Lipoprotein (a) and Genetic Polymorphisms of Clotting Factor V, Prothrombin, and Methylenetetrahydrofolate Reductase Are Risk Factors of Spontaneous Ischemic Stroke in Childhood

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Ischemic stroke is a rare event in childhood. In approximately one third of cases no obvious underlying cause or disorder can be detected. We investigated the importance of genetic risk factors of venous thromboembolism in childhood or stroke in adulthood as risk factors for spontaneous ischemic stroke in children. One hundred forty-eight Caucasian infants and children (aged 0.5 to 16 years) with stroke and 296 age-matched controls from the same geographic areas as the patients were analyzed for increased lipoprotein (a) [Lp(a)] levels >30 mg/dL; for the presence of the factor V (FV) G1691A mutation, the prothrombin (PT) G20210A variant, and the TT677 genotype of methylenetetrahydrofolate reductase (MTHFR); and deficiencies of protein C, protein S, and antithrombin. The following frequencies (patients *v* controls), odds ratios (ORs), and confidence intervals (CIs) of single risk factors were found: Lp(a) >30 mg/dL (26.4% *v* 4.7%; OR/CI, 7.2/3.8 to 13.8; *P* < .0001), FV G1691A (20.2% *v* 4%; OR/CI, 6/2.97 to 12.1; *P* < .0001), protein C deficiency (6% *v* 0.67%; OR/CI, 9.5/2 to 44.6; *P* = .001), PT G20210A (6% *v* 1.3%; OR/CI, 4.7/1.4 to 15.6; *P* = .01), and the MTHFR TT677 genotype (23.6% *v* 10.4%; OR/CI, 2.4/1.53 to 4.5; *P* < .0001). A combination of the heterozygous FV G1691A mutation with increased Lp(a) (n = 11) or the MTHFR TT677 genotype (n = 5) was found in 10.8% of cases, but only 0.3% of controls (OR/CI, 35.75/4.7 to 272; *P* < .0001). Increased Lp (a) levels, the FV G1691A mutation, protein C deficiency, the prothrombin G20210A variant, and the MTHFR TT677 are important risk factors for spontaneous ischemic stroke in childhood.
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IN CHILDREN, cerebrovascular events, half of which are ischemic strokes, occur at an estimated incidence of about 2 per 100,000 per year.[1,2] Risk factors of cerebrovascular accidents in children include congenital heart malformations, vascular abnormalities, endothelial damage, infectious diseases, and collagen tissue diseases, as well as some rare inborn errors of metabolism like Fabry's disease, homocystinuria, organic acid disorders, ornithine transcarbamylase deficiency, carbohydrate-deficient glycoprotein syndrome, and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS).[1-4] However, in about one third of affected children, no obvious cause or underlying disorder can be detected.[1-3] Hypercoagulable states may represent a risk factor for stroke in childhood. For example, deficiencies of natural anticoagulants such as antithrombin, protein C, and protein S have been found in rare cases of childhood stroke.[5-14]

There is also evidence that activated protein C resistance or its underlying genetic defect, the factor V (FV) G1691A mutation, plays a role in the early onset of childhood ischemic stroke,[15-19] which is in contrast to data obtained in adult populations.[20,21] The 20210A allele within the 3'-untranslated region of the prothrombin (PT) gene, which is a common but mild risk factor of venous thrombosis in the CNS,[22,23] has also been controversially discussed as a risk factor for arterial thrombosis, ie, myocardial infarction or stroke.[24-27] Elevated lipoprotein(a) [Lp(a)] has been identified as a genetically determined risk factor for stroke in young adults, but only preliminary data are available on its role as a risk factor for ischemic stroke in infants and children.[28-30] Finally, in adult patients, the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene, which causes a thermolabile variant of this enzyme and appears to facilitate the manifestation of hyperhomocysteinemia especially in individuals with undernutrition with folic acid, has been discussed as a genetic risk factor for vascular disease and stroke.[31-33]

The present multicenter case-control study was undertaken to unravel the role of genetic prothrombotic risk factors, elevated Lp(a), and homocysteinemia as risk factors of ischemic stroke in children who do not show additionally identifiable clinical risk factors.

MATERIALS AND METHODS

Ethics. The present prospective multicenter case-control study was performed in accordance with the ethical standards laid down in a relevant version of the 1964 Declaration of Helsinki and approved by the Medical Ethics Committee at the Westfälische Wilhelms-University, Münster, Germany.

Inclusion criteria. Infants and children aged 6 months to 16 years with first onset of spontaneous ischemic stroke were included.

