Recurrent stroke: the role of thrombophilia in a large international paediatric stroke population

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ABSTRACT

Risk factors for arterial ischaemic stroke in children include vasculopathy and prothrombotic risk factors but their relative importance to recurrent stroke is uncertain. Data on recurrent stroke from the databases held in Canada (Toronto), Germany (Kiel-Lübeck/Münster), and UK (London/Southampton) were pooled. Data were available from 894 patients aged 1 month to 18 years at first stroke (median age 6 years) with a median follow-up of 35 months. 160/894 patients (17.9%) had recurrence from 1 day to 136 months after first stroke (median 3.1 months). Among 288 children with vasculopathy, recurrence was significantly more common (hazard ratio (HR) 2.5, 95% confidence intervals (CI) 1.92-3.5) compared to children without vasculopathy. Adjusting for vasculopathy, isolated antithrombin deficiency (HR 3.9; 95%CI 1.4-10.9), isolated elevated lipoprotein (a) (HR 2.3; 95%CI 1.3-4.1), and the presence of more than one prothrombotic risk (HR 1.9; 95%CI 1.12-3.2) were independently associated with an increased risk of recurrence. Recurrence rates calculated per 100 person-years were 10 (95%CI 3-24) for antithrombin deficiency, 6 (95%CI 4-9) for elevated lipoprotein (a), and 13 (95%CI 7-20) for the presence of more than one prothrombotic risk. Identifying children at increased for second stroke events is important in intensifying measures aimed at preventing recurrent stroke.

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Introduction

Published estimates of arterial ischaemic stroke (AIS) incidence in children range from 1.2 to 8 per 100,000 children annually.[1-5] Prior diagnoses in children with symptomatic AIS are multiple and include cardiac disorders, haematological conditions (sickle cell anaemia, prothrombotic disorders), collagen tissue diseases, metabolic disorders, other chronic diseases, and acute illnesses.[6-8] Childhood infections, including *Varicella Zoster* virus, have been shown to be associated with an increased risk of AIS, with routine vaccinations being protective against AIS.[9,10] In addition, the presence of prothrombotic risk factors has been found in small case series and case control studies to be associated with ischaemic stroke in children, with this association confirmed by meta-analysis.[11] This is in contrast to perinatal stroke, in which recent studies have not shown an association with thrombophilia[12] and recurrence is relatively rare.[13,14]

In children, stroke recurrence is common and associated with significant morbidity and mortality. Five-year recurrence rates are estimated to be between 6-20%, with rates as high as 66% in certain subgroups.[15-20] Several of these studies have identified vasculopathy, in particular moyamoya, as an important factor in predicting recurrent stroke.[8,15,20] There are some early data to suggest that prothrombotic states may also enhance the risk of recurrence, but many of these studies are limited by size and scope.[7,11,15,16,21] We therefore investigated in an international cohort study the relevance of prothrombotic risk factors, as well as underlying stroke subtypes, to a second stroke in paediatric patients.

Methods

Study population, study design, and study endpoints: The present study is a multicenter cohort study to assess the rate of symptomatic stroke recurrence per 100 person-years following a first onset of AIS. The core protocol was developed by the German collaborative group and was adopted by centres in Toronto and the UK; data were pooled across these sites to determine whether the data were generalizable and to increase power for the secondary study objective, i.e. the time to recurrence. From January 1990 to January 2016, consecutively admitted in- and outpatients from each study site, i.e. Canada (Toronto: single center registry), Germany (Kiel-Lübeck/Münster: multi-center national registry: patients newly enrolled after 2002), and UK (London/Southampton: 2-centre registry) were enrolled and pooled into the paediatric stroke database located in Germany. Consecutive patients with first symptomatic AIS were recruited whether or not prothrombotic risk factors were present

and recurrence was ascertained at follow-up in survivors. Patients referred from other tertiary centres were excluded. Neonates < 1 month of age and children with sickle cell anaemia were not enrolled in the present dataset, as recurrence rates and risk factors differ markedly from other sub-types of childhood AIS. After enrolment, children with moyamoya, and those with congenital homozygous protein C or antithrombin deficiency, were excluded, since recurrence risk and therapy differ substantially from the remaining study cohort. In addition, we excluded children in whom thrombophilia screening was not performed and those lost to follow-up.

First AIS was confirmed by standard imaging methods, i.e. magnetic resonance imaging (MRI) and computerized tomography (CT).[15,16] AIS was defined as acute onset neurological deficit with an acute focal infarct in a corresponding arterial vascular territory.

