AGE-RELATED DIFFERENCES IN THROMBIN GENERATION USING A MATHEMATICAL MODEL

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Age-related differences in thrombin generation using a mathematical model

by

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Abstract

Quantitative differences in the coagulation system in children and infants, compared to adults, are well known. The purpose of this study is to investigate the coagulation system in children and infants and to examine the effect of the differences in protein levels on the resulting thrombin generation using a mathematical model. A mathematical model consisting of 77 ordinary differential equations, each representing the concentration of a specific protein, is used for this work. Thrombin-related output variables, including: endogenous thrombin potential (ETP), peak, time to peak, and initiation phase duration are presented and discussed.

The results of the study confirm literature reports that thrombin generation in children is both delayed and reduced compared to adults. Finally, the unknown impact of quantitative differences on the potential anticoagulant drug anti-thrombin heparin (ATH) on thrombin generation is examined for all age groups. Different ATH points at which thrombin generated is satisfactorily low (less than 100 nM·sec) were found for each age group, with increasing ATH resulting in decreased thrombin generated (ETP), at a rate inversely proportional to age. The principal conclusion is that ATH has a higher ability to suppress thrombin formation in children than in adults, hence lower amounts of ATH might be needed for antithrombin therapy in children and infants.

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To my friends and family, thank you for your encouraging words and support.

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Chapter 1

Introduction

In this thesis we are concerned with modelling the generation of a biological enzyme, thrombin, in children and infants. Hemostasis, the arrest of bleeding, occurs by the process of blood coagulation, in which thrombin plays a vital role in converting the protein fibrinogen to fibrin. With the help of factor XIIIa, fibrin forms a cross-linked fibrin clot. This clot helps arrest bleeding and also serves as a platform for vascular repair processes following injury.

The process of coagulation (thus of thrombin generation) has been a well researched area for many decades through the extensive work of many researchers [Owen, 2001]. Most research in this area has been focused on the coagulation system in adults. The discovery of the individual components of hemostasis over the last century has been accompanied by the realization that blood coagulation in children is significantly different from adults. It is with this background knowledge that we attempt to investigate these differences and their potential effects on the treatment of thrombotic problems in children, using a mathematical model.

1.1 The coagulation cascade

The breakthrough in understanding the coagulation cascade came when Davie and Ratnoff [1964] and Macfarlane [1965] proposed a waterfall sequence, explaining the function of protein clotting factors during the formation of a blood clot. Although the proposals were made independently, they formed the basis for the contact activation pathway, formerly known as the intrinsic pathway. Fibrin, which is a final product in the cascade, is formed after a series of reactions in which an inactive enzyme precursor (zymogen) is activated by its glycoprotein co-factor to become a serine protease that then catalyzes the next reaction in the cascade. The activation is by proteolysis, the directed digestion of proteins by cellular enzymes (proteases). Coagulation factors are denoted by Roman numerals that indicate the order of discovery of the factor, with a lowercase 'a' appended to denote the factor's active form.

The coagulation cascade has two pathways, as shown in Figures 1.1, which lead to the formation of thrombin. These are the contact activation pathway and the tissue factor pathway, previously known as the intrinsic pathway and extrinsic pathway respectively. Both the tissue factor and contact activation pathways converge to a final common pathway with the activation of factor X. Although originally these pathways were thought to be mutually exclusive and of equal importance, evidence has shown that this is not the case [Norris, 2003]. Although the contact activation pathway has a physiological role in hemostasis, it has been concluded that it plays a minor role in the initiation of coagulation. Its function is thought to be that of amplifying the coagulation activation initiated by the tissue factor pathway, which is the primary pathway of blood coagulation [Johari and Loke, 2012]. Each of the pathways will be discussed in turn below.

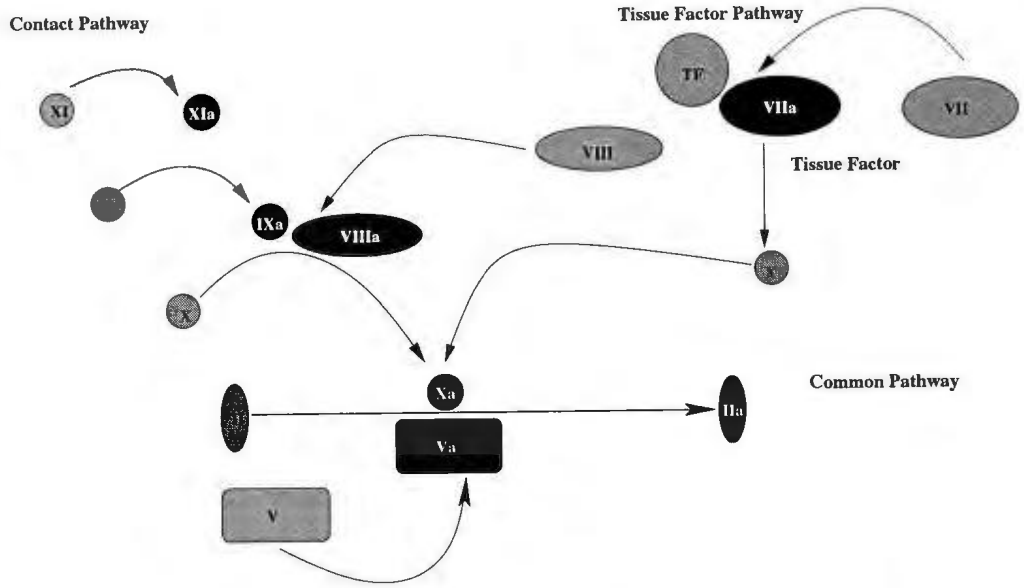

Figure 1.1: A simplified partial diagram of the coagulation cascade. The thin lines indicate the forward pro-coagulant reactions. The factors shown in a lighter shade are the precursors to the enzymes (zymogens) and the darker are the activated factors.

1.1.1 Tissue Factor activation pathway

The tissue factor pathway is activated by tissue factor (TF), a membrane glycoprotein located in the subendothelium, that comes into contact with blood following vascular injury [Maynard et al., 1975, 1977, Weiss et al., 1989, Wilcox et al., 1989]. Tissue factor, having a high affinity for factor VII that circulates in the blood, forms the complex TF-VII in the presence of calcium [Nemerson and Repke, 1985, Rao and Rapaport, 1988, Sakai et al., 1989]. When TF binds with factor VII, factor VII is activated to factor VIIa, and together they form the complex TF-VIIa. The TF-VIIa complex then converts factor X to factor Xa [Discipio et al., 1977]. A smaller amount of factor VIIa also circulates in blood plasma which also can bind with TF [Lapecorella and Mariani, 2008].

1.1.2 Contact activation pathway

The contact activation pathway begins with the formation of a complex on collagen by high-molecular-weight-kininogen (HMWK), prekallikrein (PK), and factor XII (Hageman factor). PK is converted to kallikrein and factor XII is converted to factor XIIa. Factor XIIa then converts factor XI, a plasma glycoprotein that circulates in blood, into factor XIa. Factor XIa subsequently converts factor IX to factor IXa in the presence of calcium, by hydrolysis [DiScipio et al., 1978]. Factor VIIIa is the co-factor of factor IXa, and together they form the complex IXa-VIIIa (tenase), which activates factor X to factor Xa. The contact activation pathway does not seem to play a major role in initiating clot formation. This is illustrated by the fact that patients with severe deficiencies of factor XII, HMWK, and PK do not have a bleeding disorder [Norris, 2003].

1.1.3 Final common pathway

Factor Xa (a serine protease) and factor Va (a protein cofactor) form the prothrombinase complex (Xa-Va) which cleaves prothrombin. This occurs on negatively charged phospholipid membranes in the presence of calcium ions [Tracy et al., 1981]. The complex catalyzes the conversion of prothrombin to thrombin. Thrombin has many functions in addition to its primary role in converting fibrinogen to fibrin, the building block of a blood clot. In the conversion of fibrinogen (I) to fibrin (Ia), thrombin also accelerates the conversion of factor XIII to factor XIIIa in the presence of calcium [Lorand and Konishi, 1964, Naski et al., 1991], activates factors VIII and V, as well as their inhibitor, Protein C (in the presence of thrombomodulin), and also activates factors IX and XI [Davie et al., 1991, Norris, 2003]. Following activation by the contact or tissue factor pathway the coagulation cascade is maintained in a prothrombotic

state with the continued activation of factors VIII, V, IX, and XI by thrombin in a positive feedback loop to form the tenase complex, until it is down-regulated by anticoagulants. This is illustrated in Figure 1.2 below.

