

The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke

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We mapped a gene predisposing to myocardial infarction to a locus on chromosome 13q12–13. A four-marker single-nucleotide polymorphism (SNP) haplotype in this locus spanning the gene *ALOX5AP* encoding 5-lipoxygenase activating protein (FLAP) is associated with a two times greater risk of myocardial infarction in Iceland. This haplotype also confers almost two times greater risk of stroke. Another *ALOX5AP* haplotype is associated with myocardial infarction in individuals from the UK. Stimulated neutrophils from individuals with myocardial infarction produce more leukotriene B₄, a key product in the 5-lipoxygenase pathway, than do neutrophils from controls, and this difference is largely attributed to cells from males who carry the at-risk haplotype. We conclude that variants of *ALOX5AP* are involved in the pathogenesis of both myocardial infarction and stroke by increasing leukotriene production and inflammation in the arterial wall.

Cardiovascular diseases (CVD) are the leading causes of death and disability in the developed world¹, with an increasing prevalence due to the aging of the population and the obesity epidemic. More than 1 million deaths in the US alone were caused by myocardial infarction and stroke in 2003 (ref. 2). Some of the processes underlying myocardial infarction are now understood: it is generally attributed to atherosclerosis with arterial wall inflammation that ultimately leads to plaque rupture, fissure or erosion^{3,4}. This process is known to involve diapedesis of monocytes across the endothelial barrier; activation of neutrophils, macrophage cells and platelets; and release of a variety of cytokines and chemokines^{5,6}, but the genetic basis of the process has not yet been deciphered.

Two different approaches have been used to search for genes associated with myocardial infarction. SNPs in candidate genes have been tested for association and have, in general, not been replicated or confer only a modest risk of myocardial infarction. Case-control association studies have identified several proinflammatory genes with variants that are associated with either an increased risk of myocardial infarction or a protective effect^{7–9}. Four genome-wide scans in families with myocardial infarction have yielded several loci with formidable linkage peaks, but the gene(s) underlying these loci have not yet been identified^{10–14}. In addition, one large pedigree study identified a dele-

tion mutation of a transcription factor gene, *MEF2A*, with autosomal dominant transmission¹⁴. This is an interesting cause of myocardial infarction, but the prevalence of this or other mutations in *MEF2A* outside this family remains to be determined.

Here we report a genome-wide scan of 296 multiplex Icelandic families including 713 individuals with myocardial infarction. Through suggestive linkage to a locus on chromosome 13q12–13, we identified the gene (*ALOX5AP*) encoding FLAP and found that a four-SNP haplotype in the gene confers a nearly two times greater risk of myocardial infarction and stroke. FLAP is a regulator¹⁵ of a crucial pathway in the genesis of leukotriene inflammatory mediators, which are implicated in atherosclerosis both in a mouse model¹⁶ and in human studies^{17,18}. Males had the strongest association to the at-risk haplotype, and male carriers of the at-risk haplotype also had significantly greater production of leukotriene-B₄ (LTB₄), supporting the idea that proinflammatory activity has a role in the pathogenesis of myocardial infarction. We confirmed the association of *ALOX5AP* with myocardial infarction in an independent cohort of British individuals with another haplotype. These results indicate that *ALOX5AP* is the first specific gene isolated that confers substantial population-attributable risk (PAR) of the complex traits of both myocardial infarction and stroke.

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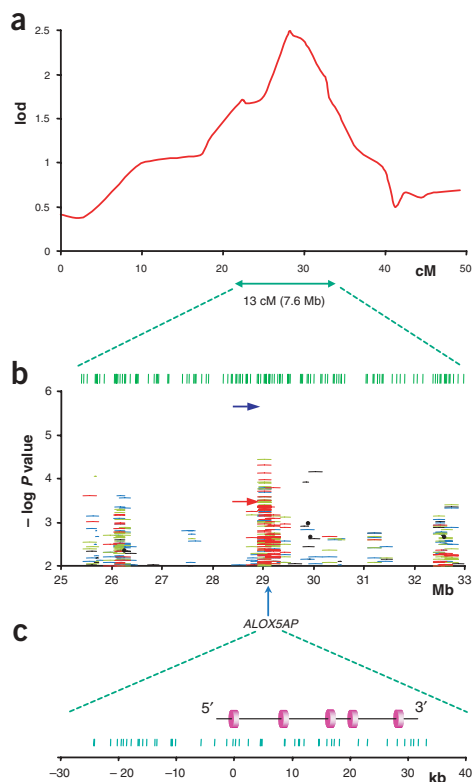


