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# Thrombosis in Children

**Olof Rask, MD**

**Doctoral thesis**



**LUND UNIVERSITY**

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Sweden, 2008

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# Thrombosis in Children

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Abstract <p>Aims: The general objective was to further elucidate thromboembolic disease in children and thereby help improve the care of these patients. More specific aims were as follows: to determine what children are affected by thrombosis; to discern any gender or age differences related to thrombosis; to identify prothrombotic risk factors; to ascertain whether autoantibodies against coagulation proteins constitute a risk factor for childhood thrombosis; to study girls receiving estrogen treatment with regard to induced hemostatic changes and efficacy of the therapy; to investigate long-term effects on the hemostatic system in children subjected to heart surgery.</p> <p>Material and methods: Children with thrombosis (Papers I and II) and children at risk of thrombosis (Papers III and IV) were investigated. In the initial study, 128 children referred for a first thrombotic event were retrospectively evaluated. In a subsequent endeavor, 57 children with thrombosis were prospectively included and evaluated for thrombotic risk factors, and patients and controls were investigated for autoantibodies. Considering children at risk, 63 girls treated with high doses of ethinyl estradiol were studied. Furthermore, 28 children with congenital heart defects were evaluated before and after Fontan surgery, and the results of follow-up global coagulation tests in patients and controls were analyzed.</p> <p>Results and conclusions: The present studies showed a bimodal age distribution in pediatric thrombosis patients in Sweden, with peaks in frequency rates during the neonatal period and in adolescence. The girls:boys ratio was 2:1. Of the children with thrombosis, 84% had acquired risk factors and they showed a significantly increased prevalence of inherited thrombophilia. Also, autoantibodies against prothrombin were significantly more often detected than in controls, suggesting immunological pathogenesis. Pediatric thrombosis often seem to be elicited by a combination of risk factors. Girls receiving high-dose estrogen treatment exhibited both pro- and anticoagulation abnormalities; the estrogen therapy was most effective when started at a younger bone age, and the risk of thrombosis was &lt;5% and considered a safe treatment in our cohort. Post-Fontan patients had a lower incidence of procoagulant abnormalities at long-term follow-up compared to before surgery, although a subset of the subjects showed evidence of elevated thrombin generation, identified by increases in APC-PCI, as compared to controls. The results indicate that prophylaxis to prevent thrombosis in these children should be individualized.</p>		
Key words: Adolescent; Autoantibodies; Child; Estradiol; Heart Defects, Congenital; Hemostasis; Protein C Inhibitor; Risk factors; Thromboembolism; Thrombophilia		
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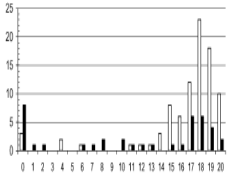
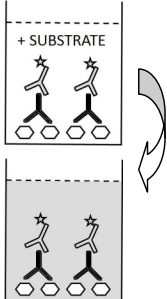
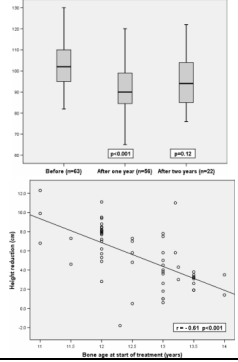
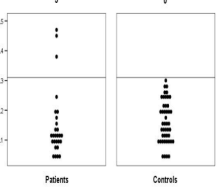
*"The clinical investigator is bathed daily in the salt water of clinical medicine and the fresh water of biology and other sciences. Relatively few choose to tolerate such daily changes in medium. /.../Accordingly, the medium for clinical investigation may well be defined as dynamic and brackish. If new discoveries submerge or elevate continental shelves of existing knowledge, there always remains an estuary where clinical medicine and biology meet, but in a new location."*

Excerpt from "The Man and Quality in Clinical Investigation" by Thomas Hale Ham (Annual Meeting of the American Society for Clinical Investigation, 1950).

*Olof Rask*



# THESIS AT A GLANCE

	QUESTION	METHOD	RESULT		CONCLUSION
I	What prothrombotic risk factors can be identified in pediatric thrombosis patients in Sweden?	128 consecutive thrombosis patients were retrospectively evaluated. Extended laboratory investigations were performed at follow-up.	A bimodal age distribution. Girls:boys ratio 2:1. Acquired risk factors in 81% (104/128). A combination of acquired and genetic risk factors occurred in 54% at follow-up and at least one risk factor in 93%.		Several risk factors are often involved in pediatric thrombosis, and specific categories of children are at greater risk.
II	Are auto-antibodies against protein S or prothrombin common in pediatric thrombosis?	57 thrombosis patients were prospectively investigated. Autoantibodies against PS and PT were examined by ELISA techniques in patients and in controls.	Anti-prothrombin antibodies were detected in 21% of the patients and 2.1% of the controls (OR 12.0, $p=0.005$ ). Multiple prothrombotic factors were found in 40% of the patients, normal screening results in 14%, and acquired risk factors in 91%.		Autoantibodies against prothrombin may be common in pediatric thrombosis. The multifactorial nature of pediatric thrombosis described in Paper I was prospectively confirmed.
III	Do coagulation disturbances occur in girls treated with high-dose estrogen?	Coagulation variables were monitored and bone age determined in 63 patients before and during treatment. Evaluation for genetic risk factors was done at follow-up, and clinical data were collected.	We found that concentrations of antithrombin and VWF concomitantly declined compared to pretreatment levels. The mean reduction in final height was 5.5 cm. No events of VTE were detected, corresponding to a risk of < 5%.		The treatment appears to have contradictory effects on coagulation, but it is effective in terms of limiting final height, more so if started at a younger bone age.
IV	Can long-term changes in hemostasis be detected after Fontan surgery for congenital heart defects?	28 children admitted for modified Fontan surgery were studied before surgery and after a mean follow-up period of 9.6 years. Results of global coagulation tests at follow-up were compared with findings for controls.	The incidence of pro-coagulant abnormalities was lower at follow-up compared to before surgery. Three of 27 patients (11%) but none of the 45 controls had APC-PCI levels above the adult reference range. There were no significant differences in thrombin generation kinetics between patients and controls.		Hemostasis appeared to be less prothrombotic in the majority of patients after surgery. New global coagulation tests may be helpful in improving identification of individuals at increased risk of thrombosis.



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## LIST OF PAPERS

- I        Rask O, Berntorp E, Ljung R.  
Risk factors for venous thrombosis in Swedish children and adolescents  
*Acta Pædiatrica* 2005; 94: 717–722
  
- II       Rask O, Hillarp A, Berntorp E, Ljung R.  
Anti-prothrombin antibodies frequently associated with thrombosis in  
Swedish children  
*Submitted for publication*
  
- III      Rask O, Nilsson KO, Berntorp E.  
Oestrogen treatment of constitutional tall stature in girls: is there a risk of  
thrombosis or bleeding?  
*Acta Pædiatrica* 2008; 97: 342–347
  
- IV      Rask O, Hanséus K, Ljung R, Strandberg K, Berntorp E  
Lower incidence of procoagulant abnormalities during follow up after  
Fontan surgery in children  
*Cardiology in the Young*, accepted for publication

# LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
Ag	antigen
APC	activated protein C
APC-PCI	activated protein C-protein C inhibitor complex
aPL	anti-phospholipid
APTT	activated partial thromboplastin time
AT	antithrombin
CI	confidence interval
CTLA	cytotoxic T-lymphocyte-associated protein
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
F	factor
HK	high-molecular-weight kininogen
Ig	immunoglobulin
IL	interleukin
IU	international unit
L	liter
LA	lupus anticoagulant
µg	microgram
mL	milliliter
MTHFR	methylene tetrahydrofolate reductase
OD	optical density
OR	odds ratio
PAI	plasminogen activator inhibitor
PK	prekallikrein
PS	protein S
SD	standard deviation
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
SPSS	statistical package for the social sciences
TAFI	thrombin activatable fibrinolysis inhibitor
TCPC	total cavopulmonary connection
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
TNF	tumor necrosis factor
t-PA	tissue plasminogen activator
VK	vitamin K
VTE	venous thromboembolism
VWF	von Willebrand factor

# INTRODUCTION

## **CURRENT CONCEPTS OF HEMOSTASIS**

Hemostasis is one of the most complex physiological self-defense systems and is still only partially understood [2]. This process controls the fluidity of blood, and, upon vascular injury, it rapidly induces the formation of a hemostatic plug to stop or limit bleeding. Inasmuch as hemostasis involves a delicate balance between procoagulant and anticoagulant forces, an imbalance in that context may result in a hypercoagulable state and thrombosis, the interruption of blood flow due to redundant intravascular coagulation [3, 4]. Hemostasis and thrombosis share cellular and biochemical mechanisms, although thrombosis is considered to be rare in children [5, 6].

Hemostasis is divided into the three stages of primary hemostasis, coagulation, and fibrinolysis, which are closely interlinked and, under normal circumstances, are also precisely regulated, as discussed in the overview of current concepts presented below. During primary hemostasis, intricate interactions between the vascular wall, platelets, and adhesive proteins lead to formation of a platelet plug. [7]. In a blood vessel, the luminal surface of normal endothelial cells exhibits antithrombotic properties, whereas the subendothelial layer contains highly thrombogenic components. If an injury occurs, local vasoconstriction slows the flow of blood, which allows the platelets to adhere to the vascular wall. This anchoring occurs through binding of specific platelet plasma membrane receptors (GPIb-IX-V and GPVI) to the collagen of the exposed vessel wall and to collagen-bound von Willebrand factor (VWF). The discoid platelets then rapidly undergo morphological modifications and become spherical, and their granules become centralized and come into contact with the membrane invaginations, leading to the secretion of active substances. Neighboring platelets are activated by secreted ADP and thromboxane A<sub>2</sub>, and receptors for fibrinogen (GPIIb/IIIa) are expressed, which subsequently cross-links the platelets. Vessel injury leads not only to the rapid binding of platelets to the subendothelium but also to activation of the coagulation cascade. These events occur concomitantly [4].

Traditionally, the coagulation process has been described as a series of proteolytic reactions that start in two alternative pathways and converge in a common route. This is called the “waterfall” model [8, 9], and it was first proposed in 1964 and has subsequently been modified [10]. The central purpose of the coagulation process is to generate thrombin from prothrombin. Eventually adequate concentrations of thrombin are produced to cleave fibrinogen, which then polymerizes into insoluble fibrin that stabilizes the emerging clot. Thrombin also activates factor (F) V and FVIII to enhance its own production.

Coagulation is initiated when the integral membrane protein designated tissue factor (TF) comes in contact with blood in what is known as the TF pathway (also called the extrinsic pathway; Fig. 1A). TF and the plasma protease FVIIa combine to form the *extrinsic* FX-activating complex (tenase), and activated FV (FVa) assembles with activated FX (FXa) on a membrane surface to form prothrombinase, which is the prothrombin-activating complex.

The contact pathway of blood coagulation (also called the intrinsic pathway; Fig. 2A) is initiated by the contact factors FXII, high-molecular-weight kininogen, and prekallikrein, which activate FXI. The activated FXI can then activate FIX, which in turn acts with its cofactor, FVIII, on a phospholipid surface (the *intrinsic* tenase complex) to activate FX. Activated FX then combines with its cofactor FVa, as previously described, to form the prothrombinase complex on a phospholipid surface that converts prothrombin to thrombin. The contact pathway represents a powerful tool for *in vitro* studies of the coagulation cascade, but it is currently not considered to be required for initiation of thrombin generation and fibrin formation *in vivo*. This is demonstrated by the fact that congenital FXII deficiency does not result in abnormal bleeding. However, this current view may be modified again in the near future, since recent experiments using mice lacking FXII or FXI have suggested that these proteases are important for formation of intravascular thrombi [11].

Since the waterfall model fails to provide insight into why patients with certain coagulation abnormalities bleed, Hoffman and Monroe [12] more recently proposed

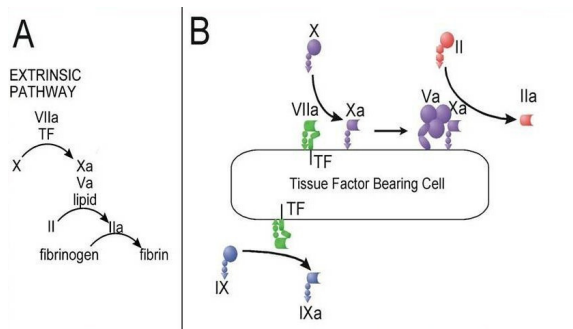
that the coagulation reactions occur as overlapping steps on *different* cell surfaces, thus introducing a “Cell-Based Model of Hemostasis” in three stages:

1. *Initiation* of coagulation takes place on TF-bearing cells, and enough FXa, FIXa, and thrombin are formed to initiate the coagulation process (Fig. 1B).
2. *Amplification* is achieved as the “action” moves from the TF-bearing cell to the platelet surface; the procoagulant stimulus is amplified as platelets adhere, are activated, and accumulate thrombin-activated cofactors (FVIIIa and FVa) on their surfaces.
3. *Propagation* is the phase during which the active proteases combine with their cofactors on the platelet surface to generate a burst of thrombin (Fig. 2B).

**Figure 1. The initiation phase of coagulation.**

(A) The classical extrinsic pathway. Deficiency of these proteins prolongs a prothrombin time assay in vitro.

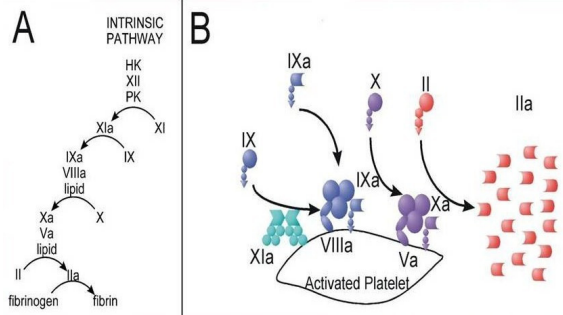
(B) In the cell-based model, FVIIa bound to TF activates both FX and FIX. FXa formed by FVIIa/TF binds to FVa on that cell and converts a small



amount of prothrombin to thrombin. (Modified from [13] with permission.)

**Figure 2. The burst of thrombin generation.**

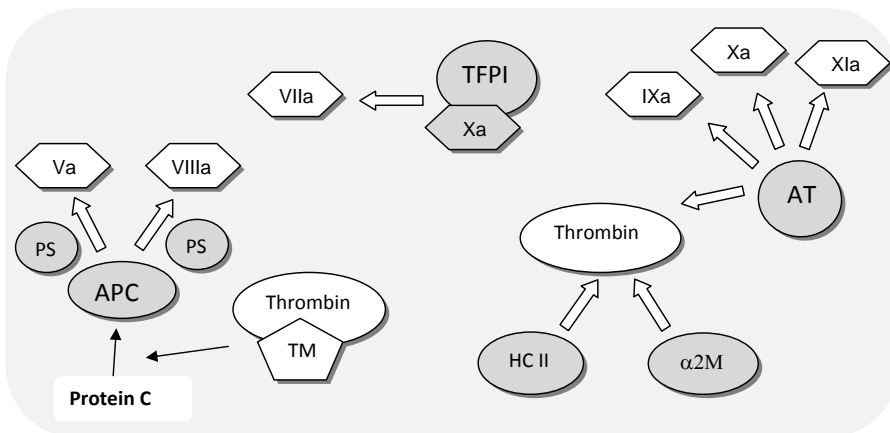
(A) The classical intrinsic pathway, with the sequence of activation proceeding from high-molecular-weight kininogen (HK) and prekallekrein (PK). Deficiency of any of these factors prolongs an activated partial thromboplastin time (APTT) assay in vitro.