Recurrence was defined as clinically symptomatic AIS events presenting with acute focal neurologic deficits with infarction in a vascular distribution on neuroimaging and beginning more than 24 hours following the first stroke onset. Fixed study end date for last follow-up was January 1, 2017. The number of patients with recurrence, and type of antithrombotic (antiplatelet or anticoagulant) therapy administered prior to recurrence were noted. The proportion of deaths following stroke recurrence was also noted. Following discontinuation of antithrombotic treatment, asymptomatic paediatric patients were followed-up every few months for the first year and at more prolonged intervals thereafter (at least yearly). All patients were seen at least once for a follow-up with a paediatric neurologist. Transient ischaemic attacks (TIAs), defined as acute onset neurological deficit lasting < 24 hours and with no associated infarct on repeat neuroimaging, were excluded from the study endpoint, as were 'silent' recurrent strokes noted on follow-up imaging without clinical manifestations.

Ethics: This study was performed in accordance with the ethical standards laid down in the updated version of the 1964 Declaration of Helsinki and was either approved or the requirement for approval was waived, by Research Ethics Boards at the Hospital for Sick Children, Toronto, the Great Ormond Street Hospital, (and UK National Health Service), and University of Münster respectively.

In the online supplement, details of stroke subtypes,[11,22] treatment modalities,[21-25] laboratory work-up[26-28] and statistical methods applied[29,30] are summarized.

Results

From January 1990 to January 2016, a total of 990 in- and outpatients consecutively reviewed at the study sites from Canada (n=308), Germany (n=461), and UK (n=221) aged >1 month and without sickle cell anaemia were enrolled and pooled into the paediatric stroke database located in Germany. After enrolment, we excluded 54 children with moyamoya, two patients with either congenital homozygous protein C or homozygous antithrombin deficiency, 5 children lost to follow-up and 35 children in whom thrombophilia screening was not performed (figure 1). We studied 894 consecutively recruited children aged 1 month to $\leq$ 18 years who survived a first episode of AIS (figure 1, table 1) and were followed for median (minimum-maximum) duration of 35 (1-256) months. Clinical characteristics are summarized in table 1. In total, 160 children (17.9%) experienced a recurrent AIS event at a median interval from initial stroke of 3.1 months (min-max: 0.1-136). Death at the time of the second AIS occurred in 15 of 160 children (9.4%). The overall recurrence rate calculated per 100 person-years was 5 (95%CI: 4-6) with a yearly incidence of 0.05%.

Data on antithrombotic prophylaxis prior to a second AIS, i.e. anticoagulation with LMWH or vitamin-K antagonists (VKA) or antiplatelet (ASA or clopidogrel) therapy, were available in a subgroup of 122 out of 160 cases on an exploratory basis: antithrombotic prophylaxis was administered independent of the presence or absence of thrombophilia (p=0.89) or different stroke subtypes (p=0.2). In children with vasculopathy prior to the second AIS, 34 of 72 index patients were on antiplatelet agents (ASA alone) and 19 were treated with LMWH, one child developed a second stroke whilst taking VKA. In patients without vasculopathy 15 out of 50 children were on ASA, twice combined with Clopidogrel, seven received LMWH and three were on oral anticoagulation with VKA. 44 of the 122 patients did not receive anticoagulation or antiplatelet therapy prior to a second AIS (vasculopathy group n=19; non-vasculopathy children n=25; p=0.05).

Vascular territory of second AIS: In the majority of cases (75%) the same vascular territory was involved as in the first AIS. The anterior circulation was involved in 62.5% and the posterior circulation in 37.5%.

Stroke subtypes (table 1): Stroke subtypes are depicted in figure 1 and table 1. 32.2% of paediatric stroke patients (n=288) had vasculopathy. Sub-classification of vascular stroke is shown in the online supplement. Imaging confirmed recurrence rates occurred in 160/880 enrolled children, for *i)* cardiac disease 23/109 (21.1%) *ii)* vasculopathy 82/284 (28.9%), for *iii)* cryptogenic 41/401 (10.2%) and for *iv)* other stroke types 14/78 (17.9%). Recurrent AIS was significantly more frequent in patients with vasculopathy (HR 2.5, 95% CI 1.92-3.5; p< 0.001) compared to patients with cryptogenic stroke. Calculated per 100 person-years in

those with vasculopathy, the recurrence rate was 8 (95% CI: 6-10) with a yearly incidence rate of 7.7%. The time to recurrence, calculated as the probability of AIS-free survival, comparing paediatric AIS patients with and without vasculopathy, is depicted in figure 2 (log rank p-value < 0.001). No statistically significant association between recurrent AIS and the remaining stroke subgroups was found: cardiac stroke (HR 1.15; 95% CI 0.7-2.0; p=0.61); non-vascular/non-cardiac/non-idiopathic (HR 1.01; 95% CI 0.5-2.0; p=0.95).

