THROMBIN REGULATION IN NEWBORN INFANTS

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ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Medical Faculty of the University of Helsinki, in the Niilo Hallman Auditorium of the Hospital for Children and Adolescents, on March 23rd, 2007, at 12 noon.

Helsinki 2007

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ISBN 978-952-92-1644-4 (paperback)
ISBN 978-952-10-3738-2 (PDF)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2007

To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals.

I. **Hyytiäinen S**, Syrjälä M, Fellman V, Heikinheimo M, Petäjä J. Fresh frozen plasma reduces thrombin formation in newborn infants. *J Thromb Haemost 2003; 1: 1189-94.*

II. **Långström S**, Wartiovaara-Kautto U, Andersson S, Heikinheimo M, Petäjä J. Exchange transfusion activates coagulation and alters the coagulation profile in newborn infants. *Thromb Haemost 2006; 96: 142-8.*

III. **Hyytiäinen S**, Wartiovaara-Kautto U, Ulander V-M, Kaaja R, Heikinheimo M, Petäjä J. The procoagulant effects of factor V Leiden may be balanced against decreased levels of factor V and do not reflect *in vivo* thrombin formation in newborns. *Thromb Haemost 2006; 95: 434-40.*

IV. **Långström S**, Rautiainen P, Mildh L, Peltola K, Wartiovaara-Kautto U, Heikinheimo M, Petäjä J. Unique regulation of thrombin generation during and after cardiopulmonary bypass in neonates. *Submitted.*

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ABBREVIATIONS

a	activated coagulation factor
alpha-2-M	alpha-2-macroglobulin
APC	activated protein C
AT	antithrombin
CPB	cardiopulmonary bypass
ET	exchange transfusion
ETP	endogenous thrombin potential
F	coagulation factor
F1+2	prothrombin fragment F1+2
FFP	fresh-frozen plasma
FVL	factor V Leiden (Arg506Gln) mutation
IVH	intraventricular haemorrhage
PC	protein C
PCC	prothrombin complex concentrate
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PS	protein S
RDS	respiratory distress syndrome
TAT	thrombin-antithrombin complex
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
VWF	von Willebrand factor

ABSTRACT

The coagulation system of newborn infants differs markedly from that of older children and adults. The activities of most coagulation factors and anticoagulants are low, leading to altered regulation in the formation of the key enzyme, thrombin. Timely and adequate generation of thrombin is essential, as thrombin activates platelets and many coagulation factors, cleaves fibrinogen into fibrin and activates the antithrombotic and anti-inflammatory protein C pathway. On the other hand, excess thrombin may promote thrombotic complications and exacerbate harmful inflammatory reactions.

Despite the characteristic features, the newborn coagulation system can be considered physiological, since healthy newborns rarely show haemorrhagic or thrombotic complications. Sick newborns, however, often encounter clinical situations that challenge their coagulation system.

The aim of this study was to clarify the behaviour of the neonatal coagulation system in selected clinical situations, with a special emphasis on the generation of thrombin. Thrombin was measured by *in vivo* thrombin generation markers and by thrombin generation potential *in vitro*. The patient groups included sick newborns undergoing intensive care and receiving fresh-frozen plasma (FFP), requiring exchange transfusions (ET) or presenting with a congenital heart defect requiring open heart surgery. Additionally, healthy newborns with inherited heterozygous factor V Leiden (FVL) mutation were studied. Thrombin generation potential was also analysed in cord plasma of healthy infants and in adults.

Healthy as well as sick newborn infants showed lower total thrombin generation potential *in vitro* but faster initiation of thrombin generation than adults. These findings were qualitatively similar when plasma was supplemented with platelets. Platelets, however, significantly altered the effect of the major anticoagulant, activated protein C (APC), on thrombin generation potential. In accordance with previous studies, thrombin generation in healthy newborn platelet-poor plasma was resistant to the anticoagulant effects of APC, but when the plasma was supplemented with platelets APC attenuated thrombin generation significantly more in newborns than in adults.

In vivo generation of thrombin was elevated in nearly all of the sick newborn infants. The low-volume FFP transfusion as opposed to the change from neonatal to adult blood in ET exerted markedly different effects on neonatal thrombin generation. FFP reduced the *in vivo* generation of thrombin in those newborns with the highest pretransfusional thrombin generation, thus acting as an anticoagulant agent. In those infants with lower pretransfusional thrombin generation, the effect of FFP on thrombin generation was fairly neutral. On the other hand, the combination of red blood cells and FFP, used to perform ET, significantly increased the *in vivo* thrombin formation and shifted the balance in the newborn coagulation system to

the procoagulant direction. Cardiopulmonary bypass (CPB) also significantly increased the *in vivo* thrombin generation, but the thrombin generation profile during CPB differed from that previously observed in adults. Escalation of thrombin at early reperfusion was not observed in newborns; in adults, its occurrence is associated with postoperative myocardial damage. Finally, in healthy newborns with FVL heterozygosity, faster initiation of thrombin generation was observed compared with controls. Interestingly, FV level was lower in FVL-heterozygous infants, possibly to counteract the procoagulant effects induced by FVL.

In conclusion, unique features regarding thrombin regulation in newborn infants were observed. These features included a novel platelet effect on the regulation of the protein C pathway. The clinical challenges mainly seemed to shift the balance in the coagulation system of newborns to the procoagulant direction. Blood component transfusions markedly affected coagulation in a manner specific to the product but that could also be altered by the clinical situation. Overall, the results highlight the need for understanding developmental haemostasis for both diagnostic and therapeutic purposes.

INTRODUCTION

Blood coagulation requires a series of reactions involving simultaneous interplay between coagulation factors, anticoagulants, platelets, endothelium and the fibrinolytic system. Coagulation is initiated by tissue factor, which activates the series of events, eventually leading to the formation of the key enzyme in coagulation, thrombin. Thrombin, in turn, activates platelets, augments its own formation by feedback activation of selected coagulation factors, cleaves fibrinogen into fibrin and activates the antithrombotic and anti-inflammatory protein C pathway. Thrombin is also an important link between coagulation and inflammation.

The coagulation system of a newborn infant is in a developmental stage. The activities of most, but not all, coagulation factors and anticoagulants are lower in newborns than in adults and older children. Some coagulation proteins exist in foetal forms. The activities of the coagulation factors and anticoagulants are dependent on both gestational age and postnatal age of the newborns. As a result, the regulation of thrombin significantly differs from that in older children and adults. However, haemostasis in healthy neonates is not clinically compromised. On the other hand, among children, sick newborns are at the greatest risk of haemorrhagic and thrombotic complications. These complications occur especially in newborns undergoing intensive care or major surgery.

Due to unique features of the newborn coagulation system, conclusions regarding management of neonatal coagulation can not be drawn from studies in older children or adults. Thus, the coagulation system of newborns was examined here in various clinically important situations, with a special emphasis on thrombin formation *in vivo* and *in vitro*. These situations included newborns receiving fresh-frozen plasma during intensive care, newborns receiving exchange transfusions, newborns with congenital heart disease requiring open heart surgery with cardiopulmonary bypass and newborns with factor V Leiden heterozygosity.

REVIEW OF THE LITERATURE

1 Haemostasis in the newborn

Haemostasis is maintained by a complicated interplay between platelets, coagulation factors, physiological anticoagulants, the fibrinolytic system and the endothelium. The most distinct features of newborn haemostasis are the low activities of most coagulation factors and physiological anticoagulants.

1.1 Coagulation from tissue factor to thrombin

Coagulation system has traditionally been presented as a cascade model with two separate pathways, extrinsic and intrinsic pathway, together leading to the formation of thrombin and the subsequent activation of fibrinogen to fibrin (Davie and Ratnoff 1964, MacFarlane 1964) (Figure 1). However, the contribution of the initiator proteins of the intrinsic pathway to haemostasis *in vivo* has been questioned since their deficiencies do not result in bleeding disorders. Importantly, the complex of tissue factor (TF) and factor VIIa (FVIIa) is able to activate factor IX (FIX) (Osterud and Rapaport 1977), and thrombin is able to feedback-activate factor XI (FXI) (Gailani and Broze 1991), intertwining the pathways (Figure 2). Thus, the critical stimulus in the activation of the coagulation system *in vivo* is tissue factor.

1.1.1 Initiation of coagulation by tissue factor

Tissue factor (TF) is the main physiological initiator of coagulation. TF is located on the membranes of adventitial fibroblasts and pericytes, forming a haemostatic envelope around blood vessels (Drake et al. 1989). Thus, the initiation of blood coagulation is presented to occur via damage to the vessel wall and subsequent exposure of blood to TF in the vessel wall (Figure 2). However, the report of Giesen et al. (1999) on active TF in blood, possibly originating from leukocytes, raised an ongoing discussion about the relative roles of vessel-wall TF and blood-borne TF in the activation and maintenance of coagulation. Since then, TF antigen has been detected in various forms in blood of healthy individuals; in procoagulant microparticles, in monocytes and platelets and as a soluble protein lacking the transmembrane domain (Rauch et al. 2000, Bogdanov et al. 2003). However, other studies have provided evidence that in healthy individuals significant amounts of active TF can not be found in whole blood (Butenas et al. 2005) and that the primary TF stimulus from the vessel wall is adequate in maintaining coagulation (Orfeo et al. 2005). Certain stimuli, such as inflammatory cytokines, are able to induce TF expression, especially in monocytes and endothelium (Moldow et al. 1993, Butenas et al. 2005).

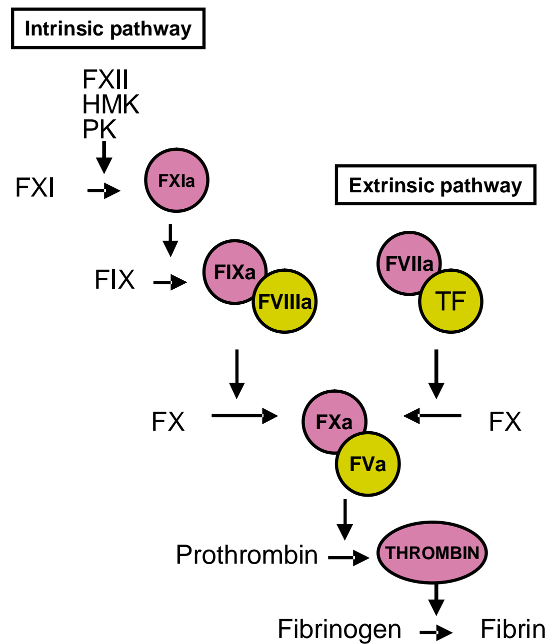


Figure 1. Traditional cascade model of the coagulation system with two separate pathways initiating coagulation. HMK= high-molecular-weight kininogen, PK= prekallikrein. The coagulation reactions require the presence of calcium and phospholipid surfaces.

TF is evidently a primary initiator of coagulation also in neonates. TF is expressed from early stages of embryogenesis in both humans and mice, and even in tissues where it can not be observed in adults (Luther et al. 1996). Thus, crucial roles for TF beyond coagulation have been proposed during human development. Interestingly, human umbilical vein endothelial cells have been reported to produce more functional TF than adult saphenous vein endothelial cells after activation by inflammatory cytokines (Grabowski et al. 2000), but the significance of this finding remains unclear.

1.1.2 Interplay of coagulation factors in the formation of thrombin

Activation of coagulation from TF to thrombin proceeds via formation of three activation complexes comprising serine proteases (FVII, FIX, FX, prothrombin) and their respective cofactors (TF, FV, FVIII) that assemble on the cell surfaces expressing negatively charged phospholipids in the presence of calcium (Figure 2). FVII, FIX, FX and prothrombin are all derived from the liver. In the liver cells, these proteins undergo vitamin-K-dependent γ-carboxylation, which is required for Ca^{2+}-dependent binding of the respective coagulation proteins to the phospolipid surface, and thus, for proper activities of these proteins (Stenflo et al. 1974).

First, a complex between TF and FVII/FVIIa is formed (Figure 2). Approximately 1% of FVII circulates in blood as the active enzyme FVIIa (Morrissey et al. 1993). TF can form a complex with both FVII and FVIIa, but only the TF-FVIIa complex is capable of activating the substrates, the coagulation factors X and IX (Rao and Rapaport 1988). FIXa and FXa, in turn, feedback-activate TF-bound FVII.

The activation complex of FIXa and FVIIIa on a phospholipid surface in order to further activate FX to FXa is referred to as the tenase complex. In this complex, FVIIIa acts as a cofactor, greatly enhancing the activation rate of FX (van Dieijen et al. 1981). Actually, the FVIIIa-FIXa complex is approximately 50 times more efficient in activating FX than TF-FVIIa, and thus, the majority of FXa is eventually generated through the FVIIIa-FIXa complex (Mann et al. 2003). The cofactor FVIII is mainly synthesized in the liver, but the extrahepatic production sites remain unclear. FVIII production in human lung microvascular cells was recently described (Jacquemin et al. 2006). FVIII circulates in blood complexed to VWF and dissociates from it upon activation. Thrombin is the most potent activator of FVIII, but activation also occurs in the presence of FXa, calcium and phospholipids (Eaton et al. 1986). The carrier of FVIII, VWF, is a large multimeric protein. VWF is expressed in endothelial cells and in megakaryocytes and platelets. Only endothelial cells express VWF constitutively, thus, nearly all circulating VWF is of endothelial origin (Bowie et al. 1986). The size of VWF multimers is regulated by ADAMTS13 on the surface of the endothelial cells (Dong et al. 2002); the largest VWF multimers are the most thrombogenic. VWF regulates the secretion and activation of FVIII, concentrates FVIII at the site of the injury and prevents its inactivation by activated protein C (APC) (Nogami et al. 2002). Another crucial role of VWF is to mediate the adhesion of platelets to subendothelial collagen via binding to platelet surface glycoprotein Ib complex. This VWF-mediated binding results in platelet tethering and rolling on the surface and further promotion of stable platelet adhesion at the site of vascular injury.

The complex of FXa and FVa on the phospholipid surface, known as the prothrombinase complex, activates thrombin from prothrombin. Once assembled, the complex converts prothrombin to thrombin 280 000 –fold faster than possible with FXa alone (Nesheim et al. 1979). The prothrombinase complex can be assembled on the surface of platelets, monocytes and activated endothelial cells (Hamilton et al. 1990, Allen et al. 1995). The cofactor FV is a glycoprotein that is synthesized in the liver and activated by FXa and thrombin via proteolytic cleavages (Monkovic and Tracy 1990). Approximately 20% of blood FV is stored in platelet α-granules and can be secreted upon platelet activation.

The activation of thrombin from prothrombin by prothrombinase complex is a key step in blood coagulation. Thrombin exerts both procoagulant and anticoagulant activities. Thrombin activates fibrinogen to fibrin, stabilizes the fibrin clot by activating FXIII, and inhibits fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor. Thrombin also activates FVIII and FV, as well as FXI, which further activates FIX. Thrombin is a potent activator of

platelets. The anticoagulant activities of thrombin are promoted via thrombin binding to thrombomodulin and subsequent activation of the protein C pathway.

The relevance of the individual coagulation factors regulating the generation of thrombin has been investigated in mice by knock-out techniques. The mice lacking TF die as embryos (Toomey et al. 1996). Lack of FVII does not appear to affect embryonic development, instead the deficient mice die during the perinatal period from severe bleeding (Rosen et al. 1997). Lack of FVIII or FIX in mice results in haemophilia-like syndrome (Bi et al. 1995, Kundu et al. 1998). Lack of FV, FX or prothrombin, in turn, leads to frequent embryonic death and fatal haemorrhage after birth (Cui et al. 1996, Sun et al. 1998, Dewerchin et al. 2000).

1.1.3 Coagulation factor activities in newborns

Maternal coagulation factors do not cross the placental barrier. However, already at five weeks of gestation, mRNA and protein for coagulation factors VII, VIII, IX and X can be detected in embryonic hepatocytes (Hassan et al. 1990), and foetal blood becomes clottable at around 11 weeks of gestation. During early foetal life, the levels of coagulation proteins are very low, gradually rising with increasing gestational age (Reverdiau-Moalic et al. 1996). The levels observed in premature newborns of corresponding gestational age are, however, not reached in the foetus (Reverdiau-Moalic et al. 1996).

At birth, the levels of most but not all coagulation factors are low compared with older children or adults (Andrew et al. 1987, Monagle et al. 2006) (Figure 2, Table 1). In general, healthy premature infants show lower coagulation factor levels than healthy term infants (Andrew et al. 1988a). In healthy term infants, vitamin K-dependent coagulation factors, FVII, FIX, FX and prothrombin circulate at approximately 40-60% of the levels observed in adults. The activity of FXI is similarly low. The levels of the cofactors FV and FVIII are, however, within the normal adult range. In foetuses and newborns, VWF circulates as ultralarge VWF multimers (Katz et al. 1989). In accordance with this, VWF activity is increased in term and preterm infants (Andrew et al. 1987, 1988a); VWF thus appears to be a major contributor to proper haemostasis in newborns. The presence of high-molecular-weight multimers has been suggested to be a result of decreased ADAMTS13 activity in newborns (Manucci et al. 2001). However, in recent studies, ADAMTS13 activity in newborns has been comparable with that in adults, although a small proportion of newborns have shown lower ADAMTS13 levels, and neonatal levels of ADAMTS13 have not correlated with VWF antigen levels (Tsai et al. 2003, Schmugge et al. 2004), contrary to earlier suggestions.

