Genetic Variation within the Interleukin-1 Gene Cluster and Ischemic Stroke

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Abstract

Background and Purpose: *Evidence is emerging that inflammation plays a key role in the pathophysiology of ischemic stroke (IS). The aim of this study was to investigate whether genetic variation in the interleukin-1α (IL-1 α), interleukin-1β (IL-1β) and interleukin-1 receptor antagonist (IL-1RA) genes (IL1A, IL1B and IL1RN) is associated with IS and/or any etiological subtype of IS.*

Methods: *Twelve tagSNPs were analyzed in the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), which comprises 844 patients with IS and 668 controls. IS subtypes were defined according to the TOAST criteria in SAHLSIS. The Lund Stroke Register (LSR) and the Malmö Diet and Cancer study (MDC) were used as a replication sample for overall IS (in total 3145 patients and 1793 controls).*

Results: *The SNP rs380092 in IL1RN showed an association with overall IS in SAHLSIS (odds ratio (OR) 1.21, 95% confidence intervals (CI) 1.02-1.43, P=0.03), which was replicated in the LSR and MDC sample. An association was also detected in all samples combined (OR 1.12, 95% CI 1.04-1.21, P=0.03). Three SNPs in IL1RN (including rs380092) were nominally associated with the subtype of cryptogenic stroke in SAHLSIS, but the statistical significance did not remain after correction for multiple testing. Furthermore, increased plasma levels of IL-1RA were observed in the subtype of cryptogenic stroke compared to controls.*

Conclusion: *This comprehensive study, based on a tagSNP approach and replication, presents support for the role of IL1RN in overall IS.*

Introduction

Ischemic stroke (IS) is a complex disease and involves a large array of biological processes which together determine the susceptibility to develop and sustain ischemic events. Accumulating evidence supports a role for inflammation.[1] One of the most potent a pro-inflammatory cytokine is interleukin-1 (IL-1), the activity of which can be inhibited by the endogenous receptor antagonist IL-1RA. Interestingly, there are experimental data suggesting a role for IL-1/IL-1RA in IS. For instance, IL-1RA has been shown to reduce, while IL-1β exacerbates brain damage in a middle cerebral artery occlusion (MCAO) model in mice.[2] IL-1 and IL-1RA may also contribute to the pathophysiology of IS by promoting vessel wall inflammation and atherosclerosis. In humans, mRNA levels of both IL-1β and IL-1RA have been found to be higher in atherosclerotic arteries than in normal arteries.[3] Furthermore, it has been suggested that IL-1 has protrombotic effects, as IL-1 can induce tissue factor and plasminogen activator inhibitor type-1 gene expression.[4, 5]

Given the putative role of IL-1/IL-1RA both in the pathogenesis of IS and in ischemic injury, this pathway has been investigated in clinical studies on IS. These studies show that patients with IS have higher plasma levels of both IL-1β and IL-1RA compared to controls.[6, 7] A smaller study also revealed increased levels of IL-1β in the cerebrospinal fluid after IS, which may indicate an importance of IL-1 locally within the brain.[8] Analysis of genetic variation within genes coding for inflammatory mediators can offer some advantage compared to analyses of the plasma protein levels, as genetic variation may affect local levels of the protein, and reflect lifelong (rather than transient) inflammation status. Genetic variation in the IL-1 gene cluster has been investigated in relation to IS, the results of which are summarized in Table S1 in the online supplement at http://stroke.ahajournals.org. No consistent picture emerges, which may be due to the fact that most previous studies are small and include a restricted number of SNPs. However, even with regards to somewhat larger samples (>200 cases), both the presence and the absence of an association with IS have been reported.[9-14] Because IS is a heterogenous disease with different etiological subtypes it is possible that these inconsistent results may reflect subtype specificity. Furthermore, as the influence of genetic factors in IS is more pronounced in younger subjects,[15] the age of the participants may play a role.

Therefore the aim of the present study was to investigate whether there is an association between genetic variation in the IL-1 gene cluster and overall IS and/or any etiological subtypes of IS using the tagSNP approach in a sample of relatively young (≤ 70 years) stroke cases and controls. Two other Swedish samples were used for replication.

Subjects and Methods

Study populations

The study population comprised participants in the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), the design of which has been reported.[16] Briefly, Caucasian patients who presented with first-ever or recurrent acute IS before reaching the age of 70 years (*n*=844) were consecutively recruited between 1998 and 2008 at four stroke units in Western Sweden. Healthy Caucasian community controls (n=668) from the same geographic area as the cases were randomly selected to match cases regarding to age and sex.[16] The patients were classified into IS etiologic subtypes according to the TOAST criteria, with minor modifications as described in the online supplement at http://stroke.ahajournals.org [17]

The Lund Stroke Register (LSR) and the Malmö Diet and Cancer study (MDC) were used as a replication sample. Sample

characteristics, data collection and clinical definitions have been described.[18, 19] Briefly, LSR is a prospective, epidemiologic register which consecutively includes all patients with first-ever stroke from the area of Lund University Hospital. Controls were selected from the same region. MDC is a prospective, population based cohort study which included 28 449 randomly selected persons at baseline examinations between 1991 and 1996 from which all incident cases of IS up to 2006 were included and matched for age and sex with stroke-free control subjects. At present, subtype data according to the same TOAST classification system as used in SAHLSIS are not available for all patients in the LSR and MDC samples.

Informed consent was obtained, and the studies were approved by the local Ethics Committees.
Functional outcome at 3 months and at 2 years after index IS was assessed according to the modified Rankin Scale (mRS) for the first 600 patients in SAHLSIS.

