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**Genetic variation in the arachidonate 5-lipoxygenase-activating protein
(*ALOX5AP*) is associated with myocardial infarction in the German
population**

Patrick Linsel-Nitschke^{1*}, MD; Anika Götz^{1*}, MSc; Anja Medack¹, MSc; Inke R. König², PhD; Petra Bruse¹, MSc; Wolfgang Lieb^{1,3}, MD; Björn Mayer¹, MD; Klaus Stark⁴, PhD; Christian Hengstenberg⁴, MD; Marcus Fischer⁴, MD; Andrea Baessler⁴, MD; Andreas Ziegler², PhD; Heribert Schunkert¹, MD; Jeanette Erdmann¹, PhD

¹ Universität zu Lübeck, Medizinische Klinik II, Lübeck, Germany

² Universität zu Lübeck, Institut für Medizinische Biometrie und Statistik, Lübeck, Germany

³ Universität Lübeck, Institut für Humangenetik, Lübeck, Germany

⁴ Klinik und Poliklinik für Innere Medizin II, Universitätsklinikum Regensburg, Germany

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*Both authors contributed equally to this work

Correspondence:

Dr. Patrick Linsel-Nitschke

Medizinische Klinik II

Universitätsklinikum Schleswig-Holstein, Campus Lübeck

Ratzeburger Allee 160

23538 Lübeck, Germany

Email: Patrick.Linsel-Nitschke@uk-sh.de

Telephone: +49 451 500 5039

Fax: +49 451 500 6437

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Abstract

Genetic variation in the genes for *ALOX5AP* and *LTA4H* has previously been shown to contribute to the risk of myocardial infarction (MI) and stroke in the Icelandic and Scottish population. Both genes encode for proteins playing a role in the synthesis of the pro-inflammatory leukotriene B, possibly providing a link between MI and inflammation. The aim of the present study was to investigate whether these associations can be confirmed in a large study of German MI patients.

Two previously described four SNP haplotypes of the *ALOX5AP* gene (termed haplotype A and B) and one SNP (rs2660899) of the *LTA4H* gene conferring the greatest risk of MI in previous studies were genotyped in 1,211 unrelated MI cases from the German MI Family Study and in 1,015 healthy married-in spouses serving as controls.

Haplotype B in the *ALOX5AP* gene was associated with an increased risk of MI in the German population, confirming previously reported associations of this haplotype with CAD in populations from Scotland and Italy.

No association with risk of MI was detected for haplotype A of the *ALOX5AP* gene nor for SNP rs2660899 representing the *LTA4H* gene. In conclusion, haplotype B of the *ALOX5AP* gene is associated with an increased risk of MI in a large German study. This study is the third independent report from a European population describing an increased risk of CAD for carriers of haplotype B of the *ALOX5AP* gene, which further substantiates a role of this gene in the pathogenesis of CAD in Europeans.

Introduction

Despite numerous advances in treatment, coronary artery disease (CAD) and its major complication myocardial infarction (MI) still represent the leading causes of mortality in the Western world[1]. Both environmental and genetic factors contribute to the pathogenesis of CAD and MI. Genetic epidemiological approaches including genome-wide linkage and association studies identified several chromosomal regions and polymorphisms related to MI and/or CAD[2-4]. However, independent validations as well as mechanistic explanation of the underlying functional basis for many of these findings are still under investigation.

In two recent studies, Helgadottir and coworkers reported a strong genetic association between the *ALOX5AP* gene and risk of MI and stroke in the Icelandic population[5, 6]. *ALOX5AP* encodes for arachidonate 5-lipoxygenase-activating protein, which plays a key role in the biosynthesis of pro-inflammatory leukotriene B mediators providing a potential link between inflammation and cardiovascular disease. In a genome-wide scan of 296 multiplex Icelandic families including 713 individuals with MI suggestive linkage lead to the identification of the *ALOX5AP* gene as a strong candidate gene for MI. Within the *ALOX5AP* gene the authors identified a four SNP haplotype (haplotype A) conferring a two-fold risk for MI. In functional studies using isolated neutrophils from carriers of haplotype A, an increased production of the pro-inflammatory leukotriene B was demonstrated[5]. However, the association of haplotypes A and B of the *ALOX5AP* gene with leukotriene B production was not confirmed in a recent study[7].