(B) In the cell-based model, FIXa formed on the TF-bearing cell can incorporate into a tenase complex on the surface of an activated platelet. Additional FIXa is formed by platelet-bound FXIa. FXa formed on the platelet surface is channeled into prothrombinase complexes, which leads to a burst of thrombin generation. The “back-activation” of FXI on the platelet surface by thrombin further increases activation of FIX. Thus, the contact factors are not required for thrombin generation *in vivo*. (Modified from [13], with permission.)

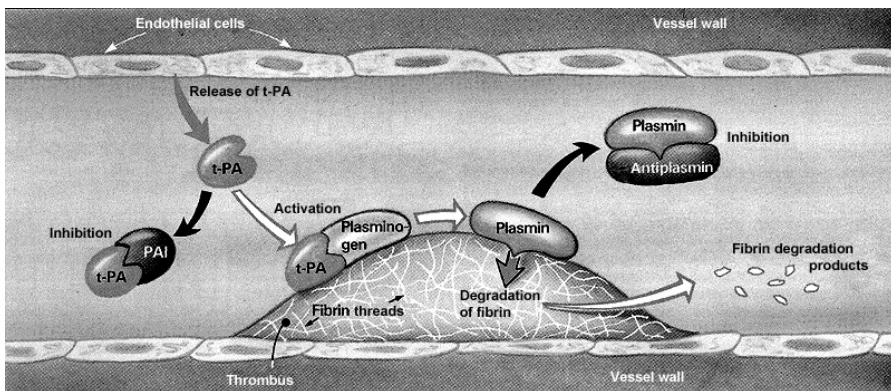
The whole system is designed to provide massive amplification of an initiating stimulus, and, if not appropriately controlled, it would rapidly convert circulating blood into a clot. This is avoided because the coagulation reactions, which take place on phospholipid surfaces, are limited in time and space by three major inhibitors [14]: tissue factor pathway inhibitor (TFPI), which shuts down TF-FVIIa once coagulation has been initiated; antithrombin, which inhibits thrombin, FIXa, FXa and FXIa; activated protein C (APC), which in the presence of protein S (PS) as a cofactor, inactivates FVa and FVIIIa. Protein C is activated on endothelium by the thrombin-thrombomodulin-EPCR (endothelial protein C receptor) complex [15]. A direct inhibition of thrombin is also mediated by circulating  $\alpha$ 2-macroglobulin and heparin cofactor II. A new vitamin-K-dependent factor Xa inhibitor, protein Z, has also been described, but its physiological role remains to be determined [16].



**Figure 3.** Major coagulation inhibitor pathways. White arrows indicate inhibition, black arrows indicate promotion of the action.

Alternative modes of initiation of coagulation have also been proposed. TF is recognized to be the sole initiator of thrombin generation and fibrin formation. However, TF is also present in circulating blood, associated with microparticles (membrane fragments released from activated or apoptotic vascular cells), and thus it has been suggested that such microparticles may induce coagulation or a hypercoagulable state. The amount of microparticles is elevated in patients with a variety of diseases [4, 17].

Once a fibrin clot has formed *in vivo*, it is modified by the fibrinolytic system [2], which constitutes the enzymatic process that leads to solubilization of such a clot by plasmin originating from fibrin-bound plasminogen. The enzyme tissue plasminogen activator (t-PA) is the principal activator of plasminogen, and t-PA-induced generation of plasmin is controlled by specific inhibitors, the most important of which is called plasminogen activator inhibitor 1 (PAI-1). Aberrant fibrinolysis is also prevented by circulating direct plasmin inhibitors such as antiplasmin and  $\alpha$ 2-macroglobulin, the latter of which can act as an inhibitor of both coagulation and fibrinolysis. Another regulator of fibrinolysis is thrombin activatable fibrinolysis inhibitor (TAFI), which is synthesized by hepatocytes, and has the capacity to decrease plasminogen binding to fibrin [18, 19].



**Figure 4.** The fibrinolytic system. In addition to the illustrated mechanisms fibrinolysis is also regulated by TAFI, a carboxypeptidase that can inhibit fibrinolysis by destroying potential binding sites for plasminogen to the surface of fibrin. (Modified from Wiman, MFR informerar 1987)

## HISTORY OF VENOUS THROMBOSIS IN PEDIATRICS -THE CHANGING PANORAMA

Thrombotic disease is one of the few diseases that is not described in the Bible, and no case compatible with the diagnosis have been found in the writings of Hippocrates,

Erasistratus, Galen,

Coelius Aurelianus,

Ibn-an-Nafiz, or

Avicenna [20]. One

could thus speculate

that the disease was

less common in ancient

days, possibly

reflecting either a

modern increase in the

population at high risk (e.g.,

an increase in the average population age) or exposure of the population to more or

new risk factors (e.g., an increase in associated medical conditions and procedures).

The first documented case of a thrombotic event found in the literature happens to be that of a young man “about the age of twenty years” who was described and illustrated in a 13th century manuscript that is now housed in the *Bibliothèque Nationale* in Paris (Fig. 5). The wording in that text is as follows: “A man named Raoul, a knight native to Normandy, who, when he was about the age of twenty years, was overtaken in the ankle of his foot on the right side by a swelling of the part which became abscessed and gave him pain, and there came there two holes, and this said illness rose from the foot up the leg...” As interpreted by Dexter and Folch-Pi in the 1970s [20], this is obviously a septic leg accompanied by characteristic venous thrombosis extending up and into the iliofemoral region.

In the mid 19th century, Virchow [21] considered factors contributing to venous thrombosis and pulmonary embolism, and discussed three broad categories: alterations in the blood flow, changes in the constitution of blood, and changes in the vessel wall. This is known as “Virchow’s triad”, and it is still useful today for illustrating the



**Figure 5.** The first documented case of a thrombotic event. (Reprinted with permission.)

pathogenesis of thrombosis. Furthermore, it is reinforced by our present understanding of the mechanisms of the disease, which include known genetic and circumstantial risk factors that have been found to affect one or more of the categories of Virchow's triad.

Although the first observation of implementing the concept of hypercoagulability in venous thrombosis was made by Virchow in 1856, the causes of the condition long remained unknown. About 50 years ago Bowie et al [22] were struck by the observation that thrombosis occurs in patients with systemic lupus erythematosus (SLE), despite the presence of a circulating "anticoagulant", a coagulation inhibitor that had been found to prolong the prothrombin time *in vitro* and subsequently was termed lupus anticoagulant (LA) [23]. In 1975, Nilsson et al. [24] discovered that this anticoagulant is also associated with recurrent intrauterine death, and LA has since become recognized as a common cause of *acquired* thrombophilia. About the same time as Bowie made his observations, Egeberg [25] reported the first cause of *inherited* thrombophilia, deficiency of antithrombin, which was found in a Norwegian family. After protein C had been isolated by Stenflo in 1976 [26] and protein S was described by DiScipio in 1977 [27], deficiencies of these proteins could be showed to be inherited risk factors for venous thrombosis in the 1980s [28, 29]. In 1993, Dahlbäck and colleagues described "familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C...", the break-through finding of APC resistance [30]. The point mutation primarily responsible for APC resistance was subsequently identified by Bertina et al. [31] and named FV Leiden, and in 1996 the same group of scientists reported a common genetic variation of the prothrombin gene, factor II G20210A, another frequent mutation associated with hypercoagulability [32].

Pediatric thrombosis as a field of clinical research is little more than a decade old. There was previously a paucity of information in that area, and most of the few studies that were published were case reports or included only a few individuals. In 1994, the pioneer investigator Maureen Andrew and her coworkers published the first data from the Canadian registry of venous thromboembolism (VTE) in children [33]. In their study of patients at tertiary care centers they could conclude that VTEs occur in a

significant number of hospitalized children. Since advances in tertiary care pediatrics have resulted in increasing numbers of children requiring antithrombotic therapy, thrombosis in this age group has been called "a new tertiary disease". Similar to the historical prevalence in adults that was previously mentioned, changes in demographics (e.g., increased neonatal survival) and risk factors (e.g., use of oral contraceptives) are now also highly relevant for children. Still, thrombosis in childhood and adolescence must be considered to be rare events, which makes studies more difficult to perform. Pediatric registry data have indicated an annual incidence of 0.7 [33]), 1.4 [34]), and 4.9 [5]) cases per 100 000 children. Since evidence-based medicine is in its infancy in this field, recommendations for antithrombotic therapy in pediatrics are largely extrapolated from suggested treatments for adults [35]. Experience from Sweden of thrombosis in pediatric cohorts has not previously been described.

## DEVELOPMENTAL ASPECTS OF HEMOSTASIS

Hemostasis is a dynamic, evolving process that begins *in utero*; indeed, the hemostatic system in infants and children is physiologically much different from adults, as summarized in Table 1. [36-44]. Clinical consequences of these circumstances are for example that prophylactic anticoagulant treatment is seldom warranted in children, whereas vulnerability to hemorrhagic disease in newborns motivates prophylactic treatment with vitamin K (VK). Also, the results of global coagulation tests such as APTT and various thrombin generation tests must be assessed in relation to age.

Healthy newborns maintain normal circulating platelet counts and a platelet ultrastructure that does not differ from adults [40, 45]. However, assessments of intrinsic platelet function *in vitro* have demonstrated transient hyporesponsiveness that is most marked in platelets from preterm infants. In contrast, there is enhanced platelet adhesion in neonatal plasma, which is not due to altered platelet function, but rather to the presence of larger, more functionally potent von Willebrand factor multimers. In the immediate newborn period, this enhanced platelet adhesion may compensate for the decreased intrinsic platelet activation seen in healthy neonates, whereas it may leave sick neonates at increased risk of bleeding. The properties of the vessel wall are also different in children as compared to adults, as it is affected by increasing age [46]. As discussed above, the intact vascular wall provides an important antithrombotic surface under physiological conditions.

The concentrations of the majority of coagulation proteins vary significantly with age (Table 2). In newborns, plasma levels of the VK-dependent factors (FII, FVII, FIX, and FX) and the contact factors (FXI, FXII, prekallikrein, and high-molecular-weight kininogen) are only approximately 50% of the corresponding adult values, whereas FV, FVIII, and VWF are increased. The VK-dependent and contact factors gradually reach approximately 80% of adult concentrations during the first six months of life, and thereafter remain lower during childhood. FV is decreased by 25% at birth, increases during the first few days, but remains slightly suppressed during childhood. FVIII show a pattern of decrease in newborns from adult levels, reaching a nadir between one month and one year of age, and then gradually increasing towards adult

levels again during the first years of life. Levels of VWF, VWF high-molecular-weight multimers, and VWF collagen-binding activity are increased during the first two months of life and then gradually decrease.

Plasma concentrations of the major coagulation inhibitors (i.e., antithrombin, protein S, protein C, and TFPI) are also lower at birth [41]. Protein S and antithrombin reach adult levels at three and six months, respectively, whereas protein C remains decreased until adolescence. Ninety percent of the plasma TFPI-pool in adults is associated with lipoproteins, and it has been shown that free TFPI is lower compared to adult levels during much of childhood, whereas the total TFPI concentration is similar to adult values [42]. Among the important physiological characteristics in this context are that protein S circulates only in its free active form in newborns, whereas in adults approximately 60% is in complex with the C4b-binding protein, and there is a relative increase in the activated form of protein C (APC) [43]. There are also additional thrombin inhibitors that probably play a significant role in infancy (fetal dermatan sulfate proteoglycan) and childhood (alpha2-macroglobulin). The net effect of these differences on thrombin generation is a reduction to about 80% of the level seen in adults [42].

Components of the fibrinolytic system interact to generate plasmin from its zymogen form plasminogen, as described in a previous section. The degree of fibrinolytic activity depends on the amounts of plasminogen as well as the plasminogen activators and inhibitors that are present. During the postnatal period plasma concentrations of plasminogen and antiplasmin are, respectively, about 50% and 80% of adult levels [44]. The lower concentration of plasminogen in plasma limits the therapeutic effectiveness of fibrinolytic agents in newborns. Furthermore, during childhood the ratio of tPA to PAI-1 is reversed (lower t-PA, higher PAI-1). A significantly lower fibrinolytic capacity has also been demonstrated in adolescents using venous occlusion stress tests [47]. This suggests that the fibrinolytic system is suppressed as compared to adults, and hence it can be speculated that the ability to eliminate thrombi in pediatric patients is reduced.

Thus, the information discussed here shows that the hemostatic differences between children and adults affect both the pathophysiology of the thrombotic process and the response to anticoagulant therapy.

**Table 1.** Features of developmental hemostasis

Hemostatic variable	Age-specific feature
Vitamin-K-dependent coagulation factors (FII, FVII, FIX, FX)	Only 50% of adult values at birth, and concentrations remain 10–20% under adult values throughout childhood.
Contact factors (FXI, FXII, prekallikrein, high-molecular-weight kininogen) and FV	Levels lower than adult values at birth, then gradually increase during childhood.
FVIII	Levels are equal to adult values at birth, decrease the first months, and then gradually increase during childhood.
VWF, VWF high-molecular-weight kininogen multimers and VWF collagen-binding activity	Levels higher than adult values during the first two months of life and then gradually decrease during childhood.
Coagulation inhibitors	Lower at birth; protein S, antithrombin reach adult levels during the first year of life; protein C and free TFPI remain low until adolescence. High levels of $\alpha$ 2-macroglobulin at birth and throughout childhood.
Fibrinolysis	Levels of plasminogen (infants), antiplasmin (infants) and t-PA lower than adult values, levels of PAI-1 higher than adult values. Decrease in over-all fibrinolytic capacity during childhood.
Platelets	Transient hypo-responsiveness and, due to potent VWF, enhanced adhesion properties at birth.
Vessel wall	Enhanced anticoagulant properties in childhood.
Thrombin generation	Reduced capacity to generate thrombin.



## **THROMBOPHILIA**

In this thesis, thrombophilia is defined as hereditary or acquired changes in circulating blood that are associated with thromboembolic disease. Some of these thrombophilic factors are discussed here, and their prevalence in the general population and increase in relative risk are summarized in Table 2. [31, 48-56]. There are also a number of candidate risk factors for thrombophilia.

Disorders of the physiological anticoagulant systems [49, 50] entail deficiencies of antithrombin, protein C, and protein S, and they represent well-established, often hereditary, risk factors for thrombosis. Other dysfunctions in the coagulation cascade also increase the risk of developing thrombosis: FV Leiden, which is the principal cause of APC resistance, is the most common genetic risk factor for VTE [57, 58] ; and the prothrombin G20210A gene variant, which is a “gain-of-function” mutation, is associated with a rise in prothrombin concentration and also with thrombosis [31, 52]. These thrombophilic traits can involve either heterozygous or homozygous inheritance.