Prothrombotic risk factors (table 2): Results derived from univariable analysis are depicted in table 2. A single prothrombotic disorder was detected in 269 children, whereas more than one prothrombotic risk factor was diagnosed in 88 cases. Heterozygous antithrombin deficiency, high lipoprotein (a), high fibrinogen, high fasting homocysteine and the presence of more than one prothrombotic disorder were associated with recurrence. Of the 7/23 patients with heterozygous antithrombin deficiency who experienced recurrent stroke, none were on unfractionated heparin or VKA: four were prescribed ASA at the time of recurrence, one patient was on LMWH, while 2 subjects were taking no prophylaxis immediately prior to re-stroke (non-compliance was not excluded). In six of 23 (26%) patients with combined defects and a second stroke, the factor 5 mutation at rs6025 was involved. Interestingly, however, the mutation at factor 2 at rs1799963 did not play a role in children with combined defects.

Examination of the roles of *i)* different stroke subtypes and *ii)* prothrombotic risk factors using multivariable Cox proportional hazards regression of variables with a p-value $\leq$ 0.15 in univariable analysis, adjusted for age, gender and study centre, demonstrated that the presence of vasculopathy (HR 2.5), antithrombin deficiency (HR 3.9), elevated Lp(a) (HR 2.3) and the presence of more than one prothrombotic risk factor (HR 1.9) were independently associated with an increased risk of recurrent stroke (table 3). The time to recurrence, i.e. recurrence-free survival comparing children with elevated Lp(a) with paediatric AIS patients with normal Lp(a) levels are depicted in figure 3 (log rank p-value < 0.039). Recurrence rates calculated per 100 person-years were 10 (95%CI: 3-24) for antithrombin deficiency (yearly incidence rate 0.1%), 6 (95%CI 4-9) calculated for elevated Lp(a) (yearly incidence rate 0.13%), and 13 (95%CI 7-20) for the presence of more than one prothrombotic risk factor (yearly incidence rate 0.13%). In the online supplement Kaplan Meier survival curves are depicted for children with multiple thrombophilias compared with normal thrombophilia status (figure 1 online material).

Based on its distribution in the Caucasian paediatric population, the number-needed-to-screen (NNS) to detect one patient with elevated Lp(a) was 10 and to detect children with more than one prothrombotic abnormality was 20.

Discussion

In our study cohort of 894 Canadian, English and German paediatric stroke patients >1 month of age, a second AIS event was diagnosed in 17.9% of patients within a median period of 3.1 months after the first stroke onset. Here we have shown, in an international cohort of children with paediatric stroke, that the presence of more than one prothrombotic risk factor is associated with AIS recurrence in children. Specifically, as demonstrated recently in children with recurrent deep venous thrombosis and thromboembolic stroke, heterozygous antithrombin deficiency is a major risk factor for second AIS events.[31] In addition, data presented here confirm an increased risk for recurrent stroke events in the subgroup of patients with underlying vasculopathy.[15,22,32-35]

The rates of recurrent cerebral thrombo-embolic events vary widely across published studies. Differences likely relate to *i)* the variable inclusion of neonates, known to have a very low rate of recurrence, *ii)* the ethnicity of patients enrolled and other variables in patient population *iii)* the definitions of recurrence and iv) the duration of follow-up. Some studies have mixed TIA and recurrent stroke in reporting the recurrence risk.[14-20] Adverse outcomes resulting from recurrent AIS are certainly more ominous than for TIA alone, with a mortality rate after second AIS of 9.4% in our patients. Keeping in mind differences in patient populations, underlying diseases as well as treatment modalities applied, and assuming that inclusion of TIAs would approximately double the recurrence risk,[16] the recurrence rate reported by us for AIS alone is within the lower rate of approximately 20% reported by other authors.[14-20] It is possible that our lower rate of recurrence reflects the use of standardized treatment protocols and institutional paediatric Stroke programs at our centres. Specialized stroke care is likely to lower the rate of recurrence through experienced selection of patients for antiplatelet/anticoagulant treatment and more consistent use of any preventative treatment.