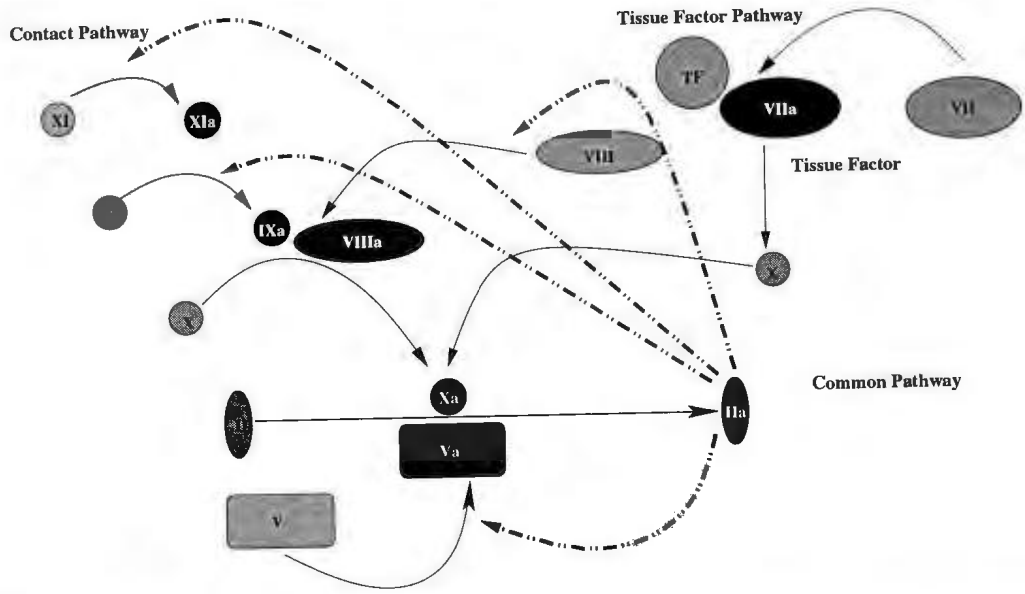

Figure 1.2: A simplified diagram of the coagulation cascade with positive feedback. Thin lines indicate the forward pro-coagulant reactions, the thick dash-dot lines are positive feedback reactions. The factors shown in a lighter shade are the precursors to the enzymes (zymogens) and the darker are the activated factors.

1.1.4 Anticoagulation

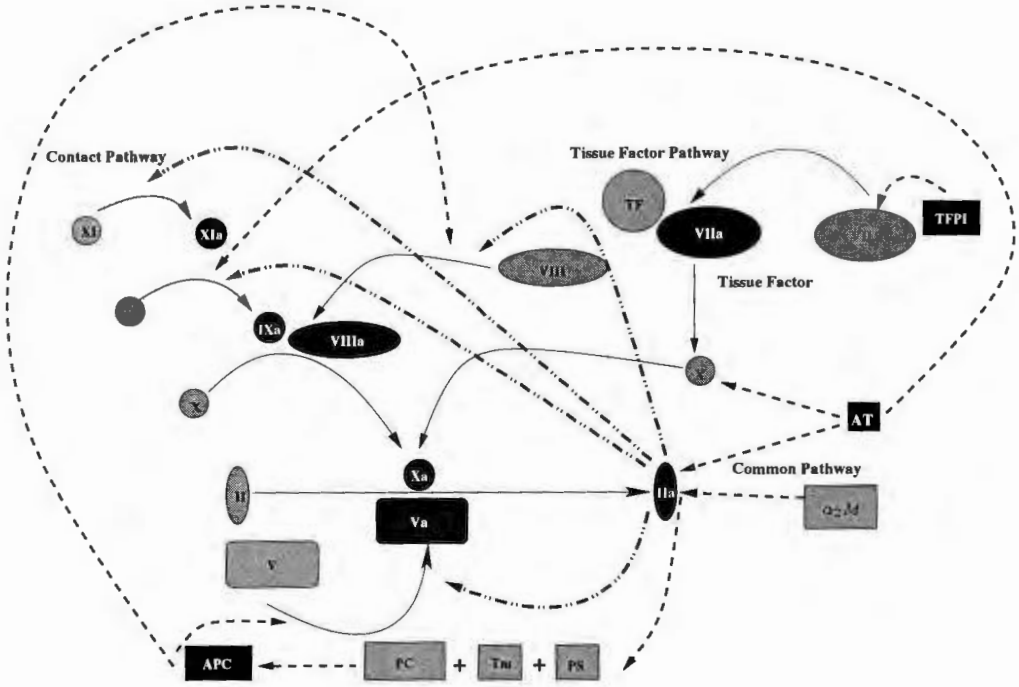

Figure 1.3: A simplified diagram of the coagulation cascade with inhibition reactions. Thin lines indicate the forward pro-coagulant reactions, the thick dash-dot lines are positive feedback reactions, and the thin dashed lines are inhibition reactions. The factors shown in a lighter shade are the precursors to the enzymes (zymogens) and the darker are the activated factors.

To limit clot extension to unaffected portions of the vascular system, the anticoagulation process depicted in Figure 1.3, has to come into play. Tissue factor pathway inhibitor (TFPI), a serine protease inhibitor, inhibits the complex TF-VIIa and is thought to be capable of shifting the cascade from the tissue factor pathway to the contact activation pathway by inhibiting the 'short burst' of thrombin in the initiation phase [Davie et al., 1991]. Antithrombin (AT) III, a protease inhibitor, is capable of blocking the activity of thrombin [Rosenberg and Damus, 1973], factors IXa [Kurachi and Davie, 1977], Xa [van't Veer and Mann, 1997], XIa, and XIIa [Rosenburg and

Bauer, 1994] via irreversible complex formation. Activated Protein C (APC), a plasma glycoprotein, inactivates both factor Va [Kisiel et al., 1977, Marlar et al., 1982] and factor VIIIa [Vehar and Davie, 1980] by proteolysis in the presence of phospholipid and calcium. This reaction is enhanced by Protein S [Walker, 1980]. Protein C is converted to APC by thrombin in the presence of co-factor thrombomodulin (Tm) [Esmon et al., 1982]. Other plasma serine protease inhibitors include heparin co-factor II [Tollefsen and McGuire, 1987], α_2-macroglobulin [Sottrup-Jensen, 1987], and trypsin [Heeb and Griffin, 1988]. α_2-macroglobulin is a natural inhibitor of thrombin, as well as factor Xa [Barret and Starkey, 1973, Meijers et al., 1987, Ignjatovic et al., 2011]. As a major plasma protease inhibitor, α_2-macroglobulin represents 2% to 4% of the total protein content in adult plasma, and also plays a role in immunity and inflammation [Sottrup-Jensen, 1989].

1.2 The hemostatic system in children and infants

While the hemostatic system in adults has been widely researched over the past few decades, the system in children and infants has been thought to be equivalent to that of adults [Monagle et al., 2010, Andrew et al., 1992]. The system in children, which begins *in utero*, appears to be incompletely developed at birth, maturing throughout infancy [Monagle et al., 2006]. Both full-term and pre-term infants are born with low levels of most procoagulant proteins, including all contact activation factors: XII, XI, HMWK and PK; and vitamin K-dependent factors: II, VII, IX, and X. Monagle and Massicotte [2011] report that differences compared to the adult system are most marked during the early stages of childhood. Similarly, levels of the major anticoagulant proteins including TFPI are low at birth. It is quite clear that although all the key components of the hemostatic system are present at birth, quantitative and

qualitative differences do exist between adults and children.

Largely due to the work by Andrew et al. in the late 80's and early 90's we have come to be aware of these differences. Monagle et al. have also done a lot of work in this area, confirming the work of Andrew et al. and reporting new information on the dynamic, age-dependent process of hemostasis, normally referred to as developmental hemostasis. For procoagulation factors, all the differences are quantitative with the exception of fibrinogen. The levels of factors II, VII, IX, X, XI, XII, HMWK and PK are low at birth and remain so until approximately 6 months of age [Andrew et al., 1992]. Levels of factor V and factor XIII are initially low but increase rapidly to those of an adult by day five [Andrew and Paes, 1987, Sell and Corrigan Jr., 1973]. In contrast, factor VIII exhibits markedly elevated levels at birth compared to adult values [Andrew and Paes, 1987, Sell and Corrigan Jr., 1973]. Fibrinogen (I) values are comparable between infants and adults, however, newborn fibrinogen is qualitatively different and exists in a fetal form for a year after birth [Andrew et al., 1990].

Levels of anticoagulant factors TFPI, AT and PC are low in full-term and pre-term newborns [Andrew and Paes, 1987, Andrew et al., 1992]. At about 3 months of age average levels of AT increase to those of adult values while Protein C remains low throughout most of childhood [Kuhle et al., 2003]. Nako et al. [1997] report that levels of thrombomodulin are increased in early childhood, decreasing to adult values by late teenage years. TFPI levels in newborns are similar to those of older children and adults [Monagle and Massicotte, 2011], although free TFPI is reportedly significantly lower in neonates [Tate et al., 1981].

Of particular interest is α_2-macroglobulin, which is increased in infants and most often reaches levels two to three-fold higher than those measured in adults [Andrew et al., 1990], seemingly compensating for the decreased levels of other anticoagulants

[Andrew et al., 1990, Ling et al., 1995]. α_2-macroglobulin contributes more to the inhibition of thrombin in children than in adults [Schmidt et al., 1989], leading to the assertion that α_2-macroglobulin is at least as important an inhibitor as AT. Within the child population, α_2-macroglobulin has been shown to be most influential in the regulation of the hemostatic system in neonates where it is a major inhibitor of coagulation, binding up to 64% of thrombin generated [Ignjatovic et al., 2011]. In adults, however, AT is the dominant inhibitor, and α_2-macroglobulin only inhibits about 7% of thrombin generated [Ignjatovic et al., 2007]. The biological basis for the increased levels of α_2-macroglobulin in infancy and childhood is not clearly understood. However, it has been shown that α_2-macroglobulin is influential in the regulation of the coagulation system in children [Ignjatovic et al., 2011].