FV Leiden is the result of a point mutation in the FV gene that renders the protein resistant to degradation by APC [51]. In addition, the mutation results in impaired degradation also of FVIIIa. The prevalence of FV Leiden in different populations varies widely, from being absent to occurring in up to 12–15% of healthy white individuals [59]. The highest prevalences of the mutation have been found in Europe, most notably in Cyprus, southern Sweden, and Germany, and this is due to a founder effect and probably also to evolutionary selective advantage of the mutation [60].

The distribution of the prothrombin mutation in Europe differs to that reported for FV Leiden, with higher prevalences in the southern than in the northern countries [61]. The mutation is found in 2% to 4% of healthy individuals in southern Europe, which is twice as high as the rate seen in northern Europe.

Raised concentrations of FVIII, FIX, or FXI have been linked to VTE [55, 62, 63], and a study of pediatric patients has indicated that FVIII concentrations > 1.50 kIU/L at the time of diagnosis are highly predictive of poor outcome [64]. The mechanisms by which elevated amounts of clotting factors cause thrombosis are poorly understood, and it is not known whether high concentrations of these factors are related to a genetic background.

Antibodies collectively termed anti-phospholipid (aPL) antibodies, of which LA and anti-cardiolipin antibodies are main classes, are, despite their name, now considered to be directed not against phospholipids as such, but instead against proteins with affinity for phospholipids. Their main antigenic targets are  $\beta$ 2-glycoprotein-I and prothrombin bound to phospholipids. These antibodies are recognized as a common cause of acquired thrombophilia in adults [56] and children [53]. When they are associated with clinical features such as thrombosis and/or fetal loss, the condition is called the antiphospholipid syndrome (APS). The aPL antibodies are commonly found in patients with autoimmune disorders such as SLE.

More specific autoantibodies against various procoagulant and anticoagulant proteins can also alter the hemostatic equilibrium. In recent years further phenotyping has been made possible. Perhaps the most well-known such autoantibodies, or inhibitors, are those causing acquired hemophilia A [65], and are directed against FVIII. Several autoantibodies may instead induce a thrombotic phenotype. Examples of coagulation proteins that may be targeted by procoagulant autoantibodies, are prothrombin, protein C and protein S [66]. The distinction between this type of potentially thrombogenic autoantibodies and aPL antibodies, that have low affinity for their protein antigen in circulation, is at times difficult to make.

Mild hyperhomocysteinemia has also been reported to be a risk factor for thrombosis [54]. This condition can be caused by genetic defects, most often homozygosity for a thermolabile mutant of methylenetetrahydrofolate reductase, or by nutritional

deficiencies in vitamin cofactors (folic acid, vitamin B6, vitamin B12), impairment of renal function, or drugs [67].

When the work on this thesis was initiated, the frequency of thrombosis and associated thrombophilic factors in the Swedish pediatric population were not known.

**Table 2.** Features of thrombophilia

Thrombophilic factor	Prevalence in the population (%)	Prevalence in adult thrombosis patients (%)	Increase in relative risk of VTE
Antithrombin deficiency	0.04–0.02	< 1	8
Protein C deficiency	0.2–0.5	2	7
Protein S deficiency	0.03–0.13	3	8
FV Leiden, heterozygosity	0–15	12–30	3–7
FV Leiden, homozygosity	0–0.5 <sup>a</sup>	1.3	50
Prothrombin mutation, heterozygosity	0–4	6–8	2–3
Prothrombin mutation, homozygosity	0–0.04 <sup>a</sup>	?	?
FVIII	11	25	5 <sup>b</sup>
Hyperhomocysteinemia	5	25	2–3 <sup>b</sup>
Lupus anticoagulant	1–5	5–20	5–16 <sup>b</sup>
Anti-cardiolipin antibodies	1–5	5–40	2–5 <sup>b</sup>

<sup>a</sup>Estimated using the Hardy-Weinberg principle

<sup>b</sup>Odds ratios

## ASSOCIATED CLINICAL CONDITIONS

Clinical risk factors for thrombosis have been identified in large-scale studies of adult patients with thrombosis [68, 69]. Multiple clinical and environmental conditions that may result in elevated thrombin generation have also been reported to contribute to the development of pediatric thromboembolic disease [33, 34, 70]. Some of these conditions are discussed here. They vary naturally with age; for example, birth-control-related risk factors are relevant for adolescents, and the use of indwelling catheters are relatively common in neonatal intensive care settings [71, 72].

A number of iatrogenic factors are associated with increased risk of thrombosis, among them the use of central lines, certain pharmaceuticals (estrogens, steroids,

chemotherapy, transenteral nutrition), surgery, and plaster casts. Several medical disorders can also be involved in thrombosis, and these include cancer, congenital heart defects, asphyxia, fetal diabetes, systemic infections, hypovolemia, inflammatory bowel disease, nephritic syndrome, hemolytic uremic syndrome and autoimmune diseases. Furthermore, thrombosis can be associated with factors such as pregnancy/puerperium, vascular malformations, immobilization, and trauma.

Systemic infection and acute inflammation are risk factors for thrombosis, and there are several pathophysiological mechanisms involved in the induction of a hypercoagulable state: during sepsis coagulation can be activated by tissue factor expression on monocytes, fibrinolysis can be inhibited by increases in PAI-1, and the anticoagulant Protein C pathway can be downregulated by inflammatory cytokines such as tumor necrosis factor (TNF) $\alpha$  [73, 74]. Thrombosis and inflammation are in fact mutually reinforcing processes as thrombin conversely activates both endothelial cells and granulocytes [75]. Microparticles bearing TF derived from tumor cells or inflammatory cells may induce thrombotic events, and therefore the presence of high levels of such microparticles warrants consideration as the predisposing cause of thrombosis in a variety of disorders [17]. It has also been suggested that TF expression by neutrophils can be induced by aPL antibodies and thus may initiate thrombosis in patients with APS [76].

In the current research, the acquired risk of thrombosis was studied under two specific clinical circumstances: in pre-adolescent girls receiving high-dose estrogen therapy and in children undergoing surgery for congenital heart defects. The overall risk of thrombosis is about threefold higher in oral contraceptive users than in non-users, and it is highest during the first year of use [77, 78]. Oral contraceptives also synergistically increase the risk of thrombosis in women from families with a natural inhibitor deficiency, or the FV Leiden or a prothrombin-mutation [78]. High doses of estrogens have been used in pediatric medicine for more than 50 years to reduce final height in constitutionally tall girls [79], but the risk/benefit ratio remains unclear. Especially when considering the observed correlation between lowering the estrogen

dose in oral contraceptives and a reduced risk of thrombosis [80], it is plausible that genetic risk factors for thrombosis in combination with changes in coagulation parameters can jeopardize the health of girls receiving high doses of estrogen for treatment of tall stature.

Patients undergoing surgery for congenital heart defects are known to be at increased risk of thrombosis. Thromboembolic events occur more frequently after modified Fontan operations (a set of staged surgical procedures used to treat children with functionally univentricular hearts) than they do following any other form of cardiac reconstruction apart from prosthetic valve replacement [81]. These events occur both early and late after surgery, and they contribute to failure of the Fontan circulation [82]. It has proven difficult to substantiate theories regarding the etiology of thromboembolism in patients with these problems. The mechanism may be a low flow state, atrial arrhythmias, adhesion to prosthetic materials, or a hypercoagulable state presumably caused by decreased synthesis of anticoagulant factors in the liver. Assessment is challenging due to the complexity of the coagulation system and the many candidate risk factors.

## AIMS OF THE STUDY

The general objective was to conduct clinical and basic research to increase our understanding of thromboembolic disease in children and thereby improve the care of these patients. The specific aims were as follows:

- To characterize epidemiological and clinical features of pediatric thrombosis patients in Sweden and identify hereditary and acquired prothrombotic risk factors (Paper I).
- To further characterize and validate epidemiological and clinical aspects of childhood thrombosis in a prospective study, and to determine whether autoantibodies against coagulation proteins can constitute a risk factor for childhood thrombosis (Paper II).
- To study girls receiving estrogen treatment with regard to induced hemostatic changes and efficacy of the therapy (Paper III).
- To investigate children who have undergone surgery for functionally univentricular hearts with respect to long-term effects on the hemostatic system and clinical outcome (Paper IV).

# METHODS

The study populations, collection of clinical and laboratory data, laboratory methods, objectives of the data analyses, and statistical methods used in the studies are reported in detail in Papers I–IV and are summarized in Table 3. All study populations comprised consecutively included cases. Annotations about some specific methods we used are given on page 31.

Table 3

Paper	Study population	Blood sampling and data collection	Laboratory methods	Objectives of data analysis	Statistical methods
I	128 thrombosis patients retrospectively evaluated	Extended thrombophilia investigation at follow-up Clinical data from patient files and interviews	All analyses performed as routine clinical assays, except the MTHFR A1298C mutation (in-house assay)	To identify thrombotic risk profiles and observed genotype frequencies compared with expected	OR with 95% CI and p-values calculated using Fisher's exact test Software: Stat Xact-5
II	57 thrombosis patients prospectively included; reference population comprising 47 healthy children	Extended thrombophilia investigation Controls investigated for anti-protein S and anti-prothrombin antibodies Clinical data from treating physician	Quantitative ELISA techniques used to determine anti-prothrombin and anti-protein S autoantibodies (in-house assays); other analyses by routine clinical assays	To identify risk factors and calculate ORs for associated thrombotic risk	OR with 95% CI and p-values calculated using Fisher's exact test. Software: R version 2.7.0
III	63 girls treated with ethinyl estradiol for constitutional tall stature	Coagulation variables during treatment, additional thrombophilia investigation at follow-up Clinical data from patient files and by interview	All analyses performed by routine clinical assays	To estimate risk of thrombosis To compare laboratory data before and during treatment To assess effect of treatment	Hanley's rule of three Paired student's t-test Bivariate analysis, Pearson correlation coefficient and p-value Software: SPSS v 13.0
IV	28 children admitted for Fontan surgery; reference population comprising 45 healthy children	Coagulation profiles before surgery and at follow-up Clinical data from treating physician and from patient files	Thrombin generation and APC-PCI levels determined by in-house assays; other analyses by routine clinical assays	To compare laboratory data before and after treatment To assess differences in thrombin generation and APC-PCI levels To obtain pediatric reference values for thrombin generation and APC-PCI	Mann-Whitney test, student's t-test Empirical 95% confidence intervals (mean $\pm$ 2 SD) Software: SPSS v 15.0



## **COMMENTS ON SPECIFIC TESTS**

### **Detection of autoantibodies**

In the study described in Paper II, in-house quantitative enzyme-linked immunosorbent assay (ELISA) techniques were employed to determine anti-protein S and anti-prothrombin autoantibodies in patient sera. In short, protein S and prothrombin antigens were purified from human plasma and coupled to the plastic surfaces of different micro titer plates. After application of patient sera to the specific plates, enzyme-conjugated polyclonal rabbit anti-human immunoglobulin was used to detect antigen-bound human antibodies. With a subsequent chromogenic reaction the amount of antibodies could be quantified. All patient and control samples were analyzed in duplicate. For anti-prothrombin, the cut-off values used to differentiate positive from negative samples was based on the mean optical density (OD) plus three standard deviations for at least 10 samples from separate healthy controls analyzed concurrently with the patient sample. For anti-protein S, an OD value based on historical data was used. The analytical specificity of all positive samples was subsequently evaluated by inhibition experiments.

### **Von Willebrand Factor**

In our investigation of girls receiving estrogen treatment (Paper III), levels of VWF antigen (VWF:Ag) were measured by applying the STA Liatest (Diagnostica Stago, Asnières, France). This is a latex enhanced immunoassay in which specific antibodies against VWF have been coupled to latex beads. In the presence of VWF, the antibodies agglutinate the beads and there is a change in turbidity.

### **Thrombin generation**

As mentioned, coagulation is not a single event, but rather involves a series of stages (initiation, amplification, and propagation), and the level of thrombin present during each stage varies. Because thrombin plays a critical role in coagulation, analytical methods have been developed to quantify the production of that key protein throughout the different stages of the clot-forming process. Such tests can also be used

as indicators of congenital or acquired hemostatic disorders. Nevertheless, caution must be observed when extrapolating data on thrombin generation *in vitro* to production *in vivo* [83]. In the study reported in Paper IV, the pharmacokinetics of the thrombin-generating capacity was determined using the method described by Varadi et al. [84], which is a fluorometric approach that allows thrombin activity in clotting plasma to be monitored continuously.

### **APC-PCI**

Due to the activation of protein C by thrombin bound to thrombomodulin, and the subsequent inactivation of APC by protein C inhibitor [85], the concentration of the APC-PCI complex is a sensitive indicator of thrombin generation and thus also the degree of activation of blood coagulation [86]. In addition, it has been suggested that the concentration of APC-PCI can be used as a marker of an increased risk of future VTE [87]. In our investigation of post-Fontan surgery children (Paper IV), we used a new and sensitive immunofluorometric assay devised by Strandberg et al. [88] to measure the APC-PCI complex.

# RESULTS AND DISCUSSIONS

## **A. STUDIES OF CHILDREN WITH THROMBOSIS**

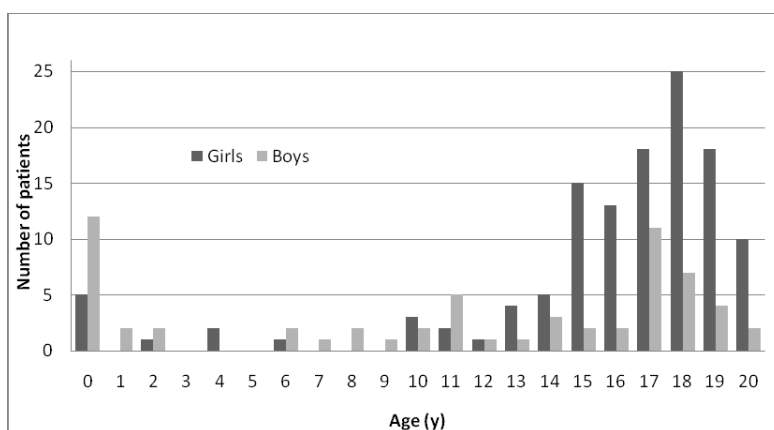
It is essential to improve our understanding of the pathophysiology of VTE in children, if the goal is to optimize prevention, diagnosis, and treatment. Experiences of thromboembolism in children in Sweden have not previously been described, and thus it has not been known whether published results concerning studies of tertiary pediatric care settings in other countries also apply to patients in Sweden. The first part of this chapter discusses the results of our first two studies, which were conducted to identify thrombotic risk profiles in children and adolescents in Sweden diagnosed with thrombosis. A prerequisite for that work was close collaboration with both the Department of Clinical Chemistry in Malmö and referring physicians.

### **Demographics**

Our results suggest that children are at greater risk of venous thrombosis before the age of one year and during adolescence, and that there is a female predominance (66% of the investigated children, in Papers I + II). Figure 6. shows demographic data on all subjects (ages 0–20 years in Paper I, ages 0–18 years in Paper II) from the first two studies (n = 185).

