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ALOX5AP Variants are Associated with In-Stent Restenosis After Percutaneous Coronary Intervention

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Abstract

Background—Use of drug-eluting stents (DES) has reduced in-stent restenosis after percutaneous coronary intervention (PCI); however, DES are associated with late stent thrombosis. There is no accurate way to predict in-stent restenosis, although risk factors for atherosclerosis overlap those for in-stent restenosis. Therefore, we evaluated atherosclerosis candidate genes for association with in-stent restenosis.

Methods—We identified 46 consecutive cases that had undergone PCI with bare-metal stents who subsequently developed symptomatic in-stent restenosis of the target lesion (≥75% luminal narrowing) within six months. Forty-six age-, race-, vessel-diameter- and sex-matched controls without in-stent restenosis after PCI with bare-metal stent were also identified. Single-nucleotide polymorphisms (SNPs, N=82) from 39 candidate atherosclerosis genes were genotyped. Multivariable logistic regression models were used to test for association.

Results—Five SNPs were associated with in-stent restenosis. Three *ALOX5AP* SNPs were most strongly associated, two with increased risk (OR 3.74, p=0.01; OR 3.46, p=0.02), and the third with decreased risk of in-stent restenosis (OR 0.09, p=0.004). Two *ALOX5AP* haplotypes were associated with in-stent restenosis (HapB: OR 3.13, p=0.03); and a haplotype similar to HapA: OR 0.14, p=0.0009).

Conclusions—*ALOX5AP*, a gene within the inflammatory leukotriene pathway linked to and associated with coronary atherosclerosis, is also associated with in-stent restenosis. Genotyping these variants may help identify those at risk for in-stent restenosis who would benefit most from use of DES.

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Keywords

Genetics; Stent Restenosis; Coronary Artery Disease; Inflammation

Percutaneous coronary intervention (PCI) has become a mainstay of treatment for coronary atherosclerosis. Unfortunately 30% to 50% of patients undergoing balloon angioplasty soon develop restenosis. The routine use of stents has decreased the incidence, but it remains a significant problem, with 10–50% of patients receiving stents developing restenosis[1]. Clinical predictors of restenosis have been described and include diabetes mellitus, multiple stents, and minimal luminal diameter[2]. However, even applying these criteria in an ideal population, 16% of patients with none of these characteristics may have restenosis.

Clinical trials have shown that drug-eluting stents (DES) can reduce in-stent restenosis rates to very low levels[3–5], resulting in widespread use of DES for PCI. More recently, however, several reports have highlighted the increased risk of late stent thrombosis with DES[6,7], and the proportional use of DES versus bare metal stents has fallen substantially (CRUSADE database, accessed at Duke Clinical Research Institute, May 2007). Furthermore, routine use of DES in all patients undergoing PCI incurs a substantial incremental cost[8].

Ideally, clinicians would be able to identify a subgroup of patients in which the risk-benefit assessment favors DES implantation. Given the lack of a strong clinical predictive model for restenosis, elucidation of non-conventional markers may help with identification of this subgroup. Genetic polymorphisms have been associated with development of coronary artery disease (CAD) and atherosclerosis risk factors (i.e. diabetes), and pathophysiologic mechanisms (i.e. inflammation and thrombosis) overlap those for restenosis. Therefore, in this study, we pursued a candidate gene association design to test the hypothesis that atherosclerosis susceptibility genetic variants are associated with risk of in-stent restenosis after PCI with bare-metal stents.

Methods

Study Population

The CATHGEN biorepository consists of subjects recruited sequentially through the cardiac catheterization laboratories at Duke University Medical Center (Durham, NC, USA). We restricted years of enrollment to 2001–2003, to ensure a time during which patients routinely and uniformly received a bare-metal stent. Cases were individuals who had undergone successful PCI with bare-metal stenting during or related to their index catheterization at enrollment in CATHGEN, who subsequently presented with symptomatic restenosis of the target lesion (≥75% luminal narrowing), documented by coronary angiography within six months post-PCI. Cases for which coronary stenting was performed in an emergent setting (i.e. ST-segment elevation MI) were excluded, as well as those receiving DES. Controls were defined as individuals who had undergone successful PCI with bare-metal stenting during or related to their index catheterization at enrollment in CATHGEN, who did not present with clinical restenosis within six months of PCI, who had at least six months of follow-up available, and who had no previous history of restenosis. Controls were matched as carefully as possible to cases on age, race, sex, and vessel-diameter (±0.5 mm). Review of medical records was performed by the same cardiologist to verify phenotypes. The Duke University Institutional Review Board approved study protocols and informed consent was obtained from each subject.

