known about their relative contribution to cardiovascular disease (CVD) risk. The minor T-allele of rs1131239, which is located in the Kozak sequence (i.e., one nucleotide upstream of the ATG initiation codon) in exon 2 of *ANXA5*, is associated with a decreased risk of myocardial infarction under the age of 45 [16] and a lower risk of a new thrombotic event during 36 months follow-up [17]. Increased plasma ANXA5 levels in rs1131239T-allele carriers were found to be protective in the occurrence of arterial thrombosis. Subsequent studies, however, were unable to replicate these findings [18,19]. Moreover, *ANXA5* intronic SNPs rs4833229 and rs6830321 are associated with increased restenosis risk in patients undergoing percutaneous coronary intervention for atherosclerosis [15]. Recently, Bogdanova and colleagues described the three *ANXA5* promoter haplotypes (N, M1 and M2) and found that the *ANXA5* M2 haplotype consisting of the minor alleles of four SNPs (rs112782763, rs28717001, rs28651243, rs113588187) reduces *ANXA5* promoter activity in a luciferase reporter gene assay [20]. While the *ANXA5* M2 haplotype has been linked to thrombotic obstetric complications [20–22], its contribution to CVD risk remains to be determined. More recently, we demonstrated that plasma ANXA5 levels in healthy individuals are affected by genetic variants in *ANXA5*. Haplotype H2 was associated with significantly decreased plasma ANXA5 levels whereas haplotype H4 was associated with increased plasma ANXA5 levels [23]. Our defined haplotypes H3 and H4 are extensions of the M2 and M1 haplotypes, and haplotypes H1 and H2 include the wild-type haplotype N described by Bogdanova et al. The clinical relevance of plasma ANXA5 levels on atherosclerotic CVD burden is yet unclear. Hypothetically, higher plasma ANXA5 levels are expected to have a protective role, whereas lower ANXA5 levels are likely to be associated with progression of atherosclerosis (i.e., an increased plaque formation and an increased CVD risk). In this context, it has been shown that circulating ANXA5 levels correlate inversely with the severity of angiographically determined coronary stenosis and the extent of atherosclerotic plaque formation [24].

In this study, we set out to test the hypothesis that plasma ANXA5 levels and *ANXA5* genetic variations are associated with carotid atherosclerosis and contribute to CVD risk in patients with familial hypercholesterolemia (FH), an autosomal dominant disease characterized by high plasma levels of low-density lipoprotein cholesterol (LDL-C) and an increased CVD risk.

2. Materials and methods

2.1. ASAP trial

ASAP (Atorvastatin versus Simvastatin on Atherosclerosis Progression) was a randomized, double-blind, two-center (Amsterdam and Nijmegen) study [25]. A total of 325 FH patients aged 30–70 years participated in the ASAP study. Eligibility of the patients was based on plasma LDL-C levels (>5.5 mmol/L) and the absence of significant clinical, hematological and biochemical abnormalities. Exclusion criteria were coronary heart disease within previous 3 months, hypertension, secondary hyperlipidemia, diabetes and other endocrine diseases [25]. The Ethics Committees of both trial centers approved the study. In the present study, we used only data collected after an 8-week placebo run-in period before starting any intervention with statins. Carotid IMT measurements were performed as described [25]. Briefly, ultrasound examinations were performed using a Biosound Phase-2 real time scanner (BiosoundEsaote, USA) equipped with a 10 MHz transducer. Three 10 mm segments were scanned bilaterally: the distal portion of the common carotid artery (CCA), the carotid bifurcation (BUL) and the proximal portion of the internal carotid artery (ICA). Both near and far walls were evaluated. Mean carotid IMT was calculated as averaged over anterior and posterior walls in the CCA, BUL and the posterior wall of the ICA, bilaterally (i.e., the mean from available 10 sites). Of the 325 participants, genomic DNA of 299 patients was available for sequencing in this study.

2.2. GIRaFH cohort

GIRaFH (Genetic Identification of Risk factors in Familial Hypercholesterolemia) was a retrospective, multicenter (27 Dutch lipid clinics) study including 2400 unrelated heterozygous FH patients of Caucasian origin as previously described [26]. The mean follow-up period was 5 years. The primary outcome of the study was the combination of cardiovascular mortality and CVD as described [26]. Genomic DNA of 1994 FH patients was available for genotyping in the present study. The Ethics Institutional Review Board of each participating hospital approved the study.