A similar bimodal age distribution has been seen in registry studies performed in other countries [33, 34], and it has been proposed that this can be explained by additional risk factors in the more affected age groups [5]. Furthermore, such risk factors probably vary between populations. For example, we did not find that teenage pregnancies represent a relevant factor in the adolescent group, as was suggested in an American registry study [5], whereas oral contraceptives did have an impact in our investigations. Our finding may also reflect less vulnerability to acquired risk factors between the neonatal and adolescent ages due to features of developmental hemostasis, and that assumption agrees with the fact that we did not observe any thromboembolic events in our pre-adolescent group treated with high-dose estrogen (Paper III).

We found a 2:1 sex ratio. That finding differs from the equal gender frequencies seen in the Canadian and Dutch registries, although both those registries had low frequencies of teenagers due to suspected referral bias. Another plausible explanation for the discrepancy is that combinations of risk factors vary between countries: in Sweden the prevalence of FV Leiden is high [59], and the combination of use of oral contraceptives and that particular genetic risk factor greatly increases the risk of thrombosis [89].



**Figure 6.** Age and gender of the 185 children with thrombosis described in Paper I (0–20 years) and Paper II (0–18 years). Two peaks in age-related frequency are seen.

Our first study involved a retrospective cohort of 128 children with thrombosis who were included over a period of 5.5 years. This corresponds to an annual referral rate of 5.2/100,000 children in the catchment population, which is somewhat higher than rates in previous reports from other countries. The patient cohort in our second study represented consecutive cases from an ongoing prospective investigation of VTE in Swedish children, which will provide updated information on incidence. For the purpose of the present research, patient data from the Hospital Discharge Register of the National Board of Health and Welfare, as well as population data from Statistics Sweden (*Statistiska centralbyrån*), were also extracted to calculate the reported

incidence of VTE in infants and children. From 1998 through 2007, the rate of diagnosis of pulmonary embolism or venous thrombosis in children/adolescents under the age of 20 years in Sweden was 4.8/100,000/year, a figure that agrees well with our findings. This may be an underestimation of the true incidence of VTE, since we found that thrombosis frequently occurred in sick patients with other diagnoses at the time of hospitalization, and thus an additional VTE diagnosis might not have been registered in all patients. Also, the rate we arrived at does not take into account that some of the children may have been diagnosed with both deep vein thrombosis and pulmonary embolism (as applied to 10% of the children described in Paper II).

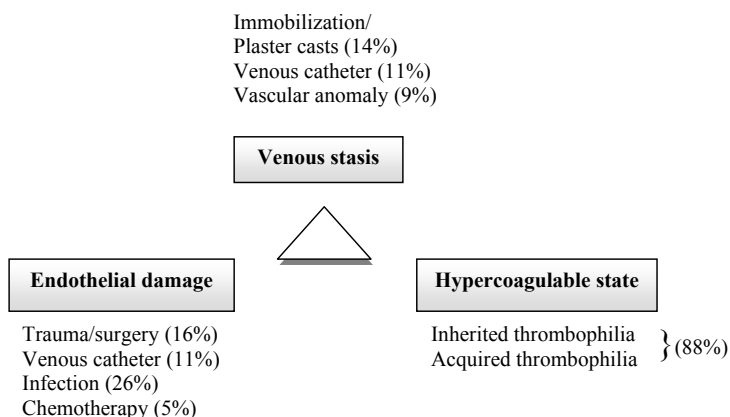
### **Clinical findings**

*Location of thrombi.* Many of the pediatric VTE patients we studied (51% Paper I, 47% Paper II) had clots in locations other than the lower venous system, and thus they differed from adults, for whom two-thirds of the thrombi occur in a leg [90]. This observation might be partly explained by the importance of central venous lines as a risk factor in children, and it also calls for increased clinical awareness on the part of the pediatrician. We found that indwelling catheters in some cases also gave rise to acute arterial impingement or/and thrombosis, a rare acute condition that was not given further consideration in our work.

*Imaging techniques.* We also found that multiple diagnostic investigations were often performed, and that ultrasound was usually chosen over venography, even though the latter method is still the “gold standard” for detecting thrombi. This finding may reflect the reluctance of physicians to perform invasive imaging in children, and it may also indicate that doctors are unaware of the low sensitivity of ultrasound for thrombi in the thorax [91].

*Associated clinical conditions.* The great majority of the patients we studied (81% Paper I, 91% Paper II) had one or more clinical prothrombotic risk factors, and many of them also had multiple co-existing risk factors. This is in contrast to thrombosis in adults, in which it seems that a significant number of first-time VTEs (approximately

25% to 50%) are idiopathic [6]. A wide spectrum of associated conditions and circumstances were identified in the patients, in our second prospective study (Paper II) most commonly systemic illnesses (31%), infections (26%), and oral contraceptive use (25%).



**Figure 7.** Findings of prothrombotic risk factors in children (Paper II) categorized according to Virchow's triad.

### Thrombophilic conditions

*Inherited thrombophilia.* The most common genetic risk factor for thrombosis, FV G1691A, was found in 30% (25/83) of the patients at follow-up in the first study (Paper I) and in 25% (14/57) of the patients included in the second investigation (Paper II). These rates are significantly higher ( $p < 0.001$  and  $p = 0.01$ , respectively) than a proportion of 11% observed in the population of southern Sweden [58], but they are similar to a prevalence of 28% found in a study of adult thrombosis patients conducted at our center [58]. These findings underline that this thrombotic risk factor is important at all ages, but in children it is apparently often revealed by the existence of additional acquired risk factors, since combinations of genetic and acquired factors were common in the patients in our two investigations. In contrast, we did not detect F II G20210A as often as was expected based on results concerning adults [52], which

suggests that this mutation is a stronger risk factor later in life. Deficiencies in the natural anticoagulants protein C, protein S, and antithrombin were found in both of our studies of children with thrombosis. Furthermore, prospective analysis indicated that the prevalence of deficiencies considered to be hereditary agreed with results obtained in studies of adults, and it seems that acquired deficiencies are also common in children.

*Acquired thrombophilia.* LA and anticardiolipin antibodies, which represent the main classes of aPL antibodies, are associated with an increased risk of thrombosis in both adults and children [53, 92], but the magnitude of the risk in children has not been established. The same is true for mild hyperhomocysteinemia, for which Kosch et al. [93] recently suggested that homocysteine levels above 8.4  $\mu\text{mol/L}$  (> 90th percentile of healthy controls) constitute a risk factor for thromboembolism in children. As described in Paper II, we found that 5% of our subjects were positive for LA, 9% were positive for IgG anti-cardiolipin antibodies, and 23% had mild hyperhomocysteinemia ( $\geq 10 \mu\text{mol/L}$ ).

*Possible thrombophilic factors.* In both investigations of pediatric thrombosis patients, we evaluated some risk factors that are less established in this age group. Most notably, we did not find significant odds ratios (ORs) for the polymorphisms in the MTHFR genotype alone (Papers I and II) or in combination with other genetic thrombophilic traits (Paper I), as compared with known prevalences in the population. Some studies have suggested that polymorphisms in the MTHFR gene represent an independent risk factor for thrombosis in children [94] or an additive risk factor when combined with other factors [95], whereas other investigations have not been able to confirm that conclusion [93, 96]. Moreover, a significant proportion of our prospectively included patients had high levels of FVIII at the time of thrombosis (Paper II), which may be an indicator of poorer outcome [64].

## Autoantibodies

At the time the present research was begun, two boys were treated at our hospital for post-varicella purpura fulminans. Both these patients developed transient deficiency of free protein S, and one of them was also homozygous for the FV G1691A mutation, and the other also had a subnormal level of protein C at the time of thrombosis. Furthermore, they both tested positive for anti-protein S antibodies, and thus it became clear that autoantibodies against coagulation proteins may have an important prothrombotic effect, especially in the presence of multiple risk factors. These cases inspired us to further study autoantibodies in children with thrombosis. Other researchers have observed that antibodies against protein S often appear in children after specific infections such as varicella [97-103], and antibodies against protein S or prothrombin have been described as part of the anti-phospholipid syndrome [66, 104] in children with autoimmune diseases such as systemic lupus erythematosus.



**Figure 8.** Post-varicella purpura fulminans in a 2.5-year-old boy positive for anti-protein S antibodies. Successful treatment included plastic surgery (courtesy of Dr. U. Tedgård, Malmö).

To address this issue, we analyzed anti-protein S and anti-prothrombin antibodies by applying methodology developed in our laboratory (Paper II). We found that qualitative immunoblotting procedures were useful for screening purposes, whereas quantitative enzyme-linked immunosorbent assay (ELISA) techniques were more reliable and specific.

We observed that 7% (4/57) of the patients and 2.1% (1/47) of the controls were positive for anti-protein S antibodies, which corresponds to a statistically non-significant OR. Precipitating varicella infections were found in 2/57 patients; both those cases were associated with protein S deficiency, and one of those children was



positive for anti-protein S antibodies. Thus it seems that the combination of anti-protein S antibodies and a low protein S concentration represents a pathogenetic risk factor in selected cases.

We found anti-prothrombin antibodies in 21% (12/57) of the patients but only 2.1% (1/47) of the controls, and the associated thrombotic risk (OR) was 12.0 (95% CI 1.7–534,  $p = 0.005$ ), which implies that anti-prothrombin antibodies represent an important risk factor for thrombosis in children. Findings of special clinical interest were as follows:

- Ten of the 12 patients positive for anti-prothrombin antibodies did not test positive for LA or anti-cardiolipin antibodies and would therefore have been classified as aPL antibody negative in a more limited thrombophilia investigation.
- Six of the nine (67%) patients with pulmonary embolism were positive for anti-prothrombin antibodies, and, conversely, pulmonary embolism was diagnosed in six of the 12 (50%) children positive for those antibodies. This suggests a more severe clinical course in the presence of such antibodies.

The pathogenic mechanism of anti-prothrombin antibodies has not been established, although it has been suggested that these proteins can enhance the binding of prothrombin to damaged endothelial cells, which in turn leads to increased formation of thrombin [105, 106]. Experiments *in vitro* have also shown that anti-prothrombin IgG can interfere with the inactivation of activated FV by activated protein C, which might result in a hypercoagulable state [107]. It is also possible that anti-prothrombin antibodies exert a prothrombotic effect due to their capacity to bind to thrombin and thereby protect it from down-regulation by its natural regulator antithrombin [108]. Data given in Paper II suggest that children who are positive for anti-prothrombin are at increased risk of thrombosis. A causal relationship can however not be assumed, it is plausible that the development of antibodies against prothrombin or protein S in genetically susceptible individuals gives rise to the thrombotic event, but also that

immune stimulatory signals evoked by the thrombosis induce antibody development. Infection does not appear to be a major cause of the anti-prothrombin antibodies, since infections were observed preceding thrombotic events in 17% (2/12) of the anti-prothrombin-positive children.

**Table 3.** Prevalence of anti-prothrombin and anti-protein S antibodies in pediatric patients with thrombosis and in healthy controls. The anti-prothrombin antibodies are significantly more prevalent in patients with thrombosis.

Autoantibody	Patients	Controls	Odds ratio (95% confidence interval, significance)
Anti-prothrombin	<b>21% (12/57)</b>	<b>2.1% (1/47)</b>	12.0 (1.7–534, $p = 0.005$ )
Anti-protein S	7% (4/57)	2.1% (1/47)	3.4 (0.3–174, $p > 0.30$ )

## **B. STUDIES OF CHILDREN AT RISK OF THROMBOSIS (PAPERS III AND IV)**

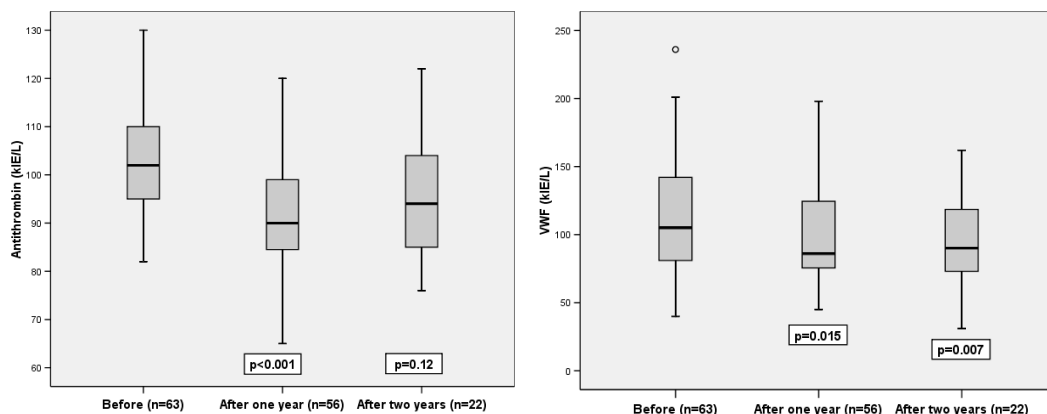
In our initial studies (Paper I), we had identified specific categories of children at increased risk of thrombosis, among them girls receiving estrogen and infants undergoing surgery for congenital heart defects. Based on those findings, we further investigated the same type of patients at a more detailed level (Papers III and IV). This work required close collaboration with the regional centers for pediatric endocrinology and pediatric cardiology, and the Department of Clinical Chemistry, Malmö.

### **Estrogen treatment during pre-adolescence**

Oral contraceptives are known to increase the risk of thromboembolism in women, whereas there are only sporadic and less comprehensive reports concerning thromboembolic complications in tall girls receiving high-dose estrogens. Women who have thrombophilic disorders and are using oral contraceptives are at even greater risk of thrombosis [77]. This risk is highest during the first year of therapy, and there is evidence of a direct relationship with the dose of estrogen given [80]. We investigated the impact of hormonal therapy for constitutionally tall stature on coagulation in 63 pre-adolescent girls (Paper III). Those girls received a median initial dose of 300 µg of ethinyl estradiol per day, which can be compared with the fact that oral contraceptives currently in use contain only 30 µg or less. To our knowledge, the present material from our single-center experience is the largest to be reported in the literature.

*Hemostatic changes.* In short, we found that levels of VWF and antithrombin were significantly decreased ( $p = 0.015$  and  $p < 0.001$ , respectively) at the end of the first year of treatment (Fig. 1 a,b). During continued therapy, there was a tendency towards restitution of antithrombin levels, whereas some patients showed a further drop in VWF. At baseline, two patients showed clearly decreased VWF levels of 0.44 and 0.40 IU/mL, and the latter concentration declined to 0.31 and 0.34 IU/mL after treatment for two and three years, respectively. There were no significant changes in the levels of PAI-1, although intraindividual variations were seen in a few patients. No correlation could be found between initial ethinyl estradiol dosage and changes in measured coagulation factors. Markers of thrombophilia were present in some of the

patients at follow-up: FV-G1691A in 12%, FII-G20210A in 0%, MTHFR-C677T (homozygosity) in 16%, and MTHFR-C677T + MTHFR-A1298C (compound heterozygosity) in 24%. Levels of protein C, protein S, and antithrombin were normal in all cases at follow-up after conclusion of treatment.