Coagulation factor levels are also influenced by postnatal age; from birth, the levels gradually increase towards adult levels, with most coagulation factors reaching nearly adult levels by six months of age (Andrew et al. 1987 and 1988a). The increase to adult levels proceeds at various speeds; e.g. FVII attains the normal adult values already at postnatal days 3-5. Further, although premature infants show lower factor levels at birth, they are still able to reach similar levels to full-term infants by the age of six months (Andrew et al. 1988a,

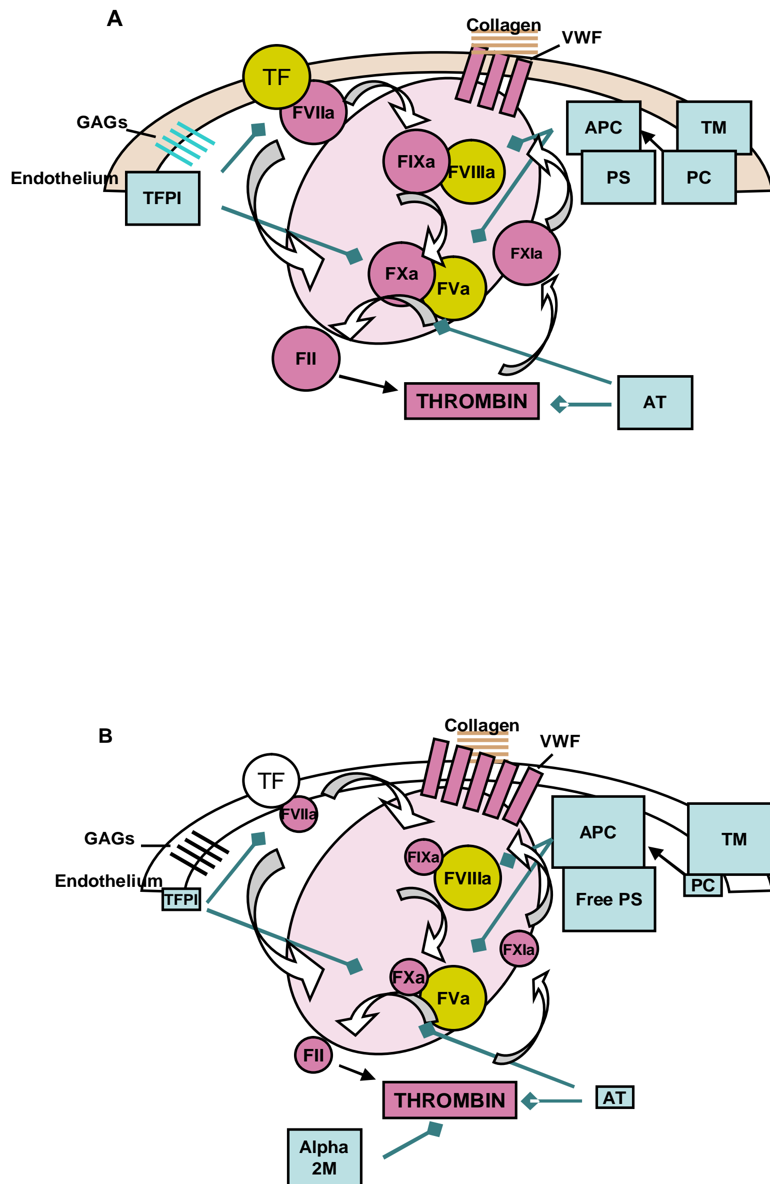


Figure 2. Coagulation reactions from the expression of tissue factor (TF), leading to formation of thrombin, and the primary anticoagulant pathways (A). The relative activities of individual coagulation factors and physiological anticoagulants in the formation of thrombin in newborns are shown in (B). The size of the symbols roughly reflect the presumed relative neonate vs. adult differences. Uncoloured symbols indicate a lack of knowledge. The levels of coagulation factors FVII, FIX, FX, FXI and prothrombin (FII) are reduced at birth, as are the levels of tissue factor pathway inhibitor (TFPI), antithrombin (AT), and protein C (PC). However, activated protein C (APC), thrombomodulin (TM) and protein S (PS, free form) similar to von Willebrand factor (VWF), are upregulated in newborns. Alpha-2-macroglobulin (alpha-2-M) is elevated in newborns and may contribute to the inhibition of thrombin. The relative expression of TF following endothelial injury in newborns as compared with adults is unknown. The role of the endothelium in the regulation of coagulation reactions remains unclear in newborn infants. Actions of thrombin in the activation of FV, FVIII and PC are not shown. GAGs = glycosaminoglycans.

Salonvaara et al. 2004).

The suggested mechanisms for low coagulation factor activities at birth include reduced synthesis, reduced secretion, accelerated clearance, synthesis of proteins with decreased functional activity and consumption at the time of birth. The coagulation factors showing low levels at birth are derived from the liver. Reduced synthesis of coagulation factors in the liver can, however, only partly explain reduced activities in plasma since, for instance, the expression of FX mRNA is similar between the foetal liver at gestation week seven and the adult liver (Hassan et al. 1990). On the other hand, the expression of FIX mRNA at that time remains at 10% of the expression observed in adults (Hassan et al. 1990). The role of the reduced secretion of coagulation proteins from foetal liver cells is supported by the observations of higher ratios of liver FVII, FX, and prothrombin protein concentrations to plasma protein concentrations in foetuses than in adults (Hassan et al. 1990, Karpatkin et al. 2000, Manco-Johnson et al. 2002). Accelerated clearance may also play a role; foetal lambs show increased fibrinogen clearance (Andrew et al. 1988b). However, in newborn rabbits, the half-life of prothrombin does not differ from adult animals (Karpatkin et al. 2000).

Post-translational modifications of coagulation proteins may affect the secretion, activity or clearance rate of the coagulation factors. Probably the most important of these modifications is the vitamin K-dependent γ-carboxylation of prothrombin, FVII, FIX and FX in liver cells. Levels of vitamin K are around 10% of maternal levels in the foetus during pregnancy, are undetectable at birth and rise to adult levels by postnatal day 4 with no vitamin K supplementation (Sutor 2003, Manco-Johnson 2005). The low vitamin K may especially affect the observed high foetal ratios of liver cell vs. plasma concentration of the vitamin K-dependent proteins since vitamin K has been shown to affect the secretion of proteins from liver cells (Jamison et al. 1992). However, although vitamin K levels in newborns rise within hours with supplemented vitamin K, the elevation in the activities of vitamin K-dependent coagulation proteins continues for up to six months. Thus, other contributing factors, in addition to vitamin K deficiency, must be involved. One of these may be the reduced capacity of the neonatal liver to utilize vitamin K (Bovill et al. 1993). Regarding other post-translational modifications, no differences between newborn and adult coagulation factor proteins have been reported, although for example protein C (PC) and some components of the fibrinolytic system may circulate in foetal form (Francis and Armstrong 1982, Greffe et al. 1989, Manco-Johnson et al. 1995).

Consumption as a significant mechanism for low coagulation factor levels in newborns is not likely. During birth the coagulation system is activated (Suarez et al. 1985, Lao et al. 1990), but levels of coagulation factors are upregulated rather than consumed since the foetal levels of coagulation factors are consistently lower than in preterm infants of the same gestational age (Andrew et al. 1988a, Reverdiau-Moalic et al. 1996). The mechanism for the birth-induced upregulation is unclear; a corticosteroid burst before the onset of labor may play a role since increases in factors II, V, VII and X have been observed in pregnant ewes after β-methasone treatment (Kisker et al. 1983).

Table 1. Levels of coagulation factors, physiological anticoagulants and components of the fibrinolytic system in newborn infants relative to adult levels (100%) on postnatal day 1 and days 3-5. The data are adapted from Andrew et al. (1987, 1990b) and Monagle et al. (2006). The levels of activated protein C were measured from cord plasma by Petäjä et al. (1998)*. The ratio of free, active protein S to total protein S was measured in cord plasma by Petäjä et al. (1998) with the adult ratio of free PS/total PS being 0.54**. TFPI was measured from cord plasma by Reverdiau-Moalic et al. (1996)***. Thrombomodulin (TM) was measured from cord plasma by Menashi et al. (1999)^. Thrombin-activatable fibrinolysis inhibitor (TAFI) was measured from cord plasma by Smith et al. (2003)^^. NA = not available; t-PA = tissue plasminogen activator; PAI-1 = plasminogen activator inhibitor-1.

	Day 1	***Days 3-5***
Coagulation factors		
Prothrombin	*50 %*	*60 %*
FV	*70-80%*	*100-120%*
FVII	*60 %*	*70-90%*
FVIII	*100-115%*	*90-100%*
FIX	*50 %*	*50-70%*
FX	*40-50%*	*50-60%*
FXI	*30-40%*	*60 %*
VWF	*170 %*	*160 %*
Anticoagulants		
Antithrombin	*60-70%*	*70 %*
Protein C	*30 %*	*40 %*
Activated protein C	*160%**	*NA*
Protein S	*35 %*	*50 %*
Free/Total protein S	*1.0***	*NA*
TFPI	*50%****	*NA*
Alpha-2M	*160 %*	*170 %*
TM	*200%^*	*NA*
Fibrin and fibrinolysis		
Fibrinogen	*100 %*	*100 %*
Plasminogen	*50 %*	*55 %*
t-PA	*170 %*	*80 %*
PAI-1	*150 %*	*70 %*
TAFI	*30%^^*	*NA*
FXIII	*80 %*	*100 %*

1.1.4 Regulation of thrombin formation by physiological anticoagulant pathways

Three major anticoagulant systems in plasma, the tissue factor pathway inhibitor (TFPI), antithrombin (AT), and protein C (PC) anticoagulant pathways, regulate the formation of thrombin (Figure 2). Thrombomodulin (TM), protein S (PS), and endothelial protein C receptor are essential constituents of the PC pathway (Figure 3).

TFPI is primarily synthesized by the endothelial cells (Bajaj et al. 1990). TFPI binds FXa to inhibit its activity, and the TFPI-FXa complex subsequently inhibits the complex of TF and FVIIa (Broze et al. 1988). The inhibitory effect on the direct activation of FX by TF-FVIIa is rapid and shifts the primary responsibility of the FX activation to the complex of FIXa-FVIIIa. Most of the circulating TFPI is associated with plasma lipoproteins; a small portion of TFPI circulates free. Platelets also carry TFPI and release it upon stimulation by thrombin or other platelet agonists (Novotny et al. 1988). Part of the TFPI pool is associated with glycosaminoglycans in the endothelium and can be released by heparin (Sandset et al. 1988).

Antithrombin (AT) is a major inhibitor of thrombin, FIXa, FXa, FXIa and FXIIa. Thrombin and FXa apparently are the most important targets for AT (reviewed by Huntington 2003). AT by itself reacts inefficiently with the coagulation factors, and its proper activity requires the presence of heparin or heparin-like glycosaminoglycans lining the endothelium (Marcum

et al. 1984). Clot-bound thrombin and FXa are protected from inhibition by AT (Weitz et al. 1990, Rezaie 2001), and thus, the physiological role of AT is to limit the coagulation activity to appropriate sites and to prevent it from spreading throughout the circulation.

Protein C (PC) is a liver-derived vitamin K-dependent protein. PC is activated by thrombin in the presence of thrombomodulin on intact endothelial cells (Esmon and Owen 1981) (Figure 3). APC proteolytically inactivates FVa and FVIIIa as well as promotes fibrinolysis by inactivation of plasminogen activator inhibitor-1 (Sakata et al. 1986). PS serves as a non-enzymatic cofactor to the anticoagulant activity of APC. PS is also a vitamin K-dependent protein. Approximately 60% of plasma PS in adults is bound to C4b-binding protein (reviewed by Rezende et al. 2004); only the free form is functionally active. One mechanism by which PS is able to enhance the activity of APC is to facilitate the affinity of APC to phospholipid membranes (Walker 1980). PS also enhances the cleavage of FVa by APC, but the efficient inactivation of FVIIIa requires the anticoagulant form of FV in addition to APC and PS (Shen and Dahlbäck 1994). The formation of this anticoagulant cofactor form of FV is derived by the cleavage of intact FV by APC (Thorelli et al. 1999).

Thrombomodulin (TM) is a membrane protein that is primarily expressed in endothelial cells. TM binds to thrombin with high affinity to prevent its procoagulant activities (Esmon et al. 1982) and to shift thrombin to express its anticoagulant activity. The binding of thrombin to TM increases protein C activation to APC over 1000-fold. Endothelial protein C receptor is yet another protein involved in the activation of protein C. Endothelial protein C receptor further enhances the activation of PC by the thrombin-thrombomodulin complex (Stearns-Kurosawa et al. 1996).

All of the primary pathways of coagulation inhibition are essential to life. Human deficiency of TFPI has not been characterized. It may not be compatible with life since mice lacking TFPI die as embryos (Huang et al. 1997). The importance of AT is demonstrated by thrombotic complications observed in inherited deficiency of AT (Mateo et al. 1998) and by embryonic lethality of mice lacking AT (Ishiguro et al. 2000). Inherited heterozygous deficiencies of PC and PS increase the risk of thrombosis (Griffin et al. 1981, Comp et al. 1984); homozygous deficiencies of these proteins result in neonatal purpura fulminans, which is fatal if untreated (Marlar and Neumann 1990). Similarly, mice lacking the PC gene experience lethal consumptive coagulopathy perinatally (Jalbert et al. 1998). Lack of TM or endothelial protein C receptor are both associated with embryonic lethality (Healy et al. 1998, Gu et al. 2000).

1.1.5 Neonatal physiological anticoagulant pathways

Similar to the coagulation factors, activities of most of the inhibitors of coagulation are diminished at birth (Andrew et al. 1987, Monagle et al. 2006) (Figure 2, Table 1). Anticoagulant alpha-2-macroglobulin is increased at birth and probably plays a more

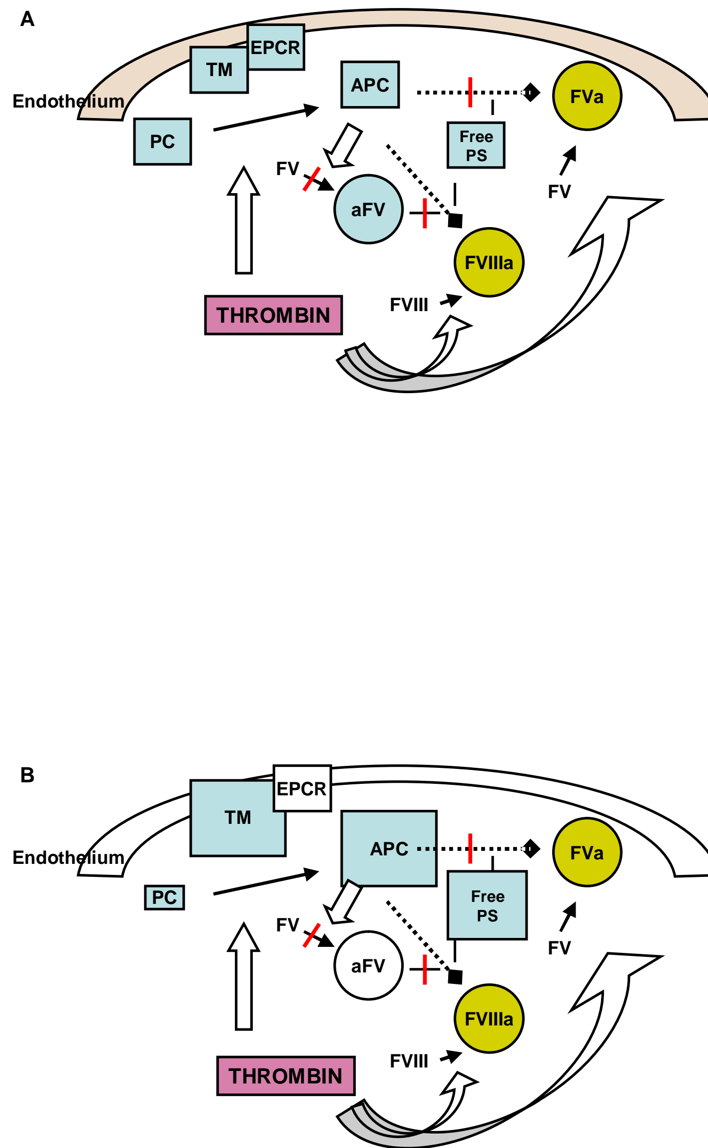


Figure 3. Protein C (PC) anticoagulant pathway. Thrombin activates PC in the presence of thrombomodulin (TM) and endothelial protein C receptor (EPCR) on the surface of the intact endothelial cells. Activated protein C (APC) inhibits the activities of FVa and FVIIIa as well as cleaves intact FV to yield an anticoagulant form of FV (aFV), which acts as a cofactor in the inactivation of FVIIIa. Activities that are deficient in the presence of Arg506Gln mutation of coagulation factor V (Factor V Leiden) are represented by red lines. In the newborn (B), despite the low level of PC, APC is increased when compared with adults. TM is also upregulated and most of the protein S is in free, active form at birth. The size of the symbol reflects the presumed relative neonate vs. adult difference. The contributions of EPCR and the anticoagulant form of FV in newborns are unclear (uncoloured symbols).

significant role in newborn infants than in adults (Schmidt et al. 1989a). The mechanisms, that contribute to decreased coagulation factor levels probably also contribute to decreased anticoagulant levels. A higher ratio of liver protein concentration to plasma protein concentration in the foetus than in adults has been observed for PC and PS, suggesting decreased secretion of these proteins from the liver (Manco-Johnson et al. 2002). This and other possible effects of vitamin K are relevant when considering the vitamin K-dependent anticoagulant proteins PC and PS. In addition, foetal PC shows unique glycosylation patterns, but the physiological significance of this observation is unclear (Manco-Johnson et al. 1995). Regarding AT, accelerated clearance in neonates has been observed (Schmidt et al. 1984).

During the foetal period, the total antigen levels of TFPI are constantly 30% of adult levels and at birth 40-50% of adult levels (Reverdiau-Moalic et al. 1996, Cvirn et al. 2003b). TFPI anticoagulant activity has been described to be 60% of adult levels in cord plasma (Cvirn et al. 2003a). The activity of AT gradually increases during the foetal period from 20% of adult levels at gestational weeks 19-23 to 40% at gestational weeks 30-38 (Reveridau-Moalic et al. 1996), finally reaching 60-70% of adult activity at birth (Andrew et al. 1987, Monagle et al. 2006). Healthy premature infants show levels around 40% (Andrew et al. 1988a). Adult activities are reached at three months of age in term infants and by six months of age in premature infants (Andrew et al. 1988a, Salonvaara et al. 2004). Despite the low levels of TFPI and AT, their functional activity *in vivo* may be upregulated: the mass of the heparane sulphates lining the endothelium and enhancing the anticoagulant effects of both TFPI and AT has been reported to be increased in the inferior vena cava of newborn pups when compared with adult rabbits (Nitschmann et al. 1998).