With respect to the inherited prothrombotic risk factors investigated, multivariable analysis provides evidence that presence of vasculopathy, heterozygous antithrombin deficiency, increased Lp(a) and the presence of more than one thrombophilia are risk factors for recurrent ischaemic stroke in paediatric patients.[14,36-38] The numbers needed to screen to detect one patient each with elevated Lp(a) or combined thrombophilic abnormalities in the present cohort were 10 and 20 respectively. The benefit of recognising a thrombophilic underlying condition, as well as the NNS to detect a carrier at risk, should be balanced against clinical impact, cost and potential insurance implications. In contrast to the literature on recurrent venous thromboembolism in a similar population of children, as well as an

association with first AIS onset,[11,38] the presence of isolated mutations in factor 5 at rs6025, factor 2 at rs1799963, protein C- and protein S were not individually significantly associated with recurrent arterial ischaemic stroke in this multicenter cohort. Despite the recurrence rate of 17.9% comprising 160/894 patients, our study may have been underpowered to find these associations. Alternatively, these factors may have been more aggressively treated with antithrombotic treatment, reducing the risk of recurrence in affected patients. Importantly, combinations of prothrombotic risk factors, including the mutation at factor 5 at rs6025, in 26% of cases are associated with recurrence, emphasizing the importance of comprehensive investigation and appropriate management.

Our study has several limitations. First, the long duration of the study means that many children were enrolled more than a decade ago, when treatments may have been selected differently than is currently recommended. While this long duration provided us with the opportunity to monitor children for longer-term recurrent AIS, our inclusion of some children with only a brief follow-up duration, as short as one month, could also have resulted in our underestimating recurrence risk, and our rate of recurrence should therefore be viewed as a minimum estimate. Secondly, the proportion of children with vascular, cardiac and cryptogenic stroke varied across countries, likely representing either differences in assignment of patients to categories (e.g. inclusion or exclusion of occlusion alone as 'vasculopathy') or different referral patterns to the three Toronto, London/Southampton and Kiel-Lübeck/Münster centres. Thirdly, we were unable to include paediatric stroke drug therapy as a main focus of this study since antithrombotic and antiplatelet agents recommended for children with stroke derived from non-randomized paediatric trials and small case series are based on a low evidence level, without adjustment for treatable prothrombotic risk factors such as heterozygous antithrombin deficiency[21-25] or routine drug monitoring to detect ASA resistance or non-drug compliance. Finally, the thrombophilia testing was done over time and in 3 different laboratory settings. However, since laboratory parameters were investigated with standard laboratory techniques and assays and were only classified as abnormal when *i)* abnormal on repeat and *ii)* confirmed by family studies or the identification of an underlying gene mutation,[26-28] it likely that our results are reliable.

In summary, recurrent AIS is relatively frequent, and is associated with significant mortality. Risk is enhanced in children who have vasculopathy, even when moyamoya is excluded, and in those with certain isolated PRs or more than one prothrombotic disorder is found. The study results emphasize the value of pooling individual patient data across geographic regions. Future studies should seek to validate our findings in additional patient cohorts of children with a first onset of AIS with stroke subtypes clearly defined according to new

paediatric stroke classifications like CASCADE.[39,40] Of note, our finding that recurrence of childhood AIS is comparable across European and North American centres supports the feasibility of multi-national recruitment strategies to sufficiently power randomized treatment studies, which could include the development of stroke recurrence prediction models. Such studies should be focused on prevention of recurrent stroke in sub-populations of paediatric patients with the highest risks for recurrent AIS. In addition, from the data reported here, prediction models could be derived combining non-moyamoya vasculopathy with the presence of multiple thrombophilic risk factors of interest. On the background of regional differences with respect to prevalence rates of thrombophilic risk factors across study populations, NNS to detect carriers at risk will allow investigators to adequately power future paediatric stroke trials. It is important to keep in mind, however, that *i)* antithrombotic and/or antiplatelet therapy may also have a significant impact on the risk of AIS recurrence in children, and *ii)* up to now, due to the lack of randomized controlled trials, paediatric stroke treatment modalities are recommended on a low evidence base;[21-25] further efforts must be undertaken to also address the latter issue.

Contributors

All investigators (deVGA, KFJ, SK, SR, KG, MM, DN, BLR, KM, AR, SM, GV, PM, SJ and NGU) took part in the design, execution, ascertainment of recurrence and/or data analysis, and in writing the report. SK and deVGA were responsible for data management in the Canadian stroke database, KFJ for the UK database and CH and NGU for the German stroke registry. TA and NGU were responsible for statistical calculations.

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Table 1: Clinical characteristics of 894 children with first AIS studied for AIS recurrence