Candidate Gene Selection

Candidate genes were selected based on (1) review of the literature showing strong association with in-stent restenosis and/or atherosclerosis (7 genes); or (2) identification as a atherosclerosis susceptibility gene from two studies of gene expression: one performed in human aortas harvested from heart donors[9], and the second in a mouse model of atherosclerosis[10] (32 genes). Single-nucleotide polymorphisms (SNPs) within each gene were selected based on previous reports in the literature, or based on an average of two SNPs per gene, focusing on functional SNPs and SNPs with a minor allele frequency of >0.05 (Supplement I).

Genotyping

DNA was extracted using PureGene (Gentra Systems, Minneapolis, MN). SNPs were genotyped using either Taqman or Illumina BeadArray systems. The 7900HT Taqman genotyping system (Applied Biosystems, Foster City, CA) incorporates a standard PCR-based, dual fluor, allelic discrimination assay. QC samples, composed of 12 reference controls, were included in each quadrant of the plate. Illumina BeadStation genotyping was performed using the 500G system (Illumina, San Diego, CA). Within each individual experiment four QC samples were included. SNPs showing mismatches on QC samples were reviewed by an independent supervisor. All SNPs were successfully genotyped for 95% or more of the individuals in the study. Error rate estimates for SNPs meeting QC benchmarks were <0.2%.

Statistical Analysis

Association of genetic variants with restenosis was assessed using logistic regression, assuming dominant (allele) and additive (genotype) models. Models were adjusted for hypertension, diabetes, dyslipidemia, smoking, and body-mass-index (BMI). Because of concerns of confounding by extent of CAD, a second model was also constructed adjusting for CAD-index, a numerical summary of the extent of angiographic CAD[11]. The Graphical Overview of Linkage Disequilibrium (GOLD) program was used to assess linkage disequilibrium. Haplotype analysis was performed using HaploStats 1.1.0 (Mayo Clinic, Rochester, MN). Power calculations used QUANTO software (http://hydra.usc.edu/gxe). As all analyses were exploratory in nature, nominal two-sided p-values unadjusted for multiple comparisons are presented. Significance was defined as p-value ≤0.05. Statistical analyses used SAS version 9.1 (SAS Institute, Cary NC).

Results

Clinical Characteristics

Forty-six cases with clinical in-stent restenosis within six months of PCI were identified, as well as 46 matched controls. Consistent with previous reports, the only strong clinical factor that differentiated cases and controls was diabetes (Table I).

Association Results

All SNPs were in Hardy-Weinberg equilibrium. A total of 82 SNPs within 39 genes were genotyped. Five SNPs within three genes were associated with in-stent restenosis in at least one of the models (Figure 1, Table II). Three of the SNPs (rs17222814 (G→A, minor allele frequency (MAF) 0.07); rs17216473 (G→A, MAF 0.19), and rs10507391 (T→A, MAF 0.49)), which were also the most significant SNPs, reside within the *ALOX5AP* gene. Rs17222814 was protective (allelic OR 0.089, p=0.004), whereas the other two *ALOX5AP* SNPs were associated with increased risk of in-stent restenosis (OR 3.743, p=0.01 and OR 3.436, p=0.02). After adjusting for CAD-index, all results remained significant suggesting

that these results are specific for restenosis, and not merely reflective of association with CAD. No SNPs were in linkage disequilibrium (pairwise $R^2<0.3$). One SNP within the *ALOX5* gene (upstream in the leukotriene pathway) was included in this study, but was not significant. One SNP each in the genes *ROR2* and *GLB1* were also weakly associated with in-stent restenosis.