Genotyping
To select a set of SNPs capturing common variation in the IL-1α, IL-lβ, and IL-1RA genes (*IL1A*, *IL1B* and *IL1RN,* respectively), we used data from the HapMap project on the CEU population (release 23). The "Tagger" program in HaploView was used to select a minimal set of tagSNP such that all alleles (MAF>0.1) to be captured were correlated at an r^2 greater than the 0.8 threshold. This resulted in 12 tagSNP (including rs16944 in *IL1B*, also known as -511). In addition, rs4251961 was selected because it is located upstream of the *IL1RN* transcription start site and has been reported to associate with plasma levels of IL-1RA.[20]

Blood sampling and IL-1RA measurements
Standardized blood sampling and isolation of plasma was performed for the first 600 patients in SAHLSIS. In the present study, we selected patients with cryptogenic stroke, together with age- and sex-matched controls, to measure the plasma levels of IL1RA. This was performed as part of the analysis of a larger panel of cytokines using a Human Antibody Bead 25-plex kit for the Luminex system (Life Technologies, Carlsbad, CA, USA).

Statistical analysis
Hardy-Weinberg equilibrium (HWE) was assessed both in controls and cases. Associations between single SNPs and case-control status or functional outcome were investigated using an additive model in binary logistic regression, primarily adjusted for age and sex. In a second model, the vascular risk factors, hypertension, diabetes, and smoking were also included as covariates. Assuming a multiplicative genetic model, the odds ratios (ORs) that can be detected with 80% power at the 5% level are in the range of 1.23 to 1.34, depending on the frequency (MAF 0.4-0.12) of the high risk allele for overall IS in SAHLSIS. Correction for multiple testing in the genetic analyses without subsequent replication was conducted using Bonferroni correction.

Differences in IL-1RA plasma values between cases and controls were examined with Mann-Whitney U-test. Time point differences in IL-1RA levels were compared using Wilcoxon Signed Ranks. Association between plasma IL-1RA values and case-control status was investigated using binary logistic regression, primarily adjusting for age and sex. In a second model, hypertension, diabetes, and smoking were also included as covariates. The reported ORs for IL-1RA were scaled to estimate the ORs associated with an increase of 1 standard deviation (SD) in the log IL-1RA plasma level. Associations between genotypes and plasma levels of IL-1RA, hsCRP, and fibrinogen were analyzed using logarithmically transformed plasma values

in a linear regression analysis adjusting for age and sex.

For further details on study populations, genotyping, protein measurements, and statistical analyses please see the online supplement at http://stroke.ahajournals.org.

Results

Baseline characteristics of the samples are presented in Table 1. The genotype distribution for all SNPs conformed to HWE (P>0.05) in controls and cases, except for rs1143634 in *IL1B* which was excluded from further analysis.

Association between overall ischemic stroke and genetic variation in IL1RN, IL1A, and IL1B

The observed genotype frequencies in SAHLSIS are presented in Table S2 (online supplement at http://stroke.ahajournals.org). The minor allele (A) of rs380092 in *IL1RN* was significantly associated with an increased risk for overall IS, when adjustments were made for age and sex (Table 2). This association remained after adjustment for hypertension, diabetes and smoking (Table 2). No SNP in *IL1A* or *IL1B* showed association with overall IS. In previous studies genetic variation in *IL1B* has shown association with obesity.[21] However, including BMI as a covariate in the above analyses did not alter the results. Haplotype analyses did not add any further information (online supplement at http://stroke.ahajournals.org, Table S3).

To investigate whether we could replicate the findings of an association between overall IS and genetic variation in *IL1RN*, we genotyped rs380092 in LSR and MDC (Table S4). A significant association was observed when adjusting for age and sex (Table 2). This association remained after adjusting also for hypertension, diabetes and smoking (Table 2). Including study site as a covariate in the analysis did not alter the result. A significant association was also observed in a joint analysis of the discovery and the replication samples (Table 2).

Association between ischemic stroke subtypes and genetic variation in IL1RN, IL1A, and IL1B

For genotype frequencies in IS subtypes in SAHLSIS, please see Table S5 in the online supplement at http://stroke.ahajournals.org. The minor alleles of rs380092, rs452204, and rs454078 in *IL1RN*, were all nominally associated with cryptogenic stroke. (Table 3). No association for *IL1RN* was detected for any of the other main IS subtypes. The SNP rs16944 in *IL1B* showed a nominal association with cardioembolic (CE) stroke (OR 0.75 (0.57-0.99), *P*=0.04) and rs1143643 in *IL1B* showed a nominal association with large-vessel disease (LVD; OR 1.37 (1.02-1.86), *P*=0.04). None of the subtype-specific results remained significant after adjustment for multiple testing.

Plasma IL-1RA levels in the ischemic stroke subtype of cryptogenic stroke

We further elucidated the role of IL-1RA in cryptogenic stroke by analyzing plasma protein levels in this subsample of SAHLSIS. Plasma IL-1RA levels, both acutely and in the convalescent phase (three months after index stroke), were elevated compared to controls (median and interquartile range 266 (183-448), 239 (180-347), and 188 (110-320), respectively). There was no significant difference in the plasma level between the two time points. Because approximately 21% of the samples had IL-1RA levels that were below the detection limit of the assay, we also tested whether the proportion of cases and controls with detectable IL-1RA plasma levels differed. IL-1RA was detected in a larger proportion of samples from patients (93% in the acute phase and 88% at convalescent phase) than from controls (69%). Binary logistic regression revealed a significant association between cryptogenic stroke and IL-1RA plasma

levels, both in the acute phase and in the convalescent phase (Table 4).

