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# Multicentre evaluation of combined prothrombotic defects associated with thrombophilia in childhood

**Abstract** To evaluate the role of multiple established and potential causes of childhood thrombophilia, 285 children with a history of thrombosis aged neonate to 18 years (first thrombotic onset) were investigated and compared with 185 healthy peers. APC-resistance (FV:$Q^{506}$), protein C, protein S, antithrombin, heparin cofactor II (HCII), histidine-rich glycoprotein (HRGP), and prothrombin (F.II), factor XII (F.XII), plasminogen, homocysteine and lipoprotein (a) (Lp(a)) were investigated. In 59% of patients investigated one thrombotic defect was diagnosed, 19.6% showed two thrombotic risk factors, while in 21.4% of children investigated no risk factor could be identified. Single defects comprised established causes of inherited thrombophilia: FV:$Q^{506}$ (homozygous $n = 10$, heterozygous $n = 69$), protein C (homozygous $n = 1$; heterozygous $n = 31$), heterozygous type I deficiency states of protein S ($n = 7$), antithrombin ($n = 7$) and homocystinuria ($n = 6$); potentially inherited clotting abnormalities which may be associated with thrombophilia: F.XII ($n = 3$), plasminogen ($n = 2$), HCII ($n = 1$), increased HRGP ($n = 4$); new candidate risk factors for thrombophilia: elevated plasma levels of Lp(a) ($n = 26$), F.II ($n = 1$). Heterozygous FV:$Q^{506}$ was found in combination with heterozygous type I deficiency states of protein C ($n = 2$), protein S ($n = 13$), antithrombin ($n = 8$) and HCII ($n = 1$), increased Lp(a) ($n = 13$), and once each with elevated levels of F.II, moderate hyperhomocysteinemia, fibrinogen concentrations $> 700$ mg/dl and increased HRGP. In addition to the association with FV:$Q^{506}$, heterozygous protein C type I deficiency was combined with deficiencies of protein S ($n = 2$), antithrombin ($n = 1$), and increased Lp(a) ($n = 3$). One patient showed protein C deficiency along with familially increased von Willebrand factor $> 250\%$. Besides coexistence with FV:$Q^{506}$ and protein C deficiency, protein S deficiency was combined with decreased F.XII and increased Lp(a) in one subject each. Furthermore, we found combinations of antithrombin deficiency/elevated Lp(a), hyperhomocysteinemia/Lp(a), deficiency of HCII/plasm-

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inogen, and plasminogen deficiency along with increased Lp(a) each in one. Increased prothrombin levels were associated with fibrinogen concentrations >700 mg/dl and with HCII deficiency in one child each. Carrier frequencies of single and combined defects were significantly higher in patients compared with the controls.

**Conclusion** In conclusion, data of this multicentre evaluation indicate that paediatric thromboembolism should be viewed as a multifactorial disorder.

**Key words** Childhood venous thrombosis · FV G1691A mutation · Protein C · Protein S · Antithrombin · Lipoprotein (a)

**Abbreviations** *F* factor · *HC* heparin cofactor · *HRGP* histidine rich glycoprotein · *Lp(a)* lipoprotein (a)

## Introduction

Within the past decade, various genetic defects of proteins regulating blood coagulation, particularly those affecting the physiological anticoagulant systems, have been well established as risk factors of cardiovascular disease in adults [7, 11, 14, 18, 25–27, 30, 33, 44, 51, 52]. Besides the high thrombotic risk reported in patients with homozygous factor V (FV):$Q^{506}$ mutation, homozygous protein C deficiency, homozygous protein S deficiency and homozygous homocystinuria due to cystathionine-β-synthase (CBS) deficiency, a high risk of early thrombotic onset is observed in patients with heterozygous antithrombin deficiency, heterozygous protein C deficiency of the so-called dominant type, and heterozygous protein S deficiency [22, 34, 35, 46, 48]. In contrast, an intermediate or low risk of developing early thromboembolism is observed in patients with heterozygous FV:$Q^{506}$, heterozygous recessive protein C deficiency and heterozygous defects of the heparin-binding site of the antithrombin molecule, moderate hyperhomocysteinemia (elevated fasting homocysteine concentrations), plasminogen deficiency and dysfunctional plasminogen or fibrinogen molecules [9, 15, 17, 45, 47]. Furthermore, the recently described 20210GA variant of the prothrombin gene seems to be a common but probably mild risk factor of arterial and venous thromboembolism [9, 45]. However, there is only scanty and conflicting information about the role in triggering thrombosis of inherited or acquired deficiency of heparin cofactor II (HCII), factor XII (F.XII), or elevated levels of histidine-rich glycoprotein (HRGP), lipoprotein (a), von Willebrand factor antigen or factor VIII:C (F.VIII:C) respectively [6, 16, 19–21, 49].