Characteristics	Canada Number [%]	Germany Number [%]	UK Number [%]	Total Number [%]
Population at first stroke onset	**294 [100.0]**	**379 [100.0]**	**221 [100.0]**	**894 [100.0]**
• Caucasian	166 [56.5]	377 [99.5]	190 [86.0]	733 [81.2]
• Black	17 [05.8]	-	4 [2.0]	
• Asian	44 [15.0]	-	27 [12.2]	
• First Nations/Aboriginal	2 [0.70]	-	-	
• Central/South American	5 [01.7]	-	-	
• Mixed ethnicity	9 [03.1]	2 [0.95]	-	
• Unknown	51 [17.3]			
Median age, years (min-max)	4.8 (0.1-17.7)	7.1 (0.2 -18)	4.5 (0.1-16.7)	6 (0.1-21)
Male	183 [59.4]	203 [54.7]	125 [56.5]	511 [56.8]
Proportion of children with a first Cryptogenic AIS	43 [14.6]	264 [69.5]	96 [43.4]	403 [45.1]
Proportion of children with a first vascular stroke	89 [30.3]	86 [22.7]	113 [51.1]	288 [32.2]
Proportion of children with a first cardiac stroke	92 [31.3]	8 [2.1]	9 [4.1]	109 [12.2]
Proportion of children with a recurrent AIS	76 [25.9]	27 [7.1]	57 [25.8]	160 [17.9]
Proportion of patients on treatment prior to second AIS	54 [71.1]	17 [63.0] #	30 [52.6]	101 [63.1]

Cohort data previously published in part (ref. 25)

Table 2: Univariable analysis: association between prothrombotic risk factors and a second stroke. A single prothrombotic disorder was detected in 269 children

Risk Factor	AIS first onset: Numbers with abnormal test /numbers tested [%]	AIS recurrence: Numbers with abnormal test /numbers tested [%]	Chi-squared p-value
Reference: no thrombophilia			
Fasting Lp(a) > 30 mg/dl	115/580 [19.8]	23/115 [20.0]	0.04
Fibrinogen	43/787 [5.5]	13/43 [30.2]	0.04
Fasting homocysteine	16/708 [2.3]	7/16 [43.8]	0.01
Antithrombin-deficiency	23/750 [3.1]	7/23 [30.4]	0.15
Protein C deficiency	26/778 [3.3]	5/26 [19.2]	0.9
Protein S deficiency	28/708 [4.0]	7/28 [28.0]	0.2
Factor 5 at rs6025	71/726 [9.8]	8/71 [11.3]	0.17
Factor 2 at rs1799963	21/631 [3.3]	3/21 [15.0]	0.84
Combined defects	88/848 [10.4]	23/88 [26.1]	0.04

Abbreviations used: F: factor; Lp: Lipoprotein

Table 3: Risk contribution to second AIS adjusted for age at onset, gender and study center (Cox proportional hazards model)

Risk Factor	Hazard Ratio	95% Confidence Interval
Stroke subtypes		
Reference: cryptogenic stroke		
Vascular stroke	2.5	1.92-3.5
Cardiac Stroke	1.15	0.7-2.0
non-vascular /non-cardiac /non-cryptogenic	1.01	0.5-2.0
Thrombophilia		
Reference: no thrombophilia		
Fasting Lp(a) > 30 mg/dl	2.3	1.3-4.1
Fibrinogen	0.9	0.3-2.8
Fasting homocysteine	3.6	0.8-15.8
Heterozygous Antithrombin-deficiency	3.9	1.4-10.9
Protein C-deficiency	1.3	0.3-5.5
Protein S deficiency	2.2	0.5-9.8
Factor 5 at rs6025	0.7	0.23-1.91
Factor 2 at rs1799963	1.8	0.4-7.8
Combined prothrombotic risk factors	1.9	1.12-3.2

Abbreviations used: F: factor; Lp: Lipoprotein

Figure 1: Patient flow chart

Figure 2 demonstrates the AIS-free survival in children with vasculopathy compared with the remainder with a normal arterial examination (p< 0.001).

Figure 3 demonstrates the AIS-free survival in children with elevated lipoprotein (a) compared with the remainder with normal lipoprotein (a) levels (p= 0.039).

electronic databases: ascertainment period 2002-2016
potentially relevant patients (first AIS onset)
aged from 1 month to 21 years

∑ = 990 [100%]

Canada n=308
Germany n=461
UK n=221

excluded: ∑ = 96 [9.7%]