Differences between the newborn infant and adults regarding the PC pathway are numerous (Figures 2 and 3). PC antigen levels and activity are very low during foetal life, with only 15% of adult activity reached in the most mature foetuses (Reverdiau-Moalic et al. 1996). At birth, PC is at 30-40% the adult levels in healthy term infants and slightly lower in healthy premature infants (Andrew et al. 1987 and 1988a, Monagle et al. 2006). Also, a foetal form of PC has been described, but its functional significance remains unclear (Greffe et al. 1989, Manco-Johnson et al. 1995). Despite the low levels of PC, APC in cord plasma is higher than in adults, suggesting an actual upregulation of the PC pathway at birth (Petäjä et al. 1998). PS in healthy term infants is 35-50% of adult levels, and at six months, 80-90% of adult levels are reached (Andrew et al. 1987 and 1988a, Monagle et al. 2006). The levels of C4b-binding protein are proportionally even lower at birth. Thus, nearly all protein S in the neonate is in the free, functional form (Malm et al. 1988, Petäjä et al. 1998).

Although TM is a membrane protein, its plasma levels can also be measured. In general, soluble TM is considered to be a marker of endothelial damage or stress (Takano et al. 1990). Foetuses show high plasma levels of TM with a peak at gestational weeks 23-26 (Menashi et al. 1999), at which time TM levels are threefold those of adults. Thereafter, TM gradually decreases and at birth the levels are twice that in the adults. Thereafter TM levels decrease but remain elevated throughout childhood (Menashi et al. 1999). Since TM levels are high in

healthy children, the elevation in foetuses, neonates and children apparently does not reflect endothelial damage, but may instead reflect the upregulation of the PC pathway.

Alpha-2-macroglobulin (alpha-2-M) is a proteinase inhibitor that can react with a variety of proteinases, e.g. FXa and thrombin. The biological role of alpha-2-M in adults is unclear, but in newborns and older children it may be a relevant anticoagulant partly balancing the low AT in thrombin inhibition (Schmidt et al. 1989a, Mitchell et al. 1991). Alpha-2-M is at levels 1.5 times adult levels at birth, with levels increasing to twice the adult levels by six months of age (Andrew et al. 1987).

1.2 Thrombin

Thrombin is the key enzyme in the coagulation system. The formation of thrombin is tightly regulated, and it exerts a number of effects on coagulation and beyond. Thrombin activates coagulation factors V, VIII, XI, XIII, and platelets, cleaves fibrinogen into fibrin, inhibits fibrinolysis, activates the PC pathway and amplifies the inflammatory response via activation of protease-activated receptors on the cell surfaces (Figure 4).

1.2.1 Measurement of thrombin

Thrombin formation in plasma can be measured by the thrombin generation potential in plasma *in vitro* after the activating stimulus from TF, or by measuring the *in vivo* markers of thrombin generation, inhibition and activity. The activation of plasma by TF produces a characteristic pattern of thrombin generation potential in time. During an initiation phase or the lag time small amounts of thrombin are formed. This is followed by a propagation phase, a thrombin burst, which reaches its peak and afterwards declines to the baseline upon the activation of inhibitors. The markers of thrombin generation *in vivo*, on the other hand, reflect the ongoing state of thrombin generation, inhibition and activity in plasma. These include prothrombin fragment F1+2, which is released upon activation of prothrombin to thrombin, thrombin-antithrombin complexes, which reflect the generated thrombin and its inhibition, and D-dimer, which reflects thrombin activity towards fibrin and the subsequent fibrinolysis (Figure 5).

1.2.2 Relevance of individual procoagulants and anticoagulants in the formation of thrombin

The formation of thrombin after the stimulus by TF can be described as occurring in two phases, the initiation phase and the propagation phase (Hoffmann and Monroe 2001, Mann et al. 2003). During the initiation phase the FVIIa-TF complex forms tiny amounts of FIXa and FXa, and further small amounts of thrombin are formed. During this phase, thrombin activates platelets, FV and FXIII, and starts to activate fibrinogen to fibrin (Brummel et al. 2002). At the end of the initiation phase, some clot formation is already observed, although 96% of

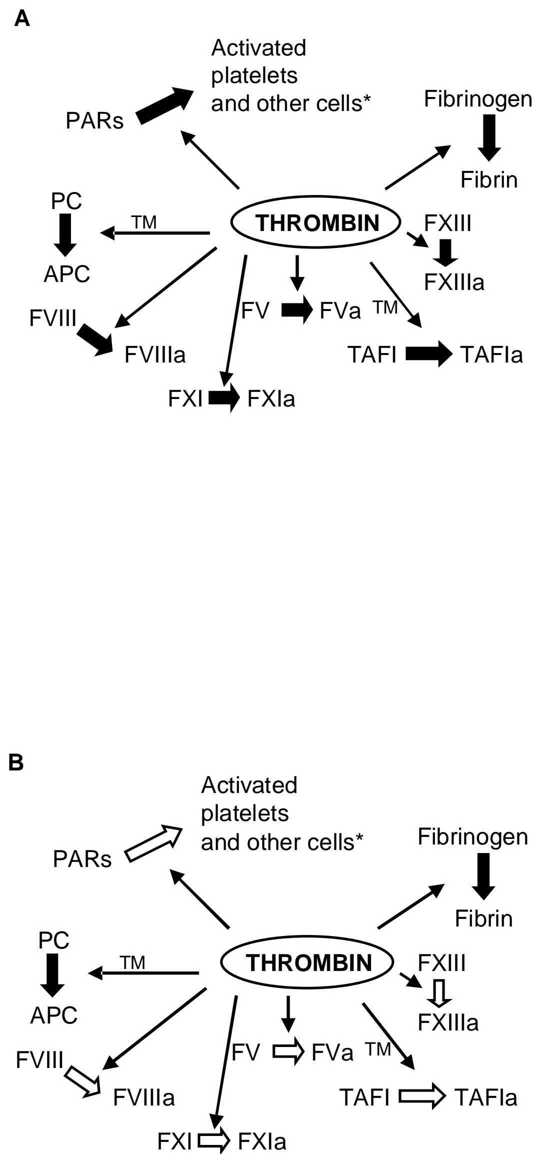


Figure 4. Functions of thrombin. In addition to platelets, thrombin activates protease-activated receptors (PARs) at least on the surfaces of endothelial cells and on smooth muscle cells*. Activation of PARs on these cells can promote e.g. inflammatory reactions. In (B) the white arrow indicates a lack of knowledge about how thrombin functions in the particular reaction in newborns. Thrombin is known to effectively activate protein C (PC) in newborns (black arrow). Thrombin-mediated activation of fibrinogen to fibrin is known to be decreased in newborns (black arrow). TAFI=thrombin-activatable fibrinolysis inhibitor. Figure (A) is adapted from a review by Huntington (2005).

thrombin is formed after the initiation phase. At the beginning of the propagation phase, TFPI downregulates the formation of FXa by TF-FVIIa to the extent that the responsibility of primary activation of FXa is switched to the FVIIIa-FIXa complex. TFPI, AT and the PC pathway synergistically attenuate thrombin generation. TFPI alone primarily regulates the initiation phase, AT in turn the propagation phase, but together, in a reconstituted plasma system, TFPI and AT provide ≈70-fold more efficient inhibition of thrombin than expected from their separate efficacies (van´t Veer et al. 1997a). In a reconstituted plasma system, activity of the PC pathway alone in diminishing thrombin generation is observed only during the propagation phase, but, together with TFPI, they can obliterate thrombin generation completely (van´t Veer et al. 1997b).

The influences of various coagulation factors and anticoagulants on thrombin generation potential have been studied in a reconstituted plasma system after the initiation of coagulation

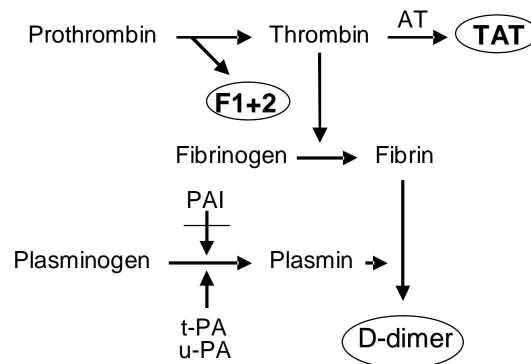


Figure 5. *In vivo* markers of thrombin generation: Prothrombin fragment F1+2, thrombin-antithrombin complexes (TAT) and D-dimer. PAI = plasminogen activator inhibitor; t-PA = tissue plasminogen activator; u-PA = urokinase plasminogen activator.

by 5 pM tissue factor (Butenas et al. 1999) as well as in patients with the respective factor deficiencies (Al Dieri et al. 2002). In the reconstituted plasma system, the alterations of prothrombin and antithrombin from 50% to 150% of normal concentration were the strongest regulators of total thrombin generation, whereas similar alterations in the concentrations of FV, FVII, FVIII, FIX, FX, TFPI, PC and PS affected total thrombin generation only mildly. Of these factors, FV, FVIII, FIX and TFPI had an observable influence on the initiation phase of thrombin generation. In plasma of patients with coagulation factor deficiencies, half-normal thrombin generation potential is observed with 2% of FVII, 5% of FX and 1% of FXI activities. In general, half-normal thrombin generation potential is seen with 10% of FVIII or

FIX activities (Dargaud et al. 2005). In prothrombin deficiency, half-normal ETP is achieved at prothrombin activity of 50%, reflecting the central role of prothrombin in thrombin generation. Similarly, in patients with antithrombin deficiency, at ~50% activity of antithrombin, thrombin generation potential was increased to 150% of normal (Wielders et al. 1997).

When platelets are added to the synthetic model of the coagulation system, prothrombin still remains the main determinant of peak thrombin generation (Allen et al. 2004), platelets apparently mostly affecting the initiation phase and the rate of thrombin generation (Vanschoonbeek et al. 2004).

1.2.3 Thrombin formation in newborns

In newborn infants, the total potential of thrombin generation was decreased to as low as 30% of adult levels when strong activated partial thromboplastin or prothrombin time reagents were used in the initiation of clotting (Schmidt et al. 1989b). However, weaker activation with small amounts of TF is considered to more accurately reflect the physiological situation and is able to better reveal the contribution of the individual coagulation factors and anticoagulants to thrombin generation (Davie et al. 1991, Butenas et al. 1999, Cvirn et al. 2003a). Consequently, when thrombin generation was initiated by low amounts of TF, the thrombin generation potential of neonates was relatively higher and even approached that of adults when the lowest TF concentrations were used (Cvirn et al. 2003a). The onset of thrombin generation was delayed in newborn infants with activated partial thromboplastin and prothrombin time reagents used in the initiation of thrombin generation (Schmidt et al. 1989b). However, thrombin generation started faster in newborns than in adults with low amounts of TF (Cvirn et al. 2003a).

In accordance with the observations in adults (Butenas et al. 1999), the total thrombin generated in newborn plasma is directly dependent on prothrombin concentration (Andrew et al. 1990a). Other procoagulants, FVII, FIX, and FX, only affected the rate of thrombin generation together with prothrombin (Andrew et al. 1990a). Similarly, recombinant factor VIIa added to cord plasma shortened the lag time to thrombin generation but had no effect on peak thrombin levels (Streif et al. 2000). The faster initiation of thrombin generation in neonates has been attributed to the low levels of inhibitors TFPI and AT (Cvirn et al. 2003a) since the increase in TFPI or AT, or both, to adult levels in newborn plasma prolongs the lag times to the beginning of the thrombin burst. Thus, thrombin generation in newborns is clinically sufficient since low prothrombin activities are balanced by the low anticoagulant activities, which allow rapid initiation of thrombin generation.

PC exerts characteristic effects on thrombin generation in newborn plasma. When exogenous TM is added to induce the activation of endogenous PC or when exogenous recombinant human APC is added to newborn plasma, the ability of APC to suppress thrombin generation

has been significantly impaired in newborns when compared with adults (Cvirn et al. 2003b and 2004).
The results on the role of alpha-2-M in thrombin inhibition in newborns are somewhat discrepant. Alpha-2-M has been shown to inhibit a larger fraction of thrombin in neonatal plasma than in adult plasma (Schmidt et al. 1989a), but in another study the increasing levels of alpha-2-M did not affect thrombin generation in neonates or in adults (Cvirn et al. 2001). Also, alpha-2-M has been proposed to exert procoagulant activity in newborns by inhibiting the complexation of APC and PS (Cvirn et al. 2002).

When thrombin generation was estimated by mathematical simulation, a slightly longer initiation phase to thrombin generation and a slightly suppressed maximum thrombin level in newborns compared with adults were suggested (Butenas et al. 2002). Thrombin generation initiated by low levels of TF in synthetic newborn plasma supplemented with platelets of unknown origin was faster in newborn than adult plasma (Butenas et al. 2002).

1.2.4 Formation of thrombin *in vivo*

In blood of healthy subjects, the levels of thrombin generation markers F1+2, TAT and D-dimer remain relatively constant, indicating low constitutive thrombin formation, inhibition and activity. Reference ranges for these markers have been defined, and elevated concentrations have been observed in for example at prothrombotic states (Bauer et al. 1985, Zöller et al. 1996) and in association with thromboembolic complications (Kornberg et al. 1992). Since one molecule of F1+2 is formed upon generation of one molecule of thrombin, it can be considered the most specific marker of thrombin generation. TAT, on the other hand, reflects both thrombin generation and inhibition. D-dimer as a marker of thrombin generation and activation is more complicated. Though fibrinogen is one of the most important targets for thrombin in plasma due to its high concentration and a rapid reaction rate between thrombin and fibrinogen (Higgins et al. 1983), the formation of D-dimer is also affected by fibrinolysis.

In vivo thrombin generation, measured as F1+2 in shed blood from capillary wounds, has been found to be stronger in healthy neonates than in adults, suggesting an adequate response to injury by the newborn coagulation system (Muntean et al. 2004). TAT formation was, however, stronger in adults, indicating a proportionally lower anticoagulant capacity of antithrombin in healthy neonatal plasma.

1.3 Functions of thrombin in fibrin formation and fibrinolysis

As a result of the activation of the coagulation system leading to thrombin formation, fibrin is formed from fibrinogen. The degradation of fibrin is controlled by the fibrinolytic system. In addition to forming fibrin from fibrinogen, thrombin also regulates fibrinolysis and, reciprocally, fibrin regulates the activity of thrombin (Figure 6).

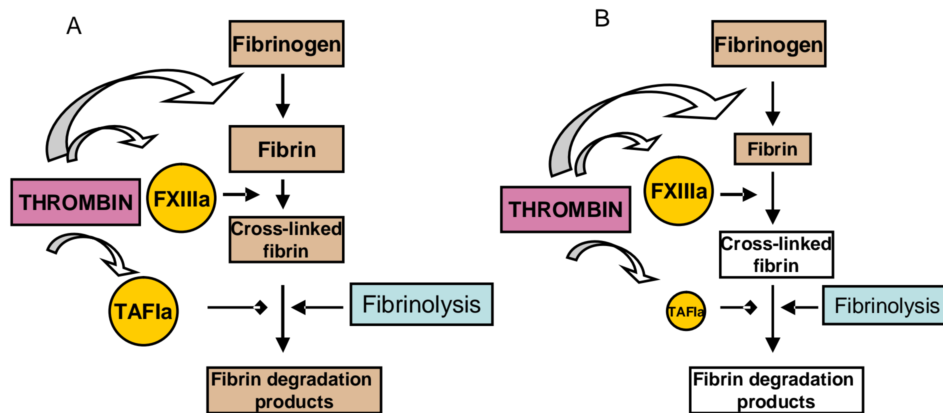


Figure 6. Activities of thrombin in fibrin formation and fibrinolysis. Thrombin activates fibrinogen to fibrin, stabilizes the fibrin net by activating FXIII and inhibits fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor (TAFI). Thrombin-mediated fibrinogen polymerization to fibrin is decreased in newborns (B). FXIII is at low adult levels at birth (Andrew et al. 1987), and TAFI is at 30% of adult activities (Smith et al. 2003). Despite the lower activation of plasminogen to plasmin, fibrinolytic activity apparently is adequate in newborns, probably contributed by the decreased inactivation of plasmin. The size of the symbol reflects the presumed relative neonate vs. adult difference. The contributions of FXIIIa and TAFIa in the formation of cross-linked fibrin and in the inhibition of fibrinolysis, respectively, are unclear in newborns.

1.3.1 Thrombin and fibrin

Fibrinogen is a large elongated molecule, that is cleaved by thrombin to yield fibrinopeptides A and B and polymerizing fibrin. The concentration of fibrinogen in adult plasma is high, ~10 µmol/l, making it the main substrate for thrombin. Upon activation, fibrin molecules initially form double-stranded fibrils, which are subsequently cross-linked with the help of FXIII to yield a stable fibrin net (reviewed by Mosesson 2005). Thrombin activates FXIII, with fibrin acting as a cofactor in the reaction by accelerating the activation of FXIII 80-fold (Janus et al. 1983). The functions of fibrin include the formation of an insoluble net at the site of injury and interactions with platelets, especially in promoting platelet aggregation via platelet surface receptor GPIIb/IIIa (reviewed by Bennett 2001). Binding of thrombin to fibrin also downregulates thrombin activity in clotting blood, and since fibrin-bound thrombin retains its proteolytic capacity fibrin localizes thrombin activity at the site of injury. The significance of thrombin binding to fibrinogen is reflected in certain dys-, hypo- and afibrinogenemias, which are paradoxically associated with thromboembolic complications and increased coagulation system activation (Mosesson 2005).

Fibrin is degraded to soluble fibrin degradation products, including D-dimer, by plasmin (reviewed by Lijnen 2001). Plasmin is activated from its precursor plasminogen by tissue-type plasminogen activator (t-PA) or by urokinase plasminogen activator (u-PA). Activation of plasminogen is greatly enhanced by binding of plasminogen and t-PA to fibrin (Hoylaerts et al. 1982). Plasminogen activation is regulated by plasminogen activator inhibitors (PAIs), PAI-1 being the most important (reviewed by Cesarman-Maus and Hajjar 2005). Thrombin regulates fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor (reviewed by Nesheim 2003). Thrombin-activatable fibrinolysis inhibitor is activated by thrombomodulin-bound thrombin and inhibits fibrinolysis by modifying fibrin to prevent the binding of plasminogen and t-PA to fibrin.

The regulation of fibrinolysis is important in preventing thromboembolic complications, but unlike the absence of the coagulation proteins, deletions of constituents of the fibrinolytic system are apparently compatible with life. Data derived from gene deletions in mouse models reveal a clinical picture from an essentially normal phenotype to spontaneous thromboses, impaired clot lysis and organ fibrin deposition, with the most serious phenotype related to the inactivation of plasminogen (Cesarman-Maus and Hajjar 2005).