Figure 1 Schematic view of the chromosome 13 linkage region showing *ALOX5AP*. (a) The linkage scan for females with myocardial infarction and the one-lod drop region that includes *ALOX5AP*. (b) Microsatellite association for all individuals with myocardial infarction: single-marker association (black dots) and two-, three-, four- and five-marker haplotype association (black, blue, green and red horizontal lines, respectively). The blue and red arrows indicate the location of the most significant haplotype association across *ALOX5AP* in males and females, respectively. (c) *ALOX5AP* gene structure, with exons shown as colored cylinders, and the locations of all SNPs typed in the region. The green vertical lines indicate the position of the microsatellites (b) and SNPs (c) used in the analysis.

this region was most likely to contribute to myocardial infarction, we typed 120 microsatellite markers in the region and carried out a case-control association study using 802 unrelated (separated by at least three meioses) individuals with myocardial infarction and 837 population-based controls. We also repeated the association study for each of the three phenotypes that were used in the linkage study: individuals with early onset, males and females with myocardial infarction. In addition to testing each marker individually, we also tested haplotypes based on these markers for association. To limit the number of haplotypes tested, we considered only haplotypes spanning less than 300 kb that were over-represented among the affected individuals.

The haplotype with the strongest association to myocardial infarction ($P = 0.00004$) covered a region that contains two known genes: *ALOX5AP* (Fig. 1b) and a gene with an unknown function called highly charged protein (*D13S106E*). The haplotype association in this region for females with myocardial infarction was less significant ($P = 0.0004$) than for all individuals with myocardial infarction, and the most significant haplotype association was observed for males with myocardial infarction ($P = 0.000002$). The haplotype associated with males with myocardial infarction was the only haplotype that retained significant association after adjusting for all haplotypes tested.

FLAP, together with 5-lipoxygenase (5-LO), is a regulator of the leukotriene biosynthetic pathway that has recently been implicated in the pathogenesis of atherosclerosis^{16–18}. Therefore, *ALOX5AP* was a good candidate for the gene underlying the association with myocardial infarction.

Screening for SNPs in *ALOX5AP* and LD mapping

To determine whether variations in *ALOX5AP* significantly associate with myocardial infarction and to search for causal variations, we sequenced *ALOX5AP* in 93 affected individuals and 93 controls. The sequenced region covers 60 kb containing *ALOX5AP*, including the five known exons and introns, the 26-kb region 5' to the first exon and the 7-kb region 3' to the fifth exon. We identified 144 SNPs, of which we excluded 96 from further analysis owing to either a low minor allele frequency or complete correlation (redundancy) with other SNPs. Figure 1c shows the distribution of the 48 SNPs chosen for genotyping, relative to exons, introns and the 5' and 3' flanking regions of *ALOX5AP*. We identified only one SNP in a coding sequence (exon 2), which did not lead to an amino acid substitution. The locations of the 48 SNPs in the National Center for Biotechnology Information human genome assembly build 34 are listed in Supplementary Table 3 online. In addition to the SNPs, we typed a polymorphism consisting of a monopolymer A repeat in the *ALOX5AP* promoter region²⁰.

The linkage disequilibrium (LD) block structure defined by the 48 genotyped SNPs is shown in Figure 2. Strong LD was detected across the *ALOX5AP* region, although at least one historical recombination seems to have occurred, dividing the region into two strongly correlated LD blocks.

RESULTS

Linkage analysis

We carried out a genome-wide scan in search of myocardial infarction susceptibility genes using a framework set of 1,068 microsatellite markers. The initial linkage analysis included 713 individuals with myocardial infarction who fulfilled the World Health Organization (WHO) MONICA research criteria¹⁹ and were clustered in 296 extended families. We repeated the linkage analysis for individuals with early onset, for males and for females separately. A description of the number of affected individuals and families in each analysis is provided in Supplementary Table 1 online, and the corresponding allele-sharing lod scores are given in Supplementary Figure 1 online. None of these analyses yielded a locus of genome-wide significance. The most promising lod score (2.86) was observed on chromosome 13q12–13 for linkage with females with myocardial infarction at the peak marker *D13S289* (Supplementary Fig. 1 online). This locus also had the most promising lod score (2.03) for individuals with early-onset myocardial infarction. After we increased the information on identity-by-descent sharing to over 90% by typing an additional 14 microsatellite markers in a 30-cM region around *D13S289*, the lod score for the association in females dropped to 2.48 ($P = 0.00036$), and the lod score remained highest at *D13S289* (Fig. 1a). In an independent linkage study of males with ischemic stroke or transient ischemic attack (TIA), we observed linkage to the same locus with a lod score of 1.51 at the same peak marker (Supplementary Fig. 2 online), further suggesting that a cardiovascular susceptibility factor might reside at this locus.