reasons for exclusion

- moyamoya n=54
- incomplete thrombophilia screening n=35
- lost to follow-up n=5
- homozygous protein C deficiency n=1
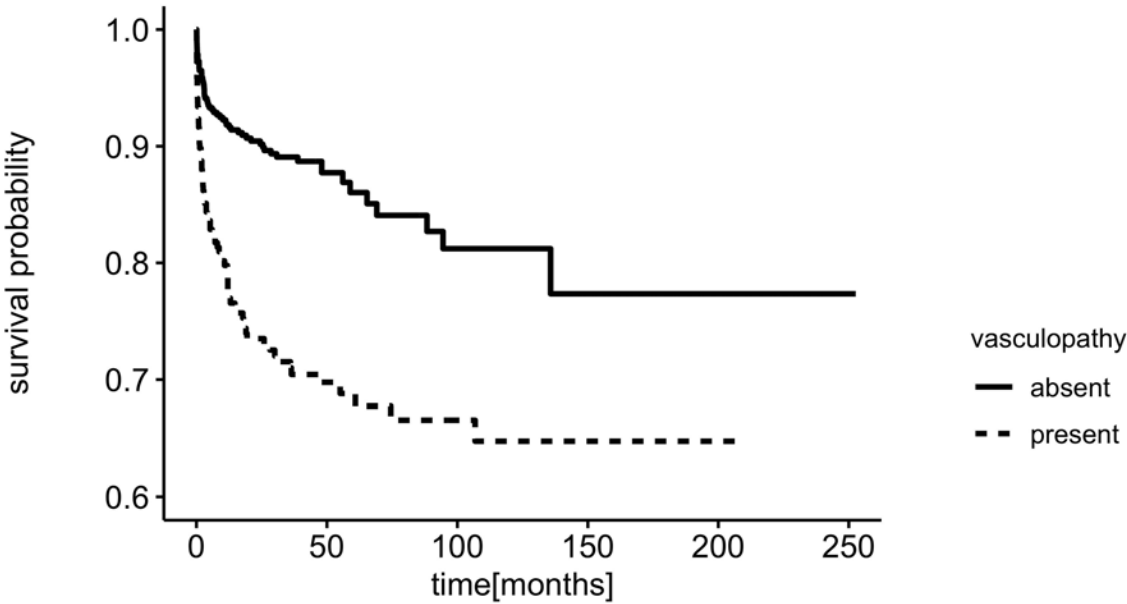- homozygous antithrombin deficiency n=1

patients included in the study
∑ = 894 [90.3%]

- vascular n=288 [32.2%]
- cardiac n=109 [12.2%]
- cryptogenic n=403 [45.1%]

- non-vascular
  non-cardiac   } *other etiologies n=94 [10.5%]
  non-cryptogenic }

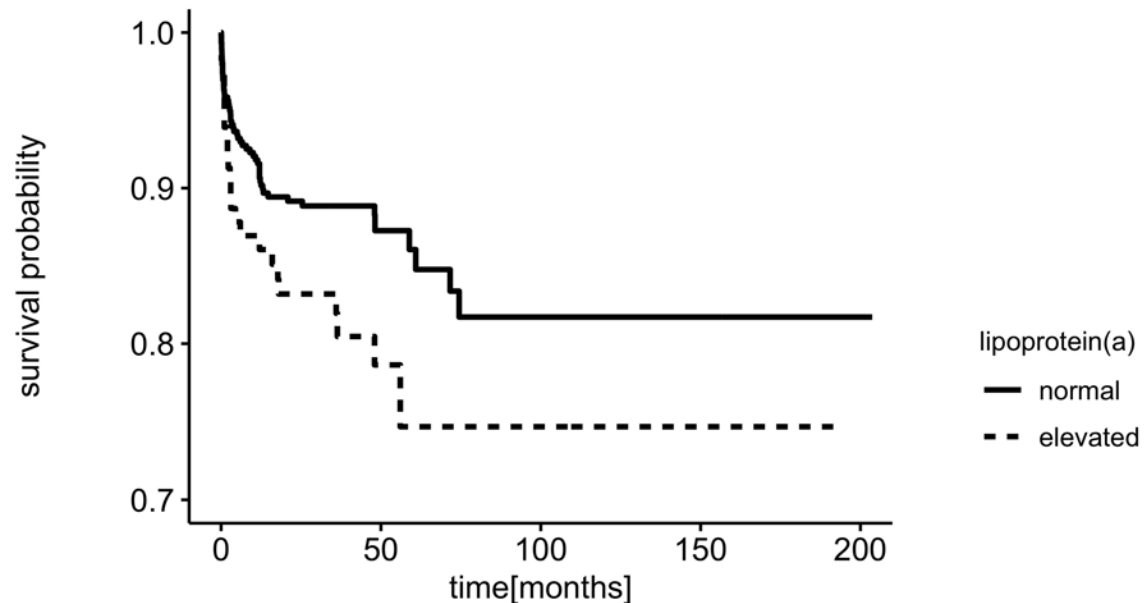* infection, post-vaccination, hemolytic-uremic syndrome, autoimmune disorders