1.3.2 Fibrin formation and fibrinolysis in newborns

Fibrinogen can be observed in the liver of five-week-old embryos (Hassan et al. 1990). The levels of fibrinogen in foetuses gradually increase during pregnancy, and at birth the antigenic levels of fibrinogen are near adult levels (Reverdiau-Moalic et al. 1996). The ability of fibrinogen to polymerize after activation by thrombin seems, however, lower than in adults (Reverdiau-Moalic et al. 1996). The existence of foetal fibrinogen with higher sialic acid contents has, indeed, been described (Francis and Armstrong 1982).

Fibrinolytic activity is observed in the foetus after the gestation week 10 (Manco-Johnson 2005). At birth, plasminogen is at 50-70% of adult levels, and as for fibrinogen, a foetal form for plasminogen exists (Edelberg et al. 1990). Foetal plasminogen has increased contents of sialic acid, its activation by t-PA is decreased, and its cell surface binding is altered. The regulators of plasminogen also differ significantly between newborn and adult plasma. In the plasma of newborns during the first days of life t-PA and PAI-1 are both increased (Andrew et al. 1990b). Their concentrations subsequently decrease to adult levels during the first month of life. The antigen level of thrombin-activatable fibrinolysis inhibitor in cord plasma is 30% of that in adults (Smith et al. 2003).

All in all, the newborn fibrinolytic system shows many unique features, with the most significant probably being the increased glycosylation of fibrinogen and various fibrinolytic proteins giving rise to foetal forms of the respective proteins. Despite the lower activation of plasminogen and its decreased activity, fibrinolytic activity seems to be clinically adequate in newborns, probably due to the decreased activation of fibrinogen and the decreased inactivation of plasmin (Ries et al. 2002).

1.4 Thrombin and platelets

Platelet activation occurs with coagulation system activation, and the processes are mutually dependent. Thrombin is a potent agonist of platelets, and platelets in turn provide procoagulant surfaces for the coagulation reactions, and provide coagulation factors. Platelet interactions with collagen via VWF and interactions with fibrin form a haemostatic plug at the site of vascular injury.

1.4.1 Activation of platelets by thrombin

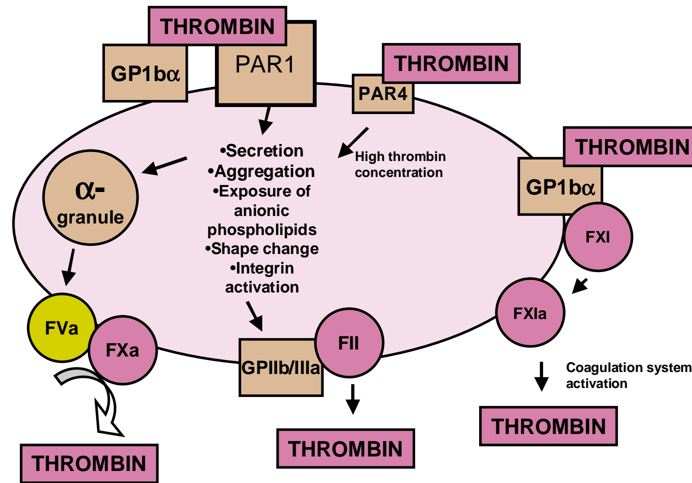
Activation of platelets by thrombin in humans is mediated by protease-activated receptors (PARs) 1 and 4, which are surface receptors on certain cell membranes that mediate the intracellular effects of coagulation factors and other proteases (Hung et al. 1992). PAR1 is likely more important in platelet activation. Thrombin also binds to surface receptor glycoprotein Ibα on the surface of platelets. Glycoprotein Ibα may localize thrombin to the platelet surface, supporting the thrombin-mediated activation of PARs (Coughlin 2005). Of the multiple functions of platelets, thrombin apparently mainly affects the release of the contents of α- and dense granules and promotes platelet aggregation (Heemskerk et al. 2002, Coughlin 2005), but also contributes to the platelet shape change, the activation of integrin receptors on the platelet surface and the exposure of anionic phospholipids on the platelet surface.

1.4.2 Platelets and coagulation reactions

Platelet membrane is the main provider of the phospholipids required in the coagulation reactions. In an unactivated state, platelets do not express significant amounts of the required negatively charged phospholipids (mainly phosphatidylserine) on their outer surface, instead those are located in the inner leaflet of the plasma membrane. During translocation of these anionic phospholipids to the platelet surface, platelets also change shape and procoagulant phosphatidylserine-containing blebs are formed (Heemskerk et al. 2002). Platelet adhesion to collagen through platelet surface glycoproteins is a potent activator of both, exposure of anionic phospholipids and bleb formation (Heemskerk et al. 1997).

Platelet membrane regulates the coagulation processes not only by providing the negatively charged phospholipid surface but also by actions of the non-lipid surface receptors. Some yet controversial evidence exists of non-lipid receptor-binding sites for all components of the tenase complex, FVIII, FIX and FX (Hoffman and Monroe 2001, Heemskerk et al. 2002). Additionally, prothrombin can bind platelet surface receptor GPIIb/IIIa (integrin αIIbβ3) independently of phospholipids (Byzova & Plow 1997), and blocking this receptor results in delayed prothrombin activation by FXa (Butenas et al. 2001). Activation of FXI by thrombin is also facilitated by binding of FXI to the platelet surface receptor GP1b complex (Baglia et al. 2002).

A



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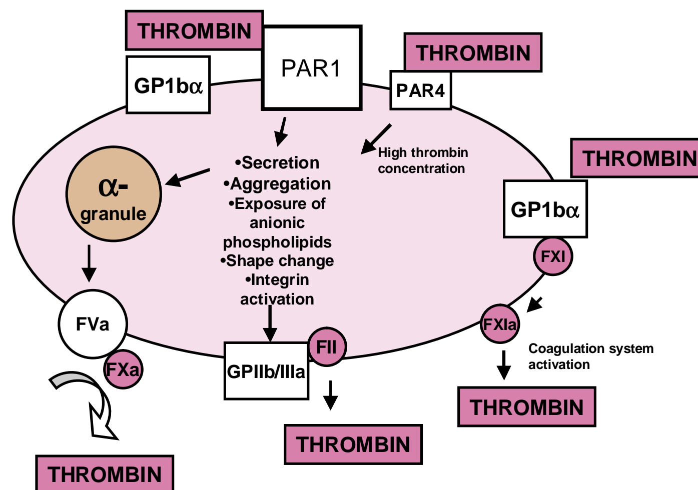

Figure 7. Interactions between platelets and thrombin. Thrombin activates platelets via activation of PARs (mainly PAR1). Glycoprotein Ibα (GP1bα) possibly supports the thrombin-mediated activation of PARs. Thrombin mainly affects the secretion and aggregation of platelets. Prothrombin binds to glycoprotein IIb/IIIa (integrin αIIbβ3) and can be activated to thrombin. Thrombin also activates FXI on the surface of platelets in the presence of GP1bα, to produce more thrombin. Platelets provide phospholipid surfaces for coagulation reactions and secrete FVa. The role of PARs and glycoprotein receptors in coagulation reactions on newborn platelet surface is unclear (B). The number of α-granules in newborn platelets may be similar to that in adults, but the secretion is decreased; the amount of FV secreted is unknown (B).

Platelets are able to secrete various coagulation factors, anticoagulants and factors of the fibrinolytic system such as FV, FXIII, TFPI, protein S, VWF, fibrinogen, plasminogen activator inhibitor-1 and thrombin-activatable fibrinolysis inhibitor. Importantly, approximately 20% of whole blood FV is stored in platelet α-granules in a partially activated state (Tracy et al. 1982). Platelet FV is essential for normal haemostasis, apparently being even more important than the plasma-derived FV. This is demonstrated as bleeding symptoms in patients with defective platelet FV in association with functional plasma FV (Tracy et al. 1984, Weiss et al. 2001), and on the other hand as normal surgical haemostasis in a patient with antibodies to plasma FV, not affecting platelet FV (Nesheim et al. 1986). There are distinct differences between platelet and plasma FV; platelet FV is more sensitive to activation by FXa (Monkovic et al. 1990b) and more resistant to inactivation by APC (Camire et al. 1998). Platelet FV is derived from plasma, but it has been shown to be modulated by megakaryocytes after endocytosis (Gould et al. 2004).

1.4.3 Thrombin and platelets in newborns

Platelets are found in foetal blood by 11 weeks of gestation and increase to adult levels by 20 weeks of gestation (reviewed by Manco-Johnson 2005). Ultrastructure and platelet counts in newborn infants do not differ from those in adults but functional differences exist.

Platelet membrane offers similar phospholipid procoagulant activity in newborns and adults when activated by thrombin but less activity when stimulated by collagen (Israels et al. 2002). The procoagulant activity measured as FV/FVa binding to platelets is similar in term neonates and adults, but is significantly less in preterm neonates, who have lower FV levels (Michelson et al. 2000). Thus, the limiting factor in the platelet procoagulant activity may be the restricted amount of coagulation factors.

The possible functions of non-lipid binding sites for coagulation proteins in newborn infants are unclear. Platelet surface receptors, such as GP1b complex and GPIIb/IIIa, which have been suggested to be involved in the interplay of platelets and thrombin, are present in foetal and cord blood platelets at similar levels as in adults (Gruel et al. 1986, reviewed by Israels et al. 2003), but their number following stimulation by agonists may be lower than in adults (Rajasekhar et al. 1994). This may contribute to the ability of the neonatal platelets to enhance coagulation reactions.

Whether differences in platelet-derived coagulation factors exist between neonates and adults is currently unknown. Stimulated secretion of platelet granule contents is decreased in term and preterm neonates (Rajasekhar et al. 1994), and this may affect for example the activity of FV in prothrombinase complex.

Although there may be limitations in neonatal platelet function regarding the procoagulant activity of platelets, the deposition of platelets to the subendothelium is enhanced (Shenkman et al. 1999, Rehak et al. 2004), bleeding time is shorter (Feusner et al. 1980) and the platelet

function analyzer PFA-100® shows shorter clotting times in newborns than in adults (Israels et al. 2000). All of these effects have been attributed to increased VWF activity in neonates.

2 Haemostasis in sick newborn infants

In sick neonates, the capacities to compensate challenges to the developing coagulation system may be limited. These challenges are provided by intensive care along with various disease states such as prematurity, respiratory distress syndrome (RDS), sepsis and asphyxia. In addition, major surgery in neonates may present with bleeding and thrombotic complications; among the most challenging is the management of coagulation during and after neonatal cardiopulmonary bypass (CPB). The effects of various transfusion therapies and haemostatic agents may differ in newborns when compared with adults. Further, newborn infants show the highest incidence of thromboembolic complications among all children.

2.1 Neonatal intensive care and the coagulation system

In neonates undergoing intensive care, the coagulation and fibrinolytic systems are activated; increased coagulation activation markers are observed in the majority of these patients (Schmidt et al. 1993). Thrombocytopenia also occurs frequently in neonatal intensive care patients. Certain disease states affect coagulation in a unique manner, and the state of the coagulation system may in turn influence the occurrence and course of certain diseases. The predominant disease states in neonates that are associated closely with alterations in the coagulation system are RDS, intraventricular haemorrhage (IVH), septic infections and asphyxia.

2.1.1 Respiratory distress syndrome

The most common disease leading to neonatal intensive care is RDS. Deficient synthesis and production of alveolar surfactant lies behind RDS, and the disease is characterized by intrapulmonary hyaline membrane formation in which fibrin is a major constituent (Gitlin and Graig 1956). Thus, especially the generation of thrombin and its inhibition by AT in neonatal RDS has been studied. During RDS, the markers of thrombin generation are increased (Schmidt et al. 1992). The activity of AT is decreased compared with age-matched premature infants who are otherwise clinically healthy (Shah et al. 1992), and further, the activity of AT is significantly lower in more severe RDS than in milder RDS (Schmidt et al. 1992). Despite the low neonatal prothrombin, the low anticoagulant capacity allows similar thrombin generation in sick newborns with RDS and in healthy newborns (Shah et al. 1992). To treat this apparent deficient ability of newborn plasma to inhibit thrombin during RDS, therapy with AT concentrate has been tested (Muntean et al. 1989, Schmidt et al. 1998). Although

thrombin generation measured as F1+2 was decreased by AT concentrate, no clinical benefit was observed (Schmidt et al. 1998).

2.1.2 Sepsis

Sepsis results from a generalized response of host inflammatory and coagulation pathways to an infection. These pathways are closely intertwined (reviewed by Esmon 2003). Thus, as a consequence of coagulation system activation, thrombin exerts its effects on micro- and macrovascular fibrin deposition as well as by exacerbating ongoing inflammation.While coagulation activation during sepsis leads to generalized consumption of pro- and anticoagulants, certain characteristic features are observed, the most distinct of which is a significant decrease in protein C (PC). The decrease in PC is observed in adult, paediatric and neonatal patients during sepsis (Roman et al. 1992, Fijnvandraat et al. 1995, Barton et al. 2004). Decreased PC is associated with increased mortality (Fijnvandraat et al. 1995), and thus, the substitution therapy of protein C in sepsis has been evaluated. Recombinant APC has reduced mortality in adult patients (Bernard et al. 2001), but its efficacy in the paediatric population is unclear, with a possible increased risk for central nervous system bleeding (Kylat and Ohlsson 2006). Thus, although PC concentrate is believed to be beneficial in neonatal sepsis (Kreutz et al. 1999), the roles of both recombinant human APC, and PC concentrate remain obscure in neonatal sepsis (Kylat and Ohlsson 2006).

2.1.3 Asphyxia

Asphyxia is one of the most common risk factors for neonatal thrombosis (Nowak-Göttl et al. 1997), but the mechanisms behind this prothrombotic state are unclear. Asphyxiated neonates show lower levels of coagulation factors (Salonvaara et al. 2003) but also lower levels of anticoagulants (El Beslawy et al. 2004) than non-asphyxiated infants. TM is also increased in asphyxiated infants, suggesting endothelial damage (Nako et al. 1997). In lambs, increased thrombin generation is observed after birth asphyxia (Andrew et al. 1988c).

2.1.4 Intraventricular haemorrhage

The prevalence of IVH in neonates weighing less than 1500 g is 15-20% and decreases with increasing gestational age. Severity of IVH is expressed by grades I-IV with better neurodevelopmental prognosis in grades I-II (Papile et al. 1978). Risk factors commonly associated with IVH include increased fragility of the vascular bed in the germinal matrix, lack of support around blood vessels, lack of muscles and type VI collagen around large capillaries, fluctuations in blood flow, increased cerebral venous pressure and increased vulnerability to hypoxic-ischaemic reperfusion injury. The role of coagulation disturbances as a risk factor for IVH has been evaluated. The question remains whether increased bleeding tendency or increased prothrombotic tendency contribute to IVH. The risk of IVH has been increased by prothrombotic FVL mutation (Petäjä et al. 2001). On the other hand, another study reported the extension of IVH grade I to be prevented by the presence of FVL or prothrombin G20210A mutation (Göpel et al. 2001). A low prothrombin level at birth has also

been associated with IVH (Salonvaara et al. 2005). Thus, therapies supplementing procoagulant activity (recombinant FVIIa), anticoagulant activity (AT concentrate), or both (FFP) have been tested in the prevention of IVH. With prophylactic FVIIa, a similar prevalence of IVH was observed as in previous studies on IVH (Veldman et al. 2006). AT concentrate seemed to lower the incidence of IVH compared with the prevalence in a similar population (Brandenberg et al. 1997). One study reported FFP to be effective in preventing IVH (Beverley et al. 1985), but no effect was observed on the incidence of IVH by FFP in a large multicentre study (Northern Neonatal Nursing Initiative Group 1996).

2.2 Blood component transfusions and coagulation in newborns

Newborn infants undergoing intensive care frequently require transfusions. The most common of these are red blood cell and platelet transfusions (reviewed by Murray and Roberts 2005), followed by transfusion of FFP, which is used in neonatal intensive care and in association with major neonatal surgery. Reconstituted blood, prepared from red blood cells and FFP, is used for exchange transfusion (ET) and can also be used during neonatal open heart surgery.

2.2.1 Red blood cells

Red blood cells are transfused commonly in neonatal intensive care to correct the haematocrit to ensure adequate tissue oxygenation. In generally, red blood cell transfusion is not considered to be associated with significant effects on the coagulation system. Red blood cells have, however, been found to increase the formation of thrombin in adult plasma in *in vitro* studies (Peyrou et al. 1999, Horne et al. 2006). Similar studies on newborn infants are not available.

2.2.2 Platelets

Platelet transfusion is reported to occur in 2-9.4% of all admissions to the neonatal intensive care unit (Murray and Roberts 2005). Platelets are typically transfused in sick newborns at platelet count of $<50 \times 10^9/l$ and in stable newborns at $<20 \times 10^9/l$. The only randomized controlled trial on platelet transfusions in newborn infants showed that platelet transfusion in moderate thrombocytopenia (platelet count 50-100 $\times 10^9/l$) did not affect the bleeding tendency (Andrew et al. 1993).

2.2.3 Fresh-frozen plasma

In addition to the treatment of specific severe coagulation disturbances as disseminated intravascular coagulation, FFP is used as a volume replacement therapy in premature infants with relatively low coagulation factors and anticoagulants (Murray and Roberts 2005). The effects of FFP on the neonatal coagulation system remain, however, largely unknown. *In vitro*, FFP increases thrombin generation in newborn plasma (Andrew et al. 1990a). FFP has

also been shown to shorten prothrombin times, but only in a minority of neonatal patients (Johnson et al. 1982). In older infants, FFP had no effect on *in vivo* thrombin generation measured as TAT and D-dimer (Halton et al. 1994).

Varying effects of FFP on the clinical state of neonates have also been reported. Earlier, a benefit from FFP in the prevention of IVH was suggested (Beverley et al. 1985), but a large multicentre study showed no benefit of prophylactic FFP administered during the first 24 hours of life on survival or on neurodevelopmental outcome of neonates (Northern Neonatal Nursing Initiative Group 1996).