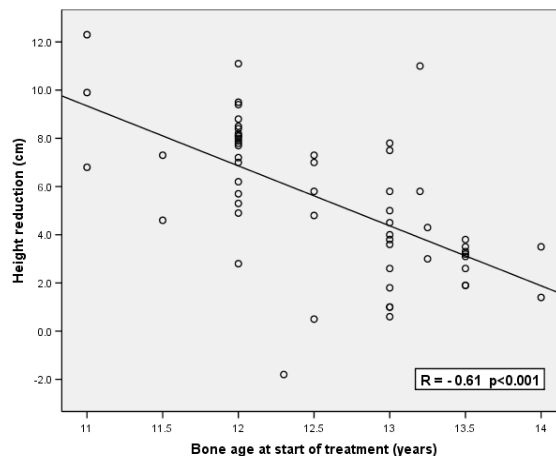


**Figure 9.** Levels of antithrombin and von Willebrand factor (VWF) before and after one and two years of treatment with high-dose ethinyl estradiol. The p-values represent comparisons of data on the same group of individuals collected at the start of treatment and at the indicated time points.

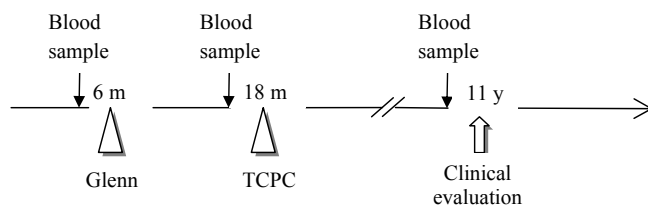
Our findings imply that a tendency towards a hypocoagulable state can be induced concomitantly to the decline in antithrombin, and hence it appears that there are contradictory influences on coagulation. These combined abnormalities may counterbalance each other to some extent and thus prevent drastic changes in the hemostatic equilibrium. Nonetheless, it is difficult to predict the overall impact on the balance in the clotting system, because we investigated only a limited number of coagulation factors due to the lengthy study period. Interestingly, two of the patients showed a marked drop in VWF that was in the range seen in von Willebrand disease [109], and in one of these cases the decrease during treatment was pronounced. To our knowledge, the possibility that such therapy can lower the level of VWF has not been

previously observed by other investigators. In a well-designed study of healthy women, van Rooijen et al. [110] found that the subjects' VWF levels declined numerically during treatment with combined oral contraceptives, although this finding was not statistically significant.

*Clinical outcome.* The patients we studied showed no thrombotic complications during the ethinyl estradiol therapy, even though they had an expected prevalence of thrombophilic traits. Consequently, according to the rule of three [111], it can be estimated that the risk of VTE during such treatment is  $< 5\%$ . We found that the treatment was effective in terms of limiting final height, with a mean reduction of 5.5 cm. As shown in Figure 10, the regimen is even more efficacious if it is started at a younger bone age.



Glenn procedure) followed by construction of a total cavopulmonary connection (TCPC), represents the definite palliative treatment for several congenital heart lesions. Thromboembolic events can occur at anytime after a Fontan procedure, but appear most often months or even years later [112-114]. The underlying mechanism may be a low flow state, atrial arrhythmias, adhesion to prosthetic materials, or a hypercoagulable state presumably due to decreased synthesis of anticoagulant factors in the liver. In the study reported in Paper IV, we evaluated long-term changes in the hemostatic system of children who had undergone surgery for functionally univentricular hearts, and we also investigated those patients compared to a healthy pediatric control population, considering several markers of activated coagulation as well as associations with adverse clinical outcome.



**Figure 11.** Timing of Fontan surgery and coagulation testing in relation to median age.

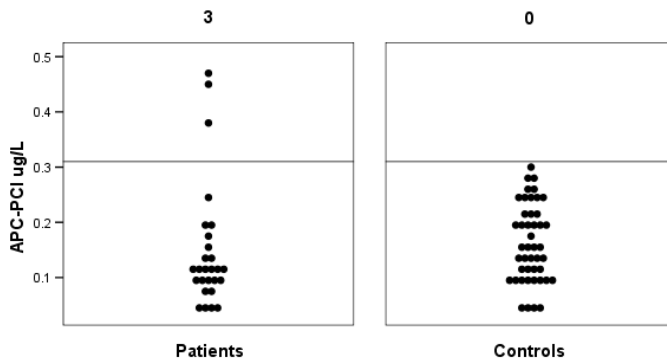
*Hemostatic changes.* The frequency of specific coagulation and fibrinolytic variables that were outside the normal range and leaning towards a prothrombotic state decreased from 72% before the Glenn procedure and 69% prior to the total cavopulmonary connection to 26% after a mean follow-up period of 9.6 years (48% when including FVIII not determined before the procedures). Detailed information concerning the different hemostatic variables is given in Appendix IV, Table 4.

Clinical implications:

- The frequency of prothrombotic changes indicates that the children we studied were less prone to thromboembolic complications at long-term follow-up after

the surgical procedures. Still, thrombophilic patterns remained in some of the patients, and thus clinical awareness of thrombosis is warranted in those children, since the risk of an event may increase with age.

Figure 12 illustrates measurements of the APC-PCI complex, which is a new and sensitive biological marker of thrombin generation [88]. Mean concentrations of APC-PCI at follow-up were 0.15  $\mu\text{g/L}$  (SD 0.11) in 27 patients and 0.16  $\mu\text{g/L}$  (SD 0.07) in 45 controls. Levels above the reference (indicated by a line in the diagram) were found in three (11%) of the patients but none of the controls, and there was a tendency towards lower values in the patient group compared to the healthy children ( $p = 0.09$ ).



**Figure 12.** Activated protein C-protein C inhibitor complex levels in patients (n = 27) and in controls (n = 45).

The kinetics of thrombin production was characterized using a thrombin generation assay [84]. Patients and controls showed no significant differences in their thrombin generation profiles (comprising lag phase, maximum rate, time to peak, peak concentration, and endogenous thrombin potential). A tendency towards an increased rate of thrombin production was noted in the patient group: time to peak 30.6 minutes versus 35.9 minutes ( $p = 0.07$ ). The highest APC-PCI concentration and the highest potential thrombin generation were observed in the same patient.

Clinical implications:

- Risk assessment by use of new global hemostatic tests appears warranted and may be helpful in identifying children who need increased anticoagulant therapy, since standard coagulation tests do not seem to be as useful for that purpose.
- Reference values obtained for the APC-PCI complex in children in this study (0.02–0.31 µg/L) correspond well with those previously found for adults (0.07–0.26 µg/L [115] ) and will be useful in future evaluations.

*Clinical outcome.* After a mean follow-up period of 9.6 years, 25 (89%) of the patients admitted for modified Fontan surgery were in the first or second class of the functional grading system of the New York Heart Association, and three (11%) were in the third class. Transthoracic echocardiography showed that ventricular function was good in 27 patients and fair in one. None of the subjects had a history of thromboembolism, and no intracardiac thrombi were detected.

Clinical implications:

- These patients were generally in good clinical condition under the currently applied therapeutic approach.



# GENERAL DISCUSSION AND FUTURE CONSIDERATIONS

Thrombosis is recognized as a major health problem for society, affecting 100 out of every 100,000 men and women each year, or 450–600 cases per 100,000 adults aged 80 [6]. It has only relatively recently been acknowledged as a clinical entity in children, and evidence-based medicine in that area is in early stages of development. Furthermore, it has been difficult to perform clinical studies in this field not only due to the lower incidence of pediatric thrombosis, but also to the diverse and complex nature of the condition and the fact that it often affects children who are seriously ill, as well as ethical considerations. Since the condition can have a great impact on the lives of affected children for many decades, it is clearly warranted to find feasible ways of providing evidence-based medicine for these patients.

The first part of this thesis presents new clinical and epidemiological data on thrombosis in children in Sweden indicating an estimated incidence of 5/100,000 children/year, which represents a higher figure than was found in the major pediatric registry studies performed in other countries [33, 34]. Several smaller studies of children with thrombosis have also been conducted, but it has been difficult to draw any conclusions about the impact of thrombophilic risk factors, mainly because of a lack of statistical power. To overcome this problem, a group of leading experts only recently performed a meta-analysis of all investigations focused on VTE in children < 20 years of age that were published from 1970 through August 2007 [116]. These researchers identified 319 potentially relevant papers in electronic databases, and 35 of those qualified for the meta-analysis in accordance with the guidelines outlined in the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement [117]. Paper I in this thesis was the only study performed in a Nordic country that was included in the meta-analysis. The results of this comprehensive assessment of the literature showed that all the traits under consideration, including antithrombin, protein C, and protein S deficiency, variants of coagulation factor V

(G1691A) and factor II (G20210A), and elevated lipoprotein (a) were significantly associated with first onset of pediatric VTE, and the highest ORs were found for combined genetic traits. For the rare event of VTE recurrence, patients derived from 6 to 13 studies showed that also recurrence was associated with inherited thrombophilia.

In the work reported in this thesis we focused on risk factors and acquired conditions that mainly influence the coagulation of blood. The role of the fibrinolytic system in the development of venous thrombosis has not yet been extensively investigated in children. In a recent population-based case-control study of adult patients with venous thrombosis, it was demonstrated that the combination of hypofibrinolysis and oral contraceptive use, immobilization, or FV Leiden results in a risk of venous thrombosis that greatly exceeds the sum of the individual risks [118]. This finding may be of particular interest for elucidation of pediatric thrombosis, because a significantly decreased fibrinolytic capacity has been demonstrated in both adolescents and neonates [44, 47]. The bimodal age distribution, with peaks of frequency in infancy and adolescence, that have been found both by us and by others [33, 34], may be explained by synergistic effects of an age-dependent intrinsic vulnerability of the hemostatic system and exposure to age-related acquired risk factors (e.g., invasive procedures in neonatal intensive care, certain childhood infections, or oral contraceptives) in genetically susceptible children. It is also plausible that hereditary variations in factors of the fibrinolytic system are of importance in this context. If such variations are discovered in the future, their clinical impact can be investigated by the use of well-defined series of children who have had thrombosis, such as our cohorts in Papers I and II.

The database of children with thrombosis generated in the current research may also have other future applications. It has been suggested that new high-throughput genome-wide DNA analysis of single-nucleotide polymorphisms (SNPs) in large well-defined patient cohorts could help identify novel common genetic risk factors for thrombosis with low individual ORs [48]. Since increasing age per se is a strong risk factor for thrombosis, children might represent an ideal group to study in that context.

Another interesting aspect is that approximately 10% of the children with thrombosis investigated in Papers I and II did not have any yet recognized genetic or acquired risk factor. These children may prove to be the ideal group for further investigations of hereditary risk factors since they lack the age- and disease related risk factors often present in studies of adults with VTE. Investigations of a large cohort of siblings may be another approach to revealing genetic and environmental risk factors for pediatric thrombosis, similar to what has been done regarding inhibitor development in hemophilia [119].

Our findings indicate that anti-prothrombin autoantibodies are associated with thrombosis in children, which implies that pediatric thrombosis to some extent displays an immunological pathogenesis mediated by antibodies directed against coagulation proteins that impact their levels and/or functions. This novel finding raises several new research questions: What are the mechanisms by which distinct autoantibodies, such as anti-prothrombin antibodies, promote a prothrombotic state? Do specific polymorphisms in immune response genes (i.e.,  $\text{TNF-}\alpha$ , IL-10, and CTLA-4) increase or decrease the risk of antibody development? Can the association that we found between this type of acquired thrombophilia and risk of pulmonary embolism be validated? Do anti-prothrombin antibodies influence other outcome variables, e.g. therapy resistance, post-thrombotic syndrome or risk of re-thrombosis? Can immune-modulating therapy be beneficial?

Notably, more than 80% of the children with thrombosis that were included in our studies had at least one clinical risk factor for VTE, and it seemed that an acquired risk factor often unmasked a congenital disorder. It is also plausible that variations in the combinations of acquired and genetic risk factors can explain the differences in clinical expression of thrombosis observed in the children. In many cases there is a lack of consensus about how to treat or prevent thrombosis in pediatric patients suffering from acquired disorders, for example during immobilization after major trauma [120], after cardiac surgery [121] or in children with APS [53]. Our results, and findings obtained in other studies, suggest that it is possible to identify high-risk

groups that have combinations of genetic and acquired risk factors and should thus be targeted for trials of primary and secondary prevention. It is plausible that the use of prophylaxis or prolonged treatment can be limited to children with such combined risk factors.

The second part of this thesis describes our studies of children at risk of thrombosis. In that work, a new global coagulation test based on measurement of the APC-PCI complex was used in pediatric patients for the first time to evaluate the risk of thrombosis. The results indicate that such assessment may aid identification of children in need of increased anticoagulant therapy in certain clinical settings, but further research is required to confirm that assumption. Our results suggest that children who underwent staged surgery for univentricular heart were in a less procoagulant state at long-term follow-up than they were before the procedure, and it seemed that estrogen treatment in girls can alter the hemostatic balance towards either bleeding or clotting. The mentioned findings have clinical implications for these groups of patients, as discussed in this thesis. Papers I and II, as well as other reports in the literature and clinical experience, have indicated several other situations in pediatric care that warrant awareness of the risk of thrombosis and also call for identification of children who are at increased risk. The ongoing research conducted by our group includes studies of thrombosis in boys with hemophilia and central venous catheters, and the preliminary results show that asymptomatic thrombosis in this group is common. In addition, work is underway to evaluate prothrombotic risk factors in prematurely born children with cerebral intraventricular hemorrhage, a condition responsible for many adverse sequelae including hydrocephalus, cerebral palsy, and death. More prospective studies are also required to define the epidemiology, pathogenesis, and management of thrombosis in children with hematologic malignancies, and in pediatric cerebral stroke. Evidence to support optimal preventative and treatment strategies is best gathered by taking part in international multicenter pediatric trials, and hence collaboration within the Nordic countries could be a feasible strategy as societies and health care in this area are similar. Moreover, our clinical findings justify refinement of local guidelines for

management of pediatric thrombosis by adding pediatric aspects. This thesis will hopefully provide the basis for future research efforts focused on venous thrombosis in children.

## CONCLUSIONS

The main conclusions that can be drawn from the present studies are as follows:

- Specific categories of children, such as neonates and adolescent girls, are at greater risk of thrombosis. It appears that pediatric thrombosis is often elicited by a combination of risk factors; more than 80% of the children with thrombosis in our studies had at least one clinical risk factor, and they showed a significantly increased prevalence of congenital prothrombotic disorders. (Papers I and II)
- Autoantibodies against prothrombin are significantly more common in children with thrombosis than in controls, and that novel finding indicates immunological pathogenesis. Anti-prothrombin antibodies seem to be clinically associated with risk of pulmonary embolism. (Paper II)
- Girls receiving high doses of estrogen are subject to changes in coagulation parameters that could favor both pro- and anticoagulation; a concomitant decrease in antithrombin and VWF levels was found. Estrogen treatment could successfully limit final height, and it was most effective when started at a younger bone age. The risk of thrombosis during such therapy was < 5%. (Paper III)
- Children with cardiac malformations who underwent Fontan surgery had a lower frequency of procoagulant abnormalities at long-term follow-up compared to before surgery. For the majority of those patients, it seems that a hemostatic equilibrium has been maintained. A subset of patients with increased thrombin generation could be identified by marked elevations in levels of the APC-PCI complex compared to controls. (Paper IV)

# SVENSK POPULÄR SAMMANFATTNING

## **ÖVERGRIPANDE SYFTE**

Det övergripande syftet med denna avhandling var att genom kliniska och grundvetenskapliga studier öka kunskapen kring trombossjukdom (blodproppsbildning) hos barn, för att därigenom skapa förutsättningar för en förbättrad vård.