2.3. Genetic analysis

In the ASAP trial, a 496-bp fragment of the *ANXA5* promoter (261 base pairs upstream and 235 base pairs downstream of the first transcription start point) was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers: forward 5′CCGAGCCCTGGACAGCTCCCCA-3′ and reverse 5′-GCCCCGCGACCACGCTCTCCTCT-3′ as described [20]. Exon 2 with flanking regions (130-bp fragment) was amplified as previously described [27]. Purified amplicons were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on the ABI 3730 PRISM DNA Analyzer (Applied Biosystems). We evaluated six SNPs within the *ANXA5* promoter (rs62319820, rs112782763, rs28717001, rs28651243, rs113588187, rs1050606) and rs1131239 located in the Kozak sequence (exon 2) to reconstruct the four known haplotypes as described [23]. Successful sequencing of the promoter region and exon 2 was possible in 284 (95.0%) and 298 (99.7%) patients, respectively.

In the GIRaFH study, we genotyped four *ANXA5* SNPs (rs62319820, rs113588187, rs1050606, rs1131239) to reconstruct the four common *ANXA5* haplotypes. Genotyping was carried out using predesigned or custom TaqMan primers with FAM or VIC as fluorophores (Applied Biosystems, USA). SNP genotyping success rates were 93.5% (1864 patients) for rs62319820, 94.6% (1886 patients) for rs113588187, 92.8% (1850 patients) for rs1050606 and 95.9% for rs1131239 (1912 patients).

2.4. Biochemical measurements

Total cholesterol, (calculated) LDL-C, high-density lipoprotein cholesterol (HDL-C) and triglycerides were determined by standard methods as previously described [25,26]. High-sensitivity C-reactive protein (hs-CRP) was measured in citrated plasma by a commercially available enzyme-immunoassay (Dako, Denmark) as described [28]. Plasma ANXA5 levels were measured in 141 participants from the ASAP study using a commercially available Zymutest ANXA5 ELISA (Hyphen Biomed). The intra- and inter-assay coefficients of variation (CV) for ANXA5 measurements were 3.1% and 3.8% respectively.

2.5. Statistical analyses

The means of continuous variables of different groups were compared by unpaired *t*-test or one-way ANOVA as appropriate; the χ^2 test was used for categorical variables. Hardy–Weinberg

equilibrium for each SNP was evaluated by the χ^2 test. Haploview software (Broad Institute, Cambridge, MA, USA) was used to estimate the degree of linkage disequilibrium (LD; r^2 values) between all SNP pairs and to determine haplotypes (H). Haplotypes were assigned manually to the subjects with complete genetic data as described [23]. Only common haplotypes (frequency >1%) were used for statistical analyses. In the present study, we included 284 FH patients from the ASAP study as well as 1730 FH patients from the GIRaFH cohort in whom common haplotypes were constructed. Power calculations were performed to determine the minimal odds ratio detectable with a power >80% (Quanto version 1.2.4 software).

In the ASAP study, the age-related IMT progression in different *ANXA5* haplotype groups was estimated by a linear regression analysis (SAS version 6.12 software). The interaction term (haplotype group * age) was entered in the regression models to account for different IMT progression rates with age between *ANXA5* haplotype groups. The variable gender associated with IMT in a univariable regression model was included in the multiple regression model. The regression coefficient β represents IMT increase with age (millimeters per year, mm/year). Comparison of the regression slopes between groups was performed by testing of the interaction terms. In the single SNP analyses, the slopes of the major allele carriers were taken as the reference. In the haplotype model, we performed one-way ANOVA for each haplotype to address the difference in regression slopes within the haplotype group. To control for the familywise error rate over the 4 haplotypes, the level of significance for each interaction test was set at $0.05/4 = 0.0125$ (Bonferroni correction for multiple testing). When the *p*-value of the interaction test was less than 0.0125, further analysis with *t*-test was warranted to assess the difference between two specific slopes (e.g. carriers and non-carriers).

The distribution of plasma ANXA5 levels was normalized by ln-transformation and used in all analyses. A one-way ANOVA followed by the Bonferroni Post Hoc test was performed to assess differences in plasma ANXA5 levels within haplotype groups. The Bonferroni threshold for correction for multiple testing was estimated at $0.05/4 = 0.0125$, taking into account the number of haplotypes (four). All values reported were reconverted to geometric means with the appropriate 95% confidence interval (CI). The association of plasma ANXA5 levels with carotid IMT was tested by a linear regression analysis and Pearson correlation. To test the relationship between circulating ANXA5 levels and total cholesterol, triglycerides, HDL-C, LDL-C and hs-CRP, we calculated Pearson's correlation coefficients. Skewed distributed variables (triglycerides and hs-CRP) were also ln-transformed prior to analysis.

In the GIRaFH study, the contribution of *ANXA5* variations to CVD risk was examined by Cox proportional hazards regression. The follow-up period started at birth and ended at the first occurrence of established fatal or non-fatal CVD event. Patients without CVD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CVD. In all analyses, we included year of birth, sex and smoking in the Cox regression models.