In a subsequent report, the same authors who first reported the association of haplotype A with MI in the Icelandic population were unable to confirm this association in a cohort of British patients. However, the authors identified another 4 SNP haplotype of the *ALOX5AP* gene, termed haplotype B, which conferred significant risk of both MI and stroke[8]. Similarly, a recent angiography based study from Italy has detected an increased risk of angiographically proven CAD for carriers of haplotype B but not for carriers of haplotype A[9]. In contrast to the aforementioned European studies no association of either haplotype A or B of the *ALOX5AP* gene with MI was detected in further studies from the US[10] and Japan[11].

The *LTA4H* gene encodes for leukotriene A 4 hydrolase, a protein in the same biochemical pathway as FLAP, the gene product of *ALOX5AP*. A subsequent study reported a moderately increased risk of MI for carriers of a haplotype in the *LTA4H* gene (haplotype K)[6] in the Icelandic population. In a similar fashion as previously demonstrated for *ALOX5AP*, the authors were again able to show an increased production of pro-inflammatory leukotriene B

associated with haplotype K of the *LTA4H* gene[6]. In the same report, the authors replicated the association of haplotype K with MI and stroke in pooled populations from three North-American cities comprising 1,591 MI cases with European ancestry and 197 MI cases with African American ancestry. The increased chance conferred by haplotype K was markedly higher in African Americans and for subjects with MI and concomitant cerebrovascular disease[6].

Since the published data on the association of *ALOX5AP* with CAD is conflicting and no further studies on the association of genetic variation in *LTA4H* with MI have been reported so far, we decided to investigate the association of genetic variation in both genes with MI in the German MI Family Study.

Methods

Study population

MI families were ascertained through patients at 17 cardiac rehabilitation centers distributed throughout Germany. All patients had suffered from a MI before 60 years of age[12]. If at least one sibling presented with MI or severe CAD (defined as percutaneous coronary intervention or coronary artery bypass grafting) before 70 years of age, the nuclear family (patient, available parents, and all affected and unaffected siblings) was contacted and invited to participate in the study. All study participants answered to a standardized questionnaire about medical history, presence of coronary risk factors, clinical events, medication, anthropometric data, and socioeconomic background. This information was validated by retrospective analyses of medical records. Additionally, all patients underwent a medical examination during a visit scheduled at their primary care physician's office. For this study, we examined 1,211 unrelated MI patients. 1,015 healthy married-in spouses served as a control group.

The study protocol was approved by the local institutional review-committee, and all subjects gave written informed consent.

Genotyping

SNPs comprising haplotype A (consisting of the common allele of rs17222814 (G), the common allele of rs10507391 (T), the common allele of rs4769874 (G), and the rare allele of rs9551963 (A)) and haplotype B (rs17216473, rs10507391, rs9315050, rs17222842) in the *ALOX5AP* gene and one SNP in the *LTA4H* gene (rs2660899) were genotyped using 5'-exonuclease activity (TaqMan) on a ABI Prism 7900HT (Applied Biosystems, Darmstadt,

Germany). TaqMan genotyping assays with probes labelled with the fluorophores FAM and VIC, respectively, were purchased from Applied Biosystems. Genotyping was performed on 384-well plates prepared with the GENESIS Freedom pipetting robot from TECAN (Crailsheim, Germany). The Universal PCR Master Mix from Applied Biosystems was used in a 5 μ l total reaction volume with 10 ng DNA per reaction. Allelic discrimination was measured automatically on the ABI Prism 7900HT (Applied Biosystems) using the Sequence Detection Systems 2.1 software (autocaller confidence level 95%).

Ten percent of all genotypes were repeated in independent PCR reactions to check for consistency and to ensure intra- and inter-plate genotype quality control. No genotyping discrepancies were detected between the repeated samples.

Statistical analysis

We performed power analyses based on the odds ratios (OR) provided from the literature for haplotypes A and B[5]. For this, we applied the two-sided exact test of Fisher at a global test-level of 5%.

Deviations of genotype distribution from and compatibility with Hardy-Weinberg equilibrium (HWE) were analyzed using a Monte Carlo χ^2 goodness-of-fit test (threshold of significance every $p > 0.05$, $HWE\chi^2$) and the exact uniformly most powerful equivalence test with $\delta_2 = 2$ and $\delta_1 = 2/3$, respectively, for all patient groups and all control groups for all markers separately[13].

Primary aim of the study was to show the association between *ALOX5AP* and *LTA4H* genes and MI. Therefore we examined for the *ALOX5AP* gene the two haplotypes A and B that are sufficient to cover genetic variation in this gene according to Helgadóttir and coworkers[5]. Given that a previous study has demonstrated that SNP rs2660899 in the *LTA4H* gene provides the best single surrogate marker of haplotype K, which is associated with the greatest risk of MI[6], we have only genotyped rs2660899 for the *LTA4H* gene. p-values of the haplotype analyses were adjusted for multiple testing according to Bonferoni-Holm[14] and were considered as significant if they were smaller than 5%.