Although qualitative differences have been clearly confirmed, what remains unclear is the extent of the qualitative changes and their implications to a growing infant. Data by Ignjatovic et al. [2011] suggests that the coagulation system in children interacts with anticoagulant drugs differently than in adults. The main question is, how do these differences affect thrombin generation? Assays show that thrombin generation in newborns is both delayed and decreased by approximately 50% compared to adults [Andrew et al., 1990, Vieira et al., 1991, Shah et al., 1992]. Although the capacity to produce thrombin increases after birth, it still remains approximately 25% less than that of adults until the late teen years [Andrew et al., 1994b, Monagle, 2004]. Also of note is that the amount of thrombin generated in plasma most closely reflects the plasma concentration of prothrombin (II) which remains 10–20% lower than adults during childhood [Andrew et al., 1992], according to the testing system used by Andrew et al. [1990]. Despite these differences in the two systems, and the perception that the hemostatic system in children is immature, it is functionally balanced with no tendency toward clotting or bleeding disorders.

1.3 Heparin

Heparin, also known as unfractioned heparin (UFH), is a widely used drug in the treatment of thrombotic disorders. Discovered in 1916 by Jay McLean, it is one of the oldest drugs currently in clinical use [Hirsh and Raschke, 2004]. It acts as an anticoagulant, preventing the formation of clots and their extension within the blood. Heparin is used for treating several conditions including deep-vein thrombosis (DVT) and pulmonary embolism (PE) [Andrew et al., 1994a, Newall et al., 2009] although it is associated with problems that include bleeding, a short half-life, unpredictable coagulant effects, impaired activity against clot-bound thrombin, heparin-induced thrombocytopenia (a low platelet count) (HIT) and osteoporosis (a thinning of the bone tissue and loss of bone density over time) [Hirsh and Buchanan, 1991, Newall et al., 2009]. It acts by binding to the enzyme inhibitor antithrombin, enhancing the inactivation of thrombin and factors Xa, IXa, XIa, and XIIa in the blood coagulation cascade [Rosenburg and Bauer, 1994]. Thrombin and factor Xa are the most sensitive to inhibition by heparin, and thrombin is about 10-fold more sensitive to inhibition than factor Xa [Hirsh and Raschke, 2004]. The rate of inactivation of thrombin and other proteases can increase up to 1000-fold due to heparin binding to AT [Bjork and Lindahl, 1982].

1.3.1 Heparin use in children

Heparin is frequently recommended in the prevention and treatment of thromboembolic disease in children [Newall et al., 2009]. There is a handful of group studies that have been carried out on UFH use in infants and children. An interesting conclusion in a study done by Andrew et al. [1994b] is that there is a disparity between UFH

doses required to achieve a target activated partial thromboplastin time (aPPT)(an indicator used to measure the effects of the intrinsic and the common pathway) in infants (28 U/kg per hour) compared with older children (20 U/kg per hour), which was the first indication that UFH use in children is age-dependent. While physiological differences in the coagulation system throughout childhood, compared to adults, are well confirmed by research, the impact of anticoagulant drugs is still not a well researched area. According to Hirsh and Raschke [2004], heparin exerts an anticoagulant action by catalysis of antithrombin inhibition of thrombin and factor Xa. This is referred to as the anti-factor IIa and anti-factor Xa effects of heparin. In work done by Ignjatovic et al. [2006], their results show that there is a clear age-related difference in aPTT which suggests that when using heparin in children, the implications of the therapeutic ranges need to be considered [Ignjatovic et al., 2006]. The evidence supporting an age-dependent response to UFH *in vitro* and *in vivo* has increased across the last half-decade [Newall et al., 2010]. Overall, the literature strongly suggests that, *in vivo*, for the same dose of UFH, the anti-factor Xa and anti-factor IIa effect, as well as the inhibition of endogenous thrombin potential (ETP), a measure of thrombin generated, is age-dependent and that these differences are not purely AT dependent. Clinical outcome studies to determine the optimal dosing for each age group have been recommended in this research area [Monagle et al., 2010].

1.4 Respiratory distress syndrome in neonates

Respiratory distress syndrome (RDS) is a breathing disorder that commonly affects premature infants born 6 weeks or more before their due dates. It is one of the most common lung disorders in infants. When thrombin is generated, it converts fibrinogen to fibrin with the help of factor XIII. While most fibrin deposition occurs

within vessels (intravascular), it also occurs outside the vessels (extravascular) in a number of organs, and contributes to morbidity of several diseases, including RDS [Conover et al., 1990, Bergstein et al., 1992].

The potential importance of fibrin in the severity of neonatal RDS is supported by the pathological presence of fibrin in the lungs of infants with RDS [Bachofen and Weibel, 1982, Gitlin and Craig, 1956]. A number of observations by these researchers suggest that fibrin may be contributing both to the severity of neonatal RDS and the subsequent development of Bronchopulmonary Dysplasia (BDP), a chronic condition involving the abnormal development of the lungs in neonates [Fukuda et al., 1987, Saldeen, 1982, Seeger et al., 1982].

The administration of an anticoagulant locally in the lungs that could prevent the formation of fibrin and not be absorbed into the system would be beneficial. The properties of an anticoagulant that could be successful include large molecular weight to prevent absorption, and the capacity to inhibit thrombin in the absence of other components of coagulation (since this will not be within the blood vessels where the components of coagulation are found).

1.5 Antithrombin Heparin (ATH)

In an attempt to synthesize an anticoagulant that had the desired properties of large weight, increased half-life and efficient anticoagulant activity, without the disadvantages of heparin, Chan et al. [1997] explored the possibility of joining heparin to the natural thrombin inhibitor, antithrombin, forming the ATH complex. Due to the problems with heparin, there have been numerous attempts to synthesize ATH by several researchers, of note: Ceustermans et al. [1982], Bjork and Lindahl [1982], Mi-

tra and Jordan [1987] and Chan et al. [1997]. Although most ATH versions have the property of increased half-life, they have not all been helpful in terms of anti-coagulation activity against thrombin and other coagulation enzymes. The most recent and successful synthesis of ATH has been reported by Chan et al. [1997].

ATH has an average molecular weight of 77000 g/mol which is considered to be large. After experimenting with ATH the researchers found that it was retained in the lungs of rabbits when administered locally and has potent anti-thrombin activity. Also, results from introducing ATH into the rabbit lungs showed that the above important properties prevented fibrin deposition in the lungs. After carrying out *in vivo* investigations with the ATH molecule involving thrombin and factor Xa inhibition, experiments showed that in comparison to AT, AT plus heparin, and AT plus high affinity heparin, ATH inhibited factor Xa and thrombin more effectively [Chan et al., 1997]. Through kinetic studies, Chan et al. reported rates at which ATH and heparin inhibit coagulation factors IIa, Xa, VIIa, IXa, XIa, and XIIa. In summary, studies have clearly demonstrated that ATH has superior antithrombic and reduced hemorrhagic properties compared to heparin, in both venous and arterial systems. Unlike heparin, ATH readily neutralizes thrombin-bound fibrin, making it suitable for treating RDS in neonates. These potential benefits of ATH call for an investigation of ATH's potential use for the treatment of thrombotic complications in newborns, including the relief of RDS, after which clinical trials may be designed.

1.6 Objectives

The objective of this work is to mathematically model the generation of thrombin in children, from which drug interactions with the blood coagulation system can be

simulated. Firstly, modifications to the currently available model [Bungay et al., 2003] are made by removing mIIa (a prothrombin to thrombin intermediate), and adding a TFPI reaction, while maintaining a physiologically reasonable model. Secondly, the model is used to compare thrombin generation between adults and children. Thirdly, drug interactions are added to the model, specifically ATH, so as to characterize real-life therapeutic interventions. This will give insight into how drugs might be best used in treatment scenarios of thrombosis and other blood complications. After adding the ATH reactions to the model we then simulate the effect of ATH on thrombin generation. In an attempt to further investigate ATH inhibition of thrombin, we vary the different coagulation parameters to gain insight into the progression of thrombin generation with increasing ATH, possibly to a point at which insignificant amounts of thrombin are produced (for example, a bleeding point). In essence we seek to understand how increasing ATH concentration affects thrombin generation and other parameters thereby laying a foundation for dose-finding studies.

1.7 Outline

In the second chapter, a discussion of the history of mathematical models of blood coagulation is provided, and the mathematical model by Bungay et al. [2003] used for this work is explained. In the third chapter, results of modelling thrombin generation in children are presented and discussed. First, the appropriate physiological thrombin generation profile of the adult model is presented and then modified to model thrombin generation in children. In the fourth chapter, the drug ATH is introduced into the model and simulated results are discussed. Finally, conclusions are presented in the fifth chapter.

