Regular article

No evidence for an association between ABO blood group and overall ischemic stroke or any of the major etiologic subtypes

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ABSTRACT

Introduction: The ABO blood group system is encoded by one gene, *ABO*. Previous studies have reported an association between blood group non-O (i.e. phenotype A, B or AB) and myocardial infarction. Studies on stroke and ABO are, however, more scarce. Therefore, we aimed to investigate whether ABO phenotype or genotype is associated with ischemic stroke and/or etiologic subtypes of ischemic stroke.

Materials and methods: The study was performed in the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), which comprises 600 patients with ischemic stroke before the age of 70 years, and 600 matched controls. Patients were classified according to the TOAST criteria.

Results: There was no significant association between ABO phenotype (blood group O vs. non-O) and overall ischemic stroke (multivariable odds ratio of 0.9, 95% confidence interval 0.7-1.2). This was also true for blood group O vs. A and O vs. B. Furthermore, no association between *ABO* genotypes and ischemic stroke was detected. The ischemic stroke subtype analysis was confined to large-vessel disease, small-vessel disease, cardioembolic stroke and cryptogenic stroke. In this analysis, there was no significant association between any ischemic stroke subtype and ABO phenotype or genotype.

Conclusions: The findings in this study suggest that ABO phenotype or genotype does not have a major impact in the pathophysiology of ischemic stroke or any of the ischemic stroke subtypes.

Key words: ABO blood group, ischemic stroke, TOAST subtype

The ABO blood group system is encoded by one gene, *ABO*, which gives rise to different glycosyltransferases that add sugar residues to the H-antigen producing A or B antigens on the surface of red blood cells [1]. These antigens produce the four phenotypes: O, A, B and AB. Polymorphisms in *ABO* give rise to a variety of alleles, and the most common alleles among Caucasians are A^1, A^2, B, O^1 and O^2 [1].

Previous studies, including a meta-analysis, have shown that the non-O phenotype (i.e. A, B or AB) is associated with myocardial infarction (MI) and coronary artery disease (CAD) [2-4]. There are also two recent genome-wide association studies (GWAS), in which significant associations between single-nucleotide polymorphisms (SNPs) at the *ABO* locus and CAD, or MI in the presence of CAD, were identified [5, 6]. Fewer studies have investigated ABO in stroke. Early studies reported a lack of association between ABO and stroke [7-10], whereas a more recent meta-analysis found a small but significant increased risk of stroke for the non-O phenotypes [3].

Against this background, we aimed to investigate whether ABO phenotype is associated with overall ischemic stroke and/or any of the ischemic stroke subtypes. In light of the recent GWAS finding of an association between the *ABO* locus and MI [6], we also examined whether we could find an association between *ABO* genotypes and ischemic stroke and/or any of the etiologic subtypes.

MATERIALS AND METHODS

Study population

Details of the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS) have been described elsewhere [11]. Briefly, this

case-control study includes 600 consecutively recruited patients with ischemic stroke before the age of 70 years. All patients were examined by a physician trained in stroke medicine and all underwent neuroimaging. Each patient was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria into the ischemic stroke etiologic categories: large-vessel disease (n=73), small-vessel disease (n=124), cardioembolic stroke (n=98), cryptogenic stroke (n=162), other determined cause of stroke including dissections (n=51), and undetermined stroke (n=92). Cryptogenic stroke was defined for cases in which no cause was identified despite an extensive investigation. The undetermined stroke group included patients for whom more than one cause was identified or for whom the evaluation was cursory. For each subject, a healthy community control, matched for age, sex and geographic area, was randomly selected from participants in a population-based health survey or the Swedish Population Register. All participants provided written informed consent prior to enrolment. For those participants who were unable to communicate, consent was obtained from the next-of-kin. This study was approved by the Ethics Committee of the University of Gothenburg.

ABO phenotyping

Fresh whole blood was unavailable for traditional antibody-based ABO phenotyping. Therefore, a DNA-based approach was used: restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) [12]. In this method, DNA is PCR amplified, digested with restriction endonucleases, and separated by agarose gel electrophoresis to determine the RFLPs according to fragment length. This method determines 15 different genotypes, based on the five most common *ABO* alleles (A^1, A^2, B, O^1 and O^2). From these genotype data, individuals can be grouped according to phenotype: phenotype O includes genotype O^1O^1, O^1O^2, and O^2O^2; phenotype A includes A^1A^1, A^1A^2, A^1O^1, A^1O^2, A^2A^2, A^2O^1, and A^2O^2; phenotype B includes BB, BO^1, and BO^2; and phenotype AB includes A^1B and A^2B.