No association between genetic variation in *IL1RN* and plasma levels of IL-1RA was detected in cryptogenic stroke. In controls, association between rs928940 in *IL1RN* and IL-1RA plasma levels was observed (P=0.02), but this association was not retained after including vascular risk factors in the model. Because hsCRP and fibrinogen levels have been observed to associate with genetic variation in *IL1RN,*[22] associations between IL-1 gene variants and plasma levels of hsCRP and fibrinogen were analyzed in the whole sample in SAHLSIS. No association between SNPs in the IL-1 gene cluster and plasma levels of these variables was observed. Levels of hsCRP and fibrinogen in relation to case-control status in SAHLSIS have been presented.[23, 24]

Functional outcome 3 months and 2 years after ischemic stroke

No association between functional outcome three months or two years after index stroke and genetic variation in the analyzed genes was detected.

Discussion

We report the largest case-control study investigating genetic variation in the IL-1 gene cluster in overall IS and IS subtypes, and our study suggests an association between genetic variation in *IL1RN* and overall IS.

In contrast to most previous genetic association studies on IS and IL-1, we used a tagSNP approach to capture the genetic variation within the IL-1 gene cluster. The main finding was that the SNP rs380092 in *IL1RN* was associated with overall IS in the primary sample, and the replication sample. We did not genotype the commonly analyzed 86-bp variable number tandem repeat (VNTR) in *IL1RN*. However, SNP rs454078, which is in strong linkage with the VNTR (r^2=0.99 [22]) did not show an association with overall IS in the present sample. This is in line with previous studies on IS, where a lack of association for the VNTR (or for rs419598; a SNP in strong linkage with rs454078) has been observed.[10-12] In contrast, in smaller, predominantly Asian studies, an association between the VNTR and IS has been reported, please see Table S1 in the online supplement at http://stroke.ahajournals.org.[14] This discrepancy could be due to the relatively small samples sizes or differences between populations.

This is the first study on genetic variation in *IL1RN* and IS that investigates the different subtypes separately in a larger sample of stroke patients. The minor alleles of rs380092, rs454078, and rs452204 in *IL1RN*, were all nominally associated with cryptogenic stroke in SAHLSIS. Interestingly, it has been previously suggested that inflammation plays a pathophysiological role in cryptogenic stroke. Even if the associations for IL1RN did not withstand correction for multiple testing, we therefore continued by analyzing plasma levels of IL-1RA in the subtype of cryptogenic stroke, and elevated IL-1RA levels compared to controls were observed. This is in concordance with studies on overall IS, where an elevated plasma level of IL-1RA has been detected in patients compared to controls.[7, 25] Although not statistically significant, the median plasma levels in the present study are lower in the convalescent than in the acute phase. Furthermore, a larger proportion of samples was below the detection limit in the convalescent phase compared to the acute phase. Thus, the present and previous data indicate that IL-1RA levels decline over time after stroke, [25, 26] and that increased IL-1RA levels in the acute phase reflect an inflammatory response. The elevated convalescent phase IL-1RA levels in our study may, however, indicate that levels were increased also before stroke onset. Results from

prospective studies support the hypothesis that inflammation is involved in the pathogenesis of stroke as elevated CRP plasma levels are found to be a risk for stroke.[27] To further elucidate the potential role of systemic IL-1RA levels in IS, subtype-specific prospective studies on IL-1RA are clearly warranted.

The potential functional role of the SNP that showed association with overall IS in the present study, rs380092 in *IL1RN,* is unknown. This SNP is located in an intron and was not associated with plasma levels of IL-1RA in our study. For the exonic synonymous SNP rs315952, which is strongly linked to rs380092 (r^2=0.8), there are conflicting data concerning its association with plasma IL-1RA levels.[20, 28] Furthermore, it cannot be ruled out that there are unknown SNPs that are linked to rs380092 that have an effect on the IL-1RA gene expression and/or protein function. It could also be speculated that the effect of the genetic variation in *IL1RN* on IS is mediated by increased plasma levels of CRP and fibrinogen, because associations between genetic variation in *IL1RN* and plasma levels of these proteins have been reported.[22] However, results from our study do not support this hypothesis.

One of the strengths of the present study is that it includes well-characterized patients together with population-based controls, and that both the initial sample and the replication sample are relatively homogenous as all participants are Caucasian and from the Southwest of Sweden. There are also some limitations. First, this study is based on hospitalized cases, but the stroke admission rate in Sweden is high with >87% of cases aged <75 years being admitted to hospital.[19] Second, the discovery sample comprising 844 patients with IS has limited power to detect associations with low odds ratios. Additionally, we had limited power in the analyses of IS subtypes and therefore we cannot exclude a role for IL-1R in the subtypes small-vessel disease, LVD, and CE stroke. Third, with regard to IL-1RA plasma levels, a detailed history of recent infections was not available, and because the measurement was conducted as part of analyzing a larger panel of cytokines the detection limit was not optimal and some samples were below this limit. Finally, the study lack data on drinking habits.

To our knowledge this is the largest study analyzing genetic variation in the IL-1 gene cluster in IS to date, and it indicates an association between genetic variation in *IL1RN* and overall IS. In addition, this study adds novel information about the IS subtype of cryptogenic stroke, as genetic variation in *IL1RN* as well as plasma levels of IL-1RA were associated with this subtype. However, as the genetic association for cryptogenic stroke did not withstand correction for multiple testing, this finding is merely hypothesis generating and requires replication.