Since the recent discovery of activated protein C resistance as a highly prevalent hereditary risk factor of venous thromboembolism, evidence has been accumulating that thrombophilia is a multigenetic disorder and that the association of multiple haemostatic defects greatly increases the risk of thrombosis in adults. Although paediatric venous thrombosis is being increasingly viewed as a multifactorial disorder as well [1, 5, 28, 36, 37, 38, 41, 42, 43, 50], information on the role of combined haemostatic defects in childhood thrombosis is limited [36]. Here, we present multicentric evaluated data on venous and arterial thrombosis in paediatric patients with regard to the frequency and combinations of several haemostatic risk factors of thrombosis in different age groups.

## Patients, materials and methods

### Subjects

At onset of this multicentre evaluation the following inclusion criteria were defined:

1. Age at first thrombotic onset less than 18 years.
2. Objective confirmation of thromboembolism by standard imaging methods.
3. To prevent results from being affected by an acute reactive process or oral anticoagulation, blood samples were obtained at least 3–6 months after the thrombotic episode and withdrawal of oral anticoagulant medication.
4. Patients found to have an abnormal protein-based laboratory test result were followed up with at least a second blood sample 6 or more weeks after the first examination.

These criteria were fulfilled by 285 out of 433 patients with a history of an objectively confirmed thromboembolic episode between the neonatal period and 18 years of age (median age at onset: 6 years). All patients had been admitted unpreferentially from different geographic areas of Germany in the acute phase for therapy and/or laboratory screening for prothrombotic haemostatic defects.

The thrombotic manifestations reported were: deep vein thrombosis ($n = 83$), central nervous thrombosis ($n = 28$), renal venous thrombosis ($n = 16$), superior caval vein thrombosis ($n = 14$), inferior caval vein thrombosis ($n = 9$), portal vein thrombosis ($n = 7$), right intracardial thrombosis ($n = 5$), splenic vein thrombosis ($n = 2$), mesenteric vein thrombosis ($n = 2$), veno-occlusive disease ($n = 2$), multiple venous thrombosis ($n = 2$), pulmonary embolism ($n = 6$), aortal thrombosis ($n = 1$), mesenteric artery thrombosis ($n = 1$), subclavian artery thrombosis ($n = 1$) and thromboembolic stroke ($n = 106$).

Duplex sonography, venography, computed tomography (CT) and magnetic resonance (MR) imaging were performed to diagnose venous thromboembolism; cerebral CT scanning, MR imaging and MR angiography or transcranial Doppler ultrasonography were performed to confirm the diagnosis of ischaemic stroke.

Besides spontaneous thromboembolism ($n = 117$) early onset of symptomatic vascular occlusion was associated with central lines ($n = 50$), bacterial or viral infection ($n = 23$), malignancy ($n = 21$), adiposity ($n = 10$), systemic lupus erythematosus ($n = 9$), underlying cardiac disease ($n = 11$), peripartal asphyxia

($n = 6$), trauma ($n = 5$), fibromuscular dysplasia ($n = 5$), dehydration ($n = 3$), and mitochondrial disease (MELAS: $n = 2$). In addition, an early intake of oral contraceptives was reported in 15 female adolescents, and nicotine abuse was known in 5 of the children investigated.

The remaining 148 infants and children could not be enrolled in this study because a complete laboratory screening programme was not performed, due mainly to the lack of blood samples for laboratory re-investigation.

In addition, to compare carrier frequencies 185 healthy age-matched children from the same geographic area were investigated as controls.