Microsatellite association study

The 7.6-Mb region that corresponds to a drop of 1 in lod score in the female-myocardial infarction linkage analysis contains 40 known genes (Supplementary Table 2 online). To determine which gene in

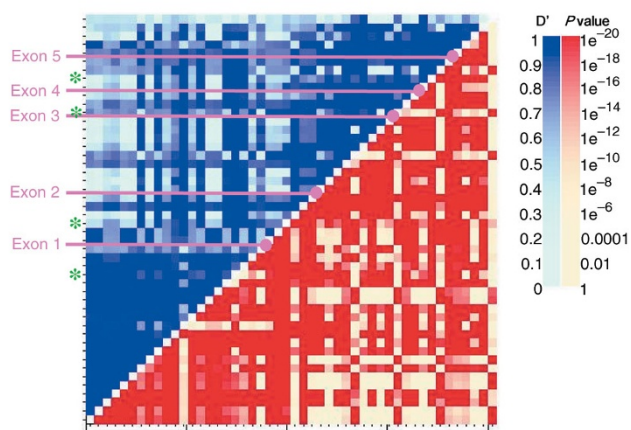


Figure 2 Pairwise LD between SNPs in a 60-kb region encompassing *ALOX5AP*. The markers are plotted equidistantly. Two measures of LD are shown: D' in the upper left triangle and P values in the lower right triangle. Colored lines indicate the positions of the exons of *ALOX5AP*, and the green stars indicate the location of the markers of the at-risk haplotype HapA. Scales for both measures of the LD strength are provided on the right.

Haplotype association with myocardial infarction

In a case-control association study, we genotyped the 48 selected SNPs and the monopolymer A repeat marker in a set of 779 unrelated individuals with myocardial infarction and 624 population-based controls. We tested each of the 49 markers individually for association with the disease. Three SNPs, one located 3 kb upstream of the first exon and the other two 1 kb and 3 kb downstream of the first exon, showed nominally significant association to myocardial infarction (Supplementary Table 4 online). After adjusting for the number of markers tested, however, these results were not significant. We then searched for haplotypes associated with the disease using the same cohorts. We limited the search to haplotype combinations constructed from two, three or four SNPs and tested only haplotypes that were over-represented in the individuals with myocardial infarction. The resulting P values were adjusted for all the haplotypes we tested by randomizing the affected individuals and controls.

Several haplotypes were significantly associated with the disease at an adjusted significance level of $P < 0.05$ (Supplementary Table 5 online). We observed the most significant association with a four-SNP haplotype spanning 33 kb, including the first four exons of *ALOX5AP* (Fig. 1c), with a nominal P value of 0.000023 and an adjusted P value of 0.005. This haplotype, called HapA, has a haplotype frequency of 15.8% (carrier frequency 29.1%) in affected individuals versus 9.5% (carrier frequency 18.1%) in controls (Table 1). The relative risk conferred by HapA compared with other haplotypes constructed from the same SNPs, assuming a multiplicative model, was 1.8 and the corresponding PAR was 13.5%. HapA was present at a higher frequency in males (carrier frequency 30.9%) than in females with myocardial infarction (carrier frequency 25.7%; Table 1). All other haplotypes that were significantly associated with an adjusted P value less than 0.05 were

highly correlated with HapA and should be considered variants of that haplotype (Supplementary Table 5 online).

Association of HapA with stroke and PAOD

Because of the high degree of comorbidity among myocardial infarction, stroke and peripheral arterial occlusive disease (PAOD), with most of these cases occurring on the basis of an atherosclerotic disease, we wanted to determine whether HapA was also associated with stroke or PAOD. We typed the SNPs defining HapA for these cohorts. We removed first- and second-degree relatives and all known cases of myocardial infarction and tested for association in 702 individuals with stroke and 577 individuals with PAOD (Table 1). We observed a significant association of HapA with stroke, with a relative risk of 1.67 ($P = 0.000095$). In addition, we determined whether HapA was primarily associated with a particular subphenotype of stroke and found that both ischemic and hemorrhagic stroke were significantly associated with HapA (Supplementary Table 6 online). Finally, although HapA was more frequent in the PAOD cohort than in the population controls (Table 1), this was not significant. Similar to the stronger association of HapA with males with myocardial infarction than with females with myocardial infarction, HapA also showed stronger association with males than with females with stroke and PAOD (Table 1).