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Skåne Competence Center, Skåne University Hospital.

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Table 1. Baseline characteristics of the control and overall ischemic stroke groups in the discovery sample and in the replication sample.

	Discovery sample (SAHLSIS)		Replication sample (LSR and MDC)	
	Control (n=668)	Ischemic stroke (n=844)	Control (n=1793)	Ischemic stroke (n=3145)
Median age, years (IQR)	58 (50-64)	59 (51-64)	72 (65-79)	74 (66-81)*
Male, n (%)	392 (59)	554 (66)†	999 (56)	1661 (53)‡
Hypertension, n (%)	230 (34)	487 (58)*	944 (53)	2143 (69)*
Diabetes, n (%)	33 (5)	153 (18)*	93 (5)	641 (21)*
Current smoking, n (%)	131 (20)	324 (38)*	282 (16)	725 (24)*
Mean BMI, (SD)	26.4 (4.0)	26.6 (4.5)		

SAHLSIS, the Sahlgrenska Academy Study on Ischemic Stroke; LSR, the Lund Stroke Register; MDC, the Malmö Diet and Cancer study; BMI, body mass index. Data are shown as mean and standard deviation (SD), median and interquartile range (IQR), or number (n) and percentage. Differences between the patients and the controls were examined with the χ^2 test for proportions, and with the Mann-Whitney U-test or t-test for continuous variables. * $P<0.001$, † $P<0.01$, and ‡ $P<0.05$ compared with the control group.

Table 2. Odds ratios and 95% confidence intervals for the associations between the SNP rs380092 in *IL1RN* and overall ischemic stroke in the discovery sample, the replication sample, and in the samples combined.

SNP	Model 1* OR (95% CI), *P*	Model 2† OR (95% CI), *P*
rs454078	0.71 (0.54-0.93), 0.01	0.69 (0.52-0.92), 0.01
rs380092	1.29 (1.02-1.64), 0.04	1.33 (1.04-1.72), 0.03
rs452204	0.76 (0.60-0.96), 0.02	0.77 (0.60-0.99), 0.04

Abbreviations are as in Table 1. Odds ratio (OR) with 95% confidence intervals (95% CI) for ischemic stroke for the minor allele. *Model 1 (adjusted for age and sex); †model 2 (adjusted for age, sex, hypertension, diabetes, and smoking).

Table 3. Odds ratios and 95% confidence intervals for the associations between SNPs in *IL1RN* and the ischemic stroke subtype of cryptogenic stroke (n=206) in SAHLSIS.

	Discovery sample (SAHLSIS)	Replication sample (LSR and MDC)	Combined sample (SAHLSIS, LSR and MDC)
OR (95% CI), *P**	1.21 (1.04-1.42), 0.02	1.09 (1.00-1.19), 0.05	1.13 (1.04-1.23), 0.03
OR (95% CI), *P*†	1.21 (1.02-1.43), 0.03	1.10 (1.00-1.21), 0.04	1.12 (1.04-1.21), 0.03

Abbreviations are as in Tables 1 and 2, *Model 1 (adjusted for age and sex); †model 2 (adjusted for age, sex, hypertension, diabetes, and smoking). The presented *P*-values are crude uncorrected *P*-values. No *P*-value is significant after Bonferroni correction (n=48).

Table 4. Odds ratios and 95% confidence intervals for cryptogenic stroke per 1 standard deviation increase in log plasma IL-1RA concentration in the acute phase and at the 3-month follow-up after index stroke.

	Model 1 OR (95% CI), *P*	Model 2 OR (95% CI), *P*
Acute phase IL-1RA	1.53 (1.15-2.02), 0.003	1.51 (1.12-2.05), 0.007
3-month follow-up IL-1RA	1.37 (1.04-1.80), 0.027	1.37 (1.02-1.84), 0.034

Abbreviations are as in Table 2. Model 1, adjusted for age and sex; model 2, adjusted for age, sex, hypertension, diabetes, and smoking.

ONLINE SUPPLEMENT

Genetic Variation within the Interleukin-1 Gene Cluster and Ischemic Stroke

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Introduction

Genetic variation in the IL-1 gene cluster has previously been investigated in relation to ischemic stroke (IS), mainly in small studies analyzing only a very restricted number of SNPs. These studies are summarized in Table S1.

Detailed description of study populations

The Sahlgrenska Study of Ischemic Stroke (SAHLSIS)

The study population comprised Caucasian patients (n=844) with first-ever (n=732) or recurrent (n=112) IS who were consecutively recruited between 1998 and 2008 at four stroke units in Western Sweden. Healthy Caucasian population controls (n=668) were randomly selected to match cases with regards to age and sex. Controls were from the same geographical region as the patients, and they were recruited through a population-based health survey or from the Swedish Population Register[1]. IS was defined as an episode of focal neurological deficits with acute onset and lasting >24 hours or until death, with no apparent non-vascular cause, and no signs of primary hemorrhage on brain imaging. Information on the subjects' vascular risk factors was collected as described elsewhere.[2] Hypertension was defined by pharmacological treatment for hypertension, systolic blood pressure ≥ 160 mm Hg, and/or diastolic blood pressure ≥ 90mm Hg. Diabetes mellitus was defined by diet or pharmacological treatment, fasting plasma glucose ≥ 7.0 mmol/L, or fasting blood glucose ≥ 6.1 mmol/L. Smoking habit was coded as current versus never or former. Information about diabetes mellitus was missing in 2 participants, hypertension in 12, smoking habits in 4, and BMI in 37.

