Genetic Variation in Members of the Leukotriene Biosynthesis Pathway Confer an Increased Risk of Ischemic Stroke
A Replication Study in Two Independent Populations

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Background and Purpose—The recent finding that genetic variants in 5-lipoxygenase activating protein and leukotriene A4 hydrolase may confer an increased risk of ischemic stroke has implicated the leukotriene family as potential mediators of cardiovascular disease. Using a case control replication methodology, all members of the leukotriene synthesis pathway and their receptors were examined for genetic variants, which may act as risk factors for all ischemic stroke and stroke subtypes.

Methods—A case control methodology using a UK stroke cohort (872 cases, 933 controls) was adopted, with additional 5-lipoxygenase activating protein genotyping and replication of positive findings undertaken in an independent stroke population from Germany (601 cases, 736 controls).

Results—Association was identified with variants in 5-lipoxygenase activating protein, leukotriene C4 synthase (leukotriene A4 hydrolase), and the leukotriene B4 receptor complex. Differing risks were identified for ischemic stroke subtypes. A variant in leukotriene C4 synthase was found to confer a 1.5-fold increase in risk of small vessel disease (RR, 1.515; 1.041 to 2.262; \( P = 0.043 \)) with replication in an independent cohort showing a similar risk (RR, 1.687; 1.065 to 2.675; \( P = 0.026 \)). A haplotype in the leukotriene B4 receptor complex was found to confer a 2.3-fold increase in risk of cardioembolic stroke (RR, 2.118; 1.194 to 3.760; \( P = 0.01 \)) and replication in a German cohort revealed a similar risk with a second distinct haplotype (RR, 2.060; 1.162 to 3.665; \( P = 0.013 \)).

Conclusions—Genetic variation in leukotriene pathway members and their receptors confer an increased risk of ischemic stroke in 2 independent populations. These risks show different magnitudes depending on ischemic stroke subtype. (Stroke. 2008;39:1109-1114.)

Key Words: genetics • inflammation • stroke

Cardiovascular disease is the leading cause of death in the developed world. Evidence suggests inflammatory mechanisms are important in stroke risk, and genetic variation in components of the inflammatory response has been implicated as a risk factor, particularly acting via interaction with proinflammatory conventional risk factors. The finding that variants in 2 members of the leukotriene synthesis pathway, 5-lipoxygenase activating protein (FLAP) and leukotriene A4 hydrolase (LTA4H), confer an increased risk of stroke has lent credence to this hypothesis.

Leukotrienes are short-lived but potent proinflammatory molecules expressed from macrophages, neutrophils, eosinophils, and mast cells (Figure). Cleavage of arachidonic acid by 5-lipoxygenase and the accessory protein FLAP produces leukotriene A4 (LTA4), which is then converted to leukotriene B4 (LTB4) by the action of LTA4 hydrolase or to leukotriene C4 (LTC4) by the action of LTC4 synthase. LTC4 is subsequently converted to leukotriene D4 (LTD4) and leukotriene E4 (LTE4). LTB4 binds to 1 of 2 receptors, LTB4R and LTB4R2 (alternatively known as BLT1 and BLT2), and acts as a potent attractor of neutrophils, induces recruitment of CD8+ T lymphocytes, and promotes leukocyte adhesion to vascular endothelium. LTC4, LTD4, and LTE4, collectively known as cysteinyl leukotrienes, bind to either cysLTR or cysLT2R and can cause altered endothelial cell permeability and vascular smooth muscle cell migration. The effects resulting from leukotriene activity are involved in the early stages of an inflammatory response, and expression of all members of the leukotriene biosynthesis pathway have been identified within human atherosclerotic...
lesions and macrophage derived foam cells, with increased expression demonstrated in advanced lesions.13

Genetic variation in the ALOX5AP gene, which encodes the FLAP protein, has been demonstrated to be an independent risk factor for myocardial infarction and stroke in an Icelandic population, where it conferred a 2-fold increase in myocardial infarction and stroke risk.6 Neutrophils from myocardial infarction cases with the at-risk haplotype produced more LTB4 than controls. A similar finding with genetic variants within LTA4 hydrolase resulting in increased LTB4 production in both the Icelandic and other populations supports the importance of the leukotriene pathway in stroke and myocardial infarction risk.7

In the present study we examined genetic variation in members of the leukotriene biosynthesis pathway and their receptors, and looked for association with ischemic stroke as a whole, as well as stroke subtypes, in a well-phenotyped cohort from the UK. In addition, replication of positive findings in a second ischemic stroke cohort from Western Germany was performed.