Haplotype association in a British cohort

In an independent study, we determined whether variants in *ALOX5AP* also affected the risk of myocardial infarction in a population outside Iceland. We typed SNPs defining HapA in a cohort of 753 individuals from the UK who had sporadic myocardial infarction and in 730 British population controls. The affected individuals and controls were from three separate study cohorts recruited in Leicester and Sheffield. We found a slightly higher frequency of HapA in affected individuals versus controls (16.8% versus 15.1%, respectively), but the results were not statistically significant. As in the Icelandic population, HapA was more common in males with myocardial infarction (carrier frequency 31.7%) than in females with myocardial infarction (carrier frequency 28.0%). When we typed an additional nine SNPs, distributed across *ALOX5AP*, in the British cohort and searched for other haplotypes that might be associated with myocardial infarction, two SNPs showed association to myocardial infarction with a nominally significant P value (data not shown). Moreover, three- and four-SNP haplotype combinations were associated with higher risk of myocardial infarction in the British cohort, and we observed the most signifi-

Table 1 Association of HapA with myocardial infarction, stroke and PAOD

Phenotype (n)	Frequency	RR	PAR	P value	P value ^a
Myocardial infarction (779)	0.158	1.80	0.135	0.0000023	0.005
Males (486)	0.169	1.95	0.158	0.00000091	ND
Females (293)	0.138	1.53	0.094	0.0098	ND
Early onset (358)	0.139	1.53	0.094	0.0058	ND
Stroke (702) ^b	0.149	1.67	0.116	0.000095	ND
Males (373)	0.156	1.76	0.131	0.00018	ND
Females (329)	0.141	1.55	0.098	0.0074	ND
PAOD (577) ^b	0.122	1.31	0.056	0.061	ND
Males (356)	0.126	1.36	0.065	0.057	ND
Females (221)	0.114	1.22	0.041	0.31	ND

^a P value adjusted for the number of haplotypes tested. ^bExcluding known cases of myocardial infarction.

Shown is HapA of *ALOX5AP* and the corresponding number of affected individuals (n), the haplotype frequency in affected individuals, the relative risk (RR), PAR and P values. HapA is defined by the SNPs SG13S25, SG13S114, SG13S89 and SG13S32 (Supplementary Table 5 online). The same controls ($n = 624$) were used for the association analysis in myocardial infarction, stroke and PAOD as well as for the analysis of males, females and individuals with early onset. The frequency of HapA in the control cohort is 0.095. ND, not done.

Table 2 Association of HapB with myocardial infarction in British individuals

Phenotype (<i>n</i>)	Frequency	RR	PAR	<i>P</i> value	<i>P</i> value ^a
Myocardial infarction (753)	0.075	1.95	0.072	0.00037	0.046
Males (549)	0.075	1.97	0.072	0.00093	ND
Females (204)	0.073	1.90	0.068	0.021	ND

^a*P* value adjusted for the number of haplotypes tested using 1,000 randomization tests.

Shown are the results for HapB that shows the strongest association in the British myocardial infarction cohort. HapB is defined by the SNPs SG13S377, SG13S114, SG13S41 and SG13S35, which have the alleles A, A, A and G, respectively. In all three phenotypes shown, the same set of 730 British controls was used and the frequency of HapB in the control cohort is 0.040. Number of affected individuals (*n*), haplotype frequency in affected individuals, relative risk (RR) and PAR are indicated. ND, not done.

cant association for a four-SNP haplotype with a nominal *P* value of 0.00037 (Table 2). We call this haplotype HapB. The haplotype frequency of HapB was 7.5% in the individuals with myocardial infarction (carrier frequency 14.4%) compared with 4.0% (carrier frequency 7.8%) in controls, conferring a relative risk of 1.95 (Table 2). This association of HapB remained significant after adjusting for all haplotypes tested, using 1,000 randomization steps, with an adjusted *P* = 0.046. No other SNP haplotype had an adjusted *P* value <0.05. The two at-risk haplotypes, HapA and HapB, are mutually exclusive; there are no instances in which the same chromosome carries both haplotypes.