All cases underwent ECG and neuroimaging with computed tomography (CT) and/or magnetic resonance imaging (MRI). Extracranial carotid and vertebral duplex ultrasound, MR angiography, catheter angiogram, transcranial Doppler ultrasound, transthoracic and/or transesophageal echo-cardiography were performed when clinically indicated. Based on clinical presentation and results from the diagnostic work-up, case were classified into IS etiologic subtypes according to modified Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.[2] In order to minimize interrater variability, the original TOAST criteria were refined according to a local protocol. Risk factors, other than atrial fibrillation and carotid stenosis (*i.e*. hypertension and diabetes), were not included in the protocol. Adjudication of subtypes were performed by two neurologists (KJ and CB)

Large-vessel disease (LVD) was defined as either occlusive or significant stenosis (corresponding to ≥50% diameter reduction according to NASCET criteria) of a clinically

relevant precerebral or cerebral artery, presumably due to atherosclerosis, or complex plaque (>4 mm thick, ulcerated or mobile) in the aortic arch. Potential causes of cardiac embolism should be excluded. Small-vessel disease (SVD) was defined as a clinical lacunar syndrome with a relevant infarct of <15 mm or normal CT/MRI in the absence of both a cardioembolic (CE) source and significant stenosis/occlusion due to atherosclerosis of an appropriate major brain artery. CE stroke was defined as the presence of atrial fibrillation, sick sinus syndrome, myocardial infarction in the past four weeks, cardiac thrombus, infective endocarditis, atrial myxoma, prosthetic mitral or aortic valve, valvular vegetations, left ventricular akinetic segment, dilated cardiomyopathy, or patent foramen ovale in combination with either atrial septal aneurysm or deep venous thrombosis. Significant stenosis/occlusion due to atherosclerosis of an appropriate precerebral or cerebral artery should be excluded. Other determined cause of stroke included those with arterial dissection, vasculitis, hemathologic disorders, monogenic syndromes and complications of cardiovascular procedures. Cryptogenic stroke was defined when no cause was identified despite an extensive evaluation. Undetermined stroke included cases for which more than one etiology was identified or when the evaluation was cursory. The distribution of subtypes was as follows: LVD n=111, SVD n=165, CE stroke n=151, other determined cause of stroke n=92, cryptogenic stroke n=206, undetermined stroke n=119. Stroke of other determined cause and undetermined stroke were not included in the subtype analysis in the present study.

Functional outcome three months and two years after IS was assessed according to the modified Rankin Scale (mRS) for the first 600 patients in SAHLSIS (missing scores for 31 and 8 patients, respectively, at the two time points). As previously described, the mRS score was dichotomized for death or dependency (mRS 3-6) versus a favourable outcome (mRS 0-2). [2]

All participants provided informed consent prior to enrolment. For participants who were unable to communicate, consent was obtained from their next-of-kin. This study was approved by the Ethics Committee of the University of Gothenburg.

Study population, the Lund Stroke Register (LSR) and the Malmö Diet and Cancer study (MDC)
Sample characteristics, data collection and clinical definitions have been described.[3, 4] IS was defined as in SAHLSIS, and all patients underwent neuroimaging or autopsy. Hypertension was defined by pharmacological treatment for hypertension, systolic blood pressure ≥ 160 mm Hg, and/or diastolic blood pressure ≥ 90mm Hg. Diabetes mellitus was defined by diet or pharmacological treatment, fasting plasma glucose ≥ 7.0 mmol/L, or fasting blood glucose ≥ 6.1 mmol/L. Smoking habit was coded as current versus never or former. In the combined sample of the Lund Stroke Register and the Malmö Diet and Cancer study 111 participants had missing data for diabetes mellitus, 77 for hypertension, and 87 for smoking habit.

All participants provided informed consent prior to enrolment. For participants who were unable to communicate, consent was obtained from their next-of-kin. The studies were approved by the Ethics Committee of Lund University.

Genotyping
In SAHLSIS, genotyping was performed as a part of the analysis of a larger panel of SNPs using the Golden Gate assay (Illumina Inc., San Diego, CA, USA) at the SNP&SEQ Technology platform (www.genotyping.se), at Uppsala University. The assay for rs16944 and rs4251961 were genotyped at the Genomics Core Facility platform at the Sahlgrenska

Academy at University of Gothenburg using the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). Genotyping for the replication in LSR and MSC (rs380092) was conducted at KBioscience (Hoddesdon, UK). The genotyping success rates were between 98 and 100%.

Blood sampling and protein measurements

In SAHLSIS, blood sampling was performed within 10 days of the index stroke (median 4 days) and at a 3-month follow-up visit, also denoted convalescent phase (median 101 days, range 85-125 days). For the control subjects, blood sampling was performed once. Blood samples were always drawn between 8:30 and 10:30 AM after an overnight fast. Plasma and serum were isolated within two hours by centrifugation 2000 x *g* 4°C for 20 minutes. The serum levels of high sensitivity CRP (hsCRP) and the plasma levels of fibrinogen were analysed as described.[5, 6]

Statistics

In logistic regression models, missing values for categorical covariates were replaced with dummy variables. Statistical analysis was performed using SPSS Ver 20 (IBM SPSS Inc., NY, USA) and HelixTree 6.3 (Golden Helix, Inc., MT, USA). To analyze the association between haplotypes and case/control status a stepwise logistic regression including all haplotypes with a frequency over 5% as well as age and sex (*P*-value cutoff of 0.1) was used. LD blocks were calculated using the method solid spine of LD in Haploview.[7] The manuscript was prepared according to the STROBE guidelines. [8]