3. Results

3.1. Patient characteristics of the ASAP study

Clinical characteristics of 284 FH patients participating in the ASAP study are shown in Table 1. Baseline characteristics of the study group were comparable to those of the total ASAP cohort (data not shown) [25]. The population consisted of Caucasian individuals with the mean age of 48.4 years; sixty percent of the subjects were female. Twenty-nine percent of the patients had a history of CVD, and 60.6% individuals were smokers (former and current).

3.2. Effect of plasma ANXA5 levels on progression of atherosclerosis in ASAP

We investigated the association between plasma ANXA5 levels and carotid IMT in a subset of subjects ($n = 141$) randomly selected from the ASAP cohort. Plasma ANXA5 levels did not differ significantly between male ($n = 50$) and female ($n = 91$) subjects (geometric mean 13.17 μg/L, 95% CI: 11.01–15.75 versus 12.81 μg/L, 95% CI: 10.98–14.94, $p = 0.82$) and were not influenced by age (linear regression: $\beta = -0.009$, $p = 0.10$). Plasma ANXA5 levels did not correlate with cholesterol (total, HDL, LDL) levels, triglycerides levels and an inflammation marker hs-CRP (Supplementary Table 1). No association was found between plasma ANXA5 and carotid IMT (linear regression: $\beta = -0.01$, $p = 0.8$).

3.3. ANXA5 SNPs and haplotypes in ASAP

Supplementary Table 2 shows the minor allele frequencies and the genotype frequencies of the seven SNPs in *ANXA5* in the ASAP cohort. All SNPs were in Hardy–Weinberg equilibrium. Haploview analysis showed a high degree of linkage disequilibrium between all SNPs except for SNP1 and SNP6 (Supplementary Fig. 1). SNP3 and SNP4 as well as SNP2 and SNP5 were completely linked ($r^2 = 1$). SNP7 was tightly linked to SNP2 and SNP5 ($r^2 = 0.85$). Based on the seven polymorphisms, the four previously reported common haplotypes were constructed (Table 2). Haplotype H1, the most frequent haplotype (51%), was composed of the major alleles of all seven polymorphisms. Haplotype H2 was discriminated from haplotype H1 by rs1050606 (SNP6). Haplotype H3 was the third major haplotype (9.9%) and consisted of the major allele of rs62319820 and the minor alleles of the other polymorphisms. Haplotype H4 compiled the minor alleles of rs62319820, rs28717001, rs28651243, rs1050606 and the major alleles of the three other SNPs. Besides the common haplotypes, three rare haplotypes (H5, H6, H7; frequency <1%) were identified in nine patients (Table 2). They were excluded for further analyses.

Table 1
Baseline characteristics of FH patients from the ASAP study.

Characteristics	$n = 284$
Age, years	48.4 ± 10.4
Gender, male/female, *n* (%)	114 (40.1)/170 (59.9)
History of CVD, *n* (%)	82 (28.9)
Smoking, *n* (%)[a]	172 (60.6)
Body mass index, kg/m^2	25.7 ± 3.5
Total cholesterol, mmol/L	10.13 ± 1.99
HDL-C, mmol/L	1.16 ± 0.31
LDL-C, mmol/L	8.17 ± 1.96
Triglycerides, mmol/L	1.64 (1.12–2.28)
Hs-CRP, mg/L	2.2 (0.8–4.6)
Carotid IMT, mm[b]	0.93 ± 0.22
CCA-IMT, mm[b]	0.87 ± 0.17

Values are presented as means ± SD or *n* (%). Triglycerides and hs-CRP are given as median (interquartile range).
CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; CCA-IMT, common carotid artery intima-media thickness; mm, millimeter.
[a] Previous and current smokers.
[b] Data of 282 patients are shown.

Table 2
Polymorphisms and haplotypes of the *ANXA5* gene in 284 FH patients from the ASAP trial.

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	
	g.-628C>T c.-390C>T	g.-467G>A c.-229G>A	g.-448A>C c.-210A>C	g.-422T>C c.-184T>C	g.-373G>A c.-135G>A	g.-302T>G c.-64T>G	g.-1C>T c.-1C>T	
dbSNP ID	rs62319820	rs112782763	rs28717001	rs28651243	rs113588187	rs1050606	rs1131239	
Haplotype								Haplotype frequency
H1	C	G	A	T	G	T	C	0.51
H2	C	G	A	T	G	**G**	C	0.29
H3	C	**A**	**C**	**C**	**A**	**G**	**T**	0.099
H4	**T**	G	**C**	**C**	G	**G**	C	0.09
H5	C	**A**	**C**	**C**	**A**	**G**	C	0.007
H6	C	G	A	T	G	**G**	**T**	0.005
H7	C	G	**C**	**C**	G	**G**	**T**	0.002

Nucleotide numbering from the ATG initiation codon; SNP, single nucleotide polymorphism; dbSNP indicates NCBI database for SNPs (http://www.ncbi.nlm.nih.gov/snp); minor alleles in bold and underlined.