More LTB4 in individuals with myocardial infarction

To determine whether individuals with a past history of myocardial infarction had greater activity of the 5-LO pathway than controls, we measured production of LTB4 (a key product of the 5-LO pathway) in blood neutrophils isolated from Icelandic individuals with myocardial infarction and controls before and after stimulation with the calcium ionophore ionomycin. We detected no difference in

LTB4 production in resting neutrophils from individuals with myocardial infarction versus controls. In contrast, LTB4 generation by neutrophils stimulated with ionomycin was substantially greater in individuals with myocardial infarction than in controls after 15 and 30 min, respectively (Fig. 3a). Moreover, the observed difference in release of LTB4 was largely accounted for by male carriers of HapA (Fig. 3b), whose cells produced significantly more LTB4 than cells from controls (*P* = 0.0042; Supplementary

Table 7 online). There was also a heightened LTB4 response in males who did not carry HapA, but this difference was of borderline significance (Supplementary Table 7 online). This could be explained by additional variants in *ALOX5AP* that have not been uncovered, or in other genes belonging to the 5-LO pathway, that may account for upregulation of the LTB4 response in some individuals without the *ALOX5AP* at-risk haplotype. We did not detect differences in LTB4 response in females (Supplementary Table 7 online), but because of the small sample size, this result is not conclusive. The elevated levels of LTB4 production in stimulated neutrophils from male carriers of the at-risk haplotype suggest that the disease-associated variants of *ALOX5AP* heighten the response of FLAP to factors that stimulate inflammatory cells.

DISCUSSION

Our results show that variants of *ALOX5AP* encoding FLAP are associated with greater risk of myocardial infarction and stroke. In our Icelandic cohort, a haplotype that spans *ALOX5AP* is carried by 29.1% of all individuals with myocardial infarction and almost doubles the risk of myocardial infarction. We then replicated these findings in an independent cohort of individuals with stroke. Furthermore, stimulated neutrophils from individuals with myocardial infarction had greater production of LTB4, one of the key products of the 5-LO pathway. When we examined this in the context of the at-risk haplotype, however, the gain of function was largely attributed to male carriers of the at-risk haplotype, who also had the strongest association with the *ALOX5AP* haplotype. Another haplotype spanning *ALOX5AP* was associated with myocardial infarction in a British cohort. Although the pathogenic variants responsible for the effects associated with the disease haplotypes are unknown, the greater production of LTB4 observed in ionomycin-stimulated neutrophils from male carriers of the at-risk haplotype suggests that the disease-associated variants increase the response of FLAP to factors that stimulate inflammatory cells.

We observed suggestive linkage to chromosome 13q12–13 with several different phenotypic groups, including females with myocardial infarction, individuals of both sexes with early-onset myocardial infarction and males with ischemic stroke or TIA. But we observed the strongest haplotype association for males with myocardial infarction or stroke. Therefore, the linkage signal in females with myocardial infarction and in individuals with early-onset myocardial infarction is not explained by the at-risk haplotype that we identified, and we expect that there may be other unidentified variants or haplotypes in *ALOX5AP*, or in other genes in the linkage region, that may confer risk of these cardiovascular phenotypes. These variants are probably rarer than HapA with relatively high penetrance, higher in women than in men.

FLAP has an important role in the initial steps of leukotriene biosynthesis¹⁵, which is largely confined to leukocytes and can be

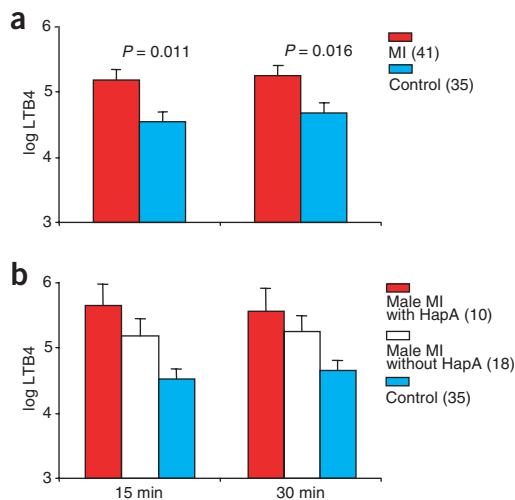


Figure 3 LTB4 production of ionomycin-stimulated neutrophils from individuals with myocardial infarction (*n* = 41) and controls (*n* = 35). The log-transformed (mean ± s.d.) values measured at 15 and 30 min in stimulated cells are shown. (a) LTB4 production in individuals with myocardial infarction (MI) and controls. The difference in the mean values between affected individuals and controls was tested using a two-sample *t*-test of the log-transformed values. (b) LTB4 production in males with myocardial infarction carrying HapA (red bars) and not carrying HapA (white bars). Mean values of controls (blue bars) are included for comparison. Males with HapA produced the highest amounts of LTB4 (*P* < 0.005 compared with controls). Data for females are shown in Supplementary Table 7 online.