Analyses of haplotypes A and B were performed using the EM-algorithm. Here, score statistics based on a χ^2 distribution with one degree of freedom and corresponding p-values were calculated as proposed by Schaid et al. (2002)[15]. We determined Monte-Carlo p-values from 1,000,000 replications.

In addition, for every SNP of haplotype A and B separately as well as for rs2660899, differences in genotype frequencies between MI cases and controls were tested using the 2-

sided Cochrane-Armitage trend test; ORs and corresponding 95% confidence intervals (CI) were calculated.

For the haplotypes A and B and rs2660899, we furthermore performed additional subgroup analyses investigating patients who were either suffering from MI before 45 or 50 years of age or those patients with MI who had a positive history of stroke or other cerebrovascular disease (history of transient ischemic attack or known supraaortic stenosis), because previous studies had indicated that the association of polymorphisms in both genes with risk of cardiovascular disease is markedly increased in individuals with early-onset MI or in individuals affected by both MI and concomitant cerebrovascular disease.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Baseline characteristics of the study population are shown in Table 1.

For the power analysis, we assumed a haplotype frequency of 15.8% for haplotype A and an odds ratio of 1.8, as reported in the initial study[5]. This resulted in sufficient power to detect differences (99.9%), even a more moderate effect of 1.4 is detectable with our study (power = 85.6%). The power to detect an odds ratio of 1.95, as reported in the initial study for haplotype B with a frequency of 7.5%, was 99.7%.

To evaluate genotyped SNPs for HWE, we checked for all patient and control groups for all markers separately if there are deviations of genotype distribution with a Monte Carlo χ^2 goodness-of-fit test (Table 2). No deviation from HWE was detected this way (every $p > 0.05$, $HWE\chi^2$). Second, we analyzed the compatibility with HWE using the exact uniformly most powerful equivalence test with $\delta_2 = 2$ and $\delta_1 = 2/3$, respectively (HWE aequi). For SNPs rs4769874 and rs9315050 we detected slightly more heterozygotes in the cases than expected. However, such findings are not unusual in cases in regions linked to a disease.

Frequencies and minor allele frequencies (MAF) of the single SNPs comprising haplotype A and B of the *ALOX5AP* gene and rs2660899 of the *LTA4H* gene are displayed in Table 2. In addition, p-values of Cochrane-Armitage trend test; and corresponding ORs plus 95% CIs are shown. No association of rs2660899 with MI was detected.

The frequency of haplotype A in our study population was comparable with those reported in the literature[5, 8, 9]. However, we detected no association between MI and haplotype A (Monte-Carlo $p = 0.2807$, Table 3a) despite sufficient power. Accordingly, neither the other haplotypes of the four SNPs comprising haplotype A, nor the global association score test

showed a significant association with MI (each Monte-Carlo $p > 0.05$). In contrast, haplotype B (consisting of the rare allele of rs17216473 (A), the rare allele of rs10507391 (A), the common allele of rs9315050 (A), and the common allele of rs17222842 (G)) was significantly associated with MI (Monte-Carlo $p < 1 \times 10^{-5}$, Table 3b). Also, the global association score test of these four SNPs showed a significant association (Monte-Carlo $p = 2 \times 10^{-5}$). Further, the last SNP of the haplotype rs17222842 probably is the most important SNP, because the single SNP analysis also gives a hint for an association ($p = 0.0175$; OR per 1 rare allele [95%CI]: 0.78 [0.63,0.96]) and the haplotype AAAA is also associated with MI (Monte-Carlo $p = 0.0112$). Contrary to the haplotype AAAG that is associated with increased risk of MI (haplotype score = 5.0), the haplotype AAAA shows a protective effect (haplotype score = -2.5).

In addition, we performed subgroup analyses for haplotype A and B of the *ALOX5AP* gene and rs2660899 of the *LTA4H* gene (Table 4). Whereas we did not detect a significant association of either haplotype A or rs2660899 in any subgroup, the effect of haplotype B was found in every subgroup investigated.