3.4. *ANXA5* haplotypes and plasma ANXA5 levels

Among the ASAP participants, a one-way ANOVA corrected for multiple testing revealed significant differences in plasma ANXA5 levels within haplotypes H2 and H4 (Table 3). Haplotype H2 was associated with decreased plasma ANXA5 levels. Post Hoc analysis indicated that homozygous H2 carriers had lower ANXA5 levels (mean 6.20 μg/L, 95% CI: 4.24–9.05) compared to heterozygous subjects (mean 13.33 μg/L, 95% CI: 11.13–15.94, $p < 0.001$) and non-H2 subjects (mean 14.25 μg/L, 95% CI: 12.18–16.69, $p < 0.001$). There were no differences in plasma ANXA5 levels between H2 heterozygotes and non-H2 individuals ($p = 0.568$).

Furthermore, non-H4 individuals (mean 10.98 μg/L, 95% CI: 9.73–12.39) had a 2-fold lower circulating ANXA5 levels compared to homozygous H4 carriers (mean 29.67 μg/L, 95% CI: 15.53–44.44, $p = 0.007$) and heterozygous H4 subjects (mean 24.48 μg/L, 95% CI: 19.99–30.02, $p < 0.001$). Plasma ANXA5 levels were not different between homozygotes for H4 and heterozygotes for H4 ($p = 0.615$).

With respect to the relationship of carotid IMT with lower plasma ANXA5 levels in H2 carriers as well as with higher plasma ANXA5 levels in H4 carriers, additional analyses did not reveal any statistical evidence for such association which might also be explained by the low number of cases in these subgroup analyses.

3.5. *ANXA5* haplotypes and progression of atherosclerosis in ASAP

A linear regression analysis approach was applied to study the association between *ANXA5* variations and age-related carotid IMT (i.e., the average of available IMT values measured in three carotid segments).

None of the *ANXA5* SNPs was associated with the age-related increase in carotid IMT (Supplementary Table 3).

Of the four haplotypes studied, the *ANXA5* haplotype H3 tended to be associated with more rapid carotid IMT increase with age. Heterozygous haplotype H3 carriers tended to have a larger arterial wall thickening over time compared with non-H3 subjects, in both the unadjusted model ($\beta = 0.0129$ mm/year versus $\beta = 0.0072$ mm/year, $p = 0.053$) and after adjustment for gender ($\beta = 0.0135$ mm/year versus $\beta = 0.0076$ mm/year, $p = 0.044$) (Table 4). However, this association did not reach Bonferroni-corrected statistical significance level. Other *ANXA5* haplotypes did not demonstrate any association with the age-related IMT progression (Table 4).

3.6. *ANXA5* haplotypes and the risk of cardiovascular events

To confirm the results from the ASAP study, we investigated the contribution of *ANXA5* variations to the risk of cardiovascular events in an independent cohort of 1730 FH patients from the GIRaFH study. Four *ANXA5* SNPs covering the four common *ANXA5* haplotypes were genotyped. All SNPs were in Hardy–Weinberg equilibrium (Supplementary Table 4). Neither individual SNPs nor the common haplotypes were associated with CVD risk (Table 5 and Supplementary Table 5). No significant associations were found either with only homozygous wild type carriers (i.e., H1H1) as a reference category (data not shown). Subgroup analysis showed no significant associations between *ANXA5* variations and the history of myocardial infarction, angina pectoris, stroke/TIA or cardiac death (data not shown).

Table 3
Association of *ANXA5* haplotypes with plasma ANXA5 levels in FH patients from the ASAP study.

Haplotype	Number $n = 141$	Geometric mean ANXA5 (95% CI), μg/L	Overall p-value
Haplotype 1			0.875
H1H1	36	13.42 (10.90–16.53)	
H1Hx	69	13.00 (11.19–15.09)	
Non-H1	36	12.33 (9.07–16.78)	
Haplotype 2			0.0005
H2H2	12	6.20 (4.24–9.05)	
H2Hx	56	13.33 (11.13–15.94)	
Non-H2	73	14.25 (12.18–16.69)	
Haplotype 3			0.018
H3Hx	30	9.89 (7.48–13.09)	
Non-H3	111	13.90 (12.24–15.77)	
Haplotype 4			<0.0001
H4H4	3	29.67 (15.53–44.44)	
H4Hx	25	24.48 (19.99–30.02)	
Non-H4	113	10.98 (9.73–12.39)	

Hx indicates all haplotypes except for the one given; CI, confidence interval. Differences were assessed by one-way ANOVA; the threshold for Bonferroni correction for multiple testing: 0.05/4 haplotype groups = 0.0125.