DNA was extracted from frozen whole blood with Maxi DNA isolation PLUS (Agowa, Berlin, Germany). Oligonucleotide primers (Applied Biosystems, Foster City, CA, USA) and protocols were used as previously described by Olsson *et al.* [12], with a few minor modifications. In brief, the PCR Master Mix was prepared using 10 ng/µl genomic DNA, 0.1 µM primer and 1 U Taq polymerase (AmpliTaq 360 DNA Polymerase, Applied Biosystems, Foster city, CA, USA), in a total volume of 11 µl. The GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) was used for thermocycling with an initial temperature of 94 ˚C for 2 min, 10 cycles at 94˚C for 20 sec, 63˚C for 30 sec, and 72˚C for 1 min. This was followed by 30 cycles at 94˚C for 20 sec, 61˚C for 30 sec and finally 72˚C for 1 min. Two U of restriction endonucleases *Kpn1* and *HpaII* (New England Biolabs, Ibswich, MA, USA) were added in a digestion mix (5 µl). The cleaved PCR product was separated by electrophoresis using a 4% agarose gel (NuSieve GTG Agarose, Lonza, Rockland, USA). For each individual, the DNA fragment pattern was used to determine the *ABO* genotype.

ABO genotyping

To capture the genetic variation at the *ABO* locus, genotype data from the CEU population in HapMap (release 22) was used to tag *ABO* with r^2=0.8 and minor allele frequency (MAF)>0.1. Eight tagSNPs (rs512770, rs625593, rs630014, rs687621, rs7853989, rs8176731, rs8176682, and rs8176747) were selected. Genotyping was performed with TaqMan assays (Applied Biosystems, Foster City, CA, USA). The assay for rs8176747 was non-functional and could not be replaced. Genotyping was performed blinded to case/control status.

Statistical analyses

ABO phenotype was analysed as a categorical variable (O, A, B, AB), or phenotype O vs. non-O. An additive model was used for analysis of tagSNPs. Associations between ABO phenotype, tagSNPs or haplotypes and overall ischemic stroke as well as ischemic stroke subtypes were investigated using uni- and multivariable conditional logistic regression, with adjustments for hypertension, diabetes mellitus, and smoking status. All statistical calculations were performed using IBM SPSS Statistics version 19 for Windows (SPSS Inc., Chicago, Illinois, USA) and HelixTree 6.3 (Golden Helix, Bozeman, MT, USA). The statistical significance level was 0.05 and *P*-values were two-tailed. For the tagSNPs, assuming a multiplicative genetic model, the odds ratios (ORs) that can be detected for overall ischemic stroke with 80% power are in the range of 1.25-1.41, depending on the MAF (0.43-0.11). Regarding ABO phenotype, ORs below 0.82 can be detected for overall ischemic stroke with 80% power for a protective effect of the O phenotype.

RESULTS

ABO phenotypes and ischemic stroke

Baseline characteristics of the participants in SAHLSIS are shown in Table 1. The genotyping success rate with RFLP-PCR was 100%. The distribution of the four most common ABO phenotypes, O, A, B and AB, is shown in Table 2. Since there were few individuals with blood group AB, no comparison between blood group O vs. AB was made. There was no significant association between ABO phenotype and overall ischemic stroke in the univariable regression analysis, for all three comparisons (i.e. O vs. non-O, O vs. A and O vs. B) (Table 2). Including vascular risk factors in the model did not change the results.

The ischemic stroke subtype analysis was confined to the four major etiologic subtypes, i.e. large-vessel disease, small-vessel disease, cardioembolic stroke, and cryptogenic stroke. None of these etiologic ischemic stroke subtypes were associated with ABO phenotype in the univariable regression analyses (Table 2). This was also true after adjustment for vascular risk factors, for all three comparisons (i.e. O vs. non-O, O vs. A or O vs. B), and for all subtypes. To increase the power in the subtype analysis, an unconditional regression analysis was also performed including the whole control population. Adjustment for age, sex, geographic area, hypertension, diabetes mellitus and smoking status was made in the multivariable analysis. In these analyses, similar results as in the uni- and multivariable conditional regression analysis were obtained (data not shown).