Exclusion criteria. Neonates and infants less than 6 months of age (n = 32), and childhood stroke patients with known underlying diseases, ie, congenital or acquired heart diseases (n = 49), cerebral vascular abnormalities (fibromuscular dysplasia, n = 12; Moyamoya, n = 4), endothelial damage (trauma or dissection, n = 12), infectious

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diseases (n = 31), collagen tissue diseases and metabolic disorders (n = 5) were not enrolled in the present study.

Study period. From October 1995 to October 1998, 148 consecutive Caucasian patients (median age at first thrombotic onset, 4.5 years; range, 6 months to 16 years; male/female distribution, 1:1.1) with spontaneous ischemic stroke were recruited from different geographic areas of Germany: 65% from the Northern and Western part of Germany, ie, catchment areas of Hamburg (10%), Münster (17%), Halle (8%), Bielefeld (5%), Düsseldorf (7%), and Frankfurt/Main (18%), and 35% of the Southern regions of Germany, the overall catchment area of Munich, respectively.

Imaging methods. Diagnoses of ischemic stroke were confirmed by an external neuroradiologist, who was unaware of the laboratory test results, upon the results of computed tomography (CT), magnetic resonance (MR) imaging, and MR angiography according to criteria previously published by Ringelstein et al.[34]

Clinical data of the patients studied. At acute onset of spontaneous ischemic stroke, the majority of patients presented with hemiparesis (n = 134) combined with aphasia (n = 22) or coma (n = 28); impairment of vision or infratentorial symptoms, ie, ataxia, was the initial symptom in the remaining 14 patients. The corresponding brain lesions with territorial infarction were predominantly found in the left medial artery (n = 89), right medial artery (n = 45), or vertebrobasilar system (n = 14).

Control population. A total of 296 age- and sex-matched Caucasian controls (potential bone marrow donors undergoing elective surgery; median age, 5 years; range, 6 months to 16 years; male/female distribution, 1:1.1) from the same geographic areas as the patients were investigated with parental consent.

Blood samples. With informed parental consent, 6 weeks to 3 months after the acute stroke event, blood samples were collected by peripheral venipuncture into plastic tubes containing 1/10 by volume of 3.8% trisodium citrate (Sarstedt, Nümbrecht, Germany), and placed immediately on melting ice. Platelet-poor plasma was prepared by centrifugation at 3,000*g* for 20 minutes at 4°C, aliquoted in polystyrene tubes, stored at −70°C, and thawed immediately before the assay procedure. The laboratory staff was unaware of whether the blood samples were those of a patient or a control.

For genetic analysis, we obtained venous blood in EDTA-treated sample tubes (Sarstedt, Nümbrecht, Germany), from which cells were separated by centrifugation at 3,000*g* for 15 minutes. The buffy-coat layer was then removed and stored at −70°C pending DNA extraction by standard techniques.

Assays for genotyping. The C677T polymorphism in the MTHFR gene, the G1691A polymorphism in the FV gene, and the G20210A polymorphism in the PT gene were blindly detected in patients and controls by polymerase chain reaction (PCR) amplification and digestion with *Hin*fI,[31] *Mnl*I,[35] and *Hin*dIII,[22] respectively, as previously reported.

Assays for the quantification of plasma proteins and metabolites. Activities of protein C and antithrombin were measured using the chromogenic substrates S-2366 and S-2765, respectively (Chromogenix, Mölndal, Sweden).[36] Free protein S antigen,[36] protein C antigen, and Lp(a) [28] were quantified by enzyme-linked immunosorbent assay (ELISA) (Asserachrom: free protein S, protein C; Diagnostica Stago, Ansieres-sur-Seine, France) and (COALIZA Lp(a); Chromogenix, Mölndal, Sweden: intraassay and interassay coefficients of variation [CVs] were <4% at 10 mg/dL and <7% at 40 mg/dL), respectively. In a subgroup of 60 randomly selected patients of all study centers and controls, fasting plasma homocysteine concentrations were measured by high-performance liquid chromatography (HPLC) using reagents and standards from Immuno (Vienna, Austria: CVs within/between days are 2.2%/3.5%).