## **ALLMÄN BAKGRUND**

Hemostas är ett sammanfattande medicinskt begrepp för att beskriva kroppens försvar mot blödningar. Det är ett dynamiskt system med åldersspecifika egenskaper. Uppgiften är att snabbt bilda en propp som stoppar blödning vid kärlskada, men samtidigt tillåta normalt blod att fortsätta cirkulera. För att klara detta krävs samverkan mellan kärlvägg, blodplättar och cirkulerande proteiner (koagulationsfaktorer). För att motverka överflödigt blodflöde finns ett flertal reglerande mekanismer, i form av bromsande (koagulationshämmande) och propplösande (fibrinolytiskt) system. Om dessa reglermekanismer inte räcker till eller sätts ur spel kan det leda till trombossjukdom. Trombos hos barn är en relativt sällsynt diagnosgrupp. Viktiga frågor angående dess epidemiologi och patogenes (sjukdomsorsaker) är därför ännu obesvarade. Betydelsen av nyligen upptäckta riskfaktorer är inte klarlagd för barn. Utvecklingen inom barnsjukvården, med en förbättrad överlevnad av sjuka barn och utökad behandlingsarsenal, kan ha medfört ett ökat antal komplikationer i form av trombossjukdom. Huruvida erfarenheter från de få utländska större studier som finns kan överföras till svenska förhållanden har inte varit känt.

## **SAMMANFATTNING AV AVHANDLINGENS STUDIER**

I denna avhandling har vi studerat barn med blodpropp (arbete I och II) samt barn med ökad risk för blodpropp (arbete III och IV).

De två första arbetena, den första retrospektiv och den andra prospektiv, omfattade sammanlagt 185 barn med trombosjukdom. Resultaten visade att barn hade högst risk att drabbas av trombos före ett års ålder samt i tonåren. Vi fann en övervikt för flickor i tonårsgruppen och för pojkar i spädbarnsgruppen. Insjuknandefrekvensen i upptagningsområdet, 5 barn per 100 000 barn och år, var högre än vad som rapporterats från andra länder. Blodpropparna var hos hälften av barnen lokaliserade till övre delen av kroppen. Den vanligaste diagnosmetoden som användes var ultraljudsundersökning. Mer än 80% av barnen hade någon form av förvärvad riskfaktor, vanliga sådana var infektion eller annan systemsjukdom, p-pilleranvändning, operation samt kärlkateter. Ärftliga eller förvärvade egenskaper i blodet medförande ökad trombosrisk påträffades hos 88% av barnen i den prospektiva studien, och två eller fler sådana riskfaktorer påvisades hos 44%. Den vanligaste ärftliga riskfaktorn för blodpropp, faktor V Leiden, påvisades hos 30% av barnen som följdes upp i arbete I och hos 25% av barnen i arbete II, vilket var signifikant vanligare än hos genomsnittet i befolkningen. I arbete II fann vi också att s.k. autoantikroppar riktade mot koagulationsproteinet protrombin var avsevärt vanligare hos barn med trombos (21%) jämfört med hos friska kontrollbarn (2,1%). Vi fann att dessa antikroppar var associerade med en ökad risk för proppbildning i lungorna (lungembolisering). Fortsatta studier krävs för att validera dessa fynd och vidare klarlägga betydelsen av anti-protrombinantikroppar. Vi fann också att antikroppar mot ett annat koagulationsprotein, protein S, verkade vara av betydelse för enstaka patienter. Våra resultat i dessa studier indikerar att trombosjukdom hos barn är multifaktoriellt betingad och i vissa fall kan ha bakomliggande immunologiska mekanismer.

I arbete III och IV studerade vi barn med förvärvade riskfaktorer för trombos närmare. I arbete III undersöktes hur flickor som behandlades med mycket höga östrogendoser mot långvuxenhet under sin uppväxt påverkades. Vi kunde visa att behandlingen hade den avsedda tillväxtbegänsande effekten, speciellt om den påbörjades vid en låg skelettmognad, men också att flickorna uppvisade behandlingsrelaterade förändringar i hemostasen. Nivåerna av både det koagulationshämmande proteinet antitrombin samt



av den s.k. von Willebrand faktorn minskade. Ingen av de 63 undersökta flickorna råkade ut för blodpropp under behandlingen, trots förekomst av ärftliga riskfaktorer i förväntad utsträckning, vilket innebar att risken för blodpropp vid sådan behandling var mindre än 5%. I arbete IV undersökte vi 28 barn med medfödda hjärtfel för att se hur hemostasen förändrades efter hjärtkirurgisk behandling enligt den s.k. Fontan-metoden. Vi fann att hemostasen snarast hade förbättrats vid långtidsuppföljning, dvs. barnen var som grupp betraktad snarast mindre blodproppsbenägna. Genom att använda nyutvecklade mätmetoder för att mäta graden av koagulationsaktivering kunde ett fåtal barn med tecken på en ökad koagulation vid jämförelse med friska kontrollbarn identifieras. Resultaten indikerar att förebyggande behandling mot blodpropp kan individualiseras för dessa barn, fortsatta studier krävs dock för att validera dessa fynd.

Sammantaget visar våra fynd att barn med blodproppssjukdom är en heterogen grupp där förvärvade riskfaktorer i de allra flesta fall kan identifieras och därigenom finns förutsättningar för att sjukdomen också till stor del ska kunna förebyggas och behandlingsinsatser individualiseras.

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I



## Risk factors for venous thrombosis in Swedish children and adolescents

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### Abstract

**Aim:** To identify prothrombotic risk profiles in children and adolescents referred to a regional coagulation centre in southern Sweden for a first thrombotic event. **Methods:** One hundred and twenty-eight consecutive children and adolescents (newborn to 20 y) referred for evaluations of a first episode of venous thrombosis were investigated. Clinical data were collected retrospectively, and the following variables were investigated: protein C, protein S, antithrombin; resistance to activated protein C; the genotypes FV-G1691A, F II-G20210A, MTHFR-C677T, MTHFR-A1298C; coagulation factors VIII and XI. **Results:** 104/128 subjects (81%) had identifiable acquired risk factors, most often indwelling catheters and hormone therapy. Predisposing genetic factors related to thromboembolic events were revealed in 53/83 (64%) of subjects who agreed to follow-up blood sampling, and 17/83 (20%) had two or more inherited risk factors. Combinations of genetic and acquired risk factors were identified in 45/83 (54%) of the subjects, and 77/83 (93%) had at least one such risk factor. Both sexes had one peak in frequency at less than 1 y of age and then an increase during adolescence, more in females than in males. Plasma values for coagulation factors VIII and XI were age appropriate and showed a normal Gaussian distribution.

**Conclusion:** This study identified prothrombotic risk profiles in almost all children and adolescents with venous thrombosis, which underlines the importance of careful evaluation of genetic and acquired risk factors.

**Key Words:** Paediatrics, risk factors, venous thrombosis

### Introduction

The incidence of venous thrombosis in the general adult population is approximately 10 in 10 000 [1]. The corresponding value for the paediatric population in Canada has been estimated to be 0.07 in 10 000 children, or 5.3 cases per 10 000 hospital admissions [2,3], whereas no such data are available for Swedish children. Little is still known about the different roles of genetic and acquired factors that predispose to thrombosis in children. Furthermore, there are very few population-based studies of venous thrombosis in children and adolescents  $\leq 20$  y of age, and new candidate genes remain to be recognized as risk factors in this age group.

Deficiency of the anticoagulant protein antithrombin was the first and for many years the only condition identified as a hereditary thrombophilic disorder [4]. Then, in the 1980s, deficiencies of two other anticoagulants, protein C and protein S, were also described [5,6]. Resistance to activated protein C (APC) was reported in 1993, and it was later found that

this intermediate phenotype is commonly caused by a single point mutation (G1691A) in the factor V gene (Factor V Leiden, FVL) [7,8]. In 1996, a mutation in the prothrombin gene (G20210A) was found to be a risk factor for thrombosis [9]. It has also been reported that high concentrations of clotting factors VIII and XI are associated with familial thrombophilia [10,11]. In addition, it appears that patients with hyperhomocysteinaemia (i.e., increased levels of the amino acid homocystein, which is not primarily linked to the coagulation system) are at increased risk of vascular diseases such as venous thrombosis. In that context, it has been reported that two common polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene, namely the thermolabile C677T and the more recently identified A1298C, may contribute to hyperhomocysteinaemia. Thus it is possible that these mutations predispose to thrombosis [12–14].

It is now presumed that venous thrombosis is a multicausal disorder that is not necessarily induced by single gene defects, unless they interact with or are accompanied by other genetic and/or acquired risk

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factors. This assumption may be particularly applicable to children, because individuals in that age group exhibit a dynamic development of haemostasis with respect to fibrinolytic versus procoagulant activities [15], and thrombotic events are rarer than later in life. It also seems that the type and frequency of acquired risk factors for venous thrombosis differ between adults and children [16]. Studies have shown that the use of central venous lines (CVLs), oral contraceptives and/or exposure to trauma or surgery represent acquired risk factors for venous thrombosis as do a number of other disorders (e.g., malignancy, congenital heart disease, SLE and infections).

The aim of our present study was to identify prothrombotic risk profiles for venous thrombosis in a cohort of children and adolescents (newborn to  $\leq 20$  y of age) referred to a regional coagulation centre in southern Sweden for a first thrombotic event.

### Patients and methods

The study was approved by the Research Ethics Committee of the Faculty of Medicine, Lund University (Sweden).

#### *Study group*

One hundred and twenty-eight consecutive patients (39 males, 89 females) fulfilled the inclusion criteria, that is, they were newborn to 20 y of age and had been referred to the Department of Coagulation Disorders, Malmö University Hospital (Malmö, Sweden) between February 1994 and August 1999 with a first episode of deep venous thrombosis verified by standard imaging techniques. The aforementioned department is the referral centre for coagulation disorders in southern Sweden, serving a population of approximately 1.6 million. Due to our close collaboration with the paediatric clinics in our catchment area, it can be estimated that close to 100% of all paediatric patients with venous thromboembolism were referred for laboratory investigation. All 128 patients were investigated for prothrombotic conditions according to the routine at the time of the thrombotic event. The 128 subjects and/or their parents/guardians were offered follow-up investigation with a second blood sample after a few years (i.e. 1–7 y). This was proposed because some hereditary risk factors in blood coagulation that are now known to be associated with venous thrombosis were not recognized at the time of the initial testing. Coagulation studies at primary investigation included in all cases evaluation at least for deficiency of protein C, protein S or antithrombin and for resistance to activated protein C; included at follow-up were also tests for the genotypes FV-G1691A, F II-G20210A, MTHFR-C677T, MTHFR A1298C and plasma

values for coagulation factors VIII and XI. A total of 83 patients were tested in the follow-up investigation; the other 45 of the original 128 patients were not included for the following reasons: nine could not be reached (i.e., they had moved abroad or could not be found), 26 chose not to participate or their parents would not give their consent, and 10 were deceased (main causes of death were underlying diseases, median follow-up time was 5 y).

#### *Methods*

Clinical data were collected from patient files and by using a standardized protocol to interview the patients and/or their parents at follow-up. In some cases the referring physician was contacted for additional information. Venous blood samples were collected in citrate-containing tubes, and plasma was separated according to standard procedures. All analyses were performed as routine assays at the Department of Coagulation Disorders. All laboratory results were interpreted in the context of published, age-matched normal values [17]. Free protein S antigen was measured using an enzyme-linked ligand-sorbent assay kit (International Laboratories, Lexington, MA, USA). Protein C and antithrombin were analysed with commercial amidolytic assay kits from Dade Behring Inc. (Newark, DE, USA). Coagulation factor XI was measured by performing a one-stage assay, using factor XI-deficient plasma as base (Dade Behring). Coagulation factor VIII and resistance to activated protein C (measured using factor V-deficient plasma) were analysed by employing chromogenic substrate methods (Chromogenix, Mölndal, Sweden). Genomic DNA from the peripheral blood was prepared according to standard procedures using EDTA-treated blood. The FV G1691A, FII G20210A, MTHFR C677T mutations were detected as previously described [18–20]. The MTHFR A1298C mutation was detected by an in-house allele-specific amplification in exon 7 using outer primers (5'-CTT TGG GGA GCT GAA GGA CTA C-3', 5'-CAC TTT GTG ACC ATT CCG GTT-3') and internal allele specific primers (5'-AGA ACA AAG ACT TCA AAG ACA CCA TC-3', 5'-GAG GAG CTG ACC AGT GAA ACA-3'), giving a product of 189 bp for all alleles, a product of 122 bp for the A allele and a product of 110 bp for the 1298 C allele. Internal positive controls for the MTHFR 1298 A/A, A/C and C/C genotypes gave expected values in each analytical batch.

#### *Statistics*

Observed genotype frequencies were compared with expected, and their odds ratios were determined. Odds ratios with exact 95% confidence limits and *p*-values, valid also for small studies, were calculated by Stat Xact-5 (Cytel Software Corporation) under the

assumption of known prevalences of the mutations in the study population. Data reported on regional prevalence for FV G1691A (11%) and FII G20210A (1.8%), and on prevalence from pooled general populations for MTHFR mutations (31.8%; homozygous 677 TT or heterozygous 677CT when in combination with 1298AC) were used [18,21–24]. This composite presentation of MTHFR mutations was done because both genotypes may cause similar elevation of the total homocystein in plasma [25]. A  $p$ -value  $<0.05$  was considered statistically significant.

## Results

Figure 1 illustrates the distribution of age and sex in the primary investigation, which included 128 thrombosis patients ages newborn to 20 y. Eight of the 11 patients in the age group  $<1$  y were males. By comparison, 39/128 of all patients in the cohort were males. Thus, the subgroup of males  $<1$  y comprised 21% (8/39) of all males in the cohort. There were 89 females in the cohort, and 77/89 (87%) of those patients were  $\geq 15$  y of age.

The sites of thrombosis were: cerebral (veins cranial to the neck veins; sinovenous thrombosis),  $n=24$  (19%); upper venous (veins of the neck (jugular), arms, thorax and abdomen including the inferior vena cava),  $n=40$  (31%); lower venous (veins distal to the vena cava inferior, including patients concurrently diagnosed with pulmonary embolism or extension of the thrombus into the upper venous system),  $n=63$  (49%); and purpura fulminans, which was diagnosed in one patient (0.8%).

Acquired risk factors could be identified in 104 (81%) of the 128 patients included in the primary investigation (and in 68 (82%) of the 83 patients included at follow-up). The most common such factors were oral contraceptives ( $n=50$ ) in members of the cohort who were  $\geq 15$  y, and in younger children

indwelling catheters ( $n=13$ ). Other acquired risk factors that were observed are accounted for in Table II.