recurrence yes vs. no

numer at risk

absent	519	109	50	16	7	1
present	290	77	41	19	1	0

recurrence yes vs. no

numer at risk

normal	461	83	34	9	1
elevated	115	22	9	2	0

Methods

Stroke Subtypes: Stroke subtypes in the enrolled children were reclassified according to explicit predefined criteria based on the TOAST criteria modified for children in which "vasculopathy" is substituted for "large vessel atherosclerosis".[11,22] Based on this classification in association with underlying diseases/co-morbidities, clinical data and the results of diagnostic studies including MR angiography, conventional angiography and Doppler ultrasonography, transthoracic and transesophageal echocardiography (ECHO) with saline contrast, and electrocardiography, the study patients were classified into four subgroups: (i) cardiac disease including persistent foramen ovale and mitral valve prolapse detected on ECHO as well as pre-diagnosed congenital heart disease (CHD) (ii) vasculopathy including dissection and other stenosis, e.g. focal cerebral and post-*Varicella* arteriopathy, post radiation and Down syndrome (iii) cryptogenic stroke, with no underlying disease/co-morbidities and no vasculopathy, and (iv) non-cardiac, non-vasculopathy and non-cryptogenic AIS, including stroke associated with other underlying diseases/co-morbidities such as systemic viral or bacterial infections including meningitis, post-vaccination, haemolytic-uraemic syndrome, malignancy or autoimmune disorders and others. Patients with both cardiac disease and vasculopathy were classified into the vasculopathy group. Ethnicity, age, gender and the proportion of stroke subtypes were collected.

Classification of vascular stroke type are as follows: vasculitis including post-*Varicella* vasculitis (n=72), dissection (n=46), transient cerebral arteriopathy (n=7), other specified vasculopathy (n=9: brain tumour n=1; post radiation n=2; Down syndrome n=3; catheter occlusion n=1; vasospasm n=2) and other unspecified vasculopathy including intracranial large vessel stenosis (n=76), short vessel stenosis with persistent transcranial Doppler-documented turbulence (n=24), large vessel occlusion (n=37) and hypoplastic arteries of un-known origin (n=17).

Antithrombotic treatment following first symptomatic stroke: At the discretion of the participating study centres, paediatric patients with a first stroke onset received antithrombotic therapy unless contraindicated by initial haemorrhagic infarction or other risk factors for haemorrhage.[21-25]

Laboratory analyses: With written or oral parental consent, the mutations in factor 5 at rs6025 and factor 2 at rs1799963 (retrospective genotyping in children with stroke diagnosed prior 1995 [factor 5] - & 1997 [factor 2]), as well as circulating levels of lipoprotein (Lp) (a), fibrinogen, factor VIIIC, homocysteine, protein C, protein S, and antithrombin were investigated with standard laboratory techniques at stroke onset and/or repeated during routine follow up visits. To rule out possible laboratory pitfalls induced by the acute stroke onset, for example consumption of antithrombin, protein C or protein S or acute phase-induced elevation of fibrinogen, factor VIII or homocysteine, repeated laboratory thrombophilia work-up was performed at least three months after the acute stroke event and was repeated if abnormal.[11] A non-homozygous type I deficiency (antithrombin, protein C) state was diagnosed when functional plasma activity and immunological antigen concentration of a protein (analysis of protein C and protein S at least three months after the index event and/or withdrawal of vitamin-K-antagonists) were confirmed to be below the age-related reference ranges.[26-28, *1a and 1b] A non-homozygous type II deficiency (antithrombin, protein C) was diagnosed when low functional activity levels were found along with normal antigen concentrations at least 3 months after the index event, repeated if abnormal. 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 at least 3 months after the index event, repeated if abnormal. Serum levels of Lp(a) > 30 mg/dl were considered elevated.[15] Since data on normal values for fibrinogen and factor VIIIC in children are sparse, fibrinogen and factor VIIIC levels > age-dependent 90th percentiles, derived from the healthy control children were used as cut-off values. Criteria for the hereditary nature of a haemostatic defect were its presence in at least one further first or second-degree family member and/or the identification of a causative gene mutation.[26-28]

Reference values 1a and 1b

1a. Nowak-Göttl U, Limperger V, Kenet G, Degenhardt F, Arlt R, Domschikowski J, Clausnizer H, Liebsch J, Junker R, Steppat D. Developmental hemostasis: A lifespan from neonates and pregnancy to the young and elderly adult in a European white population. Blood Cells Mol Dis. 2017 Sep;67:2-13. doi:10.1016/j.bcmd.2016.11.012.