Subjects and Methods

UK Cohort

Eight hundred seventy-two consecutive white patients with ischemic stroke were recruited. All patients had brain imaging with CT or MRI and imaging of the carotid arteries with duplex or MRA. Echocardiography was performed when clinically indicated. A standardized risk factor assessment was completed. Hypertension was defined as using antihypertensive therapy for high blood pressure, or having systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg. Increased cholesterol was defined as statin therapy or serum cholesterol >5.2 mmol/L. Cases were classified into stroke subtypes by the same stroke neurologist with review of original imaging using a modified TOAST classification.14 Nine hundred thirty-three white community controls, age- and sex-matched, free of symptomatic cerebrovascular disease were recruited by sampling family doctor lists. A risk factor assessment was performed for all controls. The study protocol was approved by the local research ethics committee and informed consent obtained from all participants.

German Cohort

Six hundred thirty-nine consecutive white patients presenting to a single stroke unit were recruited. All patients had cranial CT for initial imaging and an ECG immediately after hospital admission. Carotid ultrasound was performed in all patients. Four hundred ninety-two (77%) patients underwent MRI with diffusion-weighted images within the first few days after admission. Transcranial Doppler sonography and transthoracal echocardiography were performed in >90% and 60%, respectively. Ischemic stroke was diagnosed in 601 patients, and was further classified into stroke subtypes using a modified TOAST classification.14 Risk factors were assessed as described.

The control group consisted of 736 unrelated individuals selected from the KORAS2000 study, a community-based epidemiological project near Munich. Controls were sampled to match stroke patients for age and gender. The study protocol was approved by the local research ethics committees.

Choice of Variants

Variants were chosen based primarily on location and minor allele frequency >5%, and evidence of significance from previously published studies.6,15 The variants examined are listed in supplemental Table I, available online at http://stroke.ahajournals.org. In brief, we examined 18 variants in FLAP, 2 variants in 5-lipoxygenase, 1 variant in LTC4, 4 variants in LTA4H, 4 variants in CYSLTR1, 2 variants in CYSLTR2, and 5 variants spanning LTB4R and LTB4R2. Replication of variants in the 2 genes in which associations were detected in the UK population (LTC4 and LTB4R) was performed in the German cohort.

Laboratory Methods

DNA was extracted from whole blood with Nucleon kits (Tepnel Laboratory Services UK). For the UK cohort, genotyping of SNPs was performed by a commercial company (K-bioscience) using a patented protected process. For the German cohort, genotyping of SNPs was performed by primer extension of multiplex polymerase chain reaction products with detection by MALDI-TOF (Sequenom).

Statistical Methods

Analysis was undertaken using SPSS version 13. Initial univariate analysis was followed by binary logistic regression correcting for the common cardiovascular risk factors in all cases and by stroke subtype. Haplotype reconstruction was performed using Phase version 2.0.16

Table 1. Population Demographics and Risk Factor Profiles for the UK and German Stroke Cohort and Controls