FFP is widely used in neonatal cardiac surgery to prevent dilution of coagulation factors and to replace coagulation factors during the postoperative period. The results regarding the effects of FFP during CPB are also conflicting. FFP, as a component of the CPB circuit prime, limited the dilution of fibrinogen postoperatively and tended to decrease the overall exposure of infants to blood products (McCall et al. 2004). In another study, overall transfusion requirements were higher with FFP in the prime than with albumin in the prime (Oliver et al. 2005). The blood loss was, however, smaller in cyanotic patients and in those undergoing complex operations when FFP was used in the prime (Oliver et al. 2005).

2.2.4 Reconstituted blood

Reconstituted blood is prepared from red blood cells and FFP, and thus, contains no platelets. Nowadays, reconstituted blood is used to perform ET in newborn infants with severe hyperbilirubinemia. Earlier, ET was reported to increase the levels of coagulation factors and physiological anticoagulants to levels corresponding to those measured in infused blood (Nielsen 1970, Barnard et al. 1980). ET has been reported to cause coagulation system-related complications such as thromboses and haemorrhages, and even disseminated intravascular coagulation (Keenan et al. 1985, Jackson 1997, Ramasethu and Luban 2005). Despite these adverse effects, ET has been used to treat neonatal sepsis (Gross et al. 1982, Mathur et al. 1993). Reconstituted blood can also be used in neonatal cardiac surgery to prime the CPB circuit and to meet the transfusion requirements intra- and postoperatively.

2.2.5 Prothrombin complex concentrate

Prothrombin complex concentrate (PCC) is prepared from plasma and contains vitamin K-dependent coagulation factors (FVII, FIX, FX, and prothrombin) as well as PC. Indications for PCC include coagulation factor deficiencies, severe liver disease and reversal of oral anticoagulation. Use of PCC in adults is known to activate the coagulation system and can be associated with thrombotic complications (Köhler 1999). In neonates, knowledge of its effects is limited. However, due to the low volume needed, it has been used to correct haemostasis in neonatal cardiac surgery. One study on PCC in newborns showed a correction of prothrombin time with no side-effects (Turner et al. 1981).

2.3 Factor V Leiden mutation and newborns

Inherited thrombophilias are significant risk factors for thromboses and recurrent pregnancy loss. The most common known inherited thrombophilia is a resistance to activated protein C (APC) caused by Arg506Gln mutation in coagulation factor V (factor V Leiden), with a prevalence rate in general Caucasian populations of 3-15% (reviewed by Dahlbäck 2003).

Poor anticoagulant response to APC in a patient with thrombotic complications was first described by Dahlbäck et al. in 1993. The basis for the defect was found to be a mutation in factor V gene at position 1691 (G-A), inducing a replacement of Arg 506 with Gln, which is a cleavage site for APC (Bertina et al. 1994). FVL promotes thrombotic risk also by another mechanism in addition to the impaired cleavage of FVa by APC (Figure 3). Intact FV acts as a cofactor in APC-dependent inactivation of FVIIIa (Shen et al. 1994), and this cofactor activity is also deficient with FVL mutation (Thorelli et al. 1999). Apparently, the loss of this cofactor activity of FV is a significant mechanism underlying the thrombotic risk exerted by FVL (Castoldi et al. 2004).

Relative risk for the first thrombotic event has been reported to be 3- to 7-fold in heterozygous carriers of FVL and 18- to 80-fold in homozygous carriers compared with non-carriers of the mutation (Rosendaal et al. 1995, Juul et al. 2004). In addition, FVL may be a risk factor for arterial thrombosis in adults; a moderately increased risk of coronary disease in carriers of FVL has been observed (Ye et al. 2006). FVL is also associated with both early and late recurrent foetal loss (Rey et al. 2003).

Thromboembolic complications are quite rare in children. Two large studies have estimated the incidence of venous thromboembolism, with reported incidences of 0.07 and 0.14/10 000 children in Canada and in the Netherlands, respectively (Andrew et al. 1994c, van Ommen et al. 2001). In both studies, predominance in neonates and in children aged less than one year was observed.

Thromboembolic complications in neonates include both arterial and venous thromboses. The incidence of thromboembolic complication has been estimated to be 24/10 000 admissions to the neonatal intensive care unit in Canada (Schmidt and Andrew 1995) and 0.5/10 000 live births in Germany (Nowak-Göttl et al. 1997). In both studies, the vast majority of patients had a predisposing risk factor for thrombosis, often a central venous line. In neonates, thrombotic complications have been reported to be arterial in 25-30% of all thromboses, and further, of all venous thromboses, 30-50% are renal (Schmidt and Andrew 1995, Nowak-Göttl 1997).

The overall prevalence of FVL mutation in children of any age with venous or arterial thromboembolic complication, varies from 14% to 52% (Nowak-Göttl et al. 1996, Hagstrom et al. 1998, Ehrenforth et al. 1999). In neonates, FVL mutation predisposes especially to arterial thromboses and stroke in the central nervous system (Hagstrom et al. 1998, Gunther et

al. 2000). FVL may also be associated with IVH (Petäjä et al. 2001). Regarding neonatal renal venous thrombosis, 20% prevalence of FVL has been observed (Marks et al. 2005), but regarding all venous thromboses in neonates the prevalence of FVL did not differ from the prevalence in the general population (van Ommen et al. 2001).

2.4 Coagulation and cardiopulmonary bypass in newborns

Management of haemostasis during CPB in neonates remains a challenge. During CPB the blood flows from the inferior and superior vena cava to the bypass circuit to return oxygenated to the aorta, passing the heart and the lungs. Characteristic features of neonatal CPB include proportionally large priming volumes of the bypass circuit, exposure of blood to large non-endothelial surfaces, long CPB times, deep hypothermic temperatures and possibly a requirement for total circulatory arrest. As a consequence, the coagulation system becomes significantly deranged. Immediately, at the beginning of CPB, circulating coagulation factors and platelets are significantly diluted (Kern et al. 1992). Exposure to non-endothelial surfaces induces the activation of coagulation and inflammatory systems (Wan et al. 1997). As a result, transfusion requirements and bleeding as well as thrombotic complications are increased in neonates undergoing CPB when compared with older children (Petäjä et al. 1995 and 1996a, Miller et al. 1997).

To prevent the dilutional effect of the large CPB circuit, the prime in neonates often contains red blood cells and FFP separately or as reconstituted blood, in contrast to the crystalloid primes in adults. Whether FFP reduces the dilution effect and the postoperative transfusion requirements remains controversial (Oliver et al. 2003, McCall et al. 2004). Another strategy to prevent postoperative bleeding is the use of aprotinin. Aprotinin reversibly prevents the activity of plasmin and other proteolytic enzymes in plasma. It reduces the activation of coagulation, the transfusion requirements and the duration of postoperative ventilation in infants (Mössinger et al. 2003).

Proper anticoagulation during CPB is essential but poses a problem due to significant differences in responses to heparin between neonates and adults. *In vitro*, neonatal plasma has been reported to be both sensitive and resistant to heparin (Andrew et al. 1994a, Schmidt et al. 1989b, Chan et al. 2002). *In vivo,* the average amount of heparin required in neonates and older children appears to be higher than in adults (Andrew et al. 1994b, Guzzetta et al. 2005). In addition to intravenous heparin, the activation of coagulation and inflammation due to non-endothelialized surfaces has been reduced by coating the circuit surface with phosphoryl choline or heparin (Böning et al. 2004).

Despite the use of proper anticoagulant therapies, thrombin generation is increased during CPB. Adults show a distinct pattern of thrombin generation, with thrombin escalations at the beginning of CPB and at early reperfusion after opening of the aortic crossclamp (Chandler and Velan 2003, Raivio et al. 2006). The mechanism for thrombin escalation during the early

reperfusion period remains unclear; the sequelae of ischaemia-reperfusion injury in various organs may play a role. Thrombin escalation at early reperfusion is associated with postoperative myocardial damage and elevated pulmonary vascular resistance in adults (Raivio et al. 2006). Also, administration of a direct thrombin inhibitor, hirudin, at reperfusion after CPB is associated with better cardiac output and decreased systemic vascular resistance postoperatively in a porcine model (Jormalainen et al. 2004). Both of these findings suggest the clinical importance of thrombin generation at reperfusion. However, in neonates, the profile of thrombin generation in relation to repefusion is as yet undefined.

AIMS OF THE STUDY

The aim of this study was to investigate the formation of thrombin and its regulation in newborn infants in different clinical situations that challenge the coagulation system and alter the regulation of thrombin. To clarify the effects of different clinical situations on thrombin generation, healthy cord and adult plasma were also analysed.

More specifically, the following aspects were examined:

1. Thrombin generation in healthy newborn plasma in the absence and presence of platelets and its relation to thrombin generation in the respective adult plasmas.

2. *In vivo* thrombin generation and its relation to fibrinolysis in neonatal intensive care patients, in hyperbilirubinemic newborns, and in preoperative patients about to undergo cardiac surgery.

3. The effect of small-volume (fresh-frozen plasma) and large-volume (exchange transfusion) transfusion therapies on thrombin generation and on the behaviour of selected coagulation factors and anticoagulants in newborn plasma.

4. The effect of activated protein C on thrombin generation in healthy newborn plasma in the absence or presence of platelets. Further, the effect of FVL heterozygosity on thrombin generation and on selected coagulation factors and anticoagulants in newborn infants as well as the effect of APC on thrombin generation potential in FVL heterozygous newborns.

5. Thrombin generation during cardiopulmonary bypass with a special emphasis on thrombin generation during the early reperfusion period.

PATIENTS AND METHODS

1 Study populations

The studies were conducted at the Hospital for Children and Adolescents in the neonatal intensive care unit (I, II), in the operating room and the intensive care unit (IV) and in the outpatient clinic of the neonatal intensive care unit and in the Department of Obstetrics and Gynaecology (III). Written informed consent was obtained from parents prior to enrolment of the patients. The four patient groups included 33 (I), 18 (II), 27 (III), and 18 (IV) patients. The Ethics committee of the Hospital for Children and Adolescents (I, II, IV) or the Department of Obstetrics and Gynaecology (III) approved all study protocols. Blood samples at predetermined time points were obtained for coagulation analyses. Clinical information was collected from patient charts. The basic clinical data of patients is detailed in Table 2. In association with Study III, blood samples from healthy adults as well as cord plasma samples were collected from umbilical veins of nine term newborns born by vaginal deliveries to healthy women with uncomplicated pregnancies. Their median gestational age was 40.4 weeks (range 39.4-42 weeks) and median birth weight 3770 g (range 3290-4340 g). All of these infants were clinically healthy during the neonatal period. Of the healthy adult controls, four were males and six females.

2 Study protocols

2.1 Intensive care and fresh-frozen plasma transfusion (I)

The inclusion criteria were respiratory support with ventilator or nasal CPAP device, birth weight >1300 g, an indwelling arterial line and a clinically indicated FFP transfusion within 24 hours of birth. The amount of transfused FFP was 10 ml/kg, with an additional 4 ml of FFP to compensate for blood loss caused by the samples. FFP was infused in 2 h, and the blood samples were collected from the arterial line before, 15 min after, and 2h after FFP transfusion. Five millilitres of FFP was also collected from each FFP unit for later coagulation analyses.

2.2 Exchange transfusion (II)

All patients requiring an exchange transfusion were eligible for the study. Thus, 18 patients requiring a total of 22 ETs were included. All ETs were performed due to hyperbilirubinemia in the presence or absence of anaemia. Samples were obtained across only the first ET in 16,

	(I) n=33	(II) n=18	(III) n=27	(IV) n=18
Clinical information				
Gestational age (weeks)	*33.5 (29-41)*	*37.6 (23-39)*	*39.6 (37-41)*	*39.6 (36-41)*
Birth weight (g)	*2010 (1320-4120)*	*3120 (660-3980)*	*3500 (2620-4190)*	*3420 (2700-4320)*
Males	*19/33 (58%)*	*9/18 (50%)*	*17/27 (63%)*	*14/18 (78%)*
Caesarean section	*17/33 (52%)*	*8/18 (45%)*	*6/27 (22%)*	*4/18 (22%)*
Respiratory support by ventilator	*23/33 (70%)*	*5/18 (28%)*	*0/27*	*18/18*
Primary diagnoses:	*RDS 17*	*Rh-immunization 7*	*All clinically healthy*	*TGA 9*
(II: Indications for ET)	*Transient tachypnea 8*	*Other immunization 6*	*Heterozygous for FVL 11*	*TGA with other defects* 5*
	Sepsis 3	*Non-immune haemolysis 1*		*AVSD, IAA 1*
	Meconium aspiration 2	*Hyperbilirubinemia of prematurity 4*		*AAD 1*
	Hypoglycaemia 1			*CoA, HAA, VSD 2*
	PPH 1			
	Diaphragmatic hernia 1			

Gestational age and weight are expressed as medians (range).

PPH= Persistent pulmonary hypertension, TGA= Transposition of the great arteries, AVSD= Atrioventricular septal defect, IAA= Interrupted aortic arch, AAD= Arcus aortae duplex, CoA= Coarctation of the aorta, HAA=Hypoplastic aortic arch, VSD= Ventricular septal defect

*Other defects included VSD, single coronary, atrial septal defect and CoA.

Table 2. Clinical data of patients.

across both ETs in 4 and across only the second ET in 2 patients. Data of the first ETs were analysed separately in the group of all patients (n=16) and in a group of patients not requiring mechanical ventilation (n=12).

ET was performed with reconstituted blood (200 ml/kg). Blood was drawn with a 5-ml or 10-ml syringe from an arterial line while simultaneously infusing the reconstituted blood into a venous line. Reconstituted blood, prepared by mixing stored red blood cells and FFP and anticoagulated by citrate-phosphate-dextrose, was provided by the Finnish Red Cross Blood Service. The investigational sampling was performed from the arterial line immediately at the beginning of the ET, at the end of the ET and 2 h after completing the ET. Six out of 16 patients (38%) received platelet transfusion after the first ET.

2.3 Factor V Leiden in newborns (III)

Twenty-seven pregnant women with known thrombophilia and admitted to the Department of Obstetrics and Gynaecology in Helsinki were recruited into the study. All of them had low-molecular-weight heparin treatment during pregnancy to improve pregnancy outcome. Blood samples were collected from the umbilical cord and from the newborn at the age of two weeks. The newborns were grouped by Factor V Leiden (FVL) heterozygosity (n=11) and negativity (n=16). Factor V Leiden and the prothrombin (PT) G20210A mutation were checked in all newborns. The newborns whose mothers had thrombophilia other than FVL tested negative for the respective thrombophilia. These included inherited thrombophilias (prothrombin G20210A mutation (n=1), protein C deficiency type 1 (n=1)) and acquired thrombophilias (IgG-anticardiolipin antibodies (n=3), lupus anticoagulant (n=1)).

In association with Study III ten plasma samples from healthy adults were also collected from antecubital veins and cord plasma samples were collected from umbilical veins of nine term newborns born by vaginal deliveries to healthy women with uncomplicated pregnancies.

2.4 Cardiopulmonary bypass (IV)

All patients aged less than 28 days and requiring cardiac surgery with cardiopulmonary bypass for repair of congenital heart defects (two-ventricle physiology) were eligible for the study. Three of the enrolled 18 patients were excluded from the analyses since they received antithrombin concentrate during CPB. Blood samples were collected 1) preoperatively (baseline), 2) at the beginning of CPB, 3) before the release of the aortic crossclamp 4) 15 min after the release of the aortic crossclamp, 5) 30 min after protamine, 6) 6 h after protamine, 7) on the first and 8) on the fifth postoperative day.

Standardized surgical and CPB techniques were used. Repair of the heart defect required selective cerebral low-flow perfusion with 3-13 min circulatory arrest in three patients. The

CPB circuit was primed with reconstituted blood (mixed red blood cells and FFP from the Finnish Red Cross Blood Service) and/or 4% albumin with a target haematocrit value of 30%.

Aprotinin, a proteinase inhibitor possessing antifibrinolytic and anti-inflammatory activity, was given to all patients; 30 000 IU/kg in one hour after the induction of anaesthesia, 30 000 IU/kg into the prime and 8000 IU/kg/h during CPB. Before the induction of CPB, 200 IU/kg heparin was administered. During CPB anticoagulation was monitored with an activated clotting time (ACT), with ACT maintained at over 480 s with additional heparin. Heparin was neutralized by 2 mg/kg of protamine followed by additional doses (1 mg/kg) when indicated by ACT. Eleven of fifteen patients received 200 IU of PCC after protamine. PCC (200 IU) contained 200 IU coagulation factors II, IX and X, 170 IU factor VII and 135 IU of PC. One patient received 200 IU of PCC later postoperatively.

3 Coagulation studies

All blood samples for coagulation studies were collected in citrate-anticoagulated tubes. Whole blood samples were separated by centrifugation without delay, and platelet-poor plasma (PPP) was stored at -70°C until assayed in series. The experiments with platelet-rich plasma (PRP; healthy cord and adult plasmas) were carried out immediately with the platelet count of PRP adjusted to 150 x 10^9/l by using autologous PPP.

3.1 Thrombin generation

Prothrombin fragment F1+2 and TAT were analysed using Enzygnost F1+2 micro kit and Enzygnost TAT micro kit, respectively, by Behringwerke AG, Marburg, Germany. Both methods are based on the same principle of binding of F1+2 antigen or thrombin (TAT measurement) to their respective antibodies fixed to the surface of the microtitration plate. Peroxidase-conjugated antibodies to F1+2 or antithrombin (TAT) then bind the respective determinants. The colour intensity derived from the reaction of hydrogen peroxide with chromogen is determined photometrically. D-dimer concentrations were measured with an immunoturbidometric assay, which is based on the reaction of D-dimer to its antibodies on latex particles (Tina-quant D-dimer, on a Hitachi 917 analyser; Hoffmann-La Roche Ltd., Diagnostics Division, Basel, Switzerland).

Thrombogram (Thrombinoscope BV, Maastricht, Netherlands) was used to measure the TF-triggered formation of thrombin over time. The experiments were performed according to manufacturers´ instructions and published information (Hemker et al. 2000 and 2003). Thrombin generation was measured by monitoring the splitting of a fluorogenic substrate by thrombin in a TF-triggered plasma sample and by comparing this thrombin activity with the constant known thrombin-like activity of the thrombin calibrator (alpha-2-M-thrombin complex, Thrombinoscope BV) in a parallel sample. The coagulation was triggered by 5 pM

tissue factor and 4 µM phospholipids (phosphatidylserine 20 mol%: phosphatidylcholine 60 mol%: phosphatidylethanolamine 20 mol%) in PPP and by 0.5 pM tissue factor with no added phospholipids in PRP. The control experiments in PPP were additionally carried out with 1, 2.5, 10 and 20 pM tissue factor with no qualitative differences in the results compared with the experiments carried out with 5 pM TF.