Chapter 2

Mathematical models of thrombin generation

2.1 Introduction

Over the last three decades, due to the fact that the last principal reaction of the coagulation cascade was discovered in the early 1990's [Schenone et al., 2004, Williams and Wilkins, 2001], mathematical modelling has become a popular tool for understanding coagulation [Fazoil and Mikhail, 2005]. Computer simulations from mathematical models have become useful in exploring and gaining insight into thrombin generation. The objective of this chapter is to review the literature of mathematical modelling of blood coagulation and introduce the model used for the present work.

2.1.1 A short history of thrombin models

From the Davie and Ratnoff [1964] proposal (discussed in the previous chapter) sprang forth several mathematical models of blood coagulation. Seegers [1965] proposed a model for blood clotting in which he considered the process as a sequence of the formation of autoprothrombin C (FXa, thrombokinase), thrombin, and finally fibrin. Levine [1966] presented a model based on 'enzyme amplifier kinetics' in which he introduced the concept of steady-state gain of the enzyme amplifier, and further investigated the influence of small changes in the rate constants on the steady-state gain. The model was described by the following equations,

$$\frac{d[y_{1a}]}{dt} = k_1 y_1 I_a(t) - K_1 y_{1a} \tag{2.1}$$

$$\frac{d[y_{2a}]}{dt} = k_2 y_2 y_{1a} - K_2 y_{2a} \tag{2.2}$$

$$\vdots$$

$$\frac{d[y_{\mathrm{N}a}]}{dt} = k_{\mathrm{N}} y_{\mathrm{N}} y_{\mathrm{N}-1,a} - K_{\mathrm{N}} y_{\mathrm{N}a}, \tag{2.3}$$

where $I_a(t) = 1$ for $t \in [0, a]$ or 0 for $t < 0$ and $t > a$. The constant a is the activation time. The enzyme amplifier system consisted of a cascade sequence of enzyme-proenzyme reactions. During the i^{th} stage of this sequence, a proenzyme, y_i is converted to an active form, y_{ia}, which, in turn, participates in the $(i+1)^{\text{th}}$ stage. The rate of this conversion is determined by k_i. K_i is the rate at which the activated form of proenzymes are destroyed. The reaction steps are assumed to terminate at

the Nth stage. With an increase in the knowledge of the coagulation cascade, the following models modified the model by Levine.

Moro and Bharucha-Reid [1969] took the Levine model further by studying the system of differential equations he proposed. They specifically focused on the probability generalization of the Levine model that is based on the assumption that the activation time characterizing the 'enzyme amplifier system' is a random variable. Martorana and Moro [1974] went further to consider two negative physiological feedback mechanisms to the Levine model and also proposed that Levine's model and the model proposed by Seegers [1965] were identical.

Focusing on a different aspect of the coagulation cascade, Liniger et al. [1980] proposed a mathematical model of prothrombin activation. This model included a feedback mechanism of thrombin activation of factor V. The model was in agreement with the experimental data for the dependence of the rate of thrombin formation on the concentrations of factors V and Xa. It correctly predicted the existence of a maximum rate of thrombin formation at the experimentally observed concentrations of factor V and factor Xa. Nesheim et al. [1984], using their 'clot-speed' mathematical simulation, determined the distribution of the enzymatic components and substrate between the bulk fluid and phospholipid for a given set of initial concentrations of reaction components. By experimentally demonstrating inhibition by excess enzyme and phospholipids, the model was useful in rationalizing the properties of the prothrombinase complex (Xa-Va). The model also explored the properties of other factors of coagulation in addition to prothrombinase.

Nemerson and Gentry [1986] modelled the tissue factor pathway of blood coagulation and demonstrated that tissue factor and factor VII are necessary for the activation of coagulation, and that in their absence, activation is not detectable. Jones and Mann

[1994] also developed a model of the tissue factor pathway. In order to approximate the decay activity of the factor VIIIa-IXa complex, as reported by Lawson et al. [1994], their model incorporated a physical constraint associated with the stability of the complex. Leipold et al. [1995] also looked at modelling the generation of thrombin by the tissue factor pathway. This model, the first to consider the effects of exogenous inhibitors, through simulation proposed that the reason that inhibition of thrombin generation decreased dramatically as thrombin increased was due to the removal of the inhibitor through its binding to thrombin (generated in the early reaction) [Leipold et al., 1995].

Zarnitsina et al. [1996] examined the spatio-temporal dynamics of the activation of factors XI, IX, X, II, I, VIII, V and protein C. The model described the threshold behavior of coagulation, with activation at sub-threshold giving a low output of thrombin, while activation above threshold leads to an explosive amount of thrombin followed by a sharp decrease. The spatial dynamics of coagulation were analyzed for the one-dimensional case, and the model was compared with experimental data. In an attempt to confirm which process is the most important in the blood coagulation cascade, Xu et al. [2002] constructed a dynamic model of the function of platelets and showed that the pro-coagulation stimulus must exceed a threshold value, which is related to the rate of platelet activation. It was concluded that, when the level of factor IX is below 1% of normal levels, the rate of thrombin production is reduced dramatically, resulting in bleeding. They explained the bleeding tendency in most of the clinically recognized deficiencies, however, they did not discuss the reasons for thrombotic complications. Hockin et al. [2002] extended the Jones and Mann [1994] model of extrinsic blood coagulation. Their model (consisting of 34 equations) included the TFPI inhibitor, antithrombin, the activation of factor V and factor VIII by thrombin, factor VIIIa dissociation into $VIIIa_1$-L and $VIIIa_2$, the kinetic activation

steps for TF and factors VII and VIIa and the activation of factor VII by thrombin, factor Xa and factor IXa. The model displayed a non-linear dependence of thrombin generated upon TF, AT, and TFPI.

Butenas and Mann [2002] studied the dynamics of TF-initiated blood coagulation processes *in vitro* under conditions resembling those occurring *in vivo*. They used several experimental models, with their first being a 'synthetic plasma' model prepared with highly purified natural and recombinant proteins. Their second approach involved mathematical models based on reaction kinetics, allowing the prediction of the outcome of the reaction at selected parameters. The third model involved the *in vitro* study of coagulation of TF-initiated whole blood. In this model, the majority of the data agreed with the *in vivo* observation that the initiation stage is primarily influenced by the concentrations of TF and TFPI, while for the propagation phase the regulation is largely dependent on antithrombin and the protein C system.

In an attempt to explain thrombotic complications, Qiao et al. [2004] proposed a non-linear model by introducing the APC in the amplification phase (contact activation pathway) of coagulation. The model only looked at the contact activation pathway of coagulation and concluded that kinetic results showed oscillatory behavior under particularly high levels of protein C feedback inhibition.

Zhu [2007] modelled both the contact activation pathway and the tissue factor pathway. The rates of enzyme inhibition and complex formation were calculated using second-order reactions. The sensitivity of the kinetics due to a decrease in concentration of coagulation proteins was studied, and clotting times for both the contact and tissue factor activation pathways were approximated. Luan et al. [2007] used mathematical modelling to simulate platelet activation and thrombin profiles in the presence and absence of natural anticoagulants. Their work, through Monte Carlo

sensitivity analysis, identified critical mechanisms controlling the formation of thrombin, and therefore supports the hypothesis that mechanical models can be used to pinpoint key mechanisms in complex networks despite model uncertainty.

Danforth et al. [2009] used an updated Hockin et al. [2002] model to study the sensitivity of the deterministic model of blood coagulation to variations in the values of rate constants. They found that the model's predictive capacity is particularly sensitive to uncertainty in five rate constants in the regulation of the formation and function of the TF-VIIa complex. Their analysis identified specific rate constants to which the predictive capability of the model is most sensitive and thus where improvements in measurement accuracy will yield the greatest increase in predictive capacity.

The Hockin et al. [2002] updated model was also used by Mitrophanov and Reifman [2011] to examine the effects of the recombinant activated factor VII (rFVIIa) on thrombin generation by generating factor VIIa titration curves for several special cases. The effects of increasing factor VIIa concentration were observed for clotting time, thrombin peak time, and the maximum slope of the thrombin curve. Thrombin peak height was much less affected by factor VIIa titrations, and the area under the curve remained unchanged. These observations were found to match observations *in vitro*.

2.2 The model

The objective of this work is to mathematically model the generation of thrombin in children using the core chemical reactions as described in the model by Bungay et al. [2003]. The blood coagulation cascade which leads to the generation of thrombin, consists of a group of related enzymatic reactions coupled with binding of specific

proteins to lipid surfaces. These reactions are translated into a system of ordinary differential equations that model the dynamics of thrombin formation.

2.2.1 Assumptions

The model assumes a uniformly mixed, static fluid environment, which corresponds to most experimental environments. Most reactions in the coagulation cascade require the presence of calcium; it is assumed that calcium is not a limiting factor in any of the reactions. Literature values gleaned from various sources were used for the kinetic parameters. A small amount of factor VIIa was assumed to be present in the system, as reported in an experiment done by Morrissey et al. [1993].