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>UK Cases</th>
<th>UK Controls</th>
<th>German Cases</th>
<th>German Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, yr (SD)</td>
<td>65 (12.5)</td>
<td>65 (8.9)</td>
<td>65 (18.2)</td>
<td>65 (11.7)</td>
</tr>
<tr>
<td>Male gender</td>
<td>497 (59%)</td>
<td>531 (57%)</td>
<td>403 (67%)</td>
<td>447 (61%)</td>
</tr>
<tr>
<td>Stroke Subtypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large vessel</td>
<td>242 (28%)</td>
<td>...</td>
<td>186 (31%)</td>
<td>...</td>
</tr>
<tr>
<td>Cardioembolic</td>
<td>132 (16%)</td>
<td>143 (24%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Small vessel</td>
<td>153 (18%)</td>
<td>...</td>
<td>86 (14%)</td>
<td>...</td>
</tr>
<tr>
<td>Undetermined</td>
<td>224 (27%)</td>
<td>...</td>
<td>141 (23%)</td>
<td>...</td>
</tr>
<tr>
<td>Other determined</td>
<td>6 (1%)</td>
<td>...</td>
<td>0 (0%)</td>
<td>...</td>
</tr>
<tr>
<td>Combined</td>
<td>86 (10%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Figure. Leukotriene biosynthesis pathway. Solid arrows indicate the pathway, dashed arrows indicate binding of components to their respective receptors. Black arrows indicate the action of an enzyme to generate the next step in the pathway. CYSLTR indicates cysteinyl leukotriene receptor.
A replication threshold of 0.05 on unadjusted data was set for replication in the German cohort. A correction for multiple testing was applied to raw data before analysis using the spectral decomposition method of Nyholt.17 All variants were included in the correction method, which produced a lowered significance threshold to be exceeded before a significant association could be assumed. Haplotypes were treated as single variants and consequently are presented with unadjusted significance values.

Results

Demographics of the stroke and control populations for the UK and German cohorts are shown in Table 1. There was no significant difference in age or sex distribution between the UK and German populations.

Positive findings from the UK cohort are shown in Table 2 for FLAP. Only positive results are presented here. Results from all analyses are shown in supplemental Tables II and III for the UK and German cohorts respectively. All variants were within Hardy Weinberg Equilibrium in control populations (minor allele frequency reported in Table 1).

FLAP Analysis

Examining all stroke cases from the UK cohort, associations were found with FL9, FL10, FL12, and FL14 on single marker tests after adjustment for cardiovascular risk factors. The cohort size allowed meaningful study of large vessel disease, cardioembolic stroke, small vessel disease, and stroke of undetermined etiologies as distinct ischemic stroke subtypes. Associations with FL9, FL12, and FL14 were observed with large vessel disease, FL12 with cardioembolic stroke, FL9, FL10, and FL12 for stroke of undetermined etiology, and FL9, FL10, FL14, and FL18 with small vessel disease (Table 2). No variant showed a significant trend test for linearity (data not shown). Haplotype analysis was then performed (Table 3). Positive findings from the UK cohort are shown in Table 4 for other members of the leukotriene biosynthesis pathway. An analysis was also performed with 3 of the 4 variants from the previously reported HapA and HapB* (FL8 repeatedly failed to amplify by both a commercial genotyping facility and in our experience and was therefore excluded from this analysis). HapA and HapB 3 marker haplotypes showed no association with stroke or any stroke subtype (data not shown). No single marker from these haplotypes showed association with stroke or ischemic stroke subtype in our population except FL14, present as part of HapA.

The AAC haplotype of FL9, FL10, and FL12 was associated with all stroke, and showed increasing risk with number of haplotype copies an individual possesses, conferring a 1.302-fold and 2.069-fold increase in stroke risk when present at ≥1 and 2 copies, respectively (Table 3). Analysis by stroke subtype showed 2 copies of the ACG haplotype of FL9, FL12, and FL14 conferred a 2.596-fold increase in risk