4. Discussion

We investigated whether *ANXA5* gene variants and plasma ANXA5 levels were associated with atherosclerosis progression or CVD risk in FH patients. In our study, plasma ANXA5 levels were not associated with IMT. *ANXA5* haplotypes were associated with plasma ANXA5 levels, but did not correlate with carotid IMT parameters in the ASAP study or CVD risk in the GIRaFH cohort. Our data, therefore, showed a lack of association of ANXA5 protein levels as well as *ANXA5* haplotypes with carotid IMT progression or the risk of cardiovascular events in FH patients.

Table 4
Association of *ANXA5* haplotypes with the age-related carotid IMT progression in FH patients from the ASAP trial.

Haplotype	Unadjusted model		Adjusted model[a]	
	β (SE)	Overall *p*-value	β (SE)	Overall *p*-value
Haplotype H1		0.27		0.30
H1H1 ($n = 77$)	0.0062 (0.0024)		0.0066 (0.0023)	
H1Hx ($n = 128$)	0.0077 (0.0017)		0.0083 (0.0017)	
Non-H1 ($n = 68$)	0.0111 (0.0021)		0.0113 (0.0021)	
Haplotype H2		0.89		0.93
H2H2 ($n = 27$)	0.0100 (0.0038)		0.0100 (0.0037)	
H2Hx ($n = 107$)	0.0081 (0.0019)		0.0084 (0.0018)	
Non-H2 ($n = 139$)	0.0081 (0.0017)		0.0087 (0.0016)	
Haplotype H3		0.053		0.044
H3Hx ($n = 54$)	0.0129 (0.0026)		0.0135 (0.0026)	
Non-H3 ($n = 219$)	0.0072 (0.0013)		0.0076 (0.0013)	
Haplotype H4		0.48		0.55
H4H4 ($n = 4$)	0.0153 (0.0067)		0.0153 (0.0066)	
H4Hx ($n = 41$)	0.0067 (0.0027)		0.0075 (0.0026)	
Non-H4 ($n = 228$)	0.0084 (0.0013)		0.0088 (0.0013)	

Results of the linear regression analysis ($n = 273$) are shown. β indicates regression coefficient; β represents IMT increase with age (mm/year).
[a] Adjusted for gender. Hx means all haplotypes except for the one given; IMT, intima-media thickness; SE, standard error.

Table 5
Association of *ANXA5* haplotypes with CVD risk in FH patients from the GIRaFH study.

Haplotype	Number of subjects, $n = 1730$	CVD+ ($n = 548$) n (%)	HR (95% CI)[a]	*p*-value
Haplotype H1				
Non-H1	482	150 (27.4)	1.0	
H1Hx	883	278 (50.7)	0.9 (0.7–1.1)	0.5
H1H1	365	120 (21.9)	1.0 (0.8–1.3)	1.0
Haplotype H2				
Non-H2	777	257 (46.9)	1.0	
H2Hx	727	217 (39.6)	0.8 (0.7–1.0)	0.08
H2H2	226	74 (13.5)	1.0 (0.8–1.3)	0.9
Haplotype H3				
Non-H3	1358	424 (77.4)	1.0	
H3Hx	349	119 (21.7)	1.2 (1.0–1.5)	0.1
H3H3	23	5 (0.9)	0.8 (0.3–1.9)	0.6
Haplotype H4				
Non-H4	1463	465 (84.8)	1.0	
H4Hx	261	82 (15.0)	1.0 (0.8–1.3)	0.7
H4H4	6	1 (0.2)	NA	

CVD+, patients with cardiovascular disease; Hx, all haplotypes except for the one given; HR, hazard ratio; CI, confidence interval; NA, not applicable.
HRs were calculated with HxHx (i.e., non-H1, non-H2, non-H3 and non-H4) as a reference category (HR = 1.0).
[a] Adjusted for sex, year of birth and smoking.

A possible role of ANXA5 in the pathophysiology of atherosclerosis has been postulated based on the observations that ANXA5 has anti-atherogenic and anti-inflammatory properties and is found in high concentrations in atherosclerotic plaques. Of note, it has been shown that the uptake of labeled recombinant ANXA5 by atherosclerotic plaques correlates with the extent of apoptosis [29]. In this regard, reduced plasma ANXA5 levels in patients with severe coronary stenosis has been proposed to reflect the presence and extent of CVD [24]. Moreover, in systemic lupus erythematosus (SLE) patients, antiphospholipid antibody-mediated reduced binding of ANXA5 to endothelial cells was found to be associated with IMT and it has been suggested to be an important mechanism in SLE-related CVD [3]. These observations prompted us to investigate whether ANXA5 plasma levels and *ANXA5* gene variants are associated with atherosclerosis progression in a high risk population of FH patients. The FH patient population was chosen because of its homogeneity, characterized by elevated LDL-C levels and an increased risk of premature CVD [30]. We hypothesized that *ANXA5* variants associated with circulating plasma ANXA5 levels would predict clinical features of atherosclerosis and CVD risk in FH patients.