## Laboratory methods

### Blood sampling

With informed parental consent, blood samples were collected by peripheral venipuncture into 3.8% trisodium citrate (1 part anticoagulant: 9 parts blood; Sarstedt® tubes, Nümbrecht, Germany) and placed immediately on melting ice. Platelet poor plasma was prepared by centrifugation at 3000 *g* for 20 min at 4°C, aliquoted in polystyrene tubes, stored at −70°C and thawed immediately before the assay. For gene analysis we obtained venous blood in EDTA-treated S-Monovettes® (Sarstedt®, Nümbrecht, Germany), from which cells were separated by centrifugation at 3000 *g* for 15 min. The buffy coat layer was then removed and stored at −70°C until DNA extraction was performed by standard techniques.

### Assays of haemostatic factors

The co-ordinating centres Frankfurt and Münster analysed all patient plasma samples by a uniform diagnostic method using the same reagents and instruments. As routine practice Hepzym® (Dade/Baxter Diagnostics, Unterschleissheim, Germany) was added to plasma samples to eliminate the influence of heparin.

Prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time were determined in all samples by conventional methods.

According to the classification previously proposed [25], we screened three different groups of conditions predisposing for thrombophilia.

*a) Established causes of thrombosis with known inheritance: deficiency and/or dysfunction of antithrombin, protein C, protein S, APC-resistance (FV R506Q)*

The response to activated protein C (Chromogenix, Mölndal, Sweden), FV:$Q^{506}$, amidolytic protein C activity (chromogenic substrate: Chromogenix, Mölndal, Sweden), free protein S antigen, total protein S antigen and protein C antigen (Asserachrom, Stago: Asnieres-sur-Seine, France) were measured as described earlier [5, 39, 40]. Antithrombin activity was measured with chromogenic substrate (Chromogenix, Mölndal, Sweden). Partigen plates (radial immunodiffusion) used to determine antithrombin concentrations were purchased from Behringwerke, Marburg, Germany. In addition, crossed immunoelectrophoresis (Behringwerke Marburg, Germany; Dako A-S, Denmark) was performed in patients with antithrombin deficiency.

*b) Acquired/Inherited factors known to be associated with an increased risk of thrombosis (precise relative contribution as yet uncertain): hyperhomocysteinemia, elevated levels of Factor VIII:C*

F. VIII:C (normal childhood values $< 150\%$) was determined by conventional methods. Fasting plasma homocysteine concentrations were measured by high-performance liquid chromatography [4] with normal values in infancy and childhood $< 10$ μmol/l (Koch et al: unpublished data).

*c) Potentially inherited clotting abnormalities which may be associated with increased risk of thrombosis (genotype-phenotype relationships as yet unknown): deficiency of plasminogen, heparin cofactor II, Factor XII, elevated histidine-rich glycoprotein, von Willebrand factor, fibrinogen*

Heparin cofactor II (HCII) antigen was measured with ELISA technique (Asserachrom, Stago, Asnieres sur Seine, France; HCII-EIA: Diagnostik International, Karlsdorf, Germany) or with the Laurell method (Anti HC II: Behringwerke, Marburg, Germany). Plasminogen activity was measured with chromogenic substrate (Chromogenix, Mölndal, Sweden), while antigen concentrations were determined by radial immunodiffusion (Behringwerke Marburg, Germany). A one-stage clotting activity assay of F.XII was performed on the ACL 300 R (Instrumentation Laboratory, Munich, Germany) or BCT analyser (Behringwerke, Marburg, Germany) using F.XII deficient plasma (Instrumentation Laboratory, Munich, Germany). If F.XII:C was below the normal range, antigen concentrations were measured by immunoelectrophoresis according to Laurell (Anti F.XII: Behringwerke, Marburg, Germany; Enzym Research Laboratory, USA). Histidine-rich glycoprotein (HRGP) was measured with the Laurell method (Anti-HRG: Behringwerke Marburg/Germany). According to the reference boundary of 50–172%, HRGP concentrations above 172% were considered to be abnormally high. Fibrinogen, (Fg: normal childhood values (not age-dependent) 150–400 mg/dl) and von Willebrand factor (vWf:Ag) were determined by conventional methods.