Laboratory investigations identified genetic risk factors in 62/128 (54%) of the original cohort of patients. Blood tests from the 83 eligible patients at follow-up identified one or more genetic risk factors for venous thrombosis in 53/83 (64%) (if MTHFR excluded: 42/83, 51%), a single genetic risk factor in 36/83 (43%) and combined genetic risk factors in 17/83 (20%). FVL (heterozygosity) was found in 24/83 (29%) patients, FVL (homozygosity) in one (1%), FII mutation (heterozygosity) in five (6%) and MTHFR mutations (677TT or 677CT + 1298AC) in 24 (29%) (Table I). Protein S deficiency was found in four (5%), protein C deficiency in five (6%) and antithrombin deficiency in five (6%) of these subjects. Plasma values for coagulation factors VIII (mean 1.42 U/ml, SD 0.48, normal adult value 0.50–2.00 U/ml) and XI (mean 0.92 U/ml, SD 0.19, normal adult value 0.60–1.30 U/ml) showed normal Gaussian distributions.

Combinations of acquired and genetic risk factors were identified in 45/83 (54%) of the patients at follow-up, and 77/83 (93%) had at least one such risk factor.

## Discussion

This is the first study that has been conducted to identify thrombotic risk profiles in Swedish children and adolescents with venous thrombosis. In general, it can be difficult to recruit eligible patients for a retrospective re-evaluation. Our investigation included all children and adolescents  $\leq 20$  y of age and diagnosed with venous thrombosis in southern Sweden during a period of five consecutive years. Since our department is the only referral centre for coagulation check-up in that geographic area, it can be assumed that there was no bias with respect to the patients

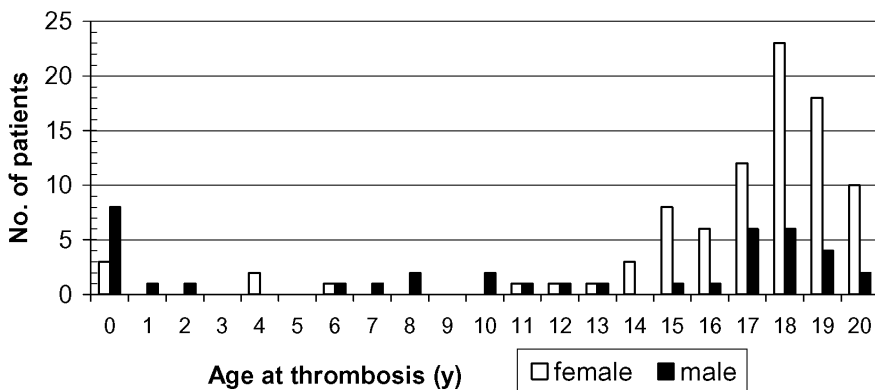


Figure 1. Age at thrombosis ( $n=128$ ).

Table I. Hereditary risk factors identified in patients  $\leq 20$  y of age with thrombosis ( $n=83$ ).

Genotype (mutations)			Cases ( <i>n</i> )	Expected ( <i>n</i> )	Odds ratio (95% confidence interval) and significance ( <i>p</i> )
F V mutation (G1691A)	F II mutation (G20210A)	MTHFR mutation (677TT or 677CT + 1298AC)			
+	+	+	1	0.1	10 (0.25–58), $p=0.19$
+	–	–	20	6.1	4.0 (2.3–6.7), $p<0.001$
–	+	–	2	1.0	2.0 (0.24–7.6), $p>0.30$
–	–	+	17	23	0.67 (0.37–1.2), $p=0.17$
+	+	–	0	0.1	0.0 (0.0–38), $p>0.30$
+	–	+	4	2.8	1.4 (0.39–3.9), $p>0.30$
–	+	+	2	0.5	4.1 (0.48–15), $p=0.18$
–	–	–	37	49.5	0.54 (0.34–0.86), $p=0.0079$

Table II. Potential acquired risk factors in patients  $\leq 20$  y of age with thrombosis ( $n=128$ ; in 49 patients one risk factor, in 44 patients two risk factors, in 11 patients three or more risk factors).

Use of oral contraceptives	50	Obesity	8
Surgery	23	Travel (> 8 h)	6
Infection	17	Prematurity (< 32 gestational weeks)	4
Central venous catheters	13	Malignancy	4
Immobilization	10	Congenital heart disorder	4
Trauma	10	Inflammatory bowel disease	4
Pregnancy or puerperium	8	Plaster cast	3
		Total parenteral nutrition	1
		Other clinical condition	4

included in the study. Accordingly, the results are probably representative of the paediatric population in that part of the country. Since not all patients were tested twice, an over- or underestimation of the frequencies of genetic risk factors is, however, possible but unlikely.

We found that both sexes had one peak in frequency at less than 1 y of age and then an increase during adolescence, more in females than in males, which agrees with findings in a paediatric population in Canada [2]. Overall incidence or prevalence of venous thrombosis in the Swedish paediatric population is not yet known; hence, larger prospective studies are needed to acquire this information.

The risk of thrombosis can be due to genetic (permanent) and acquired (permanent or transient) factors. However, if no such risk factor is found, venous thromboembolism can be referred to as idiopathic or unprovoked. We were able to identify risk factors in the vast majority of the patients in our cohort, ranging in age from newborn to 20 y, which indicates that physicians treating young patients should use the term “idiopathic thrombosis” with caution.

A majority—104/128 (81%)—of our patients had acquired risk factors, the most common of which were iatrogenic: oral contraceptives (in adolescents), surgery and indwelling catheters (in neonates). This

finding agrees with the results of previous investigations [2]. However, few population-based studies have evaluated both genetic and acquired risk factors. At follow-up we were able to identify at least one such factor in 77/83 (93%) of our patients, and combinations of genetic and acquired risk factors were identified in 45/83 (54%) of the subjects. This must be considered to be a high figure, even taking in account that the prevalence of acquired thrombotic risk factors for this age group in the general population is not known. Variation in the combinations of acquired and genetic risk factors may explain the differences in clinical expression of venous thrombosis observed in young individuals.

Our data show that the hereditary risk factors for thrombosis that are recognized in adults may also be valid for children. The most frequently encountered genetic risk factor has been reported to be the FV Leiden mutation [26], which is consistent with our findings. Since we have no control group, comparisons were made to prevalences known for thrombotic factors in the general population and figures must be interpreted with some caution. In our study group, statistical significance was reached for the presence of FVL mutation alone (OR 4.0,  $p<0.001$ ) and for the absence of all three genetic risk factors (OR 0.54,  $p=0.0079$ ). Probably due to the small size of the cohort, statistical significance was not reached when comparing other genetic combinations, and the small sample sizes make the other results difficult to interpret. However, odds ratios  $> 1.0$  were also found for mutations in the MTHFR gene when in combination with other risk factors but not alone (Table I).

Mild hyperhomocysteinaemia is an established risk factor for thrombosis. The homocysteine plasma level is determined both by non-genetic and genetic factors and increases with age. The exact role of mutations in the MTHFR gene is still a matter of debate, although homozygosity for the C677T allele, or combined heterozygosity with the A1298C allele, has been associated with hyperhomocysteinaemia [14,25,27,28].



Because we conducted a retrospective study, we tested MTHFR C677T instead of measuring plasma levels of homocysteine at the time of venous thrombosis, which would have been more accurate. Nevertheless, the above-mentioned findings support the idea that, when examining children and adolescents with venous thrombosis, it might be advisable to investigate for mutations in the MTHFR gene or hyperhomocysteinaemia, in addition to the more established hereditary defects of coagulation. We did not find any combination of FV G1691A and PT G20210A in our material.

Our findings concerning plasma values for coagulation factors VIII and XI fit well with published age-matched figures [17]; there is, however, a lack of appropriate references using the same methodology as our study.

Our results also emphasize that it is important to regularly re-evaluate previously diagnosed cases of venous thrombosis in children and adolescents, because thrombotic risk factors that were only recently identified would not have been detected in the primary investigations. Children and adolescents who have had an episode of venous thromboembolism should undergo thorough investigation for coagulation defects and it is also recommended to minimize, if possible, other non-hereditary risk factors.

To summarize, our findings confirm that several risk factors are often involved in paediatric thrombosis, and that specific categories of children and adolescents are at greater risk. Our data also show that hereditary coagulation disorders in patients  $\leq 20$  y of age who had venous thrombosis in southern Sweden are common. This implies that young patients who have had venous thrombosis should be offered re-evaluation as new genetic risk factors are discovered, and it also suggests that disorders of methionine metabolism should be considered.

## Acknowledgements

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II



III



## REGULAR ARTICLE

# Oestrogen treatment of constitutional tall stature in girls: is there a risk of thrombosis or bleeding?

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## Keywords

Bleeding tendency, Body height, Oestrogen therapy, Venous thrombosis, von Willebrand factor

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## Abstract

**Aim:** To evaluate haemostatic effects and clinical outcome of oestrogen treatment of constitutionally tall stature in girls.

**Methods:** We conducted a single-centre cohort study, 63 girls referred over a period of 15 years were investigated. The girls were given oestrogen treatment for constitutional tall stature at a median initial dose of 300 µg ethinyl estradiol/day and were consecutively examined for changes in coagulation. Medical records were retrospectively reviewed, additional data were collected at follow-up by blood sampling and interviews.

**Results:** After 1 year of treatment, levels of antithrombin and von Willebrand factor (VWF) were significantly decreased ( $p < 0.001$  and  $p = 0.015$ , respectively), whereas there was no significant change in levels of plasminogen inhibitor type 1. No venous thromboembolism (VTE) or major side effects were observed. Genetic risk factors for thrombosis were present, as was expected. The mean height reduction was 5.5 cm. The height-reducing effect was inversely correlated with chronological age ( $r = -0.44$ ,  $p < 0.01$ ) and bone age ( $r = -0.61$ ,  $p < 0.01$ ).

**Conclusions:** Changes in coagulation parameters occurred both towards pro- and anticoagulation. Treatment with high-dose ethinyl estradiol can successfully limit final height, and it is most effective when started at a younger bone age.

## INTRODUCTION

High doses of oestrogens have been used for more than 50 years to reduce final height in constitutionally tall girls. Such treatment is still widely applied (1), and new indications have recently been discussed (2). The effect of the therapy is seen as more rapid skeletal maturation and a decrease in growth velocity (3). The risk/benefit ratio remains unclear, most notably with regard to the risk of venous thromboembolism (VTE) during treatment and the risk of malignancies later in life.

Concerns about VTE are based mainly on accumulated knowledge of the use of oestrogens (e.g. ethinyl estradiol) as oral contraceptives, which is known to cause changes in haemostasis and is a well-recognized risk factor for VTE. Research has also shown that hormone replacement therapy with conjugated oestrogens doubles the risk of VTE in healthy postmenopausal women (4). However, VTE is much less common in children than in adults, and any VTE events in that young age group are usually due to a combination of inherited and acquired risk factors (5). Thrombosis during

oestrogen treatment for tall stature has been reported only sporadically, and all such cases have occurred in a clinical situation involving an elevated risk for VTE (6–9). However, the true incidence is not known because of a lack of large prospective studies, and the same applies to our understanding of the effects of this therapy on coagulation. In this context, a theoretical increase in treatment-related risk might be counteracted by important factors such as low age at the onset of therapy, and the dosage and type of sex hormones administered (combination of oestrogen and progestin).

It is plausible that genetic risk factors for thrombosis in combination with changes in coagulation parameters may jeopardize the health of girls receiving oestrogen treatment for tall stature. Thus, the main objective of the present study was to evaluate haemostatic effects in this patient group, focusing on thrombophilia and VTE. We also assessed the efficacy of the oestrogen treatment and the clinical outcome.

## PATIENTS AND METHODS

We conducted a single-centre cohort study at the University Hospital in Malmö, which is the referral facility for coagulation and paediatric endocrinology in southern Sweden, with a catchment population of about 1.2 million. Medical records were retrospectively reviewed, and additional data were collected at follow-up by blood sampling and interviews. The study was approved by the Regional Committee for Research Ethics at Lund University.

## Abbreviations

VTE, venous thromboembolism; PAI-1, plasminogen activator inhibitor type 1; VWF, von Willebrand factor; MTHFR, methylenetetrahydrofolate reductase; GP, Greulich and Pyle, BP, Bailey and Pinneau, SERM, selective oestrogen receptor modulator.

**Table 1** Clinical data at the start of treatment with high-dose ethinyl estradiol (n = 63)

Variable	Mean	Std. deviation	Minimum	Maximum
Age (years)	13.1	1.2	11.0	16.0
Maternal height (cm)	175.2	5.4	162	187
Paternal height (cm)	191.2	6.0	182	205
Height (cm)	176.9	3.4	169	184
Predicted final height BP (cm)	185.8	3.3	177.0	193.0
Body mass index	18.7	2.3	15	25

### Study population

Sixty-three girls referred to the University Hospital in Malmö (June 1984 to June 1999) and consecutively treated for constitutional tall stature were eligible for the study. Inclusion criterion was treatment >0.5 years, and the exclusion criterion was failure to follow the treatment regimen. Approximately 90% of the treated patients fulfilled these criteria. The girls were treated with ethinyl estradiol orally at a median initial dose of 300 µg/day (range: 150–500 µg), and norethisterone 5 mg/day (n = 55) or medroxyprogesterone 5 mg/day (n = 8) was added during the last 10 days of each monthly cycle. Prior to treatment, 24% of the girls had already reached menarche. Clinical baseline features are presented in Table 1. In general, treatment was stopped when two height measurements made more than 6 months apart were equal; this level was defined as the final height in the study. The mean duration of therapy was 1.8 years (SD: 0.6, range: 0.5–3.7 years). For 51 of the 63 girls, a follow-up height measurement was obtained about 1 year after the end of treatment. After informed consent, 35/63 (56%) completed a follow-up interview, and 26/63 (41%) gave an additional blood sample. Mean age at this final follow-up was 23.6 years.

### Methods

The girls included in the study were followed clinically according to a local protocol. Coagulation variables were monitored in all patients before and during treatment: antithrombin activity was analysed by an amidolytic assay (Dade Behring, Newark, DE, USA); plasminogen activator inhibitor type 1 (PAI-1) activity was assessed using Spectrolyse (pL) PAI (Biopool, Umeå, Sweden); von Willebrand Factor antigen (VWF:Ag) was measured by applying the STA Liatest (Diagnostica Stago, Asnières, France). The tests were performed according to routine standards. To detect the presence of thrombophilia in the patients, we also tested for protein C and free protein S, as well as the genotypes F V-G1691A (factor V Leiden), F II-G20210A, MTHFR-C677T and MTHFR-A1298C. Venous blood samples were collected in citrate-containing tubes, and plasma was separated according to standard procedures. Due to the long inclusion period not all tests were available before initiating treatment. All of the mentioned analyses were performed as routine assays at the time of collection at the Department of Coagulation Disorders, as previously described (5,10–12), and the laboratory results were interpreted on the basis of pub-

lished, age-matched normal values. Prolactin was assayed at the Department of Clinical Chemistry by an immunometric standard method (Access Prolactin Beckmann-Coulter).

Clinical data were collected from medical charts. Bone age determinations prior to and during treatment were made by a specialized paediatric radiologist at the referral centre (Malmö University Hospital) using the methods described by Greulich and Pyle (GP) (13), and final height predictions were done as described by Bailey and Pinneau (BP) (14). Prolactin levels were measured before, during and after treatment, and the results were compared with normal reference values. At the follow-up interview, a standardized questionnaire was used that included items that asked the patients whether they had been treated for VTE, and if they would elect to have the treatment again given the present knowledge.