The four promoter polymorphisms (rs112782763, rs28717001, rs28651243, rs113588187) evaluated in this study are known functional SNPs [20]. The rs112782763, which is located 19 nucleotides upstream from the transcription start site 1 (tsp1) in the *gGCCc* sequence, affects the zinc finger binding of the MTF-1 (metal-regulatory) transcription factor [31]. The substitution rs28717001 changes the tsp1 itself. The third polymorphism rs28651243 located 27 nucleotides downstream from the tsp1 disrupts a SP1 (specificity protein 1 transcription factor) consensus. The rs113588187 destroys a *BamHI* restriction site in the close proximity of an *AP-4 (motif B)/MED-1* consensus, which in turn is essential for the full *ANXA5* promoter activity [20,32]. The *ANXA5* M2 haplotype (including our haplotype H3) comprising the four mentioned above promoter SNPs is related to a reduced *ANXA5* promoter activity *in vitro* [20] and reduced mRNA levels in placental tissues [33]. Altogether, it is thought that through reduced ANXA5 expression on the surface of placental trophoblasts and inefficient phospholipid shielding, this haplotype contributes to a prothrombotic placental environment [20]. In addition, SNP rs1131239 located in the *ANXA5* Kozak sequence was chosen since the minor rs1131239T-allele has previously been reported to be associated with a protective role in the risk for myocardial infarction [16,17]. Furthermore, we also evaluated the rs62319820, because its minor T-allele is known to be a major contributor to higher plasma ANXA5 levels in healthy controls [23].

We initially found a trend towards a larger arterial wall thickening over time in heterozygous H3 patients compared to non-H3 carriers in the ASAP study, suggesting a possible clinical relevance of *ANXA5* H3 in susceptibility to cardiovascular events. Remarkably, such a larger arterial wall thickness progression observed in *ANXA5* H3 carriers (13.5 μm/year after adjusting for sex) should contribute to a higher incidence of cardiovascular events, resulting in an increased CVD risk in these individuals. Unfortunately, no such effect was found while studying the CVD risk in a large population of FH patients from the GIRAFH study, which was designed to substantiate genetic risk factors in FH patients. Power calculations showed that the GIRaFH study had a ~85% power ($\alpha = 0.05$) to detect a clinically relevant odds ratio of 1.5 for *ANXA5* H3 (haplotype frequency of 0.10; an assumed prevalence of the disease of 30%). Since replication in a larger study including patients with a similar disease phenotype failed, we have to consider our initial finding, a trend for an association of *ANXA5* H3 with IMT in the ASAP study, as a false-positive result or as a type I error.

Evidence for an association between *ANXA5* genetic variants and carotid IMT or CVD risk in atherosclerosis is scarce. Recently, it has been shown a moderate association between *ANXA5* intronic SNPs rs4833229, rs6830321 and the risk on restenosis in patients undergoing percutaneous coronary intervention for atherosclerosis (odds ratio 1.29, $p_{\text{allelic}} = 0.011$ and odds ratio 1.35, $p_{\text{allelic}} = 0.003$, respectively) [15]. A possible explanation for the discrepancy with this study could be explained by the pathophysiology underlying FH. Since atherosclerosis is a multifactorial disease, the contribution of genetic variations to CVD risk should be considered in the context of a chronic inflammatory disease of the arterial wall. It is known that elevated plasma LDL-C levels in FH patients maintain a chronic inflammatory environment within the arterial wall. The effects of LDL-C on the inflammatory reactions in atherosclerosis

appear to be more dominant than the small effects of endogenous ANXA5. Our observation that plasma ANXA5 levels were not associated with cholesterol levels or an inflammation marker may support the minor impact of endogenous ANXA5 on inflammation in atherosclerosis. Of note, as our study was restricted to FH patients, the role of *ANXA5* genetic variations in atherosclerosis should be examined in other patient populations in order to understand its true physiological impact.

In conclusion, the data obtained from two independent cohorts of FH patients indicate that both common genetic variants in *ANXA5* and plasma ANXA5 levels are not associated with carotid IMT parameters or CVD risk.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2014.11.023.