<table>
<thead>
<tr>
<th>Population</th>
<th>FLAP Variants</th>
<th>Haplotype</th>
<th>Copies</th>
<th>Cases (n) With Risk</th>
<th>Risk Allele</th>
<th>Risk</th>
<th>95% CI</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>FL9, 10, 12</td>
<td>AAC</td>
<td>1+</td>
<td>365/774</td>
<td>A</td>
<td>1.302</td>
<td>1.032–1.643</td>
<td>P=0.026</td>
</tr>
<tr>
<td>All cases</td>
<td>FL9, 10, 12</td>
<td>AAC</td>
<td>2</td>
<td>53/774</td>
<td>A</td>
<td>2.069</td>
<td>1.162–3.681</td>
<td>P=0.013</td>
</tr>
<tr>
<td>LVD</td>
<td>FL9, 12, 14</td>
<td>ACG</td>
<td>2</td>
<td>16/201</td>
<td>A</td>
<td>2.596</td>
<td>1.197–5.627</td>
<td>P=0.016</td>
</tr>
<tr>
<td>Undetermined</td>
<td>FL9, 10, 12</td>
<td>AAC</td>
<td>2</td>
<td>16/176</td>
<td>A</td>
<td>2.652</td>
<td>1.292–5.442</td>
<td>P=0.008</td>
</tr>
<tr>
<td>SVD</td>
<td>FL9, 10, 18</td>
<td>AAA</td>
<td>1+</td>
<td>17/138</td>
<td>A</td>
<td>1.723</td>
<td>1.163–2.554</td>
<td>P=0.007</td>
</tr>
</tbody>
</table>

CE indicates cardioembolic; LVD, large vessel disease; SVD, small vessel disease.
of large vessel disease. For small vessel disease, a haplotype containing FL9 conferred a 1.723-fold increase in risk when present at ≥1 copies.

An analysis of HAP A in the German replication population has previously been published.18 We therefore performed a replication study on those variants significant from the UK cohort and included variant FL14 to allow analysis by stroke subtype. The full results are presented in Table 3. This replication study was negative for variants FL9, FL10, FL12, and FL14 in all strokes and all stroke subtypes with the exception of presence of variant FL14 in cardioembolic stroke, which conferred a 1.884-fold increase in stroke risk (P = 0.022; 95% CI, 1.094 to 3.244). This was not a replica-

Table 4. Significant Single Variant Associations With All Strokes and by Stroke Subtype for Members of the Leukotriene Biosynthesis Pathway in Both the UK and German Cohorts

<table>
<thead>
<tr>
<th>Population</th>
<th>Gene</th>
<th>Variant</th>
<th>Copies</th>
<th>Cases (n) With Risk</th>
<th>Allele</th>
<th>Risk</th>
<th>95% CI</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK cohort</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cases</td>
<td>LTC4S</td>
<td>LTC4S_2</td>
<td>2</td>
<td>455/868</td>
<td>A</td>
<td>1.807</td>
<td>1.181–2.763</td>
<td>P = 0.006</td>
</tr>
<tr>
<td>LVD</td>
<td>LTC4S</td>
<td>LTC4S_2</td>
<td>2</td>
<td>116/217</td>
<td>A</td>
<td>2.224</td>
<td>1.107–4.469</td>
<td>P = 0.025</td>
</tr>
<tr>
<td>SVD</td>
<td>LTC4S</td>
<td>LTC4S_2</td>
<td>1+</td>
<td>92/148</td>
<td>A</td>
<td>1.515</td>
<td>1.041–2.265</td>
<td>P = 0.043</td>
</tr>
<tr>
<td>Undetermined</td>
<td>LTC4S</td>
<td>LTC4S_2</td>
<td>2</td>
<td>104/215</td>
<td>A</td>
<td>2.081</td>
<td>1.063–4.037</td>
<td>P = 0.032</td>
</tr>
<tr>
<td>All cases</td>
<td>LTB4R 1/2</td>
<td>LTB4R_2</td>
<td>1+</td>
<td>295/862</td>
<td>G</td>
<td>1.671</td>
<td>1.004–2.782</td>
<td>P = 0.048</td>
</tr>
<tr>
<td>CE</td>
<td>LTB4R 1/2</td>
<td>LTB4R_2</td>
<td>1+</td>
<td>16/73</td>
<td>G</td>
<td>2.371</td>
<td>1.282–4.387</td>
<td>P = 0.006</td>
</tr>
<tr>
<td>CE</td>
<td>LTB4R 1/2</td>
<td>LTB4R_6</td>
<td>1+</td>
<td>19/77</td>
<td>C</td>
<td>2.037</td>
<td>1.146–3.623</td>
<td>P = 0.015</td>
</tr>
<tr>
<td>German cohort</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVD</td>
<td>LTC4S</td>
<td>LTC4S_2</td>
<td>1+</td>
<td>71/86</td>
<td>A</td>
<td>1.687</td>
<td>1.056–2.675</td>
<td>P = 0.026</td>
</tr>
<tr>
<td>All cases</td>
<td>LTB4R 1/2</td>
<td>LTB4R_4</td>
<td>1+</td>
<td>60/601</td>
<td>C</td>
<td>1.531</td>
<td>1.017–2.305</td>
<td>P = 0.041</td>
</tr>
<tr>
<td>CE</td>
<td>LTB4R 1/2</td>
<td>LTB4R_1</td>
<td>1+</td>
<td>18/143</td>
<td>T</td>
<td>2.052</td>
<td>1.156–3.644</td>
<td>P = 0.014</td>
</tr>
<tr>
<td>CE</td>
<td>LTB4R 1/2</td>
<td>LTB4R_4</td>
<td>1+</td>
<td>18/143</td>
<td>C</td>
<td>2.093</td>
<td>1.177–3.722</td>
<td>P = 0.012</td>
</tr>
</tbody>
</table>