2.2.2 Formulation of equations

The mathematical model consists of 77 ordinary differential equations (ODEs) describing the time rate-of-change in the concentration of each factor or complex involved in thrombin generation and in the available lipid. The dynamic equations are formed using classical enzyme kinetics with on-rates (k_{on}), off-rates (k_{off}), and catalytic rates (k_{cat}).

The enzymatic reactions involve an enzyme (E) cleaving a substrate (S) to produce a product (P). Before the product and the enzyme are released, an intermediary enzyme-substrate (E-S), also known as a complex, is formed. This can be represented by the equation below,

$$\text{E} + \text{S} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{E-S} \xrightarrow{k_{\text{cat}}} \text{E} + \text{P}. \tag{2.4}$$

The following assumptions are made:

- the molecules in the reaction have the correct orientation for binding and they have sufficient energy upon collision to react;
- the binding of the enzyme and substrate is reversible;
- each enzyme molecule has equal accessibility to each substrate molecule;
- the reactants remain unchanged by binding, and the reversible reaction gives the original enzyme and substrate upon dissociation.

Biochemical equations of the form of (2.4) are translated into ordinary differential equations describing the change of concentration with time of the enzyme, substrate, complex, and product. According to mass-action kinetics, the rate of a reaction is proportional to the product of the reactants' concentrations, each raised to the power of the number of each species in the reaction (the stoichiometric coefficient) - in this case one. The proportionality constants, as shown in Equation (2.4), are k_{on}, k_{off} and k_{cat}. k_{on} is the rate constant for the formation of the complex E-S, k_{off} is the dissociation rate of the complex back into the enzyme and substrate, and k_{cat} is the rate constant for the formation of the product and enzyme. Using square brackets to denote concentration, the resulting system of equations for Reaction (2.4) is,

$$\frac{d[\text{E}]}{dt} = -k_{\text{on}}[\text{E}][\text{S}] + k_{\text{off}}[\text{E-S}] + k_{\text{cat}}[\text{E-S}] \tag{2.5}$$

$$\frac{d[\text{S}]}{dt} = -k_{\text{on}}[\text{E}][\text{S}] + k_{\text{off}}[\text{E-S}] \tag{2.6}$$

$$\frac{d[\text{E-S}]}{dt} = k_{\text{on}}[\text{E}][\text{S}] - k_{\text{off}}[\text{E-S}] - k_{\text{cat}}[\text{E-S}] \tag{2.7}$$

$$\frac{d[\mathrm{P}]}{dt} = k_{\mathrm{cat}}[\mathrm{E\text{-}S}]. \tag{2.8}$$

Note that the concentration is a time-dependent variable, however, time is not shown for simplicity. For example, if we take the first two equations in the model, where tissue factor reacts with both factors VII and VIIa to form complexes, we have the following:

$$\mathrm{TF} + \mathrm{VIIa} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{TF\text{-}VIIa} \tag{2.9}$$

$$\mathrm{TF} + \mathrm{VII} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{TF\text{-}VII}. \tag{2.10}$$

Applying mass action kinetics, the above equations translate to the following system of ODEs:

$$\frac{d[\mathrm{TF}]}{dt} = -k_1[\mathrm{TF}][\mathrm{VIIa}] + k_2[\mathrm{TF\text{-}VIIa}] - k_3[\mathrm{TF}][\mathrm{VII}] + k_4[\mathrm{TF\text{-}VII}] \tag{2.11}$$

$$\frac{d[\mathrm{VII}]}{dt} = -k_3[\mathrm{TF}][\mathrm{VII}] + k_4[\mathrm{TF\text{-}VII}] \tag{2.12}$$

$$\frac{d[\mathrm{VIIa}]}{dt} = -k_1[\mathrm{TF}][\mathrm{VIIa}] + k_2[\mathrm{TF\text{-}VIIa}] \tag{2.13}$$

$$\frac{d[\mathrm{TF\text{-}VII}]}{dt} = k_3[\mathrm{TF}][\mathrm{VII}] - k_4[\mathrm{TF\text{-}VII}] \tag{2.14}$$

$$\frac{d[\text{TF-VIIa}]}{dt} = k_1[\text{TF}][\text{VIIa}] - k_2[\text{TF-VIIa}]. \tag{2.15}$$

In a similar way, all reactions are translated into differential equations describing the rate of change of the concentration of all factors that participate in the reactions.

Coagulation factors that need lipids for their reactions exist in two forms: lipid-bound (denoted with a subscript l), and free (denoted with a subscript f). For example, the free prothrombin (II_f) binds reversibly to lipid to form lipid-bound prothrombin, II_l, in the following reaction:

$$\text{II}_f + \text{LBS}_{\text{II}} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{II}_l, \tag{2.16}$$

where LBS_{II} denotes a lipid binding site for prothrombin. It is assumed that the reactive lipid is provided by 10 nm radius phospholipid vesicles. The number of protein binding sites per vesicle is calculated by assuming that the concentration of phospholipid head groups is related to the vesicle concentration as follows:

$$[\text{head groups}] = [\text{vesicles}]\frac{\text{surface area of a vesicle}}{\text{surface area of a head group}}$$

where the surface area of a head group is taken to be 0.74 nm^2 (Haung and Mason, 1978). The number of head groups per binding site varies with the lipid composition and mixing in the vesicles (Heimburg et al. 1999) and with the specific factor (van der Waart et al, 1983, Catsforth et al; 1989). An average of 100 head groups per binding site has been assumed for all factors.

2.2.3 Solution of model

Systems of differential equations cannot always be solved by analytic methods, rather numerical methods are employed to find an approximate (graphical) solution. Here, the model is solved using the double precision version of the Livermore Solver for Ordinary Differential Equations (LSODE) [Radhakrishnan and Hindmarsh, 1993] which provides a selection of methods to solve initial value problems. Systems of differential equations can have components that vary on very different time scales, resulting in one or more components varies much more rapidly than others. This time-scale variability is known as stiffness, and systems exhibiting this behaviour are referred to as being stiff. In addition to ODEs, our model consists of kinetic rate constants and initial concentration values that are of varied magnitudes. As a result, the stiff solver of LSODE, based on Gear's backward differential method, was used to solve our model. The stiff solver gives a numerical approximation for the Jacobian matrix, computed with difference quotients. All simulations were performed with an absolute and relative tolerance of 1.0×10^{-7}.

2.2.4 Conservation of mass

The law of conservation of mass, also known as the principle of energy, states that the mass of an isolated system will remain constant over time. The law implies that mass cannot be created or destroyed, although it may be rearranged and changed into different types of particles. The principle is equivalent to the conservation of concentration in our model since we are dealing with a constant volume. In these biochemical processes, the sum of all products from a given factor with non-zero initial concentration must add up to the initial concentration. For example, for factor V, which becomes activated to factor Va, all amounts of V/Va are summed at every

time step and the concentration of both these species, including all complexes that contain them, must be equal to the initial concentration of factor V used (see Figure 2.1 below). This process is repeated for each factor that has a non-zero initial concentration. Conservation of mass was confirmed for the model.

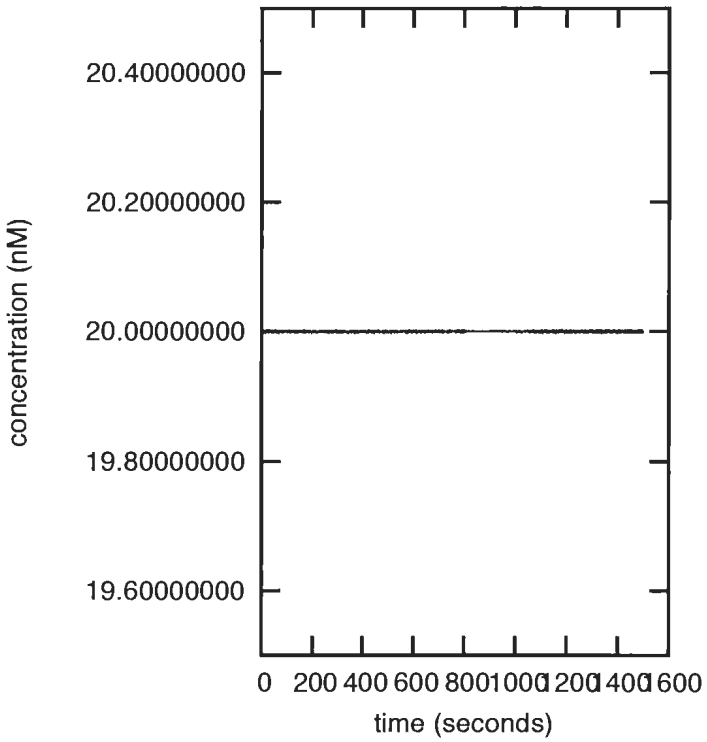

Figure 2.1: An example of the conservation of mass for the V/Va concentration amounts. The initial concentration for factor V was 20 nM.

Chapter 3

Modelling thrombin generation in children

3.1 Introduction

To our knowledge, this is the first attempt to use mathematical modelling to simulate thrombin generation in children. The work that has been done in adults is a good foundation which will be utilized in this work. In this chapter we discuss the changes made to the model, and the comparison between the adult and child models.