Haplotype results

For *IL1A* and *IL1B*, the analyzed SNPs in the respective genes were situated in the same LD block, and were thus analyzed together. No haplotype in *IL1A* or *IL1B* was associated with overall ischemic stroke. Two LD blocks were observed in *IL1RN*. The SNP rs452204 in *IL1RN* was not situated in any LD block, and was therefore not included in the haplotype analyses. The *IL1RN* haplotype H1D (including rs4251916, rs928940, rs454078, and rs380092) showed an association with overall IS (OR 1.68, 95% CI 1.12-2.53, *P*=0.01). When excluding rs380092 from the haplotype, a lack of association to overall IS was observed (OR 1.41, 95% CI 0.98-2.03, *P*=0.06). For information about the alleles included in the different haplotypes and the estimated haplotype frequencies please refer to Table S3. For graphic representations of the LD blocks in *IL1RN* please refer to Figure S1.

Table S1. Summary of previous studies analyzing genetic variation in the *IL1A, IL1B* and/or *IL1RN* gene(s) in relation to ischemic stroke

Publication	Gene, SNP	Phenotype, n	Controls, n	Population	Results
Um et al., 2003a[9]	*IL1A*, rs1800587	IS, n=360	Controls, n=519	Asian	rs1800587 associated with IS
Um et al., 2003b [10]	*IL1A*, rs1800587 *IL1B* +3953 *IL1RN*, VNTR	IS, n=363	Controls, n=640	Asian	rs1800587 associated with IS
Seripa et al., 2003[11]	*IL1B*, rs16944 *IL1RN*, VNTR	IS, n=110	Controls, n=101	European	VNTR associated with IS
Lee, et al., 2004[12]	*IL1B*, rs16944 *IL1RN*, VNTR	IS, n=152	Controls, n=165	Asian	VNTR associated with IS
Iacoviello et al., 2004[13]	*IL1B*, rs16944	IS, n=134	Controls, n=134	European	rs16944 associated with IS
Balding et al., 2004[14]	*IL1B* +3953 *IL1RN*, VNTR	IS, n=105	Controls, n=389	European	No association
Dziedzic et al., 2004[15]	*IL1B*, rs16944	IS, n=183	Controls, n=180	European	No association
Dziedzic et al., 2005[16]	*IL1B*, rs16944	LVD, n=115 SVD, n=122 CE, n=221	Controls, n=194, 227,219 (matched with LVD, SVD and CE patients, respectively)	European	rs16944 associated with SVD
Rubattu et al., 2005[17]	*IL1B*, rs16944	IS, n=115	Controls, n=180	European	No association
Lalouschek et al., 2006[18]	*IL1RN*, VNTR	IS or TIA, n=404	Controls, n=415	European	No association
Lai, et al., 2006[19]	*IL1B*, rs16944 *IL1RN*, VNTR	IS, n=112	Controls, n=95	Asian	VNTR associated with IS
Worall et al., 2007[20]	*IL1RN*, VNTR	IS, n=478 (in total) From 3 cohorts	Controls, n=261 (in total)	American, predominately Caucasian	VNTR associated with IS in Caucasian participants (n=375/208)
Zee et al., 2008[21]	*IL1A*, rs17561 *IL1B*, rs1143634, rs1143633, 16944, rs1143623, rs4848306 *IL1RN*, rs419598	IS, n=258 (only men)	Controls (only men), n=258	American, predominately Caucasian	No association

Table S1 continued

Publication	Gene, SNP	Phenotype, n	Controls, n	Population	Results
Banerjee et al., 2008[22]	*IL1A*, rs1800587	IS, n=112	Controls, n=212	Asian	rs1800587 associated with IS
Rezaii et al., 2009[23]	*IL1RN*, VNTR	IS, n=148	Controls, n=161	Asian	VNTR associated with IS
Li et al., 2010[24]	*IL1A*, *IL1B*, rs16944	IS, n=371	Controls, n=371	Asian	rs1800587 associated with IS

rs16944 is also called *IL1B* -511; rs1800587 is also called *IL1A* -889; VNTR, variable number tandem repeat. IS, ischemic stroke; LVD, large-vessel disease; SVD, small-vessel disease; CE, cardioembolic stroke.

Table S2. Genotype frequencies in the control and overall ischemic stroke groups in SAHLSIS as well as odds ratios for the associations between genetic variants and overall ischemic stroke.