References

[1] M.J. Flaherty, S. West, R.L. Heimark, K. Fujikawa, J.F. Tait, Placental anticoagulant protein-I: measurement in extracellular fluids and cells of the hemostatic system, J. Lab. Clin. Med. 115 (2) (1990 Feb) 174–181.
[2] W.L. van Heerde, P. Lap, S. Schoormans, P.G. de Groot, C.P. Reutelingsperger, T.M. Vroom, Localization of annexin A5 in human tissues, Annexins 1 (2004) 37–43.
[3] A. Cederholm, E. Svenungsson, K. Jensen-Urstad, C. Trollmo, A.K. Ulfgren, J. Swedenborg, et al., Decreased binding of annexin v to endothelial cells: a potential mechanism in atherothrombosis of patients with systemic lupus erythematosus, Arterioscler. Thromb. Vasc. Biol. 25 (1) (2005 Jan) 198–203.
[4] C.P. Reutelingsperger, J.M. Kop, G. Hornstra, H.C. Hemker, Purification and characterization of a novel protein from bovine aorta that inhibits coagulation. Inhibition of the phospholipid-dependent factor-Xa-catalyzed prothrombin activation, through a high-affinity binding of the anticoagulant to the phospholipids, Eur. J. Biochem. 173 (1) (1988 Apr 5) 171–178.
[5] W.L. van Heerde, K.S. Sakariassen, H.C. Hemker, J.J. Sixma, C.P. Reutelingsperger, P.G. de Groot, Annexin V inhibits the procoagulant activity of matrices of TNF-stimulated endothelium under blood flow conditions, Arterioscler. Thromb. 14 (5) (1994 May) 824–830.
[6] P. Thiagarajan, C.R. Benedict, Inhibition of arterial thrombosis by recombinant annexin V in a rabbit carotid artery injury model, Circulation 96 (7) (1997 Oct 7) 2339–2347.
[7] S. Ravassa, A. Bennaghmouch, H. Kenis, T. Lindhout, T. Hackeng, J. Narula, et al., Annexin A5 down-regulates surface expression of tissue factor: a novel mechanism of regulating the membrane receptor repertoir, J. Biol. Chem. 280 (7) (2005 Feb 18) 6028–6035.
[8] P. Libby, P.M. Ridker, G.K. Hansson, Progress and challenges in translating the biology of atherosclerosis, Nature 473 (7347) (2011 May 19) 317–325.
[9] K. Walsh, J.M. Isner, Apoptosis in inflammatory-fibroproliferative disorders of the vessel wall, Cardiovasc. Res. 45 (3) (2000 Feb) 756–765.
[10] C. Leon, D. Nandan, M. Lopez, A. Moeenrezakhanlou, N.E. Reiner, Annexin V associates with the IFN-gamma receptor and regulates IFN-gamma signaling, J. Immunol. 176 (10) (2006 May 15) 5934–5942.
[11] J.P. Mira, T. Dubois, J.P. Oudinet, S. Lukowski, F. Russo-Marie, B. Geny, Inhibition of cytosolic phospholipase A2 by annexin V in differentiated permeabilized HL-60 cells. Evidence of crucial importance of domain I type II Ca^{2+}-binding site in the mechanism of inhibition, J. Biol. Chem. 272 (16) (1997 Apr 18) 10474–10482.
[12] S. Kim, J. Ko, J.H. Kim, E.C. Choi, D.S. Na, Differential effects of annexins I, II, III, and V on cytosolic phospholipase A2 activity: specific interaction model, FEBS Lett. 489 (2–3) (2001 Feb 2) 243–248.
[13] L. van Tits, J. de Graaf, H. Toenhake, W. van Heerde, A. Stalenhoef, C-reactive protein and annexin A5 bind to distinct sites of negatively charged phospholipids present in oxidized low-density lipoprotein, Arterioscler. Thromb. Vasc. Biol. 25 (4) (2005 Apr) 717–722.
[14] M.M. Ewing, M.R. de Vries, M. Nordzell, K. Pettersson, H.C. de Boer, A.J. van Zonneveld, et al., Annexin A5 therapy attenuates vascular inflammation and remodeling and improves endothelial function in mice, Arterioscler. Thromb. Vasc. Biol. 31 (1) (2011 Jan) 95–101.
[15] M.M. Ewing, J.C. Karper, M.L. Sampietro, M.R. de Vries, K. Pettersson, J.W. Jukema, et al., Annexin A5 prevents post-interventional accelerated atherosclerosis development in a dose-dependent fashion in mice, Atherosclerosis 221 (2) (2012 Apr) 333–340.
[16] R. Gonzalez-Conejero, J. Corral, V. Roldan, C. Martinez, F. Marin, J. Rivera, et al., A common polymorphism in the annexin V Kozak sequence (-1C>T) increases translation efficiency and plasma levels of annexin V, and decreases the risk of myocardial infarction in young patients, Blood 100 (6) (2002 Sep 15) 2081–2086.