ABO genotypes and ischemic stroke

All tagSNPs were in Hardy-Weinberg equilibrium, and the genotyping success

rate of the SNPs was 99.8-100%. The genotype frequencies for the 7 tagSNPs in the control and overall ischemic stroke groups, as well as the TOAST subtypes, are displayed in Table 3. There was no significant association between any tagSNP and overall ischemic stroke or TOAST subtypes, in the univariable analysis (Table 3). Similar results for both overall ischemic stroke and subtypes were obtained for all SNPs in the multivariable analysis. As no significant associations were detected, correction for multiple testing was not made. When the unconditional regression analysis was performed as described above, similar results as in the uni- and multivariable conditional regression analysis were obtained (data not shown).

DISCUSSION

In the current study, we performed a comprehensive investigation of both ABO phenotype and genotype in relation to ischemic stroke. We could not detect any significant association between ABO blood group and ischemic stroke, neither by ABO phenotype nor by genotype. Furthermore, we found no association between ABO and any of the four main etiologic subtypes of ischemic stroke.

Our finding of a lack of association between ABO phenotype and overall ischemic stroke is compatible with a few earlier studies [7-10]. On the other hand, another study found that the non-O phenotypes were more frequent in patients with ischemic stroke of non-cardioembolic origin, as compared to controls [13]. Moreover, a meta-analysis showed a small but significant increased risk of stroke for the non-O phenotypes as compared with the O phenotype [3]. Although our study is underpowered to detect small effects, there are a few differences that might account for the discrepancy between our study and the meta-analysis. The meta-analysis is based on seven studies (whereof three are from the 1970s) using quite different definitions of stroke, and including patients of all ages with all types of stroke. On the contrary, our study includes well characterized patients with ischemic stroke before the age of 70 years, and all patients underwent neuroimaging. More recently, an increased risk of ischemic stroke (n=469) was reported for the B allele [14], but we could not replicate this finding in our study.

In a recent GWAS, a significant association between SNPs at the *ABO* locus and MI in the presence of CAD was identified [6]. Moreover, a recent GWA meta-analysis of CAD identified an *ABO* risk allele associated with CAD [5]. Therefore, we also investigated whether tagSNPs in *ABO* are associated with ischemic stroke, but we could not find a significant association for any of the tagSNPs. One of the top SNPs in the study by Reilly *et al*, rs687621, was also included in the present study. This SNP is located in the intronic region of *ABO* and it is not known to be functional.

As ischemic stroke is a heterogeneous disease that can be categorized into clinically distinct subtypes representing different pathophysiology, it is important to investigate potential risk factors in the etiologic subtypes separately. In the present study, we could not find significant associations between any subtype and ABO phenotype or genotype. The ischemic stroke subtype that is mechanistically most similar to CAD is large-vessel disease, since both have an atherosclerosis plaque as the main pathophysiologic mechanism. Therefore, one can speculate that the *ABO*

SNPs that associate with CAD [5, 6], would be most likely to associate with the ischemic stroke subtype of large-vessel disease. Further studies on ABO in larger samples of patients with ischemic stroke due to large-vessel disease are thus warranted.

The advantage of the present study is the thorough analysis of both ABO phenotypes and genotypes, as well as the comprehensive classification of ischemic stroke subtypes. A possible limitation is the case and control assortment, which may influence results via selection bias. However, the stroke admission rate in Sweden is high and the early case fatality rate in ischemic stroke for the age group studied here is low. Furthermore, controls were recruited by random sampling from the general population in the same geographic area as patients. It is also of note that given the sample size in the current study, small effect sizes cannot be excluded.

In conclusion, we did not find an association between either ABO phenotype or genotype and overall ischemic stroke or any etiologic subtype of ischemic stroke. These results suggest that ABO blood group is not a major contributor to ischemic stroke or any of the main ischemic stroke subtypes.

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CONFLICT OF INTEREST STATEMENT

None.

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Table 1. Baseline characteristics of controls, overall ischemic stroke and the etiologic subtypes.

	Control (n=600)	**Overall IS** (n=600)	**LVD** (n=73)	**SVD** (n=124)	**CE stroke** (n=98)	**Crypt** (n=162)
Mean age, y (SD)	56 (10)	56 (10)	59 (8)	58 (7)	57 (10)	53 (12)
Male sex, n (%)	385 (64)	385 (64)	54 (74)	77 (62)	66 (67)	95 (59)
Hypertension, n (%)	224 (37)	354 (59)	44 (60)	89 (72)	50 (51)	87 (54)
Diabetes mellitus, n (%)	33 (6)	114 (19)	25 (34)	26 (21)	19 (19)	23 (14)
Current smoking, n (%)	109 (18)	233 (39)	39 (53)	54 (44)	34 (35)	60 (37)

IS indicates ischemic stroke; LVD, large-vessel disease; SVD, small-vessel disease; CE, cardioembolic stroke; Crypt, cryptogenic stroke; SD, standard deviation.