Classification of risk cut-offs. Heterozygous protein C deficiency type I was diagnosed when functional plasma activity and immunologic antigen concentration of the protein were below the lower age-related limit.[37,38] A type II deficiency was diagnosed when functional activity was repeatedly found to be low along with normal antigen concentrations. The diagnosis of protein S deficiency was based on reduced free protein S antigen levels combined with decreased or normal total protein S antigen concentrations, respectively. Criteria for the hereditary nature of a hemostatic defect were its presence in at least 1 further first- or second-degree family member and/or the identification of a causative gene mutation.[36]

The critical cut-off level for Lp(a) concentrations was defined at 30 mg/dL, previously identified by us as the risk threshold value for venous thrombosis in childhood.[39] Moreover, this value is also widely accepted as the cut-off in the assessment of increased risk for cerebrovascular and cardiovascular events in adults.[40]

Statistics. Because of their non-Gaussian frequency distribution, continuous data are presented as medians and ranges and evaluated by nonparametric statistics using the Wilcoxon-Mann-Whitney, and 1-way analysis of variance (ANOVA) with subsequent paired comparison according to Scheffe. Prevalences of prothrombotic risk factors in patients and controls were compared by χ^2 analysis or, if necessary, Fisher's exact test. The significance level was set at .05. With respect to the number of different tests applied, a correction according to Bonferroni was performed. The critical cut-off χ^2 values for *P* less than .05 were 6.6 and .01 (Fisher's exact test), respectively. In addition, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. All statistical analyses were performed using the MedCalc software package (MedCalc, Mariakerke, Belgium).

RESULTS

Results are summarized in Table 1.

Lp(a). Caucasian children with a history of ischemic stroke had significantly higher ($P < .0001$) concentrations of Lp(a) (median, 21 mg/d; range, 0 to 162) compared with controls (median, 5 mg/dL; range, 0 to 115). Lp(a) levels greater than 30 mg/dL were found in 26.4% of cases, but only 4.7% of controls (OR/CI, 7.2/3.8 to 13.8; $P < .0001$; $\chi^2 = 41.8$).

Table 1. Distribution of Prothrombotic Risk Factors in Caucasian Patients and Controls

	Risk Factors	Controls (n = 296)	Patients (n = 148)	OR (CI)	χ^2 (*P* Value)
Single risk factor	Lp(a) > 30 mg/dL	14 (4.7%)	39 (26.4%)	7.2 (3.8-13.8)	<.0001
	FV G1691A (+/−)	12 (4%)	30 (20.2%)	6 (2.97-12.1)	<.0001
	Protein C deficiency	2 (0.67%)	9 (6%)	9.5 (2-44.6)	.001*
	PT G20210A	4 (1.3%)	9 (6%)	4.7 (1.4-15.6)	.01*
	MTHFR TT 677 genotype	31 (10.4%)	35 (23.6%)	2.64 (1.53-4.5)	<.0001
Combined risk factors	FV G1691A & Lp(a) > 30 mg/dL	1 (0.3%)	11 (7.4%)	23.6 (3-185)	<.0001*
	FVG1691A & MTHFR TT677	—	5 (3.3%)	—	.004*
Combinations (total)	FV G1691A & Lp(a) > 30 mg/dL or MTHFR TT677	1 (0.3%)	16 (10.8%)	35.75 (4.7-272)	<.0001*

*Fischer's exact test.

Factor V G1691A gene mutation. Heterozygosity for the FV G1691A mutation was diagnosed in 20.2% of cases compared with 4% of controls (OR/CI, 6/2.97 to 12.1; $P < .0001$; $\chi^2 = 28.4$).

Protein C deficiency. Six percent of patients and 0.67% of controls had protein C type I deficiency (OR/CI, 9.6/2 to 44.6; $P = .001$).

PT G20210A gene mutation. Six percent of children with stroke but only 1.3% of controls carried the PT G20210A variant (OR/CI, 4.7/1.43 to 15.6; $P = .01$).

MTHFR TT677 genotype. A total of 23.6% of cases but only 10.4% of controls were homozygous for the MTHFR TT677 genotype (OR/CI, 2.6/1.53 to 4.5; $P < .0001$; $\chi^2 = 12.5$). In the randomly selected subgroup of 60 patients, the fasting homocysteine concentrations in patients with the MTHFR TT677 genotype were significantly higher compared with the 677CT and CC genotypes (CC677: median, 5.8 μmol/L; range, 3 to 8.6 μmol/L, CT677: median, 7.0 μmol/L; range, 3 to 12 μmol/L; TT677: median, 12.1 μmol/L; range, 7 to 23 μmol/L; $P < .01$). As a consequence, fasting homocysteine concentrations were significantly higher in cases (median, 7 μmol/L; range, 3 to 23 μmol/L) as compared with controls (median, 5.5 μmol/L; range, 3 to 8.4 μmol/L; $P = .002$).