Multivariable Modeling

We sought to understand the additive predictive capabilities of these SNPs in addition to clinical factors. First, a logistic regression model inclusive of all clinical factors (diabetes, hypertension, smoking, BMI, family history of CAD, vessel-length, vessel-diameter, MI and CAD-index) was fit. In this model, only diabetes (OR (95% CI) 3.61 (1.28–10.18), p=0.02) and CAD-index (OR 1.04 (1.004–1.070), p=0.03) were predictive of restenosis. The c-statistic for this model was 0.76. A model was subsequently fit including all clinical factors, and then all significant SNPs were assessed using a backward stepwise regression fashion. In this model, two SNPs remained significantly associated with restenosis: rs17216473 (OR 5.11 (1.31–19.92), p=0.02) and rs17222814 (OR 0.07 (0.01–0.56), p=0.01), both in the *ALOX5AP* gene. The c-statistic for this model was 0.85, suggesting that these two *ALOX5AP* SNPs contribute independent information to the capacity of a clinical model to discriminate between risk of in-stent restenosis.

ALOX5AP Haplotype Analyses

Given the strong findings for *ALOX5AP*, we focused further efforts on understanding this pathway in restenosis risk. Haplotypes within *ALOX5AP* (HapA and HapB) and within a downstream gene, *LTA4H* (HapK) have previously been shown to be associated with MI[12,13]. Therefore, we genotyped additional SNPs to complete these haplotypes (Table III). The global p-value for the haplotype of the SNPs comprising HapA was significant (p=0.02), signifying that the cluster of SNPs overall is associated with restenosis. However, the combination of alleles for HapA itself was not significant, suggesting that the haplotype background of these specific HapA alleles is not associated with restenosis. Interestingly, a novel haplotype within the SNPs comprising HapA was associated (p=0.0009). This haplotype is composed of alleles that are the same as HapA except for rs17222814, where the allele is the opposite of HapA (A vs. G). This haplotype is protective for restenosis (OR 0.14), consistent with the prior HapA results (G allele associated with increased risk of MI). Within HapB SNPs, the global p-value was not significant; however, HapB itself was associated with increased risk of restenosis (OR 3.13, p=0.03). The SNPs comprising HapK were not associated with in-stent restenosis.

Additionally, three-way haplotypes composed of the individually associated *ALOX5AP* SNPs (rs17216473, rs17222814, and rs10507391) were significant (global p=0.003). The haplotype composed of alleles associated with increased risk of in-stent restenosis at each SNP (A, G, A, respectively) was significant (p=0.01). Even more significant was the background of allele G, A and T (corresponding to the opposite allele at each SNP, hence a “protective” haplotype (overall frequency 0.07; cases 0.02, controls 0.11, p=0.0009)). These results suggest that this haplotype is more strongly associated than any individual SNP, with consistency of the associated alleles. Allele frequencies are presented (Table IV).

Discussion

In this study, we have taken a candidate gene approach to assess whether atherosclerosis susceptibility genes are also associated with risk of in-stent restenosis after PCI. We report a novel finding of association of individual variants and haplotypes within the *ALOX5AP* gene with in-stent restenosis, a gene previously linked to and associated with MI in multiple

populations. These results persist even after adjustment for clinical factors, and two of the identified *ALOX5AP* SNPs add independent prognostic information and discriminative power to a model predicting restenosis inclusive of clinical factors. These results could have potentially significant clinical implications. Given the increased risk of stent thrombosis associated with DES, and lack of good clinical predictors of in-stent restenosis, identification of genetic variants for in-stent restenosis could help guide more judicious use of DES by identifying the subgroup in which the balance of risk and benefit would favor use of DES.

Previous studies have identified genes associated with in-stent restenosis[14–18]. In this study, we report a novel finding for strong association of *ALOX5AP* SNPs and haplotypes with in-stent restenosis. The individual SNPs are not known functional variants, but HapA heightens the response of 5-lipoxygenase (the protein product of the gene) to factors that stimulate inflammatory cells[12]. *ALOX5AP* was chosen for inclusion in the study based on differential expression of *ALOX5* in our study of aortic atherosclerosis[9] and previous linkage and association of *ALOX5AP*[12] with MI. Although the leukotriene pathway has not been previously implicated in in-stent restenosis pathophysiology, inflammation is known to play an important role. Targeting inflammatory pathways may have utility in preventing in-stent restenosis[19], and pharmacologic agents targeting the leukotriene pathway are available. Tranilast, an antiasthma drug that inhibits leukotriene C_4 release from mast cells and macrophages[20], and interferes with proliferation and migration of vascular SMCs[21], has been shown to reduce neointimal thickening after vascular injury in animal models[22]. Human studies have shown varying results for tranilast on cardiovascular events[2324].