*d) New candidate risk factors for thrombosis: elevated lipoprotein (a), prothrombin*

As elevated plasma levels of Lp(a) and prothrombin (F.II) were recently detected as possible causes of inherited thrombophilia, both parameters were re-evaluated additionally. Lp(a) (Chromogenix, Mölndal, Sweden) was measured in 90 unselected patients as described earlier [29, 36]. Cut-off values used in this multicentre evaluation were $> 30$ mg/dl corresponding to $< 27$ kringle IV repeats. One-stage clotting activity assays of F.II were performed on the automated Coagulation Laboratory (ACL 300 R) (Instrumentation Laboratory, Munich, Germany) or Behring BCT analyser (Behringwerke, Marburg, Germany) using F.II deficient plasma (Instrumentation Laboratory, Munich, Germany).

*Definition criteria*

Potential thrombophilic states associated with elevated HRGP, fibrinogen, vWf:Ag or F.VIII:C were established only if plasma levels of the protein investigated were clearly above the upper limit of normal in at least two different samples. For all plasma-based assays, diagnosis of a clotting abnormality was established only if the plasma level of a protein was outside the limits of its normal range in at least two different samples.

A heterozygous type I deficiency state was diagnosed when functional plasma activity and immunological antigen concentration of a protein were approximately $< 50\%$ of normal of the lower age-related limit [2, 3, 38], and a homozygous state was defined if activity levels and antigen concentrations were approximately $< 10\%$ of normal respectively.

On the other hand, a type II deficiency was diagnosed with repeatedly low functional activity levels 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 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.

For diagnostic purposes a different group of 385 healthy infants and children (elective surgery or bone marrow donors aged 0–18 years: female/male = 200/185) from the same geographic region as the patient group was previously investigated. Table 1 shows the normal values of those clotting factors which require age-adjustment. To avoid a possible overestimation of prevalences for different deficiency states the relatively low cut-off values quoted in Table 1 was chosen to define heterozygous or homozygous states in this childhood population studied.

The present multicentre evaluation 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 Westphalien Wilhelms-University, Münster, Germany.

*Statistical analysis*

Nonparametric statistics (U-test: Mann-Whitney, H-test: Kruskal-Wallis) were performed with the Stat view 4.02 programme. Chisquare ($\chi^2$) test and Fischer's exact test were used for group comparison of carrier frequencies. *P*-values of less than 0.05 were considered to be significant. With respect to the number of different tests applied a correction according to Bonferroni was performed: The critical cut-off $\chi^2$-value for $p < 0.05$ and two degrees of freedom was 9.8.

## Results

The results are shown in Table 2 and in Fig. 1.

In the population studied, 109 out of 285 infants and children presented arterial thromboembolism and 176 out of 285 subjects had suffered from venous vascular occlusion. A predominance of arterial vascular accidents in the youngest age group ($n = 52$) was observed, due mainly to thromboembolic stroke (patent foramen ovale; 24). This pattern changed to 41 arterial observations in children aged 1–9 years, while only 16 older children and adolescents presented with arterial vascular occlusion.

An established or potential cause of thrombophilia was found in 224 patients, corresponding to 78.6% of children studied ($n = 285$). One risk factor of thrombosis was diagnosed in 168 patients (59%), 56 patients (19.6%) showed two thrombotic defects, while in 61 children (21.4%) no risk factor could be identified so far.

### Single thrombotic risk factors

In the majority of cases with single thrombotic defects we diagnosed well established deficiencies with known inheritance, predominantly in the protein C anticoagulant pathway: FV:$Q^{506}$ in a homozygous ($n = 10$, 3.5%) or heterozygous ($n = 69$, 24.2%) form, protein C (heterozygous $n = 31$, 10.8%; homozygous $n = 1$, 0.35%), and heterozygous protein S ($n = 7$, 2.5%). Additionally, heterozygous deficiency of antithrombin ($n = 7$, 2.5%) was diagnosed, and inherited homocystinuria due to CBS deficiency was found in 6 (2.1%) patients. With respect to classification subgroup c (potentially inherited clotting abnormalities which may be associated with increased risk of thrombosis), deficiencies of plasminogen ($n = 2$, 0.7%), HCII ($n = 1$, 0.35%), F.XII ($n = 3{,}1\%$) and HRGP (>172%: $n = 4$, 1.4%) were found. All deficiencies were of type I with decreased functional activity and antigen concentration. The remaining defects included the new candidate risk factors for thrombosis: elevated plasma levels of Lp(a) ($n = 26$, 9.1%) and F.II ($n = 1$, 0.4%).