Combined prothrombotic defects. A combination of the heterozygous FV G1691A mutation either with increased Lp(a) (n = 11; 7.4%) or the MTHFR TT genotype (n = 5; 3.3%) was found in 16 of 148 cases with stroke, but only once among controls (OR/CI, 35.75/4.7 to 272; $P < .0001$). No further combinations of more than 2 defects were found in the population studied.

No protein S deficiency or antithrombin deficiency was found in patients and controls. In addition, mainly due to the exclusion of stroke patients with associated infectious diseases, no increased anticardiolipin IgG or IgM antibodies were detected in the population presented here.

Within the 2-year study period, 3 of 148 patients (2%) investigated had recurrent spontaneous stroke.

DISCUSSION

In this study we demonstrated that Lp(a) levels greater than 30 mg/dL (OR, 7.2), protein C deficiency (OR, 9.5), the FV G1691A mutation (OR, 6.0), the PT G20210A genotype (OR, 4.7), and the MTHFR TT677 genotype (OR, 2.6) are risk factors of spontaneous ischemic stroke in childhood. It is important to emphasize that the suspected diagnosis of ischemic stroke was blindly confirmed by an independent neuroradiologist. Moreover, underlying diseases such as cardiac malformations, including patent foramen ovale, fibromuscular dysplasia, Moyamoya disease, trauma, severe bacterial or viral septicemia, malignancy, and rheumatic diseases,[3,4] were prospectively excluded in the study patients presented here. This suggests that each of the genetic prothrombotic risk factors studied has an impact on the early onset of spontaneous cerebrovascular ischemic accidents during infancy and childhood, independently of predisposing diseases.

In adults, the FV G1691A mutation, protein C deficiency, and the PT G20210A genotype are established genetic risk factors for venous thromboembolism, but have little importance as risk factors for arterial thrombosis, ie, myocardial infarction and stroke.[20,21,25,27] In contrast, the data of our study, as well as preliminary data on case reports and small studies, indicate that these variants, mainly present in venous thrombosis, play a role as risk factors of stroke in childhood and young adults.[14-17,19,24,26] Since the children we investigated did not present with any underlying morphologic disorders of the cardiovascular system, it is also important to note that the FV G1691A mutation has been associated with cryptogenic stroke in young adults.[41] Thus, morphologic anomalies of the cardiovascular system or other underlying diseases[3,4] do not appear necessary to precipitate stroke in the presence of one of the prothrombotic defects studied.

In this study, we have shown for the first time the importance of elevated Lp(a) as a risk factor for spontaneous stroke in childhood. Lp(a) is a low-density lipoprotein that contains apolipoprotein (a) [apo(a)] as an additional protein. The primary structure of apo(a) encompasses a protease domain, a kringle V domain, and a variable number of kringle IV repeats, which are homologous to the same domains in the plasminogen molecule and which have been made responsible for the antifibrinolytic activities of Lp(a) found both in vitro and in vivo.[42,43] The genetically determined number of kringle IV domains and other polymorphisms in the apo(a) gene account for the greatest part of interindividual differences in Lp(a) levels. Lp(a) has previously been identified as an independent risk factor of both myocardial infarction and atherothrombotic stroke in young adults.[29,30,44-47] Thus, our study extends this role to childhood patients. In this context, it is interesting to note that elevated Lp(a) is also a risk factor for venous thrombosis in childhood, as well as for porencephaly and peripartal stroke in neonates.[18,28,39]

The thermolabile variant of MTHFR caused by the TT677 genotype predisposes to hyperhomocysteinemia, especially if it coincides with a reduced supply of folic acid.[48,49] Despite the well-established association between hyperhomocysteinemia and the risk of cerebrovascular and cardiovascular disease,[48,49] the role of this polymorphism as a genetic risk factor of stroke or myocardial infarction in adults is controversial.[31-33] In our study, both the TT677 genotype of the MTHFR polymorphism and slightly elevated fasting homocysteine, which interestingly did not reach homocysteine concentrations of adult stroke patients, turned out to be risk factors of stroke in childhood. However, we did not record serum levels of vitamins or daily dietary intake. Thus, the question remains whether the MTHFR TT677 genotype alone or only in combination with reduced vitamin supply is responsible for the increase of both fasting homocysteine and risk of spontaneous stroke in children of Caucasian origin.

In conclusion, in this multicenter case-control study, we have provided strong evidence that Lp(a) level greater than 30 mg/dL, protein C deficiency, the FV G1691A mutation, the PT 20210A variant, and the MTHFR TT677 genotype are risk factors of spontaneous ischemic stroke in childhood and early adolescence.

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APPENDIX

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