triggered by a variety of stimuli. In this biosynthetic pathway, unesterified arachidonic acid is converted to LTA₄ by the action of 5-LO and its activating protein FLAP. The unstable epoxide LTA₄ is further metabolized to LTB₄ or LTC₄ by LTA₄ hydrolase and LTC₄ synthase, respectively. In addition, LTA₄ can be exported to neighboring cells that are devoid of 5-LO activity and become subject to transcellular leukotriene biosynthesis^{21–23}. The leukotrienes have a variety of proinflammatory effects^{24,25}. LTB₄ activates leukocytes, leading to chemotaxis and increased adhesion of leukocytes to vascular endothelium, release of lysosomal enzymes such as myeloperoxidase and production of superoxide anions²⁵. The cysteinyl-containing leukotrienes (LTC₄ and its metabolites LTD₄ and LTE₄) increase vascular permeability in postcapillary venules and are potent vasoconstrictors of coronary arteries^{26–28}.

The importance of the 5-LO pathway is well established in asthma, and drugs inhibiting this pathway have been developed for treating asthma. The role of the 5-LO pathway in the pathogenesis of atherosclerosis has recently received attention. A study of post-mortem pathologic specimens showed an increase in the expression of members of the 5-LO pathway, including 5-LO and FLAP, in atherosclerotic lesions at various stages of development in the aorta, coronary arteries and carotid arteries¹⁸. Furthermore, 5-LO was localized to macrophages, dendritic cells, foam cells, mast cells and neutrophilic granulocytes, and the number of cells expressing 5-LO was markedly greater in advanced lesions¹⁸. The leukocytes positive for 5-LO accumulated at distinct sites that are most prone to rupture²⁹, such as the shoulder regions below the fibrous cap of the atherosclerotic lesion¹⁸. A 5-LO promoter variant is associated with abnormal carotid artery intima-media thickness and heightened inflammatory biomarkers³⁰. In addition, antagonists of LTB₄ block the development of atherosclerosis in apo-E-deficient and LDLR-deficient mice³¹, and a congenic mouse strain with a heterozygous deficiency of 5-LO shows resistance to atherosclerosis¹⁶, further supporting the idea that greater activity of the 5-LO pathway has a role in predisposition to atherosclerosis.

Our data also show that the at-risk haplotype of *ALOX5AP* has higher frequency in all subgroups of stroke, including ischemic stroke, TIA and hemorrhagic stroke. HapA confers significantly higher risk of myocardial infarction and stroke than it does of PAOD. This could be explained by differences in the pathogenesis of these diseases. Unlike individuals with PAOD, who have ischemic legs because of atherosclerotic lesions that are responsible for gradually diminishing blood flow to the legs, individuals with myocardial infarction and stroke have suffered acute events, with disruption of the vessel wall suddenly decreasing blood flow to regions of the heart and the brain.

We did not find association between HapA and myocardial infarction in a British cohort, but we did find significant association between myocardial infarction and a different *ALOX5AP* variant. The existence of different haplotypes of the gene conferring risk to myocardial infarction in different populations is not unexpected. It is not unreasonable to assume that a common disease like myocardial infarction is associated with many different mutations or sequence variations and that the frequencies of these disease-associated variants may differ between populations. It would also not be unexpected for the same mutation to arise on different haplotypic backgrounds.

Our work suggests that *ALOX5AP* has an important role in the pathogenesis of myocardial infarction and stroke in humans. Our study, together with others, may provide the necessary background to launch therapeutic trials to determine whether pharmacological inhibition of FLAP will prevent the development of myocardial infarction and stroke.

METHODS

Study population. We recruited the individuals in the study from a registry of over 8,000 individuals, which includes all individuals who had myocardial infarctions before the age of 75 in Iceland from 1981 to 2000. This registry is a part of the WHO MONICA Project¹⁹. Diagnoses of all individuals in the registry follow strict diagnostic rules based on signs, symptoms, electrocardiograms, cardiac enzymes and necropsy findings.