We also found that one SNP each in the *ROR2* and *GLB1* genes was associated with in-stent restenosis, though neither gene has any known role in cardiovascular disease. The protein encoded by *ROR2* is a type I transmembrane protein and may be involved in the early formation of chondrocytes[25]. *GLB1* encodes beta-galactosidase-1, a lysosomal hydrolase, and a variant within this gene causes GM1-gangliosidosis.

Our study has some limitations. None of the individual SNPs (lowest p=0.003) would survive a conservative Bonferroni adjustment for multiple comparisons at the gene level (p=0.001), but the HapA-related haplotype and the three-way haplotype would (p=0.0009). We do not believe this is a spurious result, as genes were carefully chosen from a comprehensive study of atherosclerosis, and the identification of three independent SNPs within the same gene lends further credence. Further, there was consistency of the associated allele both with previous studies, and across haplotypes that were associated. Power calculations demonstrate that our study has 80% power to detect an effect size of ≥2.3 for common SNPs (MAF 0.50), and effect sizes ≥4.0 for rarer SNPs (MAF 0.05.), therefore, we may not have had sufficient power for rarer SNPs or with more modest effect sizes. Our study lacks a true validation, however, prospective validation of these results are difficult given the routine use of DES. Furthermore, our results corroborate previous studies showing that these *ALOX5AP* SNPs and haplotypes are associated with MI, and thus our study is partially validated by other studies showing this pathway to be important in related atherosclerotic disease. Our study is remarkable for a very well-phenotyped cohort, and we took great care to adjust for potential confounders in our analyses. Finally, our results show that variants in the *ALOX5AP* gene can contribute significantly to predictive models of in-stent restenosis, adding independent information in addition to clinical factors.

In conclusion, we report herein a novel finding of association of *ALOX5AP* variants with risk of in-stent restenosis after PCI. These results could have significant clinical implications. In conjunction with clinical factors, genotyping of these *ALOX5AP* SNPs