In infants and children with a single thrombotic risk factor (P) the following carrier frequencies were significantly higher compared with the controls (C): heterozygous FV:$Q^{506}$ (P = 69; C = 15: $x^2 = 19.8$), protein C deficiency (P = 32; C = 1: $x^2 = 19.6$) and increased

**Table 1** Age-dependent normal reference values (median, range) for haemostatic parameters established from 385 healthy infants and children. In addition, cut-off values (heterozygous/homozygous deficiency state) are shown

| Parameter<br>Number | Neonate<br>55 | 3 Months<br>50 | 6 Months<br>60 | 1–5 Years<br>60 | 6–9 Years<br>58 | 10–18 Years<br>52 |
|---|---|---|---|---|---|---|
| F.II % | 45 (22–70) | 70 (45–105) | 80 (50–110) | 85 (60–110) | 80 (65–108) | 83 (65–106) |
| Cut-off | >100 | | | >150 | | |
| F.XII % | 45 (15–75) | 58 (25–98) | 70 (35–110) | 92 (59–125) | 90 (60–130) | 90 (60–130) |
| Cut-off | <7/<2 | <12/<2 | <20/<5 | <30/<5 | <30/<5 | <30/<5 |
| vWf:Ag % | 150 (60–250) | 110 (55–200) | 105 (50–190) | 88 (50–150) | 90 (52–153) | 95 (55–150) |
| Cut-off | >300 | >250 | >150 | >150 | >150 | >150 |
| APCR 1:11Dil | 2.6 (2–3.5) | 2.8 (2.05–3.5) | 2.9 (2–3.6) | 3.0 (1.8–3.8) | 2.9 (1.8–3.2) | 2.8 |
| Cut-off | <2/<1.5 | <2/<1.5 | <2/<1.5 | <1.7/<1.4 | <1.7/<1.4 | <1.7/<1.4 |
| AT % | 52 (30–85) | 90 (55–120) | 98 (65–126) | 101 (85–140) | 100 (85–136) | 98 (84–139) |
| Cut-off | <15/<2 | <25/<5 | <35/<5 | <45/<10 | <45/<10 | <45/<10 |
| HC II% | 35 (15–58) | 58 (35–70) | 80 (45–105) | 85 (55–120) | 87 (54–120) | 95 (60–125) |
| Cut-off | <7/<2 | <20/<5 | <25/<5 | <30/<10 | <30/<10 | <30/<10 |
| Protein C Ac. % | 35 (14–55) | 55 (25–82) | 60 (38–95) | 75 (45–102) | 84 (64–125) | 88 (62–128) |
| Cut-off | <7/<2 | <13/<2 | <20/<5 | <25/<5 | <32/<5 | <32/<5 |
| Protein C Ag. % | 30 (12–50) | 50 (22–75) | 55 (40–100) | 70 (45–98) | 80 (55–120) | 82 (55–120) |
| Cut-off | <7/<2 | <13/<2 | <20/<5 | <25/<5 | <30/<5 | <30/<5 |
| Free Prot.S Ag. % | 38 (15–55) | 55 (35–92) | 77 (45–115) | 78 (62–120) | 80 (62–130) | 85 (60–140) |
| Cut-off | <7/<2 | <20/<5 | <25/<5 | <30/<10 | <30/<10 | <30/<10 |
| Total Prot.S Ag. % | 35 (14–55) | 58 (35–90) | 75 (50–110) | 85 (60–120) | 82 (59–118) | 80 (60–115) |
| Cut-off | <7/<2 | <20/<5 | <25/<5 | <30/<10 | <30/<10 | <30/<10 |
| Plasminogen % | 50 (35–70) | 68 (45–95) | 87 (65–100) | 98 (63–123) | 95 (68–120) | 90 (70–115) |
| Cut-off | <20/<5 | <25/<5 | <35/<10 | <35/<10 | <35/<10 | <35/<10 |