We used genotypes from 713 individuals with myocardial infarction and 1,741 of their first-degree relatives in the linkage analysis. For the microsatellite association study of the locus associated with myocardial infarction, we used 802 unrelated (no first- or second-degree relatives) individuals with myocardial infarction (233 females, 624 males and 302 with early onset) and 837 population-based controls. The females studied were post-menopausal. Over 90% of the individuals were taking aspirin or other nonsteroidal anti-inflammatory drugs. For the SNP association study in and around *ALOX5AP*, we genotyped 779 unrelated individuals with myocardial infarction (293 females, 486 males and 358 with early onset). The control group for the SNP association study was population-based and comprised of 624 unrelated males and females 20–90 years of age whose medical history was unknown. The stroke and PAOD cohorts used in this study have previously been described^{32–34}. For the stroke linkage analysis, we used genotypes from 342 males with ischemic stroke or TIA that were linked to at least one other male within and including six meioses in 164 families. For the association studies, we analyzed 702 individuals with all forms of stroke (329 females and 373 males) and 577 individuals with PAOD (221 females and 356 males). Individuals with stroke or PAOD who also had myocardial infarction were excluded. Controls used for the stroke and PAOD association studies were the same as used in the myocardial infarction SNP association study.

The study was approved by the Data Protection Commission of Iceland and the National Bioethics Committee of Iceland. We obtained informed consent from all study participants. Personal identifiers associated with medical information and blood samples were encrypted with a third-party encryption system as previously described³⁵.

Statistical analysis. We carried out a genome-wide scan as previously described³³, using a set of 1,068 microsatellite markers. We used multipoint, affected-only allele-sharing methods³⁶ to assess the evidence for linkage. All results were obtained using the program Allegro³⁷ and the deCODE genetic map³⁸. We used the S_{pairs} scoring function^{39,40} and the exponential allele-sharing model³⁶ to generate the relevant 1-degree-of-freedom statistics. When combining the family scores to obtain an overall score, we used a weighting scheme that is halfway on a log scale between weighting each affected pair equally and weighting each family equally. In the analysis, all genotyped individuals who were not affected were treated as 'unknown'. Because of concern with small-sample behavior, we usually computed corresponding P values in two different ways for comparison and report the less significant one. The first P value was computed based on large sample theory, $Z_{\text{lr}} = \sqrt{(2 \log_e(10) \text{lod})}$, and is distributed approximately as a standard normal distribution under the null hypothesis of no linkage³⁶. A second P value was computed by comparing the observed lod score with its complete data sampling distribution under the null hypothesis³⁷. When a data set consisted of more than a handful of families, these two P values tended to be very similar. The information measure we used, which is implemented in Allegro, is closely related to a classical measure of information and has a property that is between 0 (if the marker genotypes are completely uninformative) and 1 (if the genotypes determine the exact amount of allele sharing by descent among the affected relatives)^{41,42}.

For single-marker association studies, we used Fisher's exact test to calculate two-sided P values for each allele. All P values are unadjusted for multiple comparisons unless specifically indicated. We present allelic rather than carrier frequencies for microsatellites, SNPs and haplotypes. To minimize any bias due to the relatedness of the individuals who were recruited as families for the linkage analysis, we eliminated first- and second-degree relatives. For the haplotype analysis we used the program NEMO³², which handles missing genotypes and uncertainty with phase through a likelihood procedure, using the expectation-maximization algorithm as a computational tool to estimate haplotype frequencies. Under the null hypothesis, the affected individuals and controls were assumed to have identical haplotype frequencies. Under the alternative

hypotheses, the candidate at-risk haplotype was allowed to have a higher frequency in the affected individuals than in controls, and the ratios of frequencies of all other haplotypes were assumed to be the same in both groups. Likelihoods were maximized separately under both hypotheses, and a corresponding 1-degree-of-freedom likelihood ratio statistic was used to evaluate statistical significance³². Although we only searched for haplotypes that increased the risk, all reported *P* values are two-sided unless otherwise stated. To assess the significance of the haplotype association corrected for multiple testing, we carried out a randomization test using the same genotype data. We randomized the cohorts of affected individuals and controls and repeated the analysis. This procedure was repeated up to 1,000 times, and the *P* value we present is the fraction of replications that produced a *P* value for a haplotype tested that was lower than or equal to the *P* value we observed using the original affected individual and control cohorts.