Other Leukotriene Pathway Members
Significant single marker associations for the remaining leukotriene biosynthesis pathway members in the UK cohort can be seen in Table 4. For all stroke, after adjustment for cardiovascular risk factors, the LTC4S2 variant in LTC4 conferred a 1.807-fold increase in stroke when present at 2 copies, whereas the LTB4R_2 variant in the LTB4R/LTB4R2 gene region conferred a 1.671-fold increase in stroke risk when present at ≥1 copies. Analysis by subtype revealed the same LTC4 variant to confer a 2.224-fold increase in large vessel disease risk when present at 2 copies, a 1.515-fold increase in risk of small vessel disease when present at ≥1 copies and a 2.081-fold increase in risk of ischemic stroke of undetermined etiology when present at 2 copies. Two variants in the LTB4R/LTB4R2 region, LTB4R_2 and LTB4R_6, conferred relative risks of 2.371 and 2.081 in cardioembolic stroke when present at ≥ copies. No variant showed a significant trend test for linearity (data not shown).

Haplotype analysis of variants in the LTB4R region revealed the risk alleles from the 2 significant variants to be in almost complete linkage disequilibrium, with the frequencies of the other 2 possible haplotypes being <0.1%. As such these 2 variants act as a single variant. The risk associated with the GC haplotype was 2.118 (1.194 to 3.760; P = 0.01), equivalent to the risk seen with each of the variants independently (Table 4).

Replication
Replication of the effects seen in the UK cohort with the LTC4 and LTB4R/LTB4R2 variants was performed in the second population from Germany (Table 4). Examining all cases, no effect was identified for the LTC4 variant. An effect of an LTB4R/LTB4R2 variant, LTB4R_4, was found for all cases with a relative risk of 1.531. Ischemic stroke subtype analysis showed a significant risk associated with the LTC4 variant in small vessel disease but not large vessel disease or stroke of undetermined etiology, whereas 2 LTB4R/LTB4R2 variants, LTB4R_1 and LTB4R_4, showed a 2.052- and 2.093-fold increase in risk of cardioembolic stroke, respectively, compared to controls. Haplotype analysis of these 2 variants again revealed almost total linkage disequilibrium between them, with the TC haplotype conferring a relative risk of 2.060 (1.162–3.665; P = 0.013), equivalent to the risk seen when analyzing each allele independently.