Plasma sample (80µl) with the trigger (20µl) or the calibrator (20µl) was pipetted into 96-well plates. Twenty microlitres of FluCa (containing 2.5 mM fluorogenic substrate, Z-Gly-Gly-Arg-AMC and 100 mM $CaCl_2$) was then added into the wells, and colour formation at 390 and 460 nm was monitored for one hour. Measurements were taken every 20 s. During the experiment, the Thrombinoscope software compares the absorbance readings from triggered wells with the corresponding calibrator wells. In triggered wells, increasing fluorescence reflects not only free thrombin activity but also thrombin-alpha-2-M activity (Hemker et al. 1993). The thrombin-alpha-2-M activity was mathematically subtracted from the total amidolytic activity by a computerized algorithm, yielding a free thrombin generation curve over time. Alpha-2-M activity is increased in newborns (Andrew et al. 1987). The amount of alpha-2-M is ≈4.5 µmol/l in newborns and ≈2.5 µmol/l in adults (Cvirn et al. 2001). In control experiments, adding up to 2.0 µmol/l alpha-2-M to adult plasma had no influence on the thrombin generation curves, confirming the validity of the algorithm also with high alpha-2-M levels, as in neonatal plasma (Figure 8).

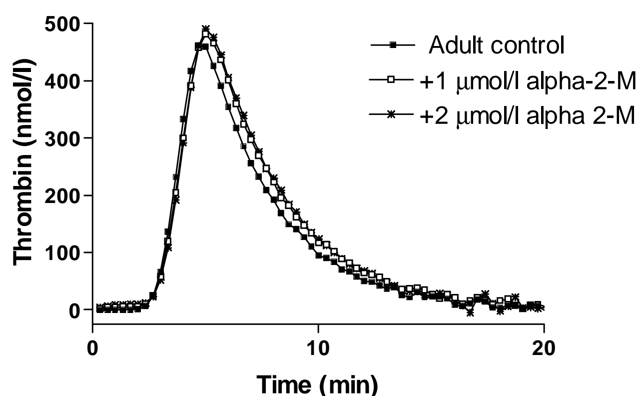

Figure 8. Thrombin generation potential in healthy adult plasma supplemented with 0, 1 and 2 µmol/l of alpha-2-macroglobulin (alpha-2-M) to correspond to the levels in newborn infants.

When applicable, APC (final concentration 0.5, 1, 2.5, 3.75, 5, 10 or 20 nmol/l) was mixed with the coagulation trigger just before adding the trigger to the plate. APC (Hyphen BioMed, Neuville sur Oise, France) was produced by activation of plasma-derived human protein C by agarose-bound thrombin. An aliquot of pooled internal control plasma was used in each Thrombogram assay.

3.2 Other coagulation studies

Coagulation factor V and VII clotting activities were measured by prothrombin time-based clotting assays utilizing commercial factor-deficient plasmas (Thromborel S, Dade Behring, Liederbach, Germany). FVIIIc activities were assayed by one-stage APTT-based assay and FVIII-deficient plasma (Pathromtin SL, Clotting Factor VIII-Deficient Plasma, Dade Behring). Antithrombin and protein C activities were measured by chromogenic assays (Berichrom Antithrombin III and Berichrom Protein C, Dade Behring, Liederbach, Germany). For measurement of free protein S antigen, a latex-based method was used (IL Test Free Protein S, Instrumentation Laboratory). Von Willebrand factor antigen was determined using STA-Liatest VWF-kit (Diagnostica Stago, Asnières, France), and VWF ristocetin cofactor activity was determined using Packs-4 aggregometer (Helena BioSciences Europe, Sunderland, UK) with lyophilised platelets (Helena BioSciences Europe) and Ristocetin A SO_4 (America Biochemical and Pharmaceutical Ltd., Marlton, NJ, USA). The variants FV Leiden and PT G20210A were detected using an automatic PCR-based solid minisequencing method and standard primers. Lupus anticoagulant was excluded by means of APTT (IL Test APTT SP-reagent, Instrumentation Laboratory, Lexington, MA, USA) and dRVVT (dilute Russell's Viper Venom test, American Diagnostica, Pfungstadt, Germany) assays supplemented with a plasma mixing test if needed. A Varelisa EIA kit supplied by Pharmacia Diagnostics, Freiburg, Germany, was used to test for cardiolipin IgG antibodies.

4 Statistics

Non-parametric tests were used for all comparisons. Mann-Whitney *U*-test and Wilcoxon test were used for non-paired and paired comparisons, respectively. Kruskall-Wallis test and Friedman test were used for non-paired and paired multiple comparisons, respectively. Dunn´s test was used as a post-hoc test when required. Spearman R correlation coefficients were calculated. Data are expressed as means and standard errors of mean (SEM) unless otherwise stated. All box and whiskers plots depict median, 25th and 75th percentile, and minimum and maximum. Data were analysed using GraphPad Prism 3.0 (San Diego, USA).

RESULTS AND DISCUSSION

1 Diminished but fast thrombin generation in healthy newborns (II, III)

Thrombin generation was studied in cord plasma collected from vaginally-born healthy newborns and compared with that of healthy adults. The analyses were carried out in platelet-poor as well as in platelet-rich plasma initiated by 5 pM and 0.5 pM TF, respectively. The measured parameters included ETP (area under the thrombin generation curve), peak thrombin, lag time to the beginning of thrombin generation (time when 10 nmol/l of thrombin is formed) and time to peak thrombin. All of these parameters are relevant when measuring the generation of thrombin. Lag time reflects the time required for assembly of efficient prothrombinase complexes. At the end of the lag time, fibrin clot formation is already observed, although over 95% of thrombin is yet to be formed. ETP reflects the total capacity of plasma to generate thrombin and exert its activities, while peak thrombin reflects the maximal level of thrombin that can be generated in triggered plasma.

ETP and peak thrombin were significantly lower in newborn PPP and PRP than in the respective adult plasmas (Figure 9). Lag time to the initiation of thrombin generation and time to peak thrombin generation were, however, significantly shorter in newborns than in adults in both plasma preparations (Figure 9). ETP was similar in newborn PPP (1296±34 nmol/l*min) and PRP (1278±28 nmol/l*min). In addition, adult PPP (2073±94 nmol/l*min) and PRP (2061±107 nmol/l*min) showed similar ETPs. Thus, newborn ETP remains at 60% of adult ETP in both PPP and PRP ($p<0.0001$ for both comparisons). Similarly, newborn peak thrombin was at 70% of adult peak thrombin in both plasma preparations. Thrombin generation started 55% and 40% earlier in newborn PPP and PRP, respectively, when compared with adult plasmas ($p<0.001$ in both comparisons).

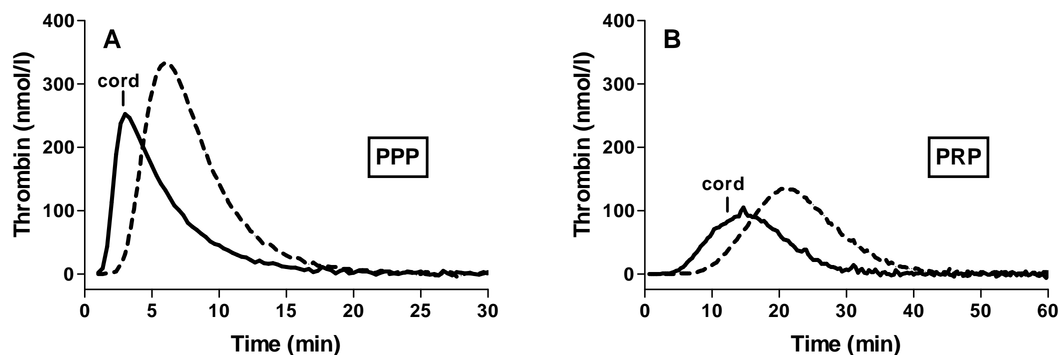

Figure 9. *Thrombin formation in platelet-poor (A) and platelet-rich (B) cord (solid line) and adult (dotted line) plasma initiated by 5 pM (PPP) or 0.5 pM (PRP) TF.*

In earlier measurements, when thrombin generation was initiated by strong activated partial thromboplastin time and prothrombin time reagents, newborn infants showed significantly lower and delayed thrombin generation compared with adults (Schmidt et al. 1989b). However, initiation of thrombin formation with small amounts of TF is considered to better reflect the *in vivo* situation (Davie et al. 1991, Butenas et al. 1999, Cvirn et al. 2003a). With low amounts of TF, thrombin generation started earlier in newborn infants than in adults, while the total amount of thrombin generated was still lower in newborns (Cvirn et al. 2003a). Faster thrombin generation and lower total thrombin generation potential in newborns than in adults were confirmed in the current Studies II and III. Thus, the coagulation system of newborn infants appears to be efficient in its response to injury and to be able to quickly generate the fibrin clot; these observations are in line with a clinically uncompromised haemostasis in healthy newborn infants.

Previous studies on thrombin generation in newborns have been carried out in PPP. The only report on thrombin generation in plasma supplemented with platelets was carried out in synthetic plasma with platelets of unknown origin (Butenas et al. 2002). In that study, faster thrombin generation in newborns was reported, but the role of neonatal platelets in the formation of thrombin remained unclear. In coagulation reactions, the role of platelets is increasingly recognized to be more significant than a mere provider of the required phospholipids. Thus, including platelets in the measurement of thrombin generation better reflects the *in vivo* regulation of thrombin. Similar to PPP thrombin generation in newborns´ PRP started faster than in adult PRP, but ETP and peak thrombin remained lower than in adults. The differences between newborn and adult thrombin generation in PRP were similar to those observed in PPP. Fast thrombin generation in cord PPP has been suggested to derive from the low synergistic anticoagulant capacity of AT, TFPI and PC (Cvirn et al. 2003a and 2003b), whereas low total and peak thrombin generation have been attributed to low prothrombin levels (Andrew et al. 1990a). Apparently, low prothrombin and low anticoagulant capacity are the main determinants of low but fast thrombin generation also in newborn PRP since the differences between newborn and adult plasma are fairly similar in PRP and PPP. Phospoholipid composition of adult platelets regulates the initiation phase of thrombin generation in adult PRP (Vanschoonbeek et al. 2004) and may thus in part also contribute to the faster initiation of thrombin generation in newborn PRP.

2 Elevated thrombin generation in sick newborns *in vivo* (I, II, IV)

Thrombin turnover *in vivo* was estimated by measuring the levels of F1+2, TAT and D-dimer in different clinical patient groups, including neonatal intensive care patients during the first 24 h of intensive care, patients with severe hyperbilirubinemia and patients with congenital heart defect about to undergo open heart surgery. F1+2 reflects the production of thrombin, TAT the production and inhibition of thrombin and D-dimer the effect of thrombin on

fibrinogen and subsequent fibrinolysis, with *in vivo* half-lives of 90 min and 15 min for F1+2 and TAT, respectively (Bauer 1993). Reported half-lives of D-dimer are inconsistent, being probably at least 3 h (van der Putten et al. 2006). Adult reference ranges for TAT and D-dimer (Table 3) have been considered valid also in newborn infants (Schmidt et al. 1993). Recently, one study reported reference ranges for D-dimer in newborn infants at postnatal days 1 and 3, with values ranging from 0.4 µg/ml to 2.5 µg/ml (mean values 1.3-1.5 µg/ml) when adult levels were below 0.5 µg/ml (Monagle et al. 2006). Stable infants about to undergo cardiac surgery have typically shown F1+2 levels within the adult reference range (Petäjä et al. 1996b). Accordingly, in neonatal intensive care patients, during the first 24 h of intensive care and prior to FFP infusion, F1+2 was elevated in 97% of patients and TAT in 81% of patients. In neonates with severe hyperbilirubinemia, prior to ET, F1+2 and TAT were elevated in all patients. In neonates about to undergo open heart surgery, F1+2 was elevated in 56% and TAT in 78% of patients. When compared with the adult reference ranges, D-dimer was elevated in nearly all patients in all patient groups studied. Upon consideration of the results of Monagle et al. (2006), D-dimer seemed to be elevated in neonates in the intensive care unit and in neonates with hyperbilirubinemia, but not necessarily in preoperative patients (Table 3).

Table 3. Thrombin formation in vivo in newborn infants undergoing neonatal intensive care (NICU, n=32), in severe hyperbilirubinemia (n=15) and in preoperative neonates (age < 28 days) with a congenital heart defect requiring open heart surgery (n=18).

	NICU	***Hyperbilirubinemia***	***Heart defect***	***Adults***
F1+2 *(nmol/l)*	3.24±0.40	2.0±0.2	1.7±0.3	<1.1
TAT *(µg/l)*	23.7±4.69	10.1±1.3	11.8±3.3	<4.0
D-dimer *(mg/l)*	3.4±0.5	2.2±0.5	1.5±0.4	<0.5

Since F1+2 was considered the most specific marker of thrombin generation, the correlations of F1+2 with TAT and D-dimer in the different patient groups were calculated. The correlations between F1+2 and TAT were high in intensive care unit patients (R=0.70, p<0.0001) and in patients with severe hyperbilirubinemia (R=0.71, p=0.003), but non-significant in preoperative patients (R=0.37, p=0.13). The correlation between F1+2 and D-dimer was high in preoperative patients (R=0.83, p<0.0001), but lower in intensive care patients (R=0.42, p=0.01), and no correlation was observed in neonates with severe hyperbilirubinemia.

The effect of different clinical situations on thrombin generation was also analysed in patients undergoing intensive care. Prior to FFP transfusion, the presence of infection, RDS or the requirement for respiratory support by ventilator as opposed to nasal-CPAP device did not affect thrombin generation measured as F1+2 (Figure 10). Further, four infants had IVH and their F1+2 levels prior to FFP ranged from 1.8 nmol/l to 5.7 nmol/l. These levels did not differ from the F1+2 levels of the other infants.

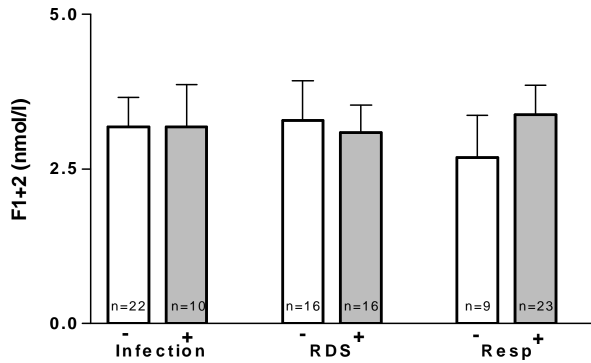

Figure 10. Prothrombin fragment F1+2 levels prior to FFP transfusion were similar in different clinical groups. Infection=proven or suspected infection; RDS= respiratory distress syndrome; Resp= respiratory support by ventilator (+) or nasal-CPAP device (-).

The birth process is known to activate the coagulation system (Suarez et al. 1985, Yuen et al. 1989). In healthy infants, the activation attenuates with no clinical complications, but in sick newborns the markers of ongoing thrombin formation are often elevated (Schmidt et al. 1993). In the current patient groups, nearly all patients had increased levels of the thrombin generation markers. The only exception was preoperative patients; 50% of these patients showed normal F1+2, consistent with previous observations in a comparable patient population (Petäjä et al. 1996b), and their D-dimer levels possibly approached the age-specific reference range (Monagle et al. 2006).

Of the markers of thrombin generation, F1+2 reflects the activation of prothrombin, TAT the generation and inhibition of thrombin and D-dimer the effect of thrombin on fibrinogen and the subsequent fibrinolysis. High correlations between F1+2 and TAT in intensive care patients and hyperbilirubinemic patients suggest that at least transiently AT was able to follow the increasing thrombin. However, since high thrombin activation was present and plasma of sick newborn infants generally shows decreased anticoagulant capacity (Shah et al. 1992), the capacity of AT may eventually be overwhelmed by the ongoing thrombin activation.

In all patient groups, thrombin activity was, among other possible targets, directed to fibrin formation, as evidenced by the observed D-dimer levels. However, the correlations between F1+2 and D-dimer varied from absent to very high, suggesting different balance between thrombin generation and the regulation of fibrinolysis in the different patient groups. Variations in the ability of AT to inhibit thrombin may contribute, as can the clinical stage and the age of patients; the fibrinolytic system undergoes profound changes during the first days of life (Andrew et al. 1990b). In addition, the balance between thrombin generation and fibrinolysis may be better regulated with a lower activation state of the coagulation system, as in preoperative patients with lower activation of thrombin and possibly near-normal levels of D-dimer.

3 Blood component transfusions affect neonatal haemostasis (I, II, IV)

Thrombin generation, reflected as F1+2, TAT, and D-dimer, was measured over FFP transfusion, exchange transfusion and infusion of prothrombin complex concentrate. Additionally, the effects of the transfusions on selected pro- and anticoagulants were measured. Thrombin formation potential was measured at the beginning and at the end of ET in platelet-poor plasma.

3.1 Procoagulants and anticoagulants

Procoagulants FV and FVII as well as anticoagulants AT and PC were measured over all the transfusions. All of these pro- and anticoagulants significantly increased during the transfusions, except for FV during ET and AT during PCC. Further, the pro- and anticoagulant levels measured after FFP and ET were compared with expected levels. The expected levels after FFP were calculated using the following formula: Factor $level_{PostFFP}$ = (Plasma volume x Factor $level_{PreFFP}$ + FFP volume x Factor $level_{FFP}$)/ (Plasma volume + FFP volume). The infant blood volume was estimated to be 100 ml/kg, and the plasma volume of the whole blood volume was calculated based on the average haematocrit value of the patient population in question (47.5%). The expected levels after ET were the levels measured from the transfused plasma of each patient. Of these pro- and anticoagulants, all reached the expected levels except PC. PC levels were significantly lower than expected levels after FFP and after ET ($p<0.001$ in both comparisons) (Figure 1 in I and Table 2 in II).