**Table 2** Coexistence of established and potentially inherited conditions predisposing for thrombosis found among 285 symptomatic children (absolute and relative frequencies)

| | FV:$Q^{506}$ | Protein C | Protein S | Antithrombin | Lp(a)↑ | HCII | F.XII | HRGP↑ |
|---|---|---|---|---|---|---|---|---|
| FV:$Q^{506}$ | | 2<br>0.7% | 13<br>4.6% | 8<br>2.8% | 13<br>4.6% | 1<br>0.35% | – | 1<br>0.35% |
| Protein C | 2<br>0.7% | | 2<br>0.70% | 1<br>0.35% | 3<br>1.05% | – | – | – |
| Protein S | 13<br>4.6% | 2<br>0.7% | | – | 1<br>0.35% | – | 1<br>0.35% | – |
| Antithrombin | 8<br>2.8% | 1<br>0.35% | – | | 1<br>0.35% | – | – | – |
| F.II↑ | 1<br>0.35% | – | – | – | – | 1<br>0.35% | – | – |
| Plasminogen | – | – | – | – | 1<br>0.35% | 1<br>0.35% | – | – |
| Hyperhomocysteinemia | 1<br>0.35% | – | – | – | 1<br>0.35% | – | – | – |

Lp(a) ($P = 26$; $C = 8$: $x^2 = 33.7$). However, antithrombin deficiency ($P = 7$; $C = 0$: $x^2 = 4.6$) was not significantly different when the Bonferroni correction was performed.

No HCII or plasminogen or F.XII deficiency, hyperhomocysteinemia, elevated levels of vWf:Ag, fibrinogen, F.II >150% or F.VIII was found in the healthy children investigated.

## Combined thrombotic risk factors

Thrombophilic states including more than one established or potential factor predisposing to thrombosis are shown in Table 2. Besides the combined defects shown in Table 2, we found repeatedly increased fibrinogen concentrations >700 mg/dl associated with FV:$Q^{506}$ or elevated F.II levels, and protein C deficiency along with familially increased vWf:Ag >250% in one child each.

Combined defects within the protein C pathway (FV:$Q^{506}$/protein C or protein S: $P = 15$; $C = 0$: $x^2 = 10.05$) or FV:$Q^{506}$/Lp(a) ($P = 13$; $C = 0$: $x^2 = 12.7$) were not found in the control population, leading to significant different frequencies between patients and controls. The association between FV:$Q^{506}$ and antithrombin ($P = 8$; $C = 0$: $x^2 = 5.2$) was not significantly different when the Bonferroni correction was performed.

## Overall frequencies of single and combined thrombotic risk factors

The overall frequencies of established and potential haemostatic risk factors predisposing to thrombosis found in 224 out of 285 patients (168 singly affected, 56 doubly affected) were as follows: FV mutation 42.1% ($n = 120$), protein C deficiency 14.4% ($n = 41$), protein S deficiency 8.4% ($n = 24$), antithrombin deficiency 6% ($n = 17$) and elevated Lp(a) (46 out of 90 children investigated: 51%).The remaining clotting abnormalities, i.e. deficiency states of F.XII, plasminogen and HCII or elevated levels of vWf:Ag, fibrinogen, HRGP and F.II related to the patient population investigated were negligibly low, totalling 8.4%.