1b. Nowak-Göttl U, Junker R, Hartmeier M, Koch HG, Münchow N, Assmann G, von Eckardstein A. Increased lipoprotein(a) is an important risk factor for venous thromboembolism in childhood. Circulation. 1999 Aug 17;100(7):743-748.
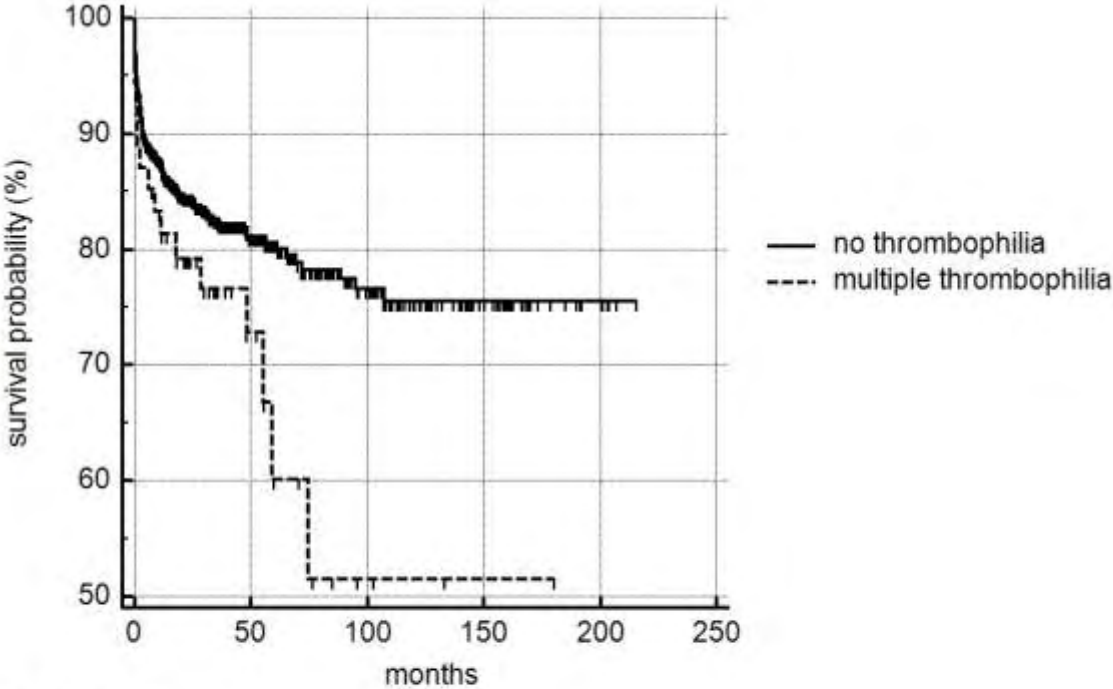
Statistics: Patients with recurrent stroke were compared with patients without stroke recurrence. For the primary study objective, we calculated the symptomatic recurrence rate per 100 person-years and yearly incidence rates, based on the recruitment period and a median follow-up period of 35 months. Predictors possibly influencing symptomatic recurrence were defined *a priori* based on literature data and included stroke subtypes and prothrombotic risk factors (PR) significantly associated with a first AIS onset.[11] Using a rule of

thumb for proportional hazards analysis including approximately 10 outcomes for each independent predictor,[29] the final statistical model aimed to include seven predictors: calculated were the possible role of stroke subtypes on the recurrence rate per 100 person-years and the possible influence of thrombophilic risk factors on recurrent AIS. In order to evaluate an independent contribution to the risk of recurrent AIS and to adjust for further potential confounders (age at onset, gender, study centre) the hazard ratio (HR) together with 95% confidence intervals (CI) were estimated from Cox's proportional hazards model. Variables were removed from the backward model if p was > 0.16. Stroke groups (cardiac, vascular, non-cryptogenic/-cardiac,-vascular) were compared to cryptogenic stroke. Patients with no thrombophilia were compared with those with thrombophilia. Statistical analyses were performed using the MedCalc software bvba (version 16.4.3, Ostend, Belgium) and StatView 5 software packages (SAS Institute Inc.). The recurrence rates were calculated as the number of recurrent events per 100 person-years.

For the secondary study objective, i.e. the time to recurrence, we calculated the probability of AIS-free survival (AFS) as a function of time utilizing the method of Kaplan and Meier (univariate analysis). The log rank test was used to test for differences in recurrence-free survival between groups. Patients were withdrawn from the survival analysis (censored cases) either at death unrelated to AIS recurrence or at loss to follow-up using data of the last clinical follow-up visit. To further test the relationship between independent and dependent variables, the likelihood ratio test was performed. Because of their apparently non-Gaussian distribution, continuous data are presented as median and minimum-maximum (min.-max.) values and were evaluated by non-parametric statistics including the Wilcoxon-Mann-Whitney U test. To compare frequency distributions of fatal outcome, $\chi 2$ test and, if necessary, Fisher's exact test was performed. In addition, numbers needed to screen (NNS) were calculated as previously described.[30]

Results

Figure 1 (supplement): Stroke–free survival with respect to thrombophilia status (none versus combined) is depicted (p=0.039).