Table 2. Phenotype frequencies in controls, overall ischemic stroke and etiologic subtypes, and univariable and multivariable odds ratios (ORs) and 95% confidence intervals (CI) for overall ischemic stroke and subtypes.

	Control (n=600)	**Overall IS** (n=600)	**LVD** (n=73)	**SVD** (n=124)	**CE stroke** (n=98)	**Crypt** (n=162)
Phenotype O, n (%)	214 (36)	206 (34)	30 (41)	45 (36)	32 (33)	52 (32)
Phenotype A, n (%)	269 (45)	284 (47)	30 (41)	60 (48)	51 (52)	69 (43)
Phenotype B, n (%)	77 (13)	78 (13)	9 (12)	15 (12)	11 (11)	26 (16)
Phenotype AB, n (%)	40 (7)	32 (5)	4 (6)	4 (3)	4 (4)	15 (9)
OR for O vs. non-O* (95% CI)	ref	0.9 (0.7-1.2)	1.2 (0.6-2.1)	1.2 (0.7-2.0)	0.7 (0.4-1.3)	0.9 (0.6-1.5)
OR for O vs. non-O* (95% CI)†	ref	0.9 (0.7-1.2)	1.7 (0.6-4.4)	1.1 (0.5-2.1)	0.7 (0.3-1.4)	0.9 (0.5-1.5)
OR for O vs. A (95% CI)	ref	1.1 (0.8-1.4)	0.8 (0.4-1.6)	0.9 (0.5-1.6)	1.6 (0.8-3.0)	1.1 (0.6-1.8)
OR for O vs. A (95% CI)†	ref	1.1 (0.8-1.5)	0.6 (0.2-1.6)	1.0 (0.5-2.1)	1.5 (0.7-3.0)	1.0 (0.6-1.9)
OR for O vs. B (95% CI)	ref	1.1 (0.7-1.5)	0.9 (0.3-2.2)	0.8 (0.3-1.7)	2.2 (0.7-6.9)	1.0 (0.5-1.9)
OR for O vs. B (95% CI)†	ref	1.1 (0.7-1.6)	0.6 (0.1-3.0)	1.2 (0.4-3.9)	1.8 (0.6-6.0)	1.0 (0.5-2.1)

IS indicates ischemic stroke; LVD, large-vessel disease; SVD, small-vessel disease; CE, cardioembolic stroke; Crypt, cryptogenic stroke. Conditional logistic regression was used for overall IS and subtypes. *Non-O includes phenotype A, B and AB. †Adjusted for hypertension, diabetes mellitus, and smoking status.

Table 3. Genotype frequencies for seven *ABO* tagSNPs in controls, ischemic stroke and etiologic subtypes, and univariable and multivariable odds ratios (ORs) and 95% confidence interval (CI) for overall ischemic stroke and subtypes.