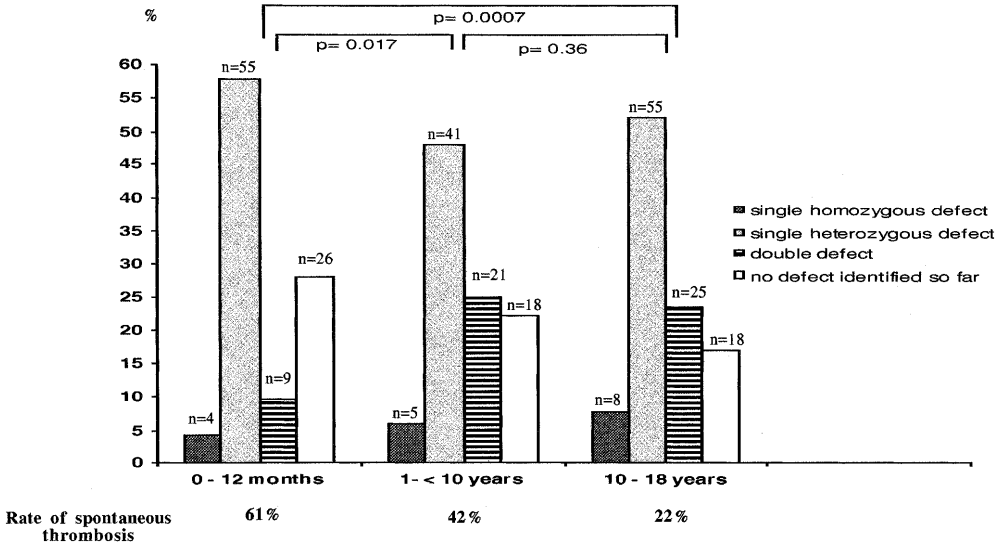


**Fig. 1** Distribution (absolute number and percentage of investigated children) of double and single thrombophilic risk factors found in children with vascular occlusion at different ages. The prevalence of combined compared to single haemostatic defects differed in the three age groups analysed, with a significantly higher prevalence of combined defects in 1–9-year-olds ($p = 0.017$) and 10–18-year-olds ($p = 0.0007$) respectively when compared with children in the first year of life. As also shown in this figure, the highest rate of spontaneous thrombosis was found in infancy (61% compared with 42% in 1–9-year-olds and 22% in 10–18-year-olds

In all three subjects with elevated prothrombin values >150% the prothrombin 20210A polymorphism was identified in a heterozygous state (Junker et al: personal communication).

The absolute and relative frequencies of single and combined thrombotic risk factors with respect to patients' age at first thrombosis are shown in Fig. 1. The prevalence of combined compared to single haemostatic defects differed in the three age groups analysed, with a significantly higher prevalence of combined defects in 1–9-year-olds ($p = 0.017$) and 10–18-year-olds ($p = 0.0007$) respectively when compared with children in the first year of life ( = infancy). As also shown in Fig. 1, the highest rate of spontaneous thrombosis was found in infancy (61% compared with 42% in 1–9-year-olds and 22% in 10–18-year-olds; $p < 0.0001$). However, besides the higher rate of spontaneous thrombosis observed in infants affected mainly by relatively strong inherited thrombotic risk factors, i.e. deficiency of protein C, protein S or antithrombin, no statistically significant differences were found regarding the relationship between clotting abnormalities diagnosed and site or type of thrombosis.

The female/male ratio changed from male predominance (1:1.26) in the first year of life to female predominance (1:0.5) in children aged 10–<18 years.

## Discussion

In recent years, the relations of various hereditary haemostatic abnormalities contributing to the risk of venous thromboembolism – in particular a deficiency of protein C, protein S or antithrombin, activated protein C resistance and hyperhomocysteinemia – have been well established [7, 11, 14, 18, 22, 25–27, 30, 33–35, 44, 46, 48, 51, 52]. In addition, the recently described polymorphism of 20210GA in the prothrombin gene has been identified as a common but probably mild thrombotic risk factor in adults [9, 32, 45]. Based on several recent trials, mainly in adults but also in paediatric patients, evidence is accumulating that familial thrombophilia may be due to a combination of clotting defects. Although the importance of inherited thrombophilia in children with thromboembolism has recently been established [1, 5, 28, 36, 37, 39, 41, 42, 43, 50], the role of combined haemostatic defects is still unclear. The results obtained from adult trials cannot be extrapolated to children, due mainly to age-dependent physiological differences in haemostasis, the frequency of thrombosis and the risk factors involved [36, 42, 43]. Additionally, in a number of children affected, an acquired risk masks the inherited deficiency [1, 37, 41, 41]. Therefore we performed this multicentre evaluation to assess to what extent single and combined clotting abnormalities influence thrombophilia in paediatric patients.

Among the 285 infants and children investigated, one or more established or potential thrombophilic haemostatic abnormalities were found in 78.6%. The majority of thrombophilic defects diagnosed in all affected patients were caused by an established hereditary trait (deficiency of protein C, protein S, antithrombin, FV:$Q^{506}$, homocystinuria due to CBS-deficiency) and concerned mainly the protein C pathway. The clotting abnormalities identified in the remaining patients such as deficiency of HCII, F.XII, plasminogen, or elevation of homocysteine, Lp(a), HRGP and vWf:Ag respectively, are viewed as possibly inherited or acquired [25].