Gene	Genotype and OR	Control (n=668)	Ischemic stroke (n=844)
IL1A	**rs3783550**		
	AA, n (%)	315 (47)	415 (49)
	AC, n (%)	291 (44)	360 (43)
	CC, n (%)	59 (9)	65 (8)
	OR (95% CI)*	1	0.93 (0.79-1.09)
	OR (95% CI)†	1	0.93 (0.78-1.11)
	rs2856838		
	GG, n (%)	274 (41)	310 (37)
	AG, n (%)	298 (45)	402 (48)
	AA, n (%)	94 (14)	126 (15)
	OR (95% CI)*	1	1.11 (0.96-1.29)
	OR (95% CI)†	1	1.10 (0.93-1.29)
	rs1800587		
	GG, n (%)	304 (46)	390 (47)
	AG, n (%)	288 (43)	363 (43)
	AA, n (%)	74 (11)	84 (10)
	OR (95% CI)*	1	0.96 (0.82-1.12)
	OR (95% CI)†	1	0.96 (0.82-1.14)
IL1B	**rs1143643**		
	GG, n (%)	316 (47)	359 (43)
	AG, n (%)	280 (42)	386 (46)
	AA, n (%)	70 (11)	95 (11)
	OR (95% CI)*	1	1.14 (0.98-1.33)
	OR (95% CI)†	1	1.14 (0.97-1.35)
	rs16944		
	GG, n (%)	276 (42)	363 (44)
	AG, n (%)	297 (46)	382 (46)
	AA, n (%)	77 (12)	81 (10)
	OR (95% CI)*	1	0.92 (0.79-1.08)
	OR (95% CI)†	1	0.93 (0.78-1.09)
IL1RN	**rs4251961**		
	TT, n (%)	253 (38)	318 (39)
	CT, n (%)	309 (47)	373 (45)
	CC, n (%)	102 (15)	130 (16)
	OR (95% CI)*	1	0.99 (0.85-1.15)
	OR (95% CI)†	1	0.98 (0.83-1.16)
	rs928940		
	AA, n (%)	491 (74)	628 (75)
	AC, n (%)	162 (24)	188 (22)
	CC, n (%)	13 (2)	24 (3)
	OR (95% CI)*	1	0.98 (0.80-1.20)
	OR (95% CI)†	1	0.95 (0.76-1.19)

Table S2 continued

Gene	Genotype and OR	Control (n=668)	Ischemic stroke (n=844)
IL1RN	**rs454078**		
	TT, n (%)	332 (50)	469 (56)
	AT, n (%)	286 (43)	313 (37)
	AA, n (%)	48 (7)	57 (7)
	OR (95% CI)*	1	0.86 (0.73-1.01)
	OR (95% CI)†	1	0.86 (0.72-1.03)
	rs380092		
	TT, n (%)	313 (47)	351 (42)
	AT, n (%)	293 (44)	386 (46)
	AA, n (%)	60 (9)	103 (12)
	OR (95% CI)*	1	1.21 (1.04-1.42)‡
	OR (95% CI)†	1	1.21 (1.02-1.43)‡
	rs452204		
	GG, n (%)	208 (31)	315 (38)
	AG, n (%)	349 (52)	389 (46)
	AA, n (%)	109 (16)	134 (16)
	OR (95% CI)*	1	0.87 (0.75-1.01)
	OR (95% CI)†	1	0.87 (0.74-1.02)
	rs315951		
	CC, n (%)	318 (48)	373 (44)
	CG, n (%)	290 (43)	377 (45)
	GG, n (%)	58 (9)	90 (11)
	OR (95% CI)*	1	1.13 (0.97-1.33)
	OR (95% CI)†	1	1.13 (0.95-1.34)
	rs9005		
	GG, n (%)	333 (50)	447 (53)
	AG, n (%)	287 (43)	323 (38)
	AA, n (%)	46 (7)	70 (8)
	OR (95% CI)*	1	0.96 (0.82-1.13)
	OR (95% CI)†	1	0.95 (0.80-1.13)

Odds ratio (OR) with 95% confidence interval (95% CI) for ischemic stroke for the minor allele. *Model 1 (adjusted for age and sex); †model 2 (adjusted for age, sex, hypertension, diabetes, and smoking). ‡ $P<0.05$

Table S3. Haplotype frequencies

Gene	Haplotype	Alleles	Estimated frequency
IL1A	H1A	CGG	0.30
	H1B	AAG	0.38
	H1C	AGA	0.32
IL1B	H1A	GG	0.35
	H1B	AG	0.32
	H1C	GA	0.32
IL1RN	H1A	TCTA	0.14
	H1B	CATT	0.37
	H1C	TAAT	0.25
	H1D	TATA	0.18
	H2A	CA	0.28
	H2B	CG	0.40
	H2C	GG	0.32

SNP order in *IL1A* rs3783550, rs2856838, and rs1800587; in *IL1B* rs1143643, and rs16944; and in *IL1RN* rs4251961, rs928940, rs454078, and rs380092 in the first LD block, and rs315951 and rs9005 in the second LD block.

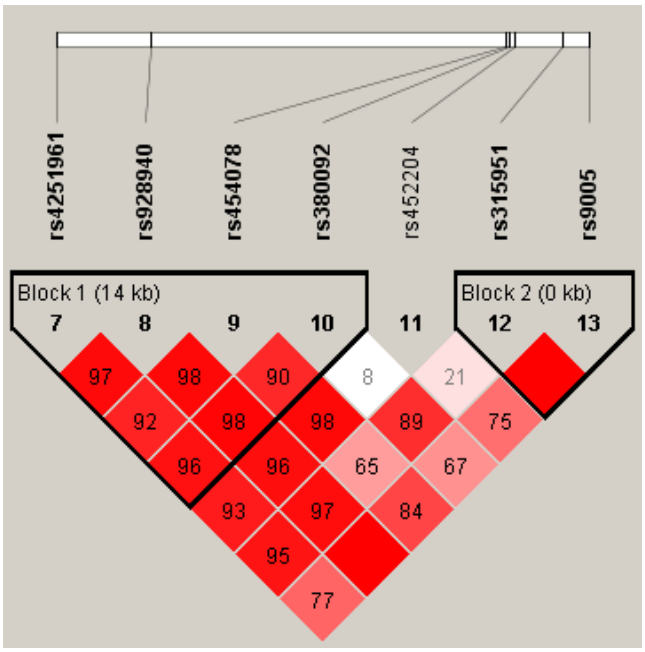

Figure 1S.
Graphic representation of the linkage disequilibrium (LD) structure in *IL1RN* for the analyzed SNPs. The LD blocks were defined by using Haploview 4.1 and the method solid spine of LD.