Discussion
This study examined the significance of the previously identified stroke risk gene arachidonate 5-lipoxygenase-activating protein (ALOX5AP) in a UK cohort of ischemic stroke and extended this analysis to other members of the leukotriene biosynthesis pathway and the leukotriene receptors for all ischemic strokes and by ischemic stroke subtype. A replication study of significant findings was then conducted in a second white stroke cohort from Germany.

We found evidence of associations with a number of FLAP SNP, although not with the previously reported haplotype identified by the DeCode study.6 An examination of the previously reported HapA and HapB in ALOX5AP in our cohort was hampered by the inability to genotype variant FL8 using 2 different genotyping protocols. Of the remaining single markers and 3 marker haplotypes, only FL14 was
significant in the UK stroke cohort overall when present at \( \geq 1 \) copies. This failure to replicate previous findings, although possibly attributable to the absence of FL8, is more likely attributable to population differences for the cohorts undergoing investigation and the incidence of disease within them. Despite this, associations outside HapA and HapB were identified in this population, most notably with FL9 in all strokes and several ischemic stroke subtypes. Both FL9 and FL12 appear to show gene dosage effects with increasing copies of the variants giving increasing risk of stroke. This was also shown for haplotypes including FL9 and FL12, although no variant or haplotype gave bigger risks than the presence of FL9 alone.

This study is the first to our knowledge to investigate ischemic stroke subtypes for an effect of ALOX5AP variants. The finding that a single variant, FL9, was associated with a number of ischemic stroke subtypes indicates a likely common causative mechanism attributable to this variant, or one in which it is in strong linkage disequilibrium with the. Leukotrienes are potent inflammatory mediators, and inflammation has been implicated as a mechanism underlying stroke risk. Increased production of leukotrienes therefore is likely to enhance an inflammatory cascade in response to external stimuli and consequently increase stroke risk. Previous studies have shown increased LT4 production to be associated with ALOX5AP variants. Although serum was not available for patients in this cohort to investigate this, confirmation of this finding in patients carrying the FL9 variant should now be performed to assess whether variant FL9 has a biological effect.

The HapA haplotype identified by DeCode has previously been examined in our replication cohort.\(^{18}\) We therefore restricted our replication of ALOX5AP to those variants significant in the UK stroke cohort. No variant replicated was also significant in the replication cohort other than presence of variant FL14 in cardioembolic stroke, although after correction for multiple testing this also became nonsignificant.

Although the specific variants examined in our replication cohort were not positive, significant variants in ALOX5AP from the same cohort have been identified as risks for ischemic stroke.\(^{18}\) A number of studies have now investigated ALOX5AP and ischemic stroke with conflicting results, and meta-analysis or large-scale studies on specific ischemic stroke subtypes should be performed to identify whether ALOX5AP variants are true genetic risk factors.

Examination of the remaining leukotriene biosynthesis pathway members revealed associations only with a variant in LTC4 synthase and 2 variants within the LT4R/LTB4R2 gene region. Associations were identified in all cases and in ischemic subtypes. The minor allele of variant LTC4S\(_{2}\), however, was found to be protective in this cohort, making the observation of 1 or 2 copies combined in small vessel disease requiring cautious interpretation attributable to the small number of individuals carrying 2 copies of the minor allele. Replication in a dedicated small vessel disease population will be necessary to confirm this finding. The risk identified with 2 copies of the major allele in all cases, and in large vessel disease, are more robust, but replication is again advisable.

An increased stroke risk was also observed with variants in the LT4R/LTB4R2 gene region for all stroke, and most noticeably cardioembolic stroke in particular. These 2 receptors show differential expression, with LT4R being expressed predominantly in leukocytes and LTB4R2 showing ubiquitous expression.\(^{19}\) The 2 receptors appear to have different functions, with LT4R showing a higher affinity for LT4 and initiating inflammatory responses,\(^{11}\) whereas LTB4R2 has been implicated in regulating the fate of hematopoietic stem cells.\(^{20}\) The close proximity of the 2 genes in the genome means the risk variants identified in this study cannot be ascribed to either of the genes alone. A single coding variant in each of the LT4R and LTB4R2 genes is detailed in dbSNP (rs17849864 and rs1950504, respectively). However, additional genotyping in both the UK and German cohorts revealed them to be monomorphic in both populations (data not shown). An examination of further promoter variants in an effort to identify causal variants and examination of downstream pathway members of LT4R and LTB4R2 therefore is likely to be required to further elucidate how these increased stroke risks are manifest.