Whereas the association between FV:$Q^{506}$ and other well established causes of inherited thrombophilia has been investigated in greater detail, there is only scanty information about associations between FV:$Q^{506}$ and other potential genetic factors predisposing to thrombosis, like the commonly observed increase of Lp(a) [19, 36, 49], which is located on chromosome 6 close to the gene for plasminogen and is closely homologous with plasminogen [31]. However, our data indicate that elevated Lp(a) concentrations, clearly correlated with low molecular weight apo(a) glycoprotein isoforms, classified by the number of kringle IV repeats [23, 29], combined with FV:$Q^{506}$, protein C deficiency or deficiency of protein S and antithrombin led also to an early thrombotic manifestation in paediatric patients. However, since the measurement of Lp(a) could not be re-evaluated in the entire study population presented here, further studies are needed to confirm the potential role of increased Lp(a) in childhood venous and arterial thromboembolism.

Furthermore, the data obtained in the Leiden Thrombophilia Study (LETS) showed that the level of F.VIII:C is an important, independent risk factor for venous thrombosis and that in addition high levels of vWf:Ag were associated with an increased risk of venous thrombosis [20, 32]. In addition, evidence of the potential importance of HRGP in thrombosis and fibrinolysis is provided by recent reports that high levels of HRGP are associated with thrombotic disease and also familial thrombophilia, indicating the possible hereditary nature of this disorder [8, 10, 12, 13]. However, in the population presented we identified familially increased vWf:Ag in only one subject and high levels of HRGP in 5 out of 285 infants and children investigated. Thus, our findings did not indicate a significant, independent effect of elevated vWf:Ag or HRGP on thrombotic risk in childhood, as it seems to be the same for deficiencies of HCII, F.XII and plasminogen diagnosed in 4 cases each. However, further prospective studies are required to evaluate their potential role as so-called "triggering" factors for thrombosis in childhood.

With respect to the parameters investigated, the prevalence of combined thrombotic risk factors reported here was clearly higher compared with results recently reported in adult populations [18, 30, 44]. The high frequency of more than one established or potential cause of thrombophilia found in the paediatric population studied (19.6%) supports the hypothesis

that the presence of combined haemostatic risk factors might contribute to a high incidence of thromboembolic manifestations observed during childhood and adolescence [25, 28, 36, 39, 42, 43, 50]. Although more combined defects occurred in the older age group, a higher rate of spontaneous thrombosis was observed in younger children and infants. This observation might be due to age-dependent physiological differences in haemostasis [1–3] and the prevalence of relatively strong inherited thrombotic risk factors, like deficiencies of protein C, protein S or antithrombin. Both circumstances might lead to an early clinical onset of thrombosis within the first year of life with a lower rate of additional exogenous risk factors required for manifestation. In contrast, the impact of two or more weaker clotting abnormalities is stronger with the presence of additional exogenous risk factors. The latter are increasingly prevalent with increasing age and during puberty, leading to a later onset of thrombosis and a higher prevalence of thromboembolism after triggering events, thus decreasing spontaneous thrombosis.

Although symptomatic patients included in our retrospective study had been admitted unpreferentially from different geographic areas of Germany, the general validity of the results presented might be a point of discussion. We could not exclude the possibility that physicians who admitted symptomatic patients for thrombophilia screening ''unpreferentially'', were more likely to investigate patients with spontaneous and unusual or familial thrombosis at a young age. This fact could bias the results presented here towards a higher prevalence of single or combined clotting abnormalities found in children with a first thrombotic onset.

In conclusion, paediatric thrombosis should be viewed as a multifactorial disorder. Whether combinations of two or more thrombophilic risk factors along with early thromboembolism during infancy and childhood are associated with a higher frequency of recurrent thrombotic events, and whether a consequent primary prophylactic anticoagulation in well-known risk situations such as immobilisation, cancer treatment or severe infection might reduce the early thrombotic onset in this high-risk population are issues being dealt with in an ongoing prospective multicentre study.

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