Discussion

In analogy to two previous reports from Great Britain[8] and Italy[9], we detected an increased risk of MI for carriers of haplotype B of the *ALOX5AP* gene in our German study whereas no association of haplotype A with MI risk was observed. Haplotype frequency, odds ratio and direction of effect for haplotype B in our population proved to be very similar to the previous reports from Britain[8] and Italy[9] but different to the North American study in which no difference in frequency of haplotype B was detected between MI cases and controls[10]. The fact that the association of haplotype B with risk of CAD has now been replicated three times in European populations but proved negative in the North American study might hint at differences in genetic structure, namely differences in SNP linkage disequilibrium and haplotype structure between European and North American populations.

For the *LTA4H* gene our study did not find any association of SNP rs2660899 representing haplotype K with risk of MI in the German population. In the original report the risk associated with haplotype K was particularly pronounced in individuals suffering from MI with concomitant cerebrovascular disease and in African American individuals. However, a subgroup analysis for individuals with MI and concomitant stroke in our German study did not yield any additional information. The lack of association between rs2660899 representing haplotype K with MI in the German MI study consisting entirely of Caucasians might again

be due to population specific effects. Further studies in populations of different ethnic origins might be needed in order to better understand the association of genetic variation in *LTA4H* with MI and stroke.

In summary, our study was able to confirm the association between a common haplotype of *ALOX5AP* and CAD in the European population. There is now increasing evidence from several studies for a role of *ALOX5AP* in the pathogenesis of CAD, particularly in subjects of European descent.

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Table legends

Table 1: Baseline characteristics of the study population stratified by affection status. Displayed are means \pm standard deviations and frequencies in percent.

Table 2: Overview of single-SNP analyses in the *ALOX5AP* and *LTA4H* genes. Genotype abbreviation: 0= homozygous for common allele, 1=heterozygous, 2=homozygous for rare allele; HWE χ^2 = pvalue of *Monte Carlo* χ^2 goodness-of-fit test for deviation from HWE; HWE aequi = confidence intervals for heterozygotes for compatibility with HWE using the exact uniformly most powerful equivalence test with $\delta_2 = 2$ and $\delta_1 = 2/3$, respectively. P-trend: unadjusted, 2-sided p-values of Cochran-Armitage trend test; OR indicates odds ratio; 95% CI indicates 95% confidence intervals of OR.

Table 3a: Association of haplotype A in *ALOX5AP* with MI. Displayed are global and specific haplotype scores. Analyses were performed in 1,211 MI Cases and 1,015 controls. Haplotype A is shaded in grey.

Table 3b: Association of haplotype B in *ALOX5AP* with MI. Displayed are global and specific haplotype scores. Analyses were performed in 1,211 MI Cases and 1,015 controls. Haplotype B is shaded in grey.

Table 4: Subgroup analyses of haplotypes A and B in *ALOX5AP* and rs2660899 in *LTA4H*. Displayed are global and specific haplotype scores for the haplotypes. For rs2660899 MAF, 2-sided p-values of Cochran-Armitage trend test; ORs and 95% CI are presented. Each subgroup was analysed separately versus the 1,015 controls.

Table 1

	MI Cases	Controls
N	1211	1015
Men (%)	782 (64.6)	377 (37.1)
Age, years (%)	58.2 ± 8.6	56.8 ± 9.9
Body mass index, kg/m²	27.5 ± 3.7	26.7 ± 4.2
Systolic blood pressure, mm Hg	138.0 ± 19.7	134.0 ± 17.5
Diastolic blood pressure, mm Hg	82.4 ± 10.3	82.1 ± 9.8
Total cholesterol, mg/dl	227.7 ± 46.0	238.3 ± 42.3
HDL cholesterol, mg/dl	50.6 ± 13.3	60.5 ± 15.3
LDL cholesterol, mg/dl	151.1 ± 42.5	147.5 ± 34.5
Smoking status (%)	822 (67.9)	509 (50.1)
Diabetes (%)	197 (16.3)	56 (5.5)
Antihypertensive medication (%)	1066 (88.0)	382 (7.6)
Lipid lowering medication (%)	801 (66.1)	94 (9.3)

Table 2.

	RS17222814		RS10507391		RS4769874		RS9551963	
Position	30197553		30210096		30224441		30230547	
Major/Minor Allele	G/A		T/A		G/A		C/A	
Gene	<i>ALOX5AP</i> - Hap A		<i>ALOX5AP</i> - Hap A, Hap B		<i>ALOX5AP</i> - Hap A		<i>ALOX5AP</i> - Hap A	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
0	903	819	488	463	971	862	302	245
1	216	153	474	389	101	76	526	455
2	10	9	120	100	1	3	290	190
freq of rare allele	10.5	8.7	33.0	30.9	4.8	4.4	49.5	46.9
HWE χ^2	0.5232	0.5751	0.7849	0.1741	0.5064	0.4154	0.0538	0.4164
HWE aequi	[196,220]	[147,159]	[384,562]	[329,473]	[97,99]	[76,78]	[436,682]	[349,539]
p Trend	0.05675		0.1656		0.5027		0.1118	
OR [95% CI]*	1.22	[0.99,1.51]	1.10	[0.96,1.25]	1.11	[0.82,1.49]	1.11	[0.98,1.25]
OR [95% CI]**	1.50	[0.99,2.27]	1.20	[0.93,1.56]	1.23	[0.68,2.22]	1.22	[0.95,1.56]