3.2 Profile of thrombin generation

From the adult model, the profile of thrombin generation (thrombin concentration vs. time curve) must be reasonable and comparable to experiment. Thrombin generation occurs in three distinct phases, the initiation phase, the propagation phase, and the termination phase [Danforth et al., 2009]. These phases are illustrated in Figure 3.1.

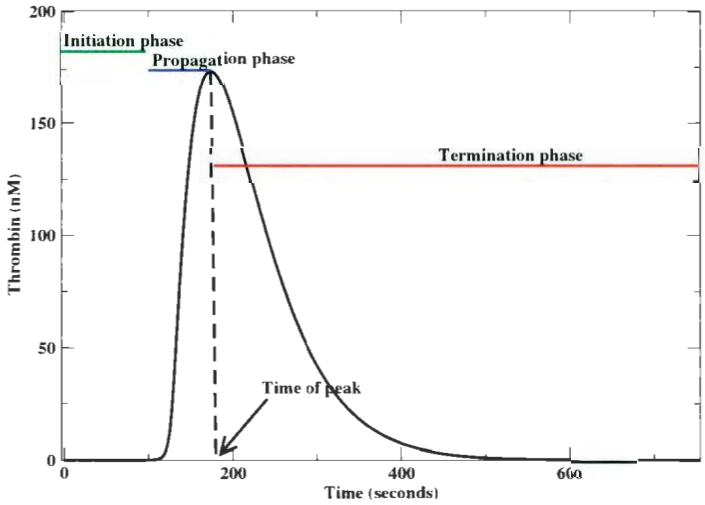

Figure 3.1: A graphical illustration of the three phases of thrombin generation: initiation, propagation, and termination.

Associated with these three phases, various measures are often used to quantitatively describe the thrombin generation profile. The following measures will be used in this work:

- endogenous thrombin potential
- peak height
- time to peak
- duration of the initiation phase
- slope of the propagation and termination phase

The endogenous thrombin potential (ETP) is defined as the amount of thrombin that can be generated after the *in vitro* activation of coagulation, with tissue factor as the trigger, in the presence of phospholipids [Hemker et al., 1993]. Computationally, it is measured by the area under the thrombin generation curve [Petros et al., 2006]. The peak height is the highest level of thrombin (or other proteins) reached in the

simulation, and the 'time to peak' is the time (in seconds) at which the peak occurs (shown as the 'time of peak' in Figure 3.1). The 'duration of the initiation phase' is the time it takes (in seconds) for the thrombin concentration to surpass 10 nM. Two slopes for each simulated graph were measured by linear regression on selected regions of the corresponding propagation and termination phases, as a measure of the rate of each phase.

3.3 Changes to the model

The model of Bungay et al. [2003], was modified to include an additional TFPI reaction and to remove mIIa while still maintaining a reasonable profile of thrombin generation in adults. Each of these changes are discussed in turn in the following two sections.

3.3.1 Removal of mIIa

We removed mIIa from the Bungay et al. [2003] model since its presence was thought to be unnecessarily complicating the model, making it difficult to use rate constant values from the literature. The following reactions,

$$\text{Va-Xa}_l + \text{II}_l \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{Va-Xa-II}_l \tag{3.1}$$

$$\text{Va-Xa}_l + \text{mII}_l \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{Va-Xa-mIIa}_l \tag{3.2}$$

$$\text{Va-Xa-II}_l \xrightarrow{k_{\text{on}}} \text{Va-Xa-mIIa}_l \xrightarrow{k_{\text{cat}}} \text{Va-Xa}_l + \text{IIa}_f + \text{LBS}_{\text{mIIa}} \tag{3.3}$$

were replaced with the single reaction

$$\text{Va-Xa}_l + \text{II}_l \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{Va-Xa-II}_l \xrightarrow{k_{\text{cat}}} \text{Va-Xa}_l + \text{IIa}_f + \text{LBS}_{\text{IIa}}. \tag{3.4}$$

with reaction rates $k_{\text{on}} = 0.1$, $k_{\text{off}} = 100$ and $k_{\text{cat}} = 15$ [Krishnaswamy et al., 1987].

This led to the deletion of reactions 25, 26, 29 and 33 from the original model equations, in which factor V and VIII, in the presence of lipid, are activated by mIIa, to Va and VIIIa respectively. Figure 3.2 shows the thrombin generation with the removal of mIIa from the original model.

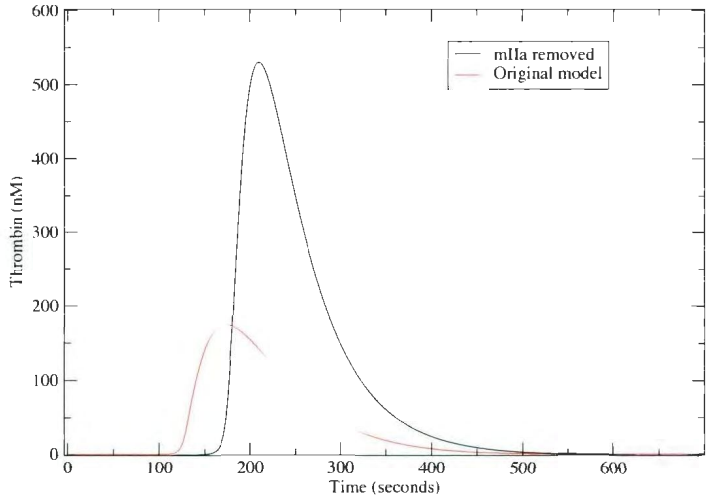

Figure 3.2: Thrombin generation in adults after the removal of mIIa from the original model.

Here, thrombin generation increases from 21,947 nM·sec to 51,571 nM·sec when mIIa is removed from the original model. The thrombin peak also increases from 174 nM to

530 nM. The initiation phase increases from 124 seconds to 175 seconds. The removal of mIIa also increases both the propagation and termination phase slopes four-fold from 4.54,-1.12 to 17.3,-4.52, respectively (see Table 3.1). Hence the presence of mIIa significantly affects the rate of thrombin generation and its termination.

3.3.2 Addition of a TFPI reaction

There are two pathways to the factor Xa dependent inhibition of TF-VIIa by TFPI. In the first pathway, factor Xa generated by TF-VIIa binds with TFPI, which in turn binds to TF-VIIa, forming the quaternary complex TF-VIIa-TFPI-Xa, as follows:

$$\text{TFPI} + \text{Xa} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{TFPI-Xa} \tag{3.5}$$

$$\text{TFPI-Xa} + \text{TF-VIIa} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{TFPI-Xa-TF-VIIa}. \tag{3.6}$$

In the second pathway, TFPI directly binds to a complex containing TF-VIIa, and factor Xa that has not yet dissociated from TF-VIIa following its activation [Broze, 2003]. Kinetic studies strongly suggest that the physiological effect of TFPI is predominantly mediated through the second pathway [Baugh et al., 1998], therefore the following equation was added to the model,

$$\text{TF-VIIa-Xa} + \text{TFPI} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{TFPI-Xa-TF-VIIa}, \tag{3.7}$$

with reaction rates $k_{\text{on}} = 0.01$ and $k_{\text{off}} = 0.0011$[Jesty et al., 1994, Baugh et al., 1998].

Figure 3.3 shows the thrombin generation with the addition of Reaction (3.7) to

the original model. Thrombin generation increases slightly from 21.947 nM·sec to 21.994 nM·sec. The thrombin peak changes slightly from 174.16 nM to 174.48 nM. The duration of the initiation phase increases from 124 seconds to 129 seconds. The addition of this extra TFPI reaction does not seem to affect either the propagation or termination phase slopes as the values 4.56 and -1.15 are not significantly different from those of the Bungay et al. [2003] model, 4.54 and -1.12, respectively. While the addition of the TFPI reaction does result in a slight increase in ETP, the removal of mIIa (in the previous section) results in a much higher ETP value of 51.571 nM·sec and peak value of 530 nM.

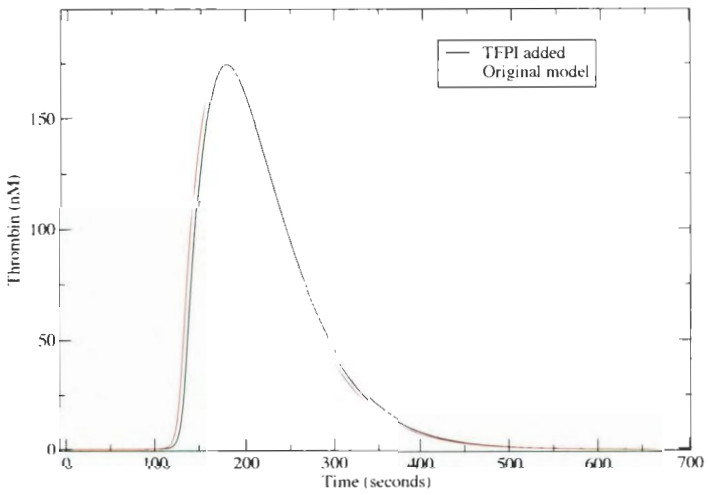

Figure 3.3: Thrombin generation in adults with the addition of the TFPI reaction (3.7) to the original model, in comparison with the original model.