could help guide clinical decision-making and directed use of DES for PCI. Further studies and validation are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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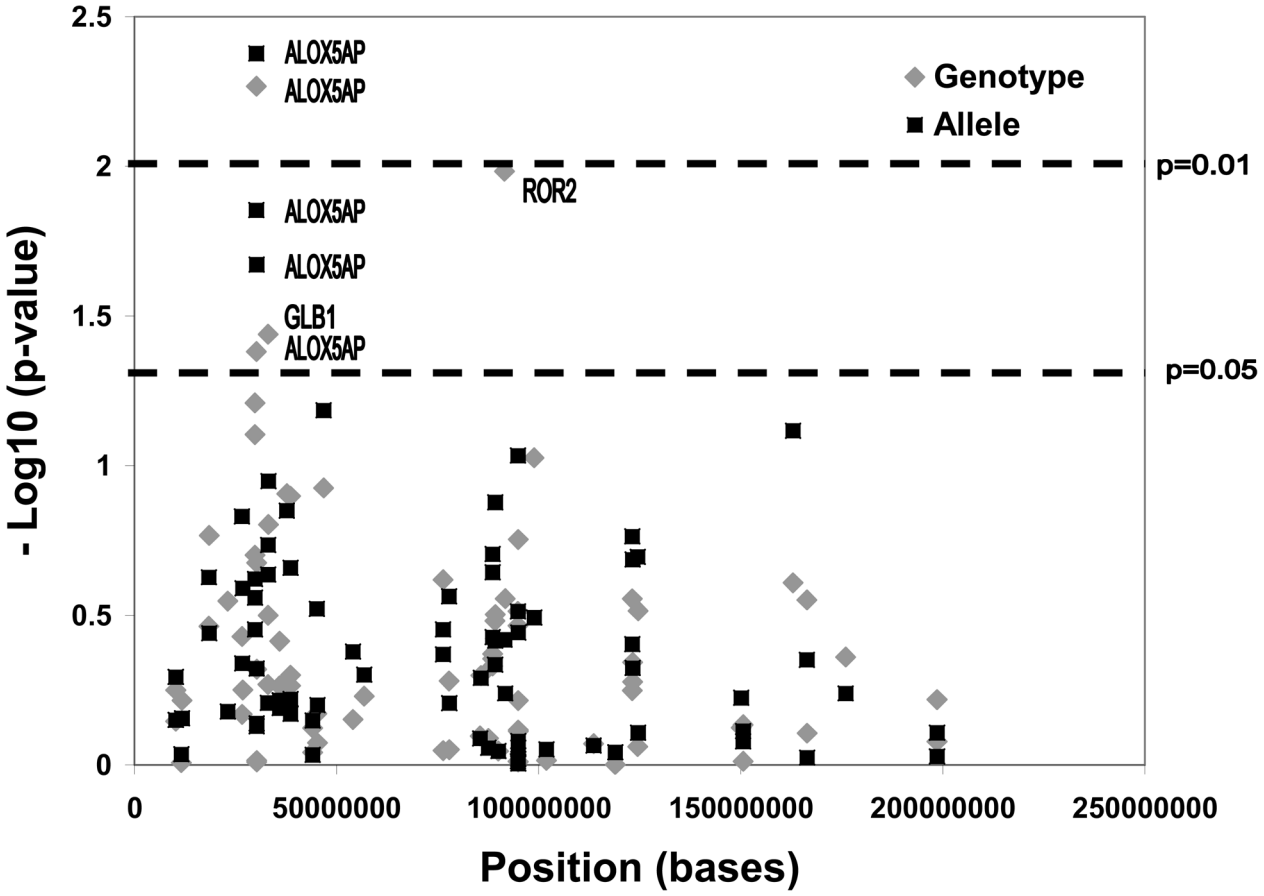

Figure 1. Association results, all SNPs

Results for all SNPs for association with restenosis are displayed. Genomic position (X-axis) and negative log10 of the p-value (Y-axis) are noted. Gene annotations are made for SNPs meeting statistical significance.

Table I

Baseline characteristics.

Variable	Overall (N=92)	Cases (N=46)	Controls (N=46)	p-value
Age (mean, SD)	61.88 (10.64)	62.09 (10.52)	61.67 (10.88)	0.8
Sex (% female)	33.7%	34.8%	32.6%	0.8
Dyslipidemia	65.2%	73.9%	56.5%	0.08
Lipids (mean, SD)				
Total cholesterol	188.42 (49.99)	186.92 (57.41)	190.23 (40.20)	0.6
Triglyerides	190.18 (144.18)	210.36 (172.26)	165.97 (98.34)	0.7
HDL cholesterol	41.69 (11.99)	40.58 (13.70)	43.03 (9.61)	0.2
LDL cholesterol	104.56 (40.42)	91.45 (30.32)	123.29 (46.49)	0.06
Hypertension	78.3%	80.43%	76.1%	0.6
Diabetes mellitus	33.7%	47.8%	19.6%	0.004
CAD-index (mean, SD)	44.27 (17.46)	48.62 (19.24)	40.02 (14.51)	0.02
Number of diseased vessels				
1	42.9%	33.3%	52.2%	0.09
2	33.0%	33.3%	32.6%	
3	24.2%	33.3%	15.2%	
Vessel-diameter mm (mean, SD) *	2.74 (0.48)	2.64 (0.49)	2.85 (0.45)	0.04
Vessel-length mm (mean, SD) *	15.44 (5.64)	15.07 (5.27)	15.82 (6.04)	0.6
BMI (mean, SD)	30.68 (5.56)	30.90 (5.83)	30.45 (5.34)	0.7
Smoking	63.0%	58.7%	67.4%	0.4
History of MI	50.0%	43.5%	56.5%	0.2
Race (% Caucasian)	69.6%	67.4%	71.7%	0.4
Family history CAD	45.7%	41.3%	50.0%	0.4

* for vessel that underwent PCI.