	Control (n=600)	**Overall IS** (n=600)	**LVD** (n=73)	**SVD** (n=124)	**CE stroke** (n=98)	**Crypt** (n=162)
rs512770						
CC	403 (67)	416 (69)	48 (66)	79 (64)	68 (69)	116 (72)
CT	173 (29)	169 (28)	22 (30)	40 (32)	29 (30)	42 (26)
TT	24 (4)	15 (3)	3 (4)	5 (4)	1 (1)	4 (2)
OR (95% CI)	ref	0.9 (0.7-1.1)	0.9 (0.5-1.7)	1.1 (0.7-1.7)	0.9 (0.6-1.6)	0.8 (0.5-1.2)
OR (95% CI)*	ref	0.9 (0.7-1.1)	1.7 (0.7-4.2)	0.9 (0.5-1.6)	0.9 (0.5-1.7)	0.8 (0.5-1.3)
rs625593						
CC	346 (58)	368 (61)	42 (58)	71 (57)	59 (60)	104 (64)
CT	219 (37)	206 (34)	26 (36)	46 (37)	35 (36)	52 (32)
TT	33 (6)	25 (4)	5 (7)	7 (6)	4 (4)	6 (4)
OR (95% CI)	ref	0.9 (0.7-1.0)	1.0 (0.6-1.8)	1.0 (0.7-1.5)	0.9 (0.6-1.5)	0.8 (0.6-1.2)
OR (95% CI)*	ref	0.8 (0.7-1.0)	1.4 (0.6-3.7)	0.8 (0.5-1.4)	0.9 (0.5-1.5)	0.9 (0.6-1.3)
rs630014						
CC	195 (32)	210 (35)	23 (32)	36 (29)	40 (41)	58 (36)
CT	299 (50)	278 (46)	33 (45)	66 (53)	40 (41)	73 (73)
TT	105 (18)	110 (18)	17 (23)	22 (18)	18 (18)	31 (19)
OR (95% CI)	ref	1.0 (0.8-1.1)	1.0 (0.7-1.6)	1.1 (0.8-1.6)	1.0 (0.6-1.4)	1.0 (0.7-1.3)
OR (95% CI)*	ref	0.9 (0.7-1.1)	1.1 (0.6-2.2)	1.0 (0.6-1.8)	0.9 (0.6-1.4)	0.9 (0.7-1.4)
rs687621						
TT	240 (40)	211 (35)	31 (42)	44 (35)	34 (35)	54 (33)
TC	276 (46)	298 (50)	31 (42)	65(52)	48 (49)	80 (49)
CC	84 (14)	90 (15)	11 (15)	15 (12)	16 (16)	28 (17)
OR (95% CI)	ref	1.1 (1.0-1.3)	1.0 (0.6-1.5)	0.9 (0.7-1.3)	1.2 (0.7-1.8)	1.3 (1.0-1.9)
OR (95% CI)*	ref	1.2 (1.0-1.5)	0.9 (0.5-1.8)	1.0 (0.6-1.6)	1.2 (0.7-1.9)	1.4 (1.0-2.1)
rs7853989						
GG	475 (79)	481 (80)	60 (82)	101 (81)	81 (83)	120 (74)
GC	119 (20)	115 (19)	13 (18)	22 (18)	16 (16)	42 (26)
CC	4 (1)	3 (1)	0 (0)	1 (1)	1 (1)	0 (0)
OR (95% CI)	ref	0.9 (0.7-1.2)	1.1 (0.5-2.6)	0.8 (0.4-1.5)	0.9 (0.5-1.8)	1.0 (0.7-1.6)
OR (95% CI)*	ref	1.0 (0.7-1.3)	0.8 (0.2-2.9)	1.4 (0.6-3.3)	0.9 (0.5-1.9)	1.1 (0.7-1.8)
rs8176682						
CC	234 (39)	228 (38)	32 (44)	50 (40)	34 (35)	62 (38)
CT	273 (46)	289 (48)	27 (37)	57 (46)	55 (57)	80 (49)
TT	93 (16)	81 (14)	14 (19)	17 (14)	8 (8)	20 (12)
OR (95% CI)	ref	1.0 (0.8-1.2)	1.0 (0.6-1.6)	1.1 (0.8-1.5)	0.9 (0.6-1.4)	0.9 (0.6-1.3)
OR (95% CI)*	ref	1.0 (0.8-1.2)	0.9 (0.5-1.8)	1.2 (0.7-2.0)	0.9 (0.6-1.5)	0.8 (0.5-1.2)
rs8176731						
TT	259 (43)	283 (47)	32 (44)	55 (44)	45 (46)	72 (44)
TC	270 (45)	255 (43)	33 (45)	54 (44)	45 (46)	74 (46)

CC	71 (12)	61 (10)	8 (11)	15 (12)	8 (8)	16 (10)
OR (95% CI)	ref	0.9 (0.7-1.0)	1.1 (0.6-1.9)	1.0 (0.7-1.4)	1.0 (0.6-1.4)	0.9 (0.6-1.2)
OR (95% CI)*	ref	0.9 (0.7-1.0)	1.4 (0.6-3.2)	0.9 (0.5-1.5)	0.9 (0.6-1.4)	1.0 (0.7-1.4)

IS indicates ischemic stroke; LVD, large-vessel disease; SVD, small-vessel disease; CE, cardioembolic stroke; Crypt, cryptogenic stroke. Conditional logistic regression was used for overall IS and subtypes.
*Adjusted for hypertension, diabetes mellitus, and smoking status.