### Statistics

The results were analysed using the Statistical Package for Social Sciences (SPSS v 13.0). To compare laboratory data before and during treatment, paired Student *t*-test was performed for each of the variables. The effect of the treatment was assessed using bivariate analysis, and the Pearson correlation coefficients were calculated. A *p*-value <0.05 was considered significant. For estimating the risk of thrombosis Hanley's rule of three was used (15).

## RESULTS

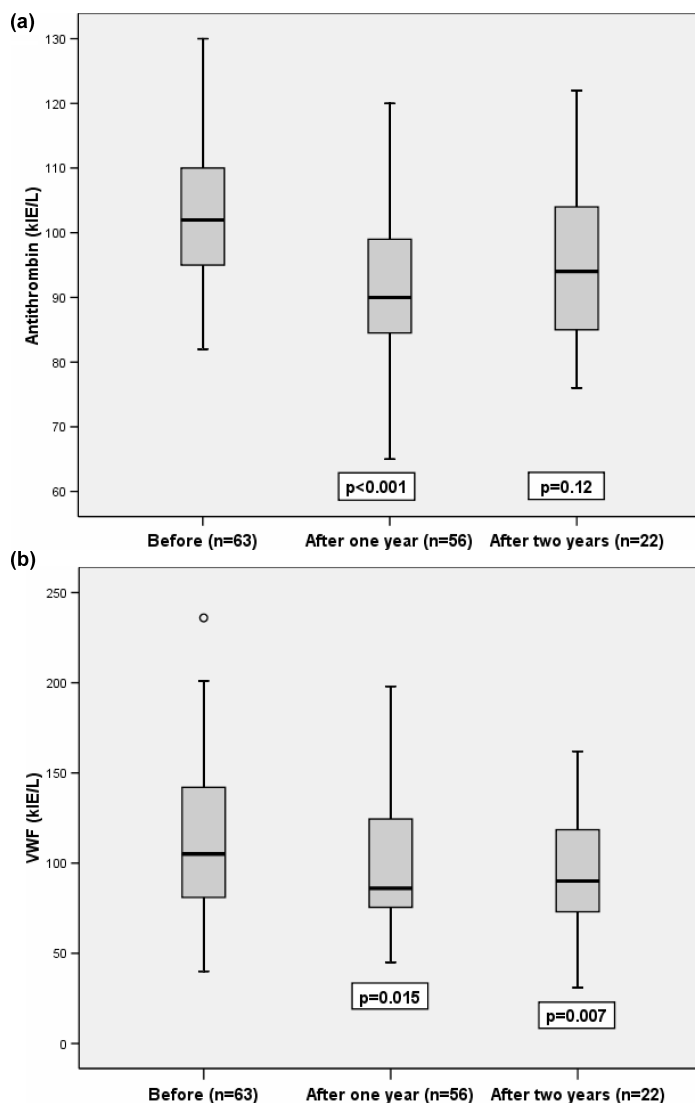
### Coagulation

Levels of VWF and antithrombin were significantly decreased (*p* = 0.015 and *p* < 0.001, respectively) at the end of the first year of treatment (Fig. 1a,b). During continued therapy, there was a tendency towards restitution of antithrombin levels, whereas some patients showed a further decrease in VWF. Two patients had clearly decreased VWF levels at baseline, 0.44 and 0.40 IU/mL of whom the latter had a decline in levels to 0.31 and 0.34 IU/mL at 2 and 3 years, respectively, after the start of treatment. There were no significant changes in the levels of PAI-1, although intraindividual variations were seen in a few patients. Supernormal levels of PAI-1 (>16 E/mL) were seen in 5% (3/63) of the patients before treatment and in 2% (1/56) after 1 year of treatment. No correlation between initial ethinyl estradiol dosage and changes in coagulation factors measured could be found (data not shown). Markers of thrombophilia were present in some of the patients at follow-up: F V-G1691A in 12%, F II-G20210A in 0%, MTHFR-C677T (homozygote) in 16%, and MTHFR-C677T (heterozygote) + MTHFR-A1298C (heterozygote) in 24%. Levels of protein C, protein S and antithrombin were normal in all cases at follow-up.

### Auxology

Auxological parameters of all patients are summarized in Table 2. The mean height reduction (estimated as the difference between predicted and final height) was 5.5 cm (SD: 3.0 cm; range: from –1.8 to 12.3 cm). The mean height





**Figure 1** Levels of antithrombin (a) and von Willebrand factor (VWF) (b) before and after 1 and 2 years of treatment with high-dose ethinyl estradiol. p-values represent comparisons of data on the same group of individuals at the start of treatment and at indicated time points.

increase observed after the start of treatment (3.5 cm) was limited to 39% of the mean predicted remaining growth at the start of treatment (9 cm). The height-reducing effect was inversely correlated with chronological age ( $r = -0.44$ ,  $p < 0.01$ ) and bone age ( $r = -0.61$ ,  $p < 0.01$ ) (Fig. 2) and positively correlated with predicted remaining growth ( $p < 0.01$ )

at the onset of treatment. Median residual growth 1 year after the end of treatment was 1.0 cm ( $n = 51$ , range: 0–5.5 cm). No correlation between initial ethinyl estradiol dosage and height reduction could be found (data not shown). The mean height reduction in the premenarcheal girls was 5.6 (SD: 3.2) cm, which was not statistically

**Table 2** Clinical data at the end of treatment with high-dose ethinyl estradiol (n = 63)

Variable	Mean	Std. deviation	Minimum	Maximum
Age (years)	14.9	1.2	12.0	18.0
Height (cm)	180.4	2.7	175.0	186.0
Height reduction(cm)	5.5	3.0	-1.8	12.3
Body mass index	20.9	1.8	18	27

different from the corresponding mean of 5.1 cm (SD: 2.6) recorded for the postmenarcheal girls.

### Side effects of treatment

Treatment was not accompanied by any major side effects. Leg cramps at night (20%) and headache (18%) were most frequently reported. However, it was necessary to discontinue the therapy in only one girl due to increased migraine symptoms. Fifty-four percent of the patients gained  $\geq 10$  kg body weight. The mean prolactin level increased from 7.2 ug/L (SD: 2.4, range: 2–13) to 25.3 ug/L (SD: 13.4, range: 10–80) at the end of treatment (normal <15 ug/L). All values were normal at follow-up. Spontaneous cyclic bleeding occurred in all girls within the first 9 months after conclusion of treatment, and 87% of them experienced such bleeding within 3 months. At follow-up interview, 86% of the patients were satisfied with the therapy and reported that, given their present knowledge, they would choose to

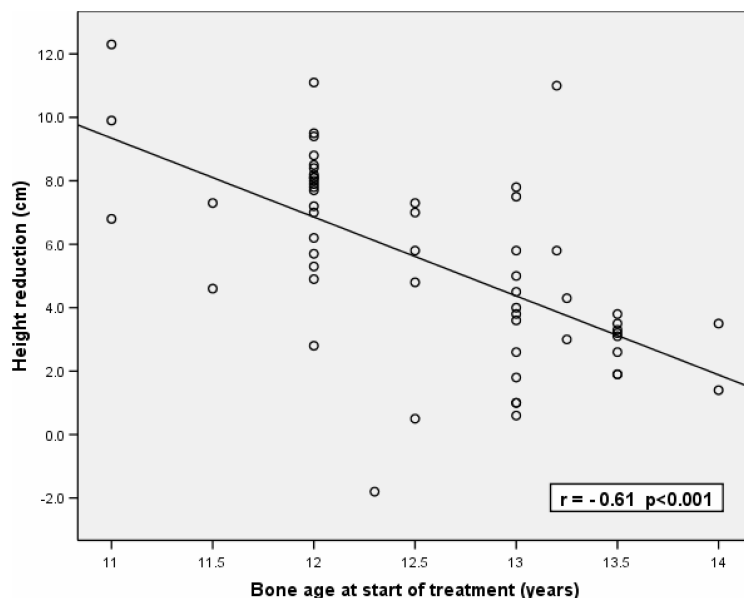
be treated again, whereas 6% indicated that they would not accept the therapy again, and 8% did not know.

None of the patients were diagnosed with VTE during the oestrogen therapy. The risk of thrombosis was calculated to 0–4.7% using a 95% confidence interval. One patient, who had been on oestrogen treatment for tall stature for 2.0 years, reported having a VTE 10 years after the end of that treatment. At the time of the thrombosis, she had been taking oral contraceptives for 6 years. Investigation for thrombophilia showed that she was a heterozygote carrier of the FV-G1691A mutation, but she had no family history of VTE.

### DISCUSSION

There is increasing evidence that oestrogen treatment can induce a hypercoagulable state in women, but there is a lack of large studies that have examined the impact of hormonal therapy for constitutionally tall stature on coagulation in girls. To our knowledge, the present material from our single centre experience is the largest reported.

Antithrombin is a potent inhibitor of several coagulation proteases, and the presence of a low level of that glycoprotein is a substantial risk factor for deep vein thrombosis (16). The patients in our study showed a significant decrease in antithrombin levels during the first year of treatment with high-dose ethinyl estradiol. Previous reports regarding the effects of such therapy have been conflicting. In two early studies, patients were treated with ethinyl estradiol at



**Figure 2** Effect of oestrogen treatment on height reduction (cm) by bone age (Greulich and Pyle) at the start of therapy.

500 ug/day and 250–1000 ug/day (17,18), respectively. In the latter investigation a significant decrease in antithrombin was seen. More recently, van Ommen et al. (9) found no significant decrease in antithrombin levels in 24 patients given 200–300 ug of ethinyl estradiol whereas a significant decrease in protein S was reported indicating a prethrombotic state.

PAI-1 is an endothelial factor that is sensitive to metabolic and hormonal changes, and high levels of this protein have been reported to be associated with thrombosis in both the arterial and the venous vessel system (19,20). Most patients in our study had normal PAI-1 levels, given that they were within normal range. We can however not preclude the presence of an abnormal circadian rhythm. On the other hand, therapy did result in a decrease in VWF, which to our knowledge has not been previously reported by other investigators. In a recent, well designed study on healthy women, the VWF level declined numerically, although not statistically significant, during treatment with combined oral contraceptives (21). Our finding suggests that a tendency towards a hypocoagulable state can be induced concomitantly to the decline in antithrombin, and that there thus appears to be contradictory influences on coagulation. The overall impact on the balance of the clotting system is however difficult to predict as the number of investigated coagulation factors were restricted in this study due to the long study period. Notably, two of the patients showed a marked decrease in VWF that was in the range seen in von Willebrand disease and in one of these the decrease was pronounced during treatment.

The frequency of genetic thrombophilic markers was as expected in the study participants. No events of VTE were detected during the treatment period, even in girls with genetic markers for thrombophilia. According to the rule of three, it can consequently be estimated that the risk of VTE during treatment is <5%. Interestingly, one of the patients with thrombophilia showed no sign of thrombosis during the high-dose oestrogen therapy for tall stature, but she was treated for VTE at follow-up after 6 years of taking oral contraceptives. This could suggest that oestrogen treatment for tall stature is not a strong risk factor for VTE at this young age.

The young age of the subjects and the relatively short treatment period do not preclude that girls receiving such treatment are at increased risk of VTE or bleeding episodes. Therefore, before implementing the oestrogen therapy, we advise that a careful medical history be taken that includes hereditary factors and focuses on symptoms related to a clotting disorder. Based on our data, it may also be prudent to measure VWF and antithrombin levels prior to and during treatment, because that might help determine the risk of bleeding or thrombosis. Enrollment into the cohort we studied was started in 1984, and since then routine investigation for thrombophilia has been expanded and can now be recommended to include pretreatment analysis of additional thrombophilic markers, primarily protein C, protein S and factor V Leiden. This is particularly important if there is any indication of thrombophilia in close relatives.

The treatment decision must consist of a careful weighing of available facts – such as predicted final height and potential treatment risks – and the values of the girl, her family, the doctor and society (22). Over the years various methods have been developed to predict final height, of which the method of BP is the most commonly used. In two recent large studies in girls with constitutional tall stature (23,24), the mean (SD) errors of BP prediction were found to be rather small, 0.5 cm (2.7) and –0.14 cm (3.1), respectively, and thus indicating that the BP method is clinically acceptable for height prediction in these girls. If treatment is applied, the effect of treatment is generally evaluated by subtracting the achieved final height from the predicted final height. In studies including controls, the effect of treatment might be corrected by subtraction of the mean prediction error, which has commonly been performed in past studies.

In the present study, which did not include controls, the mean reduction in final height was 5.5 cm, which agrees with previously published data indicating mean decreases (corrected and uncorrected) in the range 2.1–10.0 cm (for review, see Ref. 3). However, it is difficult to achieve adequate comparisons of the results of different investigations due to disparities regarding the initial clinical data (chronological age and bone age), duration of treatment, therapeutic regimen and time of final height assessment. In our study, the height reduction was more pronounced when treatment was started at a younger bone age, which is in accordance with most previous observations (5). Nonetheless, we found no statistically significant difference in height reduction between premenarcheal and postmenarcheal girls, which is supported by some earlier studies but not by others (3). Furthermore, we did not find any correlation between initial oestrogen dosage and outcome, which concurs with previous reports (25). In our study, where the oestrogen treatment was generally stopped when two height measurements made more than 6 months apart were equal, an additional mean growth of 1.0 cm was observed. This increase is less than the mean post-treatment growth of 1.8–3.3 cm reported by other researchers (23,24,26). Once treatment has been initiated, we recommend that the therapy be continued until complete closure of the epiphyses has occurred in order to avoid considerable residual growth.

It is known that oestrogens are potent stimulators of prolactin secretion, and occurrence of a prolactinoma has been described in a girl receiving oestrogen therapy for height reduction (27). In our study, the mean serum prolactin concentrations increased but never reached excessively high levels, and they returned to normal after discontinuation of therapy. Only minor side effects were observed during treatment. At final follow-up (mean age of subjects 23.6 years), the great majority of the patients expressed satisfaction with the treatment. Nevertheless, concerns have been raised that oestrogen treatment might increase the risk of malignancies later in life and also seems to reduce fertility (28). Hence, it might be desirable to develop new approaches to the pharmacological treatment of constitutionally tall stature in young females. Recent discussions on this topic have concerned possible treatment with selective oestrogen

receptor modulators (SERMs), which act as oestrogen agonists with respect to growth plate fusion but function as oestrogen antagonists in relation to breast and uterine tissue (29). Preliminary findings also seem to suggest that, compared to oestrogens, SERMs have fewer unfavourable effects on haemostatic variables in women (30).

## CONCLUSIONS

In summary, we report a single centre evaluation of a relatively large cohort of adolescent females receiving oestrogen treatment for constitutionally tall stature. We found a concomitant decline from pretreatment levels of both antithrombin and VWF. We conclude that there appears to be contradictory influences on coagulation. We also conclude that the treatment with high-dose ethinyl estradiol is effective in terms of limiting final height, and that the regimen is even more efficacious if the therapy is started at a younger bone age.

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IV