Table II

Odds ratios for significant SNPs.

Gene	SNP	Type of SNP	Nucleotide	Adjusted*				Adjusted for CAD-index†			
				Genotype		Allele		Genotype		Allele	
				OR (95% CI)	p-val	OR (95% CI)	p-val	OR (95% CI)	p-val	OR (95% CI)	p-val
ALOX5AP	RS17222814	Upstream	G→A	0.089 (0.016–0.506)	0.004	0.089 (0.016–0.506)	0.004	0.058 (0.009–0.383)	0.003	0.058 (0.009–0.383)	0.003
ALOX5AP	RS17216473	Upstream	G→A	2.566 (1.054–6.251)	0.04	3.743 (1.331–10.518)	0.01	3.082 (1.205–7.881)	0.02	5.097 (1.673–15.528)	0.004
ALOX5AP	RS10507391	Upstream	T→A	1.452 (0.791–2.666)	0.21	3.436 (1.197–9.861)	0.02	1.644 (0.864–3.129)	0.13	3.605 (1.190–10.924)	0.02
ROR2	RS10820899	Intronic	G→A	2.341 (1.215–4.510)	0.01	1.537 (0.575–4.106)	0.39	3.095 (1.467–6.532)	0.003	1.838 (0.646–5.228)	0.25
GLB1	RS9861960	Intronic	G→A	0.513 (0.275–0.957)	0.04	0.525 (0.204–1.352)	0.18	0.464 (0.242–0.890)	0.02	0.406 (0.147–1.089)	0.07

*adjusted for smoking, body-mass-index, diabetes, hypertension and dyslipidemia, and

†also adjusted for CADindex.

Table III

Leukotriene pathway haplotypes.

Haplotype	Global p-value†	SNPs										Haplotype Frequency			P-value†
												Overall	Cases	Controls	
ALOX5AP Three-way haplotype*	0.003	1	2	3											
		G	**A**	**T**								**0.06**	**0.02**	**0.11**	**0.0009**
		G	G	A								0.30	0.31	0.29	0.81
		G	G	T								0.45	0.43	0.46	0.85
		A	**G**	**A**								**0.19**	**0.24**	**0.14**	**0.01**
ALOX5AP	0.02	1	2	3	4										
HapA		**A**	**T**	**G**	**A**							**0.066**	**0.109**	**0.022**	**0.0009**
		G	T	G	C							0.316	0.149	0.109	0.48
		G	A	G	C							0.129	0.351	0.280	0.59
		G	A	A	C							0.049	0.099	0.142	0.47
		G	T	G	A							0.121	0.033	0.067	0.28
		G	A	G	A							0.319	0.260	0.380	0.07
ALOX5AP	0.24	1	2	3	4										
		G	T	A	C							0.496	0.560	0.430	0.10
		G	A	G	C							0.105	0.187	0.214	0.86
		A	A	A	T							0.079	0.109	0.100	0.89
		G	A	A	C							0.201	NA	0.014	0.64
		G	T	A	T							0.006	0.076	0.079	0.42
		A	A	G	C							0.008	0.000	0.016	0.23
HapB		**A**	**A**	**A**	**C**							**0.105**	**0.068**	**0.146**	**0.03**
LTA4H	0.44	1	2	3	4	5	6	7	8	9	10				
HapK		C	G	T	G	A	T	T	C	G	G	0.027	0.070	NA	0.15
		T	G	T	G	G	C	G	T	A	G	0.025	0.039	NA	0.22