3.4 Original versus modified model

Henceforth, the 'original model' is used to refer to the model of Bungay et al. [2003], while the original model with both the changes stated above will be referred to as

the 'modified model'. Figure 3.4 shows the thrombin generation profile of an adult with the Bungay et al. [2003] model and with the modified model. Also shown are the endogenous thrombin potential (ETP) values of both profiles.

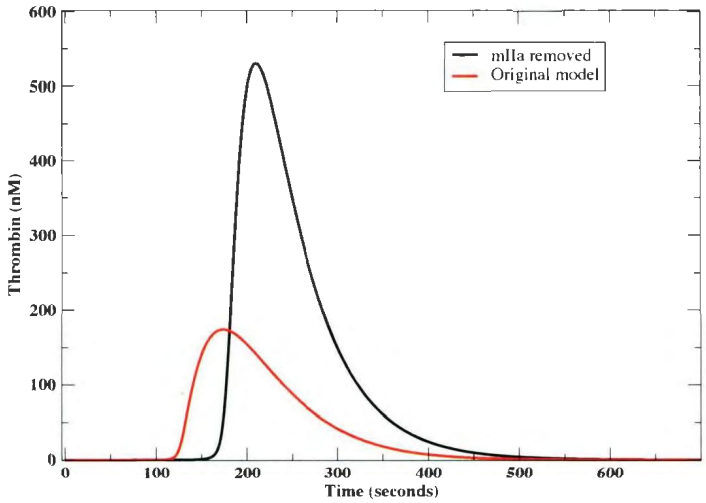

Figure 3.4: Thrombin generation in adults for the Bungay et al. [2003] model and the modified model.

Table 3.1 shows the propagation and termination phase slopes for the Bungay et al. [2003] and the modified models shown in Figure 3.4 above, as well as the slopes of both phases after the removal of mIIa, and separately after the addition of the TFPI reaction.

Table 3.1: Thrombin generation slopes for the adult model.

	Propagation phase (nMs^{-1})	Termination phase (nMs^{-1})
Bungay et al. [2003]	4.54	-1.12
TFPI	4.56	-1.15
mIIa	17.21	-4.89
Modified	17.31	-4.52

The addition of a TFPI reaction, as shown in Figure 3.3, shows a minimal change in

the initiation phase, therefore the delay in the initiation phase of the modified model compared with the Bungay et al. [2003] model is likely due to the removal of the activation of factors V and VIII by mIIa. In the initiation phase, factors V and VIII are activated, leading to the formation of small amounts of factors Xa and IXa. The rate of thrombin generation in the propagation stage is more or less the same between the Bungay et al. [2003] model and the model with another TFPI reaction added and also between the Bungay et al. [2003] model with mIIa removed and the modified model. The Bungay et al. [2003] model and the modified model have different times to peak of 174 seconds and 219 seconds respectively.

The Bungay et al. [2003] model has an endogenous thrombin potential (ETP) value of 21,947 nM·seconds while the modified model has an ETP value of 51,569 nM·seconds, more than twice the thrombin generated by the Bungay et al. [2003] model. In the Bungay et al. [2003] model, Va-Xa participates in two separate reversible reactions with prothrombin and meizothrombin to produce Va-Xa-II and Va-Xa-mII respectively. Va-Xa is directed to these two reversible reactions in the Bungay et al. [2003] model while in the modified model the same Va-Xa is directed towards a single reversible reaction, which is less effective in converting the Va-Xa-II back to Va-Xa. This leads to more thrombin being generated in the modified model.

All attempts to strictly validate the model (in the quantitative sense) with existing experimental data did not produce satisfactory results (not shown). It should be noted that it is difficult to obtain experimental data that is directly and easily comparable to the model results. However, the modified model gives a more reasonable thrombin profile based on the results observed by Hockin et al. [2002]. A comparison of mathematical models of thrombin generation [Hemker et al., 2012] indicate that the original model produced a thrombin peak that was much lower than other published

models, while the modified model increases the thrombin peak to a more comparable value.

It appears that in the original model, the equations chosen for the prothrombin to thrombin conversion sequestered the mIIa unnecessarily, resulting in thrombin production that was slow enough to allow inhibition to take over earlier. Given the time constraints of this research project, we proceed with the modified model and draw relative comparisons throughout the rest of this work. However, it is noted that firm validation of the model is required (see Chapter 5.2). From this point onwards, the modified model is used and is referred to as the 'model'.

3.5 Differences between adult and child plasma

Both qualitative and quantitative differences exist between blood coagulation proteins in adults and children [Andrew and Paes, 1987, Andrew et al., 1990, 1992]. Here, we focus on the effect of the quantitative differences using our mathematical model. These quantitative differences are summarized in Tables 3.2 and 3.3 which present the data from two labs [Andrew and Paes, 1987, Andrew et al., 1990, 1992, Monagle et al., 2006]. The data in the tables show concentration values derived from measurements of plasma samples that were obtained from healthy children and adults with no medication or past history of bleeding or thromboembolic disease. The data values were grouped into seven categories, day 1 infant, day 3 infant, 1 month–1 year, 1–5 years, 6–10 years, 11–16 years, and adults. For the purposes of this study, we consider only three of the categories, 1 day infant ('infant'), 6–10 years ('child'), and the adult. These values, referred to as reference ranges, are expressed in units per milliliter, a measure of enzyme activity. At least 20 blood samples were tested by Monagle et al.

[2006], Andrew and Paes [1987], and Andrew et al. [1988, 1992] for each analyte in each age group and an average value reported, including its lower and upper boundary values encompassing 95% of the population. Expressing the reference values as a proportion of the adult reference value, we find the percentage reference value for a particular protein for each age-group relative to the adult. We convert the infant and child references to concentration values by multiplying the percentages for each protein in each age group by the known initial concentration level of the adult [Butenas et al., 1999]. In this way we obtain concentration levels of all proteins (in nanomolar) for both infants and children, as expressed in Tables 3.2 and 3.3. Both data sources confirm age-related changes in coagulation protein levels throughout childhood, and show a trend that agrees with the developmental haemostasis concept. The reasons for the observed differences between children and adults are still uncertain and likely reflect differences in rates of protein synthesis. Despite its difference from the adult, the haemostatic system in children is considered physiologic.

Table 3.2: Coagulation factor levels (in nM) as reported in Andrew and Pacs [1987], and Andrew et al. [1988, 1992]

Species	1 day infant	6 to 10 years	adults
Prothrombin	622	1141	1400
Tissue Factor	0.005	0.005	0.005
Factor V	14	17	20.0
Factor VII	6	8	10.0
Factor VIII	0.7	0.7	0.7
Factor IX	44	62	90
Factor X	64	120	170
Factor XI	12	27	30
Protein C	22	43	60
TFPI	2.5	2.5	2.5
Antithrombin	2,142	3774	3400
Protein S	133	289	300
α_2-Macroglobulin	4202	5109	2600

Table 3.3: Coagulation factor levels (in nM) as reported in Monagle et al. [2006]

Species	1 day infant	6 to 10 years	adults
Prothrombin	687	1133	1400
Tissue Factor	0.005	0.005	0.005
Factor V	14	17	20.0
Factor VII	5	9	10.0
Factor VIII	0.7	0.5	0.7
Factor IX	33	66	90
Factor X	75	133	170
Factor XI	8	30	30
Protein C	21	55	60
TFPI	2.5	2.5	2.5
Antithrombin	2692	4038	3400
Protein S	144	436	300
α_2-Macroglobulin	4202	5109	2600

For both data sources, most coagulant factors increase in concentration from the infant to the adult levels. For example, prothrombin is 622 nM, 1141 nM, and 1400 nM for the Andrew and Pacs [1987], Andrew et al. [1990, 1992] data and 687 nM, 1133 nM and 1400 nM for the Monagle et al. [2006]. Tissue factor, factor VIIa (0.1 nM) and TFPI values are the same for both the data sets as they are assumed to be the same as in the adult. Differences between the data sets are found in the factor VIII, AT and Protein S levels. For the Andrew and Pacs [1987], Andrew et al. [1988, 1992] data, factor VIII concentration is 0.7 nM for all three age groups while in the Monagle et al. [2006] data the value decreases to 0.5 nM in the child age group. In the Andrew and Pacs [1987], Andrew et al. [1988, 1992] data, Protein S concentration progressively

increases to 300 nM for the age groups, while in the Monagle et al. [2006] data the middle child group value of 436 nM is significantly higher than the adult value of 300 nM. AT concentration follows a similar trend where the child level is greater than the adult for both data sources although the absolute values are higher in the Monagle et al. [2006] data. The differences in the values between the two sets of data can be attributed to different measuring devices and reagents. However, both show a trend consistent with the developmental haemostasis principle.

3.6 Simulation using the Andrew et al. data

Figure 3.5 shows the thrombin profiles of all age groups (adults, children and infants) using data from Andrew and Paes [1987], and Andrew et al. [1988, 1992].