Table S4. Genotype frequencies in the control and overall ischemic stroke groups for rs380092 in *IL1RN* in SAHLSIS and the replication sample.

	SAHLSIS		Replication sample (LSR and MDC)	
Genotype and OR	Control	Ischemic stroke	Control	Ischemic stroke
	(n=668)	(n=844)	(n=1793)	(n=3145)
rs380092				
TT	313 (47)	351 (42)	797 (45)	1335 (43)
AT	293 (44)	386 (46)	774 (44)	1381 (45)
AA	60 (9)	103 (12)	186 (11)	377 (12)

SAHLSIS, the Sahlgrenska Study on Ischemic Stroke; LSR, the Lund Stroke Register; and MDC, the Malmö Diet and Cancer study

Table S5. Genotype frequencies in the control and the four main ischemic stroke subtypes groups in SAHLSIS.

Gene	SNP	Control (n=668)	Crypt. (n=206)	LVD (n=111)	SVD (n=165)	CE (n=151)
IL1A	**rs3783550**					
	AA, n (%)	315 (47)	94 (46)	53 (49)	86 (52)	80(53)
	AC, n (%)	291 (44)	93 (45)	48 (44)	64 (39)	62 (41)
	CC, n (%)	59 (9)	18 (9)	8 (7)	14 (9)	8 (5)
	rs2856838					
	GG, n (%)	274 (41)	72 (35)	36 (33)	55 (34)	60 (40)
	AG, n (%)	298 (45)	106 (52)	56 (51)	85 (51)	64 (43)
	AA, n (%)	94 (14)	26 (13)	17 (16)	25 (15)	26 (17)
	rs1800587					
	GG, n (%)	304 (46)	101 (49)	55 (50)	78 (48)	64 (43)
	AG, n (%)	288 (43)	85 (41)	44 (40)	70 (43)	65 (44)
	AA, n (%)	74 (11)	19 (9)	10 (9)	16 (10)	19 (13)
IL1B	**rs1143643**					
	GG, n (%)	316 (47)	86 (42)	40 (37)	67 (41)	69 (46)
	AG, n (%)	280 (42)	100 (49)	55 (50)	84 (51)	59 (39)
	AA, n (%)	70 (11)	20 (10)	14 (13)	13 (8)	22 (15)
	rs16944					
	GG, n (%)	276 (42)	92 (45)	50 (45)	74 (46)	74 (50)
	AG, n (%)	297 (46)	95 (47)	49 (45)	74 (46)	62 (42)
	AA, n (%)	77 (12)	16 (8)	11 (10)	14 (9)	12 (8)
IL1RN	**rs4251961**					
	TT, n (%)	253 (38)	71 (36)	46 (41)	63 (38)	55 (38)
	CT, n (%)	309 (47)	105 (52)	39 (35)	80 (49)	62 (43)
	CC, n (%)	102 (15)	24 (12)	26 (23)	21 (13)	27 (19)
	rs928940					
	AA, n (%)	491 (74)	150 (73)	79 (72)	123 (75)	108 (72)
	AC, n (%)	162 (24)	50 (24)	26 (24)	38 (23)	36 (24)
	CC, n (%)	13 (2)	6 (3)	4 (4)	3 (2)	6 (4)
	rs454078					
	TT, n (%)	332 (50)	122 (59)	65 (60)	87 (53)	93 (62)
	AT, n (%)	286(43)	74 (36)	36 (33)	66 (40)	45 (30)
	AA, n (%)	48 (7)	9 (4)	8 (7)	11 (7)	12 (8)
	rs380092					
	TT, n (%)	313 (47)	79 (38)	49 (45)	73 (45)	62 (41)
	AT, n (%)	293 (44)	102 (50)	43 (39)	71 (43)	67 (45)
	AA, n (%)	60 (9)	25 (12)	17 (16)	20 (12)	21 (14)
	rs452204					
	GG, n (%)	208 (31)	79 (38)	41 (38)	59 (36)	61 (41)
	AG, n (%)	349 (52)	100 (49)	49 (45)	78 (48)	61 (41)
	AA, n (%)	109 (16)	26 (13)	19 (17)	27 (16)	27 (18)

Table S5 continued

Gene	SNP	Control (n=668)	Crypt. (n=206)	LVD (n=111)	SVD (n=165)	CE (n=151)
	rs315951					
	CC, n (%)	318 (48)	91 (44)	50 (46)	76 (46)	65 (43)
	CG, n (%)	290 (43)	95 (46)	43 (39)	73 (45)	65 (43)
	GG, n (%)	58 (9)	20 (10)	16 (15)	15 (9)	20 (13)
	rs9005					
	GG, n (%)	333 (50)	114 (55)	64 (59)	80 (49)	90 (60)
	AG, n (%)	287 (43)	80 (39)	38 (35)	65 (40)	46 (31)
	AA, n (%)	46 (7)	12 (6)	7 (6)	19 (12)	14 (9)

SAHLSIS, the Sahlgrenska Study on Ischemic Stroke; Crypt., cryptogenic stroke; LVD, large-vessel disease; SVD, small-vessel disease; CE, cardioembolic stroke. Data are shown as the number (n) and percentage.

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