The influence of the cardioembolic subtype on the overall stroke finding can be seen by examining the degree of variance explained by the genotype in question. The LT4R variants have an \( R^2 \) value of 0.016 in cardioembolic stroke, equivalent to a 1.6% contribution to cardioembolic stroke risk. When examining all strokes in the absence of cardioembolic stroke, however, this figure decreases to 0.1%.

Replication is an essential prerequisite of any candidate gene study of this nature, with the role of ALOX5AP variants in stroke risk having now been replicated in several independent populations. The findings for LTC4 and LT4R/LTB4R2 in this study are novel, however. A replication study in a German stroke cohort has been performed and failed to show an association with LTC4 in all stroke cases, although an association was confirmed in the small vessel disease subtype, with a similar risk to that observed in the UK cohort.

Replication of the association between LT4R/LTB4R2 and all strokes, and in particular cardioembolic stroke, adds credence to the initial finding in the UK cohort. Similar risks were identified in both cohorts, with a relatively modest risk of 1.5 to 1.7 observed in all cases, strengthening to risks of 2- to 2.4-fold in cardioembolic stroke. It has been suggested, however, that to be called a true replication the same variant should be identified in both populations as the risk allele. Under these criteria, this should be referred to as supportive evidence rather than direct evidence of replication. At the gene level, however, variants within the same gene are shown to give equal levels of increased risk in the 2 independent populations investigated.

Multiple testing is a concern in candidate gene studies, with a total of 36 variants examined as part of this study. Applying a Bonferroni correction to analyses is generally recognized as being overly severe because the 36 variants are not independent events. An alternative correction method has been proposed based on the number of genes undergoing investigation and the degree of linkage disequilibrium between variants spanning each gene.\(^{17}\) This spectral decomposition method corrects for linkage disequilibrium between
variants and returns a significance level designed to keep the type I error rate at 5%. Although the results presented for the LTB4R gene region are unadjusted, using this spectral decomposition method of Nyholt indicates that a significance threshold of 0.048 needs to be exceeded when examining variants LTB4R_2 and LTB4R_6 alone in cardioembolic stroke, and a threshold of 0.02 when examining all LTB4R_1/ LTB4R_2 variants in all cases and controls. Both the initial population and the replication cohort of cardioembolic strokes exceed this threshold. Comparable Bonferroni correction thresholds would be 0.05 divided by 6, or 0.008. Only LTB4R_2 in the UK cardioembolic cohort would exceed this threshold. There is no clear consensus on how to correct for multiple testing, with suggestions ranging from the most conservative (Bonferroni) through to no correction but cautious interpretation. Until a clear consensus is agreed, replication and confirmation from further functional studies must remain the benchmarks of confirmation in genetic association studies.

In conclusion, this study has confirmed that variants in ALOX5AP confer an increased risk of ischemic stroke and show that these variants may contribute differing risks to different ischemic stroke subtypes. In addition, variants around the LTB4R/LTB4R2 gene region have been identified that contribute a similar risk to ischemic stroke risk as ALOX5AP variants, and a greater risk to cardioembolic stroke. Together these findings implicate the leukotriene biosynthesis pathway in the underlying pathogenesis of ischemic stroke, and suggest genetic variation within leukotriene biosynthesis pathway members may account for this increased risk. To our knowledge this is the first such investigation into ischemic stroke subtypes, and it suggests that novel mechanisms may be implicated in different subtypes of ischemic stroke.

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None.

References
Genetic Variation in Members of the Leukotriene Biosynthesis Pathway Confer an Increased Risk of Ischemic Stroke: A Replication Study in Two Independent Populations

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