Haplotype	Global p-value†	SNPs										Haplotype Frequency			P-value†
												Overall	Cases	Controls	
		C	G	T	A	A	T	T	T	A	A	0.014	0.017	0.011	0.25
		C	G	T	G	A	C	G	C	G	G	0.008	NA	NA	0.28
		C	G	C	G	A	T	T	T	A	G	0.006	0.012	NA	0.34
		C	G	C	G	A	T	G	C	G	A	0.006	0.011	NA	0.38
		C	G	T	G	A	C	T	T	A	G	0.012	0.019	NA	0.44
		C	G	T	G	A	C	T	T	G	G	0.020	0.023	0.024	0.58
		T	G	T	G	G	C	G	C	G	G	0.058	0.076	0.064	0.59
		T	A	T	G	A	C	G	T	A	A	0.104	0.108	0.100	0.74
		T	G	T	G	G	C	G	T	A	A	0.050	0.054	0.046	0.76
		C	G	T	A	A	T	T	T	A	G	0.032	0.035	0.000	0.82
		C	G	T	G	A	T	T	T	G	G	0.324	0.324	0.312	0.93
		C	G	T	G	A	C	T	C	G	G	0.012	NA	0.022	0.91
		C	G	C	G	A	T	G	T	A	G	0.006	NA	0.013	0.71
		C	G	T	G	A	T	T	T	A	G	0.062	0.054	0.080	0.60
		C	G	T	A	A	T	T	C	G	G	0.006	NA	0.011	0.57
		C	G	T	A	A	T	T	C	A	G	0.028	0.000	0.028	0.55
		C	G	C	G	A	C	T	T	G	G	0.006	NA	0.011	0.44
		C	A	T	G	A	C	G	T	A	A	0.158	0.142	0.188	0.36
		T	G	T	G	A	T	T	T	A	G	0.006	NA	0.011	0.31
		C	G	C	G	A	T	G	T	A	A	0.025	NA	0.046	0.01

*Three-way haplotype composed of the individually associated *ALOX5AP* SNPs (rs17216473, rs17222814, rs10507391).

†Adjusted for hypertension, dyslipidemia, diabetes, smoking, BMI and CADindex.

**
ALOX5AP (HapA): rs17222814 (1), rs10507391 (2), rs476874 (3), rs9551963 (4)
ALOX5AP (HapB): rs17216473 (1), rs10507391 (2), rs9315050 (3), rs17222842 (4)
LTA4H (HapK): 12p0557 (1), rs2660880 (2), rs6538697 (3), rs1978331 (4), rs17677715 (5), rs2247570 (6), rs2660898 (7), rs2540482 (8), rs2660845 (9), rs2540475 (10)

NA: numbers too small to converge on estimate

Table IV

Minor Allele Frequencies for significant SNPs/haplotypes and for SNPs composing the haplotypes.

Gene	SNP	Allele	MAF (overall)	MAF (cases)	MAF (controls)
ALOX5AP	RS17222814**	G→A	0.07	0.02	0.11
ALOX5AP	RS17216473††	G→A	0.19	0.23	0.14
ALOX5AP	RS10507391†	T→A	0.49	0.54	0.43
Three-way haplotype*	--	--	0.07	0.02	0.11
HapB (*ALOX5AP*)	--	--	0.11	0.15	0.07
ALOX5AP novel haplotype	--	--	0.07	0.02	0.11
ROR2	RS10820899	G→A	0.47	0.56	0.39
GLB1	RS9861960	G→A	0.44	0.37	0.51
SNPs composing HapA (*ALOX5AP)*	RS4769874	G→A	0.05	0.07	0.03
	RS9551963	A→C	0.48	0.43	0.53
SNPs composing HapB (*ALOX5AP)*	RS9315050	A→G	0.11	0.11	0.11
	RS17222842	C→T	0.08	0.09	0.08
SNPs composing HapK (*LTA4H*)	12P0557	C→T	0.21	0.19	0.23
	RS2660880	G→A	0.06	0.06	0.05
	RS6538697	T→C	0.11	0.13	0.08
	RS1978331	G→A	0.50	0.51	0.49
	RS17677715	A→G	0.15	0.13	0.16
	RS2247570	T→C	0.34	0.31	0.36
	RS2660898	T→G	0.31	0.31	0.30
	RS2540482	T→C	0.25	0.26	0.24
	RS2660845	A→G	0.30	0.30	0.30
	RS2540475	G→A	0.22	0.23	0.20

*Haplotype composed of the three individually associated *ALOX5AP* SNPs (rs17216473 (G), rs17222814 (A), rs10507391 (T)).

**SNP also included in HapA

†SNP also included in both HapA and HapB

††SNP also included in HapB