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Table 2 (continued)

	RS17216473		RS9315050		RS17222842		RS2660899	
Position	30201965		30234045		30238117		94932888	
Major/Minor Allele	G/A		A/G		C/T		G/T	
Gene	<i>ALOX5AP</i> - Hap B		<i>ALOX5AP</i> - Hap B		<i>ALOX5AP</i> - Hap B		<i>LTA4H</i>	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
0	820	740	966	834	889	735	770	622
1	270	228	162	125	170	188	322	278
2	29	12	3	8	10	10	43	34
freq of rare allele	14.7	12.9	7.4	7.3	8.9	11.1	18.0	18.5
HWE χ^2	0.2787	0.2592	0.1901	0.1583	0.5730	0.6235	0.2283	0.7381
HWE aequi	[248,298]	[200,230]	[148,158]	[125,133]	[164,178]	[170,192]	[290,364]	[244,306]
p Trend	0.09297		0.8659		0.0175		0.6534	
OR [95% CI]*	1.16	[0.98,1.39]	1.02	[0.81,1.29]	0.78	[0.63,0.96]	0.96	[0.83,1.13]
OR [95% CI]**	1.35	[0.95,1.93]	1.04	[0.65,1.66]	0.6	[0.40,0.92]	0.93	[0.68,1.27]

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Table 3a.

SNPs		rs17222814 (G/A), rs10507391 (T/A), rs4769874 (G/A), rs9551963 (C/A)						
Global Score	Nominal p	0.2067						
	Simulated p	0.2058						
Specific Score	Haplotype	GTGC	GTGA	GAGA	ATGA	GAGC	GAAC	
	Haplotype frequency	42.5	15.4	23.3	9.5	4.5	4.2	
	Haplotype Score	-1.9	-1.1	1.2	1.9	0.5	0.4	
	Nominal p	0.0579	0.2804	0.2257	0.0532	0.62244	0.6906	
	Simulated p	0.0577	0.2807	0.2249	0.0533	0.62312	0.6907	

Table 3b.

SNPs		rs17216473 (G/A), rs10507391 (T/A), rs9315050 (A/G), rs17222842 (G/A)						
Global Score	Nominal p	1 x 10 ⁻⁵						
	Simulated p	1 x 10 ⁻⁵						
Specific Score	Haplotype	GTAG	GAAG	AAAA	AAAG	GAGG	GTGG	GTAA
	Haplotype frequency	63.8	13.3	8.1	5.3	4.9	2.2	1.3
	Haplotype Score	-1.5	0.6	-2.5	5.0	0.0	0.1	-0.1
	Nominal p	0.1376	0.5733	0.0113	< 0.0001	0.9822	0.9281	0.8826
	Simulated p	0.1377	0.5737	0.0112	< 0.0001	0.9822	0.9285	0.8833

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Table 4.

	MI	MI + cerebrovascular disease	Early onset MI ≤ 45 years	MI ≥ 50 years
Number of affected individuals	1211	279	304	509
Haplotype A	Haplotype frequency	15.4	15.9	16.1
	Haplotype Score	-1.1	-0.5	0.1
<i>ALOX5AP</i>	Nominal p	0.2804	0.5859	0.8960
	Simulated p	0.2807	0.587	0.8962
Haplotype B	Haplotype frequency	5.3	4.1	4.3
	Haplotype Score	5.0	3.7	4.2
<i>ALOX5AP</i>	Nominal p	< 0.0001	0.0002	< 0.0001
	Simulated p	< 0.0001	0.0003	< 0.0001
	MAF	18.0	18.3	18.7
rs2660899	p trend	0.6534	0.8895	0.9138
	OR per 1 rare allele [95% CI]	0.96 [0.83,1.13]	0.98 [0.77,1.26]	1.01 [0.80,1.28]
	OR per 2 rare alleles [95% CI]	0.93 [0.68,1.27]	0.97 [0.59,1.58]	1.01 [0.83,1.23]

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