[17] V. Roldan, F. Marin, R. Gonzalez-Conejero, J. Corral, V. Vicente, Prognostic value of annexin A5 -1 C/T polymorphism in a long term follow-up after premature myocardial infarction, J. Thromb. Haemost. 5 (4) (2007 Apr) 862–863.
[18] M. Kozak, Not every polymorphism close to the AUG codon can be explained by invoking context effects on initiation of translation, Blood 101 (3) (2003 Feb 1) 1202–1203.
[19] W.L. van Heerde, H. Kenis, S. Schoormans, P. Lap, C.P. Reutelingsperger, The -1C>T mutation in the annexin A5 gene does not affect plasma levels of annexin A5, Blood 101 (10) (2003 May 15) 4223–4224.
[20] N. Bogdanova, J. Horst, M. Chlystun, P.J. Croucher, A. Nebel, A. Bohring, et al., A common haplotype of the annexin A5 (ANXA5) gene promoter is associated with recurrent pregnancy loss, Hum. Mol. Genet. 16 (5) (2007 Mar 1) 573–578.
[21] H. Miyamura, H. Nishizawa, S. Ota, M. Suzuki, A. Inagaki, H. Egusa, et al., Polymorphisms in the annexin A5 gene promoter in Japanese women with recurrent pregnancy loss, Mol. Hum. Reprod. 17 (7) (2011 Jul) 447–452.
[22] G. Tiscia, D. Colaizzo, E. Chinni, D. Pisanelli, N. Scianname, G. Favuzzi, et al., Haplotype M2 in the annexin A5 (ANXA5) gene and the occurrence of obstetric complications, Thromb. Haemost. 102 (2) (2009 Aug) 309–313.
[23] L. Hiddink, M.C. de Visser, W.L. van Heerde, Polymorphisms in the annexin A5 gene influence circulating annexin A5 levels in healthy controls, Thromb. Res. 129 (6) (2012 Jun) 815–817.
[24] L.J. van Tits, W.L. van Heerde, G.M. van der Vleuten, J. de Graaf, D.E. Grobbee, L.P. van de Vijver, et al., Plasma annexin A5 level relates inversely to the severity of coronary stenosis, Biochem. Biophys. Res. Commun. 356 (3) (2007 May 11) 674–680.
[25] T.J. Smilde, S. van Wissen, H. Wollersheim, M.D. Trip, J.J. Kastelein, A.F. Stalenhoef, Effect of aggressive versus conventional lipid lowering on atherosclerosis progression in familial hypercholesterolaemia (ASAP): a prospective, randomised, double-blind trial, Lancet 357 (9256) (2001 Feb 24) 577–581.
[26] A.C. Jansen, E.S. van Aalst-Cohen, M.W. Tanck, M.D. Trip, P.J. Lansberg, A.H. Liem, et al., The contribution of classical risk factors to cardiovascular disease in familial hypercholesterolaemia: data in 2400 patients, J. Intern. Med. 256 (6) (2004 Dec) 482–490.
[27] B. de Laat, R.H. Derksen, I.J. Mackie, M. Roest, S. Schoormans, B.J. Woodhams, et al., Annexin A5 polymorphism (-1C->T) and the presence of anti-annexin A5 antibodies in the antiphospholipid syndrome, Ann. Rheum. Dis. 65 (11) (2006 Nov) 1468–1472.
[28] S. van Wissen, M.D. Trip, T.J. Smilde, J. de Graaf, A.F. Stalenhoef, J.J. Kastelein, Differential hs-CRP reduction in patients with familial hypercholesterolemia treated with aggressive or conventional statin therapy, Atherosclerosis 165 (2) (2002 Dec) 361–366.
[29] F.D. Kolodgie, A. Petrov, R. Virmani, N. Narula, J.W. Verjans, D.K. Weber, et al., Targeting of apoptotic macrophages and experimental atheroma with radiolabeled annexin V: a technique with potential for noninvasive imaging of vulnerable plaque, Circulation 108 (25) (2003 Dec 23) 3134–3139.
[30] J.J. Kastelein, F. Akdim, E.S. Stroes, A.H. Zwinderman, M.L. Bots, A.F. Stalenhoef, et al., Simvastatin with or without ezetimibe in familial hypercholesterolemia, N. Engl. J. Med. 358 (14) (2008 Apr 3) 1431–1443.
[31] X. Chen, M. Chu, D.P. Giedroc, MRE-Binding transcription factor-1: weak zinc-binding finger domains 5 and 6 modulate the structure, affinity, and specificity of the metal-response element complex, Biochemistry 38 (39) (1999 Sep 28) 12915–12925.
[32] M.T. Carcedo, J.M. Iglesias, P. Bances, R.O. Morgan, M.P. Fernandez, Functional analysis of the human annexin A5 gene promoter: a downstream DNA element and an upstream long terminal repeat regulate transcription, Biochem. J. 356 (Pt 2) (2001 Jun 1) 571–579.
[33] A. Markoff, S. Gerdes, S. Feldner, N. Bogdanova, V. Gerke, E. Grandone, Reduced allele specific annexin A5 mRNA levels in placentas carrying the M2/ANXA5 allele, Placenta 31 (10) (2010 Oct) 937–940.