For both single-marker and haplotype analysis, we calculated relative risk (RR) and PAR assuming a multiplicative model^{43,44} in which the risk of the two alleles of haplotypes a person carries multiply. We calculated LD between pairs of SNPs using the standard definition of *D'* (ref. 45) and *R*² (ref. 46). Using NEMO, we estimated frequencies of the two marker allele combinations by maximum likelihood and evaluated deviation from linkage equilibrium by a likelihood ratio test. When plotting all SNP combinations to elucidate the LD structure in a particular region, we plotted *D'* in the upper left corner and the *P* value in the lower right corner. In the LD plots we present, the markers are plotted equidistantly rather than according to their physical positions.

Identification of DNA polymorphisms. We identified new polymorphic repeats (dinucleotide or trinucleotide repeats) with the Sputnik program. We subtracted the lower allele of the CEPH sample 1347-02 (CEPH genomics repository) from the alleles of the microsatellites and used it as a reference. We detected SNPs in the gene by PCR sequencing exonic and intronic regions from affected individuals and controls. We also detected public polymorphisms by BLAST search of the National Center for Biotechnology Information SNP database. We genotyped SNPs using a method for detecting SNPs with fluorescent polarization template-directed dye-terminator incorporation⁴⁷ and TaqMan assays (Applied Biosystems).

Isolation and activation of peripheral blood neutrophils. We drew 50 ml of blood from each of 41 individuals with myocardial infarction and 35 age- and sex-matched controls into vacutainers containing EDTA. All blood was drawn at the same time in the early morning after 12 h of fasting. We isolated neutrophils using Ficoll-Paque PLUS (Amersham Biosciences).

We collected the red cell pellets from the Ficoll gradient and then lysed red blood cells in 0.165 M ammonium chloride for 10 min on ice. After washing them with phosphate-buffered saline, we counted neutrophils and plated them at 2×10^6 cells ml⁻¹ in 4-ml cultures of 15% fetal calf serum (GIBCO BRL) in RPMI-1640 medium (GIBCO BRL). We then stimulated cells with maximum effective concentration of ionomycin (1 M). At 0, 15, 30, 60 min after adding ionomycin, we aspirated 600 l of culture medium and stored it at -80 °C for the measurement of LTB₄ release as described below. We maintained cells at 37 °C in a humidified atmosphere of 5% carbon dioxide-95% air. We treated all samples with indomethasine (1 M) to block the cyclooxygenase enzyme.

Ionomycin-induced release of LTB₄ in neutrophils. We used the LTB₄ Immunoassay (R&D systems) to quantify LTB₄ concentration in supernatant from cultured ionomycin-stimulated neutrophils. The assay we used is based on the competitive binding technique in which LTB₄ present in the testing samples (200 l) competes with a fixed amount of alkaline phosphatase-labeled LTB₄ for sites on a rabbit polyclonal antibody. During the incubation, the polyclonal antibody becomes bound to a goat antibody to rabbit coated onto the microplates. After washing to remove excess conjugate and unbound sample, a substrate solution was added to the wells to determine the bound enzyme activity. We stopped the color development and read the absorbance at 405 nm. The intensity of the color is inversely proportional to the concentration of LTB₄ in the sample. Each LTB₄ measurement using the LTB₄ Immunoassay was done in duplicate.

British study population. We recruited three separate British cohorts as described previously^{48,49}. The first two cohorts comprised 549 individuals from

among those who were admitted to the coronary care units of the Leicester Royal Infirmary, Leicester (July 1993–April 1994), and the Royal Hallamshire Hospital, Sheffield (November 1995–March 1997), and satisfied the WHO criteria for acute myocardial infarction in terms of symptoms, elevations in cardiac enzymes or electrocardiographic changes⁵⁰. We recruited 532 control individuals in each hospital from adult visitors of individuals with noncardiovascular disease on general medical, surgical, orthopedic and obstetric wards to find subjects representative of the source population from which the affected individuals originated. Individuals who reported a history of coronary heart disease were excluded.

In the third cohort, we recruited 204 individuals retrospectively from the registries of three coronary care units in Leicester. All had suffered a myocardial infarction according to WHO criteria before the age of 50 years. At the time of participation, individuals were at least 3 months from the acute event. The control cohort comprised 198 individuals with no personal or family history of premature coronary heart disease, matched for age, sex and current smoking status with the cases. We recruited control individuals from three primary care practices located in the same geographical area. In all cohorts, individuals were white of Northern European origin. Local research ethics committees approved all the studies, and individuals provided written informed consent for use of samples in genetic studies of coronary artery disease.

URLs. The Sputnik program is available at <http://espressosoftware.com/pages/sputnik.jsp>. The National Center for Biotechnology Information SNP database is available at <http://www.ncbi.nlm.nih.gov/SNP/index.html>.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

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