Factor VIIIc exceeded the expected levels after ET (FVIIIc after ET 1.13±0.05 U/ml; FVIIIc in the infused blood 0.83±0.05 U/ml; $p<0.001$). Prior to ET, VWF activity and antigen were above normal adult levels (1.32±0.14 U/ml and 1.54±0.14 U/ml, respectively), and in contrast to FVIIIc, VWF activity and antigen levels both decreased during ET ($p<0.01$). Post-ET VWF was at the levels measured in infused blood.

Of the measured factors, two factors behaved unexpectedly during the transfusions. First, PC increased less than other pro- and anticoagulants during both FFP and exchange transfusions, suggesting enhanced consumption of PC during the transfusions. Second, FVIIIc increased more than expected during ET. Apparently, the increase in FVIIIc did not result from endothelial activation since the carrier of FVIII, VWF, decreased. Either activation of FVIII by increasing thrombin or the role of FVIII as an acute phase reactant may have contributed.

3.2 *In vivo* thrombin generation

FFP, ET and PCC each exerted specific effects on the markers of thrombin generation *in vivo*. F1+2 was either lowered, practically unchanged, or significantly increased after the different

transfusions (Figure 11). FFP influenced F1+2 in a different manner depending on the pretransfusional F1+2 level; thus, patients receiving FFP were divided into two groups according to pretransfusional F1+2 level (Figure 11A). After FFP transfusion, the patients with the highest pretransfusional F1+2 ($\geq$ 2.35 nmol/l) showed a significantly lower F1+2 level than before the transfusion ($p<0.01$), whereas in the patients with lower pretransfusional F1+2 the effect on F1+2 was neutral ($p=0.05$). In contrast to FFP, exchange transfusion induced a significant increase in F1+2 ($p<0.01$). A marked increase in F1+2 generation was observed also after PCC infusion ($p<0.01$).

The lowering effect of FFP on F1+2 ceased after the end of transfusion since no change in F1+2 was observed in the 2-h post-transfusion period (Figure 11A). The increase induced by ET attenuated during the 2 h after the transfusion, but at that time F1+2 still remained higher than at the beginning of ET ($p<0.05$). After PCC infusion, F1+2 started to decrease, but was still elevated at 6 h after PCC.

TAT was affected similarly by the transfusions as F1+2 (Figure 11B). TAT was significantly decreased by FFP in those patients with the highest pretransfusional F1+2 ($p=0.01$), with no observable effect in patients with low pretransfusional F1+2. ET induced a marked increase in TAT; the median increase from pre-ET levels was 14-fold ($p<0.0001$). PCC induced an increase in TAT that was not, however, as pronounced as the effect observed in F1+2. The effect of FFP on TAT ceased after the transfusion. The strong increase induced by ET attenuated during the 2-h post-transfusion period, but TAT was still elevated at 2 h after ET when compared with preoperative levels ($p<0.05$). The effect of PCC on TAT seemed to be attenuated by 6 h after PCC.

FFP decreased D-dimer in both groups, with a more pronounced decrease in the group with higher pre-FFP F1+2 ($p<0.01$) (Figure 11C). D-dimer was decreased at the end of ET ($p<0.01$), in contrast to significant increases in F1+2 and TAT. The effect of PCC on D-dimer was modest.

FFP is generally administered to substitute procoagulant factors. Anticoagulants are obviously also included. *In vitro*, FFP has been observed to slightly increase the thrombin peak when added to cord plasma (Andrew et al. 1990a). *In vivo*, in children aged 3-17 years FFP had no effect on TAT or D-dimer (Halton et al. 1994). Similarly, only a slight effect on F1+2, TAT and D-dimer was observed in the current study in those intensive care patients with moderate activation of the coagulation system prior to the transfusion. However, in patients with the highest pretransfusional activation of coagulation, FFP decreased thrombin generation. Thus, during FFP transfusion, although the activities of all the measured pro- and anticoagulants increased, anticoagulant activity in newborn plasma increased proportionally more than the procoagulant activity. One of the major anticoagulants, PC, seemed to simultaneously be consumed more than expected. Thus, PC may play a significant role in the thrombin-attenuating effect of FFP, but possibly with the exhaustion of the PC pathway. All in all, the finding of decreasing thrombin generation with FFP supplementation emphasizes that the

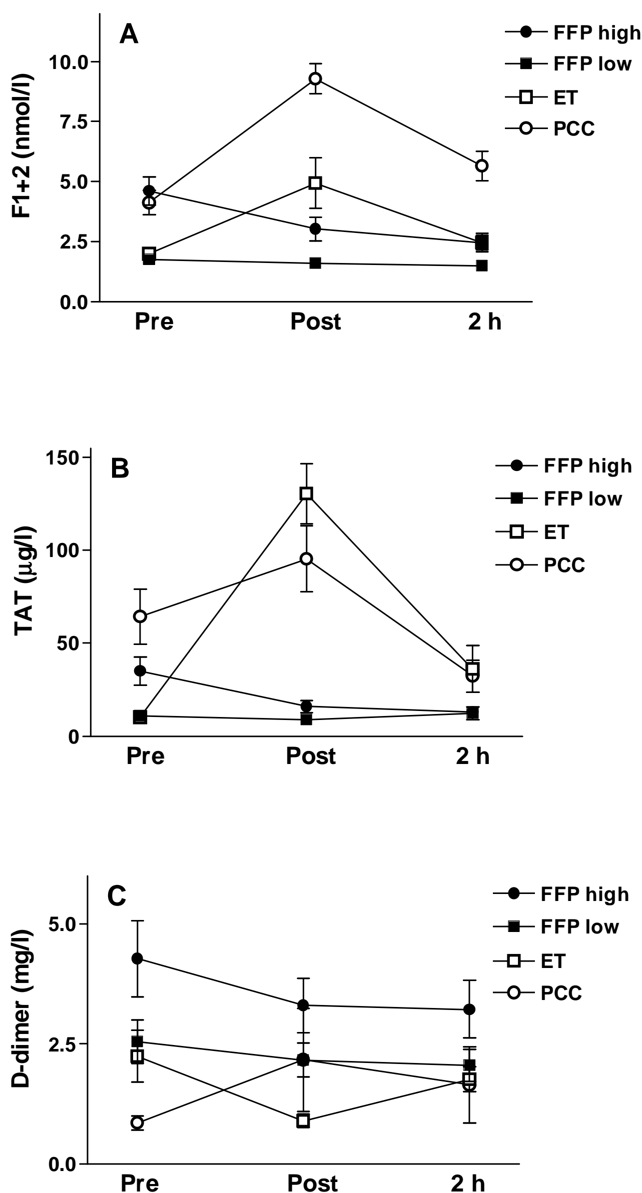

Figure 11. Effects of FFP (n=16 + 16), ET (n=15), and PCC (n=11) on F1+2 (A), TAT (B) and D-dimer (C). Blood samples for FFP and ET were obtained before and after transfusions, and 2 h after the end of transfusions. Samples for PCC were obtained 27± 5 minutes prior to PCC and 30 min and 6 h (presented at 2 h) after PCC. FFP high = newborns with high pretransfusional F1+2 (pretransfusional F1+2 > 2.35 nmol/l, n=16), FFP low = newborns with low pretransfusional F1+2 (pretransfusional F1+2 < 2.35 nmol/l, n=16).

regulation of coagulation in neonates is unique and that the activated coagulation system can be significantly affected by a small-volume plasma transfusion.

In marked contrast to the effect of FFP on thrombin generation, ET significantly activated the coagulation system. In addition to FFP, reconstituted blood used in ET contains red blood cells but no platelets. Thus, after the observed neutral or thrombin-reducing effect of small-volume plasma transfusion along with thrombocytopenia resulting from performing ET with reconstituted blood, the strong activating effect of ET on coagulation was unexpected. The infused reconstituted blood contained very low levels of F1+2 and TAT, and although an increase in all pro- and anticoagulants was observed during ET the median 14-fold increase in

TAT could not be predicted on the basis of the significantly more modest increases in individual pro- and anticoagulant levels.

The endothelium as a possible contributor to coagulation activation during ET was investigated since FVIII was observed to increase more than expected. However, the carrier of FVIII, VWF, which is released from the endothelium, did not increase but instead decreased to the levels observed in infused blood. Red blood cells may participate in thrombin generation (Peyrou et al. 1999, Horne et al. 2006), but whether the adult red blood cells could contribute to activation of coagulation in neonatal plasma remains to be elucidated. ET did not increase the level of D-dimer. The efficient complexation of thrombin with antithrombin or attenuated fibrinolysis may have contributed.

PCC significantly increased thrombin generation. The same phenomenon has also been observed in adults (reviewed by Köhler 1999), but the effects of PCC in newborn plasma are unclear. The infants who received PCC also received other transfusions, such as red blood cells, platelets or FFP, during the time period from the sample obtained prior to PCC to the sample obtained after PCC. The effect of PCC was, however, profound and not observed in patients receiving no PCC, despite their having received other transfusions (Figure 2 in IV). Thus, the increase in F1+2 and TAT by the sample obtained after PCC was likely mostly attributed to PCC. PCC did not affect D-dimer, but aprotinin probably still attenuated the fibrinolysis at that time.

All in all, the blood components affect newborn coagulation differently, with variable effects exerted by the same product depending on the state of the coagulation system. FFP decreased or exerted a neutral effect on thrombin generation, whereas ET and PCC significantly increased thrombin generation. The blood components are special as medicines in that their effects have been studied fairly little. Since newborn infants receive adult blood components, it is important to know the effects that may be expected; blood components can either induce or alleviate coagulopathy in the newborn.

3.3 Exchange transfusion and thrombin generation potential in newborns

At the beginning of ET, newborns showed an essentially similar thrombin generation curve to cord PPP with respect to all the parameters studied (ETP, peak thrombin, lag time, time to peak thrombin) (Figure 12). ET significantly increased ETP and peak thrombin to adult levels in newborn plasma ($p<0.0001$ between pre-ET and post-ET measurements). However, at the end of the ET lag time to the start of thrombin generation and the time to peak thrombin were significantly shorter in patient plasma than in control plasma ($p<0.001$). Six patients underwent a second ET the next day. In these patients, measurements at the beginning of the second ET showed that ETP and peak remained at adult levels in newborn plasma for at least one day (Figure 4 in II).

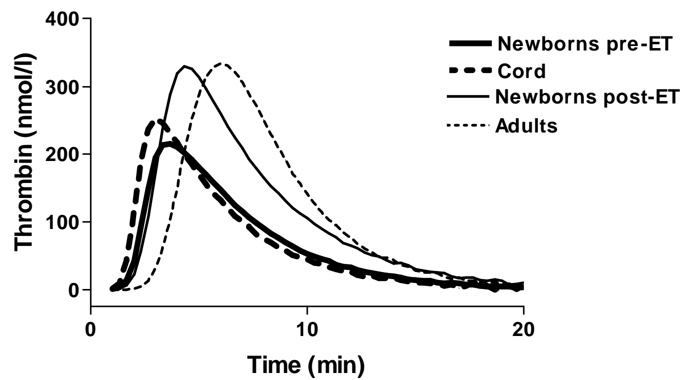

Figure 12. Thrombin generation potential in newborn infants before and after exchange transfusion compared with cord and adult plasma.

The exchange transfusion carried out with adult blood was able to increase ETP and peak thrombin to adult levels in newborn infants. Interestingly, however, the lag time and the time to peak thrombin were still significantly shorter in newborn plasma than in adult plasma after ET. The main determinant of total and peak thrombin, prothrombin (Andrew et al. 1990a, Butenas et al. 1999), probably affected these parameters and the persistence of adult-like ETP and peak thrombin levels since the half-life of prothrombin is rather long, over two days (Mann 1999). However, since fast thrombin generation in newborns is apparently associated with low anticoagulant capacity, this low anticoagulant capacity seemed to prevail in newborn plasma even after the change to adult plasma. All in all, ET seemed to combine the procoagulant features of adult and newborn plasma, leaving the newborn with a unique thrombin formation-favouring plasma milieu.

4 Protein C and thrombin regulation in newborns (I, II, III)

The PC pathway is an essential anticoagulant and anti-inflammatory pathway. Due to its physiological importance and previous observations of its specific developmental features, it was given special attention. The behaviour of APC in healthy cord PPP and PRP was studied first. The observations were then extended to the effects of APC in cord PPP of newborn infants with FVL mutation and in PPP of newborn infants undergoing ET.

4.1 Anticoagulant effects of activated protein C in newborns

APC dose-dependently decreased ETP and peak thrombin in cord plasma as well as in adult plasma. The effects were, however, significantly different in adult vs. cord plasma and in PPP vs. PRP. Addition of APC at concentrations of 1, 2.5 and 5 nmol/l to PPP resulted in

significantly more pronounced suppression of ETP in adult than in cord plasma (Figure 13). In contrast in PRP, the suppression of ETP by APC was significantly more pronounced in cord plasma at APC concentrations of 2.5, 5 and 10 nmol/l (Figure 13). The effects of APC on peak thrombin were comparable with the effects on ETP (data not shown).

In adults, PRP was significantly ($p<0.0001$) more resistant to the anticoagulant effects of APC than PPP at APC concentrations of 2.5 and 5 nmol/l. In cord plasma, there was a tendency towards more resistant PRP with 2.5 nmol/l ($p=0.05$) and 5 nmol/l ($p<0.05$) APC concentrations when the APC effect on ETP was analysed. When the effect of APC on peak thrombin was analysed, cord PRP was significantly more resistant to the anticoagulant effects of APC than cord PPP ($p<0.01$).

The lag time was dose-dependently prolonged with APC when analyses were carried out in PRP (Figure 4 in III). The lag time in cord PRP was significantly more prolonged by APC at concentrations of 5 and 10 nmol/l than in adult plasma. Time to peak thrombin was significantly more prolonged by APC in cord PRP at an APC concentration of 10 nmol/l (data not shown). APC also prolonged lag time in cord PPP, but the effect was saturated at 3.75 nmol/l of APC (Figure 4 in III). APC slightly shortened the lag time in adult PPP.

The observations of the resistance of thrombin generation in newborn PPP to the anticoagulant effects of APC are in line with previous studies in cord PPP. APC, whether generated from endogenous PC by adding exogenous thrombomodulin (Cvirn et al. 2003b) or added in the form of recombinant preactivated APC (Cvirn et al. 2004), has been able to decrease thrombin potential and to prolong clotting time significantly less in neonatal than in adult plasma. This resistance of APC has been attributed to the low synergistic capacity of anticoagulants AT, TFPI and APC in neonatal plasma since when AT and TFPI were added *in vitro* the resistance to APC in neonatal plasma disappeared (Cvirn et al. 2003b and 2004).

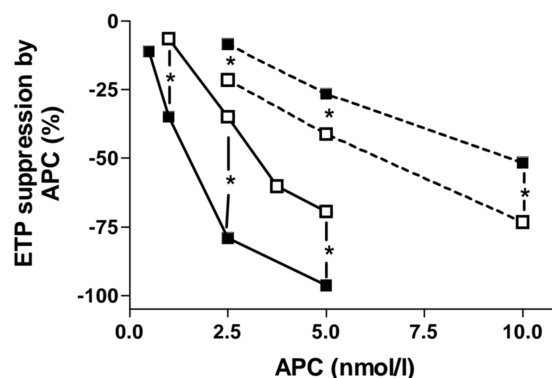

Figure 13. The anticoagulant effect of activated protein C (APC) on the suppression of ETP in cord (open square) and adult (black square) PPP (solid line) and PRP (dotted line). Anticoagulant effects of APC differed between cord and adult plasma in both PPP and PRP ($p<0.05$)*. Thrombin generation in cord PRP was more susceptible to APC than in adult PRP, whereas thrombin generation in cord PPP was more resistant to APC than in adult PPP.

In contrast, thrombin generation in cord PRP was found to be more susceptible to the anticoagulant effects of APC than that in adult PRP. Sensitivity in newborn plasma to APC was consistent in all the parameters of thrombin generation, i.e. ETP, peak, lag time, and time to peak thrombin. This finding is novel. The finding of APC sensitivity is consistent with earlier observations of other upregulated features of the neonatal protein C pathway (Malm et al. 1988, Petäjä et al. 1998, Menashi et al. 1999). The mechanisms behind APC sensitivity in cord PRP may be various. In adults, glycosylation patterns of FV modulate its sensitivity to APC (Fernandez et al. 1997), and adult platelet FV is more resistant to APC than adult plasma FV (Gould et al. 2004). Furthermore, APC activity is affected by the phospholipid composition on the platelet surface (Smirnov et al. 1994). Thus, differences in the modulation of FV in neonates vs. adults may exist, as well as differences in the platelet surface components that regulate the activity of APC. Neonatal platelet FV might be uniquely regulated to allow more efficient cleavage by APC.

4.2 Factor V Leiden heterozygosity induces a procoagulant effect in newborns

Neonates with or without FVL heterozygosity showed similar ETP and peak thrombin levels, but lag time and time to peak thrombin were significantly shorter in FVL-heterozygous newborns than in FVL-negative newborns (Figure 14).

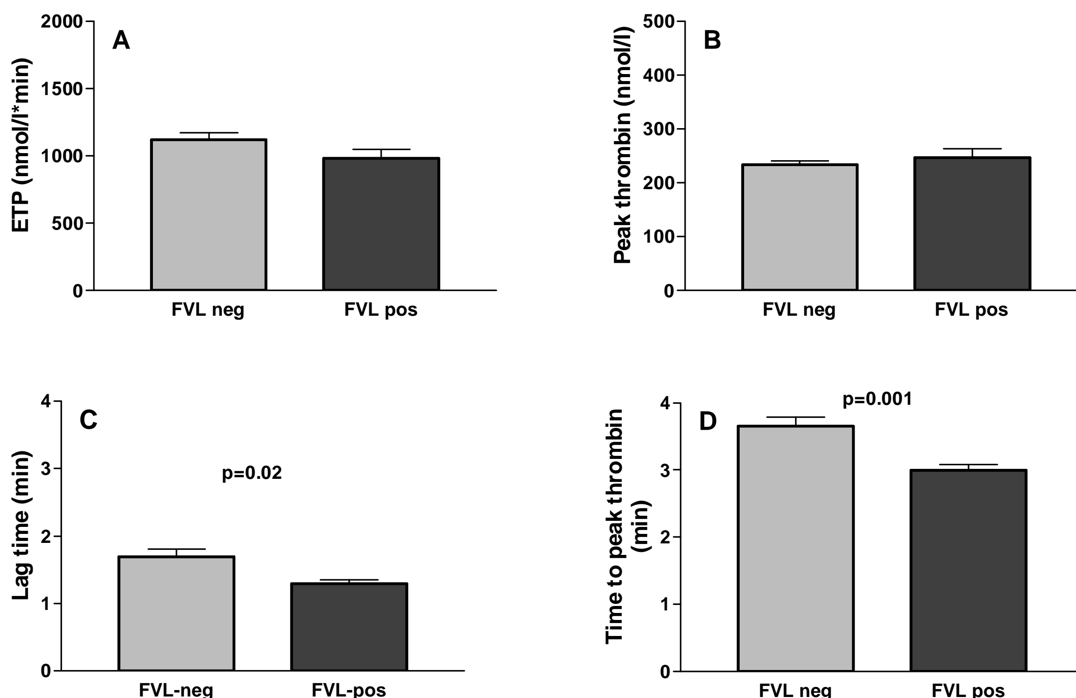

Figure 14. Thrombin generation potential in newborn infants with or without FVL heterozygosity. ETP and peak thrombin were comparable between the groups (A, B), but lag time to the beginning of thrombin formation and time to peak thrombin were significantly shorter in FVL-heterozygous newborns (C,D).

The effect of APC in cord plasma of neonates with FVL heterozygosity was studied in PPP. When compared with the internal control (healthy adult plasma), the effect of APC on ETP and peak thrombin was modest in cord PPP in both FVL-negative and FVL-heterozygous neonates. FVL-negative neonates, however, showed a significantly more pronounced suppression in ETP than FVL-heterozygous neonates (Figure 5 in III). Also, APC prolonged the lag time significantly more in FVL-negative than in FVL-heterozygous infants (Figure 5 in III).

The only difference in the measured pro- and anticoagulants (FV, FVII, AT, PC, PS) between FVL-negative and FVL-heterozygous infants was a lower level of FV in FVL-heterozygous infants in cord plasma (FV 0.82±0.07 U/ml vs. 0.98±0.05 U/ml, p=0.03) and at the age of two weeks (FV 1.15±0.04 U/ml vs. 1.32±0.05 U/ml, p=0.03).

FVL mutation is associated with a different pattern of thrombotic complications in newborns than in adults. In adults, thrombotic complications occur especially in the veins. In newborns, FVL predisposes to arterial complications (Hagstrom et al. 1998, Gunther et al. 2000). The mechanisms underlying this difference are unclear, as are the effects of FVL on the coagulation system of newborns. Thus, all of the current findings on FVL and neonatal haemostasis are novel.

In neonates with FVL, the finding of shorter lag times to thrombin generation and time to peak thrombin reflect an altered balance of pro- and anticoagulants to the procoagulant direction in FVL-heterozygous neonates. Another novel finding regarding FVL and the protein C pathway is the observed lower activity of FV in FVL-heterozygous infants. The lower FV in FVL heterozygotes may be a compensation mechanism to reduce the procoagulant capacity in neonatal plasma. The influence of FVL heterozygosity on FV levels has not been reported in adults.

4.3 Resistance to activated protein C in newborns prevails after exchange transfusion

During ET, ETP and peak thrombin increased to adult levels in newborn plasma, but the lag time and time to peak thrombin were significantly shorter in newborns than in adults (Figure 12). The anticoagulant effect of APC in suppressing ETP was similar in newborn infants before (-31±5%) and after (-35±4%) ET, thus significantly (p<0.001) decreased compared with the suppression observed in adults (-79±4%).

The remaining resistance to the anticoagulant effects of APC in post-ET plasma may reflect the lower synergistic anticoagulant capacity of AT and TFPI with APC even after ET. By increasing the levels of AT and TFPI, the resistance of newborn plasma to APC disappeared (Cvirn 2003b). During ET, the level of AT was increased, with no effect on the capacity of APC to reduce thrombin generation potential. Thus, the mechanism behind the resistance of

newborn PPP to APC may be more complicated and involve other factors in addition to AT and TFPI. All in all, the remaining resistance to APC after ET is yet another reflection of reduced anticoagulant capacity and increased procoagulant capacity in post-ET newborn plasma.

4.4 Enhanced consumption of protein C in the activated coagulation system of newborns

PC behaved similarly during FFP and ET; while the low pro- and anticoagulant levels increased, a proportionally lower increase in PC was observed when compared with the other pro- and anticoagulants (Figure 1 in I and Table 2 in II). Also, at the end of CPB, at the point at which coagulation was strongly activated, a negative correlation between PC and TAT was noted (IV).

These findings suggest a relatively higher consumption of PC over the other measured pro- and anticoagulants in the activated coagulation system of newborns. A similar finding of proportionally decreased PC activity is observed in severe sepsis, in which the coagulation system is strongly activated, in both children and adults (Roman et al. 1992, Fijnvandraat et al. 1995, Barton et al. 2004). This current consistent finding in newborns suggests that similar mechanisms may lie behind the regulation of coagulation and especially the regulation of thrombin in coagulation system activation resulting from causes other than severe sepsis. All in all, the behaviour of PC highlights the physiological importance of PC pathway and its vulnerability in the regulation of thrombin in newborn infants.

5 Cardiopulmonary bypass and thrombin generation in neonates (IV)

Cardiopulmonary bypass activated coagulation significantly immediately at the beginning of CPB, and the increase of F1+2 and TAT continued during CPB (Figure 15). Due to substantial haemodilution, F1+2, TAT and D-dimer were corrected for haemodilution (calculation in IV). Compared with baseline, median F1+2 increase was 2.5-fold and TAT 5-fold at the beginning of CPB. Before opening of the aortic crossclamp F1+2 was 4-fold and TAT 9-fold compared with baseline. Opening of aortic crossclamp was not associated with a further increase in F1+2 or TAT; when the haemodilution-corrected levels were analysed, decreases in F1+2 ($p=0.03$) and TAT ($p=0.002$) were observed (Figure 15D). D-dimer did not increase during CPB.

Birth weight, gestational age, duration of CPB, aortic crossclamp time, heparin dose or levels of cardiac biomarkers creatinine kinase MB mass and troponin T measured on the first postoperative day were not correlated with F1+2 or TAT at any of the sampling points.

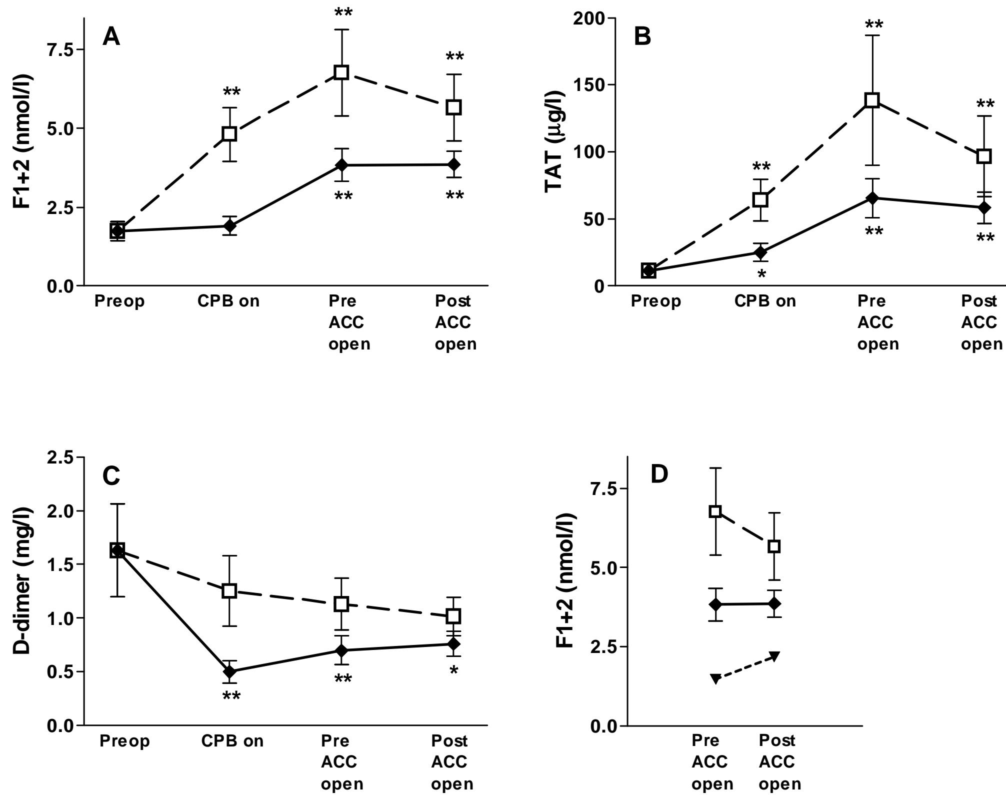

Figure 15. Measured (black diamonds) and haemodilution-corrected (open squares) prothrombin fragment F1+2 (A,D), thrombin-antithrombin complexes (TAT, B), and D-dimer (C) during cardiopulmonary bypass (CPB) in 15 neonates. A significant increase in both F1+2 and TAT was observed during CPB (*p<0.05, **p<0.001) when compared with corresponding preoperative levels (A,B). D-dimer, in contrast, decreased (C). Opening of the aortic crossclamp (ACC) was associated with an actual decrease in F1+2 in neonates (haemodilution-corrected levels - open squares) in contrast to the previously observed increase in F1+2 in 100 adult patients undergoing coronary artery bypass grafting surgery with CPB (black triangles; Raivio et al. 2006) (D). Samples were obtained preoperatively, at the beginning of CBP, before opening ACC, and 15 minutes after opening ACC.

Coagulation in neonates during CPB was invariably activated. The profile of neonatal thrombin generation, however, differed from that previously observed in adults. During adult cardiac surgery, thrombin formation is enhanced especially at the beginning of CPB and during reperfusion after cardiac ischaemia (Chandler and Velan 2003, Raivio et al. 2006). In neonates, escalation of thrombin was present immediately at the beginning of CPB, followed by a continued increase over the course of CPB. In marked contrast to adults, however, no increase in thrombin generation was observed during the early reperfusion period.

The activation of coagulation during CPB is mainly initiated by the TF-dependent mechanism (Boisclair et al. 1993), but the precise mechanism of the systemic thrombin burst in adults during the early reperfusion remains incompletely characterized. The sequelae of ischaemia-reperfusion injury in various organs may play a role. Activation of coagulation may partly derive from the coronary circulation (Petäjä et al. 1999). However, since the nature of thrombin burst after opening of aortic cross-clamp in adults is apparently systemic, other

vascular beds likely contribute. The pulmonary vascular bed, which is not perfused during the time of aortic cross-clamping, might be involved since ischaemia-reperfusion in the lungs results in thrombin generation at early reperfusion (Farivar et al. 2006). A rapid release of endotoxin from splanchnic microcirculation has been observed in adults after the release of the aortic cross-clamp (Jansen et al. 1992). Endotoxin, in turn, is capable of inducing TF expression on monocytes and the endothelium (Gregory et al. 1989, Moldow et al. 1993). Thus, while a thrombin burst immediately after the release of the aortic cross-clamp was previously observed in adults, no thrombin burst occurred in neonates. The definitive underlying mechanisms are beyond the scope of this study, but this finding may suggest differences in tolerance to reperfusion between adults and neonates.

The exposure to non-endothelial surfaces at the beginning of CPB resulted in immediate thrombin escalation in neonates, consistent with observations in adults. Thereafter, thrombin generation remained high. From the beginning of CPB, TF is available due to inflammatory response (Wan et al. 1997) and due to surgical trauma to the blood vessels (Boisclair et al. 1993), but the reinfusion of TF-activated blood aspirated from the operational field may be the strongest individual activator of coagulation during CPB (Chung et al. 1996, Eisses et al. 2006). All of these factors likely contributed to the enhanced thrombin generation during neonatal CPB. Aspirated blood was reinfused in all patients, the inflammatory response during CPB may even be enhanced in neonates (Alcaraz et al. 2005) and substantial tissue damage to the heart and the large vessels occurs during the repair of the congenital heart defects. Additionally, the majority of the neonates in the current study had cyanotic heart defects, which are associated with the activation of coagulation system (Chan et al. 1997). Further, the reconstituted blood used in the prime and infused during CPB may contribute to coagulation system activation.

GENERAL CONCLUSIONS

- Thrombin generation potential in both newborn PPP and PRP is reduced, but thrombin generation starts faster than in adults. Low ETP and peak thrombin probably result from low prothrombin activity in neonates, and the low anticoagulant capacity allows the rapid start of thrombin generation, with a possible additional contribution to enhanced thrombin generation from phospholipid composition of platelets in neonatal PRP. As a result, despite the low total thrombin generation capacity, rapid thrombin generation induces fast generation of fibrin clot, which may contribute to clinically uncompromised haemostasis in healthy newborn infants.

- Activation of the coagulation and fibrinolytic systems is common in sick newborns. Different clinical situations may produce a different balance between thrombin generation, fibrin formation and fibrinolysis.

- FFP induced an anticoagulant effect in neonatal plasma, which was most clearly observed in the patients with the highest pretransfusional thrombin generation. Protein C may play a marked role in the anticoagulant activity of FFP.

- In contrast to FFP, ET induced a procoagulant effect that could be observed in various measurements. *In vivo* markers of thrombin generation significantly increased during ET. *In vitro* thrombin generation potential in neonates increased to adult levels during ET, but the generation of thrombin started significantly earlier in neonates than in adults even at the end of ET. The anticoagulant effect of APC was reduced in pre-ET and in post-ET plasma. The post-ET levels of FVIII exceeded the levels expected. The observed multifactorial procoagulant state may predispose neonates to thrombotic complications.

- Platelets significantly affect the anticoagulant capacity of APC in neonatal plasma. The susceptibility of thrombin generation in newborn PRP to the anticoagulant activity of APC, in contrast to the resistance of newborn PPP to APC, suggests an actual upregulation of the protein C pathway in neonates. Differences in neonatal vs. adult platelet surface or in the modulation of FV by neonatal vs. adult platelets may contribute.

- FVL heterozygosity induced a procoagulant effect in neonatal plasma. Lower levels of FV in FVL-heterozygous infants than in healthy infants may be a feasible adaptation mechanism to counteract the procoagulant effect induced by FVL.

- Thrombin generation during neonatal cardiopulmonary bypass was escalated immediately at the beginning of CPB. In contrast to previous observations in adults,

thrombin was not increased during the early reperfusion period in neonates, suggesting different effects of ischaemia-reperfusion on coagulation in neonates vs. adults.

ACKNOWLEDGEMENTS

This study was carried out at the Pediatric Graduate School of the Hospital for Children and Adolescents, University of Helsinki, 2000-2007. I wish to thank all colleagues, collaborators and participating families for making this work possible. I wish to express my gratitude to the Head of the Hospital for Children and Adolescents, Dr. Veli Ylitalo, and to Professors Christer Holmberg, Mikael Knip, Erkki Savilahti and Martti Siimes for providing excellent research facilities.

I am deeply indebted to my two excellent supervisors, Docent Jari Petäjä and Professor Markku Heikinheimo. Jari, I thank for his continuous enthusiasm and ideas and for sharing his extensive knowledge in the field of haemostasis, providing the foundation for this work. Markku, I thank for his encouraging and positive attitude and his vast interest and knowledge in science. I also wish to thank Markku for his interest in the well-being of us graduate students in his role as the Director of the Pediatric Graduate School. Finally, Jari and Markku, thank you for being so easy to work with and for finding the time to help whenever I came to you with problems and questions.

Docent Liisa Lehtonen and Docent Anne Mäkipernaa are gratefully acknowledged for reviewing this work and for constructive and valuable comments.

Members of my thesis committee at the Pediatric Graduate School, Docent Sture Andersson and Docent Kim Vettenranta are also warmly acknowledged for their constructive comments as well as for the positive and encouraging meetings during the course of this study. I also thank Sture for his enthusiasm and fruitful collaboration as a co-author.

All of my co-authors are thankfully acknowledged. Concerning the first part of this thesis, I thank Professor Vineta Fellman for valuable comments and Docent Martti Syrjälä for introducing me to the laboratory techniques of haemostasis. The second part of this work was partly carried out at the Department of Obstetrics and Gynaecology. In this department, I am particularly grateful to Docent Risto Kaaja and Dr. Veli-Matti Ulander for recruiting patients to the study. Drs. Paula Rautiainen, Leena Mildh and Kaija Peltola at the Department of Anesthesia and Intensive Care at the Hospital for Children and Adolescents are gratefully acknowledged for making the last part of this thesis possible. Their collaboration in recruiting patients and introducing me to the complex world of neonatal cardiovascular anaesthesia has been invaluable. Finally, I thank Dr. Ulla Wartiovaara-Kautto not just for crucial collaboration concerning the laboratory methods of the study but also for her positive attitude and for delightful discussions also on other aspects of life.

I am grateful for the technical assistance of Kaisa Kuukka, Riikka Maunula, Ellen Saarela, and Liisa Tulikallio at the HUSLAB Laboratory of Haematology. I especially wish to thank Kaisa and Riikka for showing me how to perform the laboratory work myself, and Ellen and

Liisa for carrying out numerous analyses in addition to their everyday work. I also wish to thank Marita Suni at the neonatal intensive care unit for helping me in so many ways from the very beginning of the project. In addition I thank the personnel in the neonatal intensive care unit, in the operating room and in the intensive care unit of the Hospital for Children and Adolescents as well as the personnel in the delivery room of the Department of Obstetrics and Gynaecology and the laboratory staff in both departments for their assistance during the study. My gratitude is owed to Carol Ann Pelli for skilful language editing of this manuscript.

I also appreciate the many discussions with my colleagues at the old office on Lastenlinnantie and at the new office in Biomedicum 2 about everything related or unrelated to research work.

I wish to send special thanks to my friends; some of whom I have known as long as I can remember, some with whom I have shared great moments since high-school and some with whom I have shared the all-time best class in medical school! I really appreciate the relaxing times with all of you far away from work.

I owe the warmest gratitude to my parents, Sirkka and Seppo, for their love and support and for encouraging me in life as well as in this project.

Finally, I thank my dear husband and my closest friend, Mika, who has been there and supported me, always.

Financial support provided by the Finnish Pediatric Research Foundation, the Helsinki University Research Funds, the Finnish Society of Haematology, the Sigrid Jusélius Foundation and the Lilly Foundation is gratefully acknowledged.

Helsinki, February 2007

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