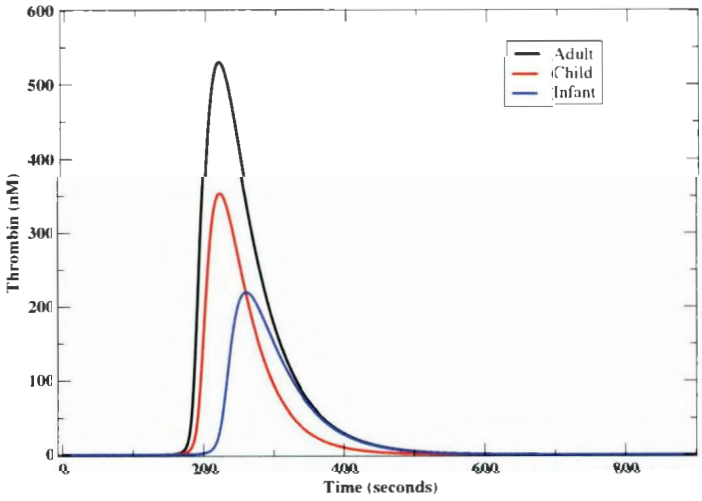

Figure 3.5: Simulated thrombin generation in three age groups with initial data taken from Andrew and Paes [1987], and Andrew et al. [1988, 1992] (see Table 3.2).

Table 3.4 shows the ETP, time to peak, peak thrombin, and the duration of the

initiation phase for all age groups for the results displayed in Figure 3.5.

Table 3.4: Model results for Andrew and Pacs [1987], and Andrew et al. [1988, 1992] data.

	ETP (nM·sec)	Peak (nM)	Time to Peak (seconds)	Initiation phase (seconds)
Adult	51,569	530	219	175
Child	28,900	354	223	184
Infant	22,290	220	261	215

Table 3.5 shows the propagation and termination phase slopes for the adult, child and infant age groups for the Andrew and Pacs [1987], and Andrew et al. [1988, 1992] data. For both the propagation and termination phases, the magnitude of the slopes nearly triples from infants to adults.

Table 3.5: Thrombin propagation and termination phase slopes for the Andrew and Pacs [1987], and Andrew et al. [1988, 1992] data.

	Propagation phase (nMs^{-1})	Termination phase (nMs^{-1})
Adult	18.81	-5.08
Child	12.96	-4.10
Infant	6.97	-2.00

3.6.0.1 Thrombin generated and peak

From Figure 3.5, we see that the child thrombin peak is lower than the adult peak, while the infant thrombin peak is lowest. This pattern matches the pattern of total amount of thrombin generated as evidenced by the ETP values in Table 3.4. Overall, the simulation results are consistent with the developmental haemostasis principle by Andrew et al. [1994b], and in the case of the infant, also the observation that the amount of thrombin generated is proportional to the initial amount of the prothrombin. The prothrombin concentration in infants and children is 44% and 82% of that in adults, while the calculated ETP values for the infant and children are 43.2% and

56% of adults respectively. Both prothrombin and ETP have similar percentages at the infant category of about 44% while the child's percentages of 82% and 56% for prothrombin and ETP respectively, are clearly different. Hemostasis is a progressive system that develops with age, and importantly, the ability to produce thrombin increases with age, yet the increase does not necessarily correspond in percentages with prothrombin concentration as there are other factors influencing thrombin generation.

3.6.0.2 Initiation phase

The infant profile has the longest initiation phase, followed by the child, and then the adult profile. In the initiation phase activated factor V and factor TF-VIIa play an important role, with TF-VIIa generating small amounts of factor Xa and factor IXa. This leads to the formation of picomolar amounts of thrombin (less than 10%) when prothrombin is activated [Mann et al., 2003]. In these results, the different initiation phase lengths are due to the trend in concentration levels of factors V and VII. However, factor VII concentration levels are the lowest for the infants, leading to a lower TF-VIIa amount, hence the observation of the longest initiation duration for the infant.

To further investigate which of the two, factors V or VII, has a bigger effect on the initiation phase, we changed the factor V concentration to the adult value, for both infants and children, while the factor VII concentration was unchanged. In the second case we changed the factor VII concentration to the adult value for both infants and children, while the factor V concentration was unchanged. The results for these simulations are presented in Figures 3.6 and 3.7, and Tables 3.6 and 3.7 below.

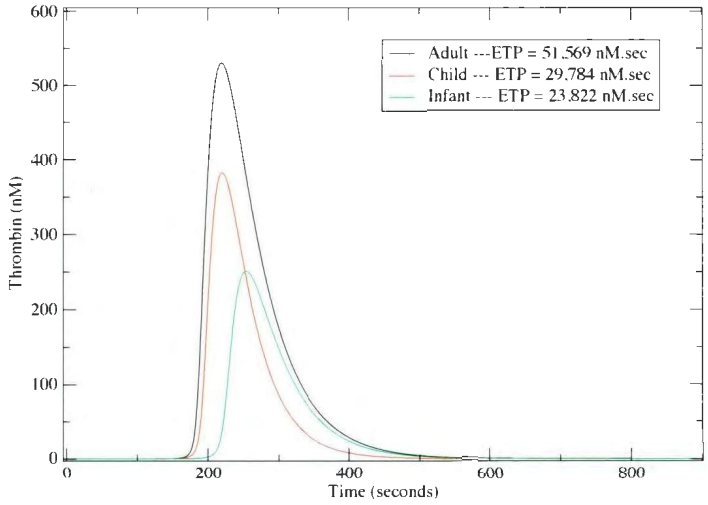

Figure 3.6: Simulated thrombin generation, with initial data taken from Andrew and Paes [1987], and Andrew et al. [1988, 1992] (see Table 3.2) but with factor V concentration set to the adult value for all age groups.

Table 3.6: Factor V test results for Andrew and Paes [1987], and Andrew et al. [1988, 1992] data.

	ETP (nM·sec)	Peak (nM)	Time to Peak (seconds)	Initiation phase (seconds)
Adult	51.569	530	219	175
Child	29.784	382	220	182
Infant	23.822	251	254	211

When factor V is held 'constant' (at adult concentration values for all age-groups), initiation duration for the child and infant relative to the Figure 3.5 simulation (see Table 3.4) decreases by 2 seconds and 4 seconds respectively. However, ETP values for the child and infant both increase by 3.1% (884 nM·sec) and 6.9% (1,532 nM·sec), respectively. Similarly, there is an increase in peak of 7.9% (28 nM) and 14.1% (31 nM) for the child and infant, respectively.

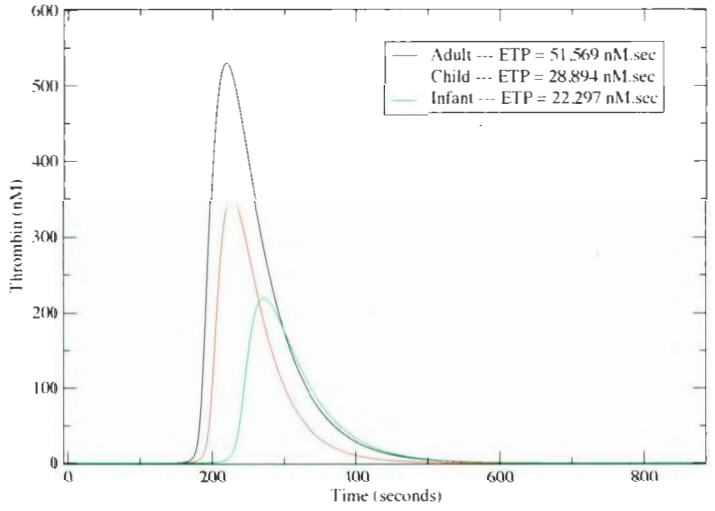

Figure 3.7: Simulated thrombin generation, with initial data taken from Andrew and Paes [1987], and Andrew et al. [1988, 1992] (see Table 3.2) but with factor VII concentration set to the adult value for all age groups.

Table 3.7: Factor VII test results for Andrew and Paes [1987], and Andrew et al. [1988, 1992] data.

	ETP (nM·sec)	Peak (nM)	Time to Peak (seconds)	Initiation phase (seconds)
Adult	51.569	530	219	175
Child	28.894	353	228	188
Infant	22.297	220	278	227

For 'constant' factor VII, the initiation duration increases for the child and infant by 4 seconds and 12 seconds respectively. The ETP value of the child decreases (by 0.021%) from 28.900 nM·sec to 28.894 nM·sec while that of the infant increases (by 0.031%) from 22.290 nM·sec to 22.297 nM·sec. The infant peak remains the same (220 nM), while the child peak decreases by 0.28% from 354 nM to 353 nM. From these observations, it seems that factor VII has a bigger effect on the initiation duration while factor V has a more significant effect on the thrombin generated, as measured by the ETP values.

The factor X concentration is quite different in adults and children (see Table 3.2). To investigate the effect of this difference, we changed the factor X levels to those of an adult in children and infants, with the results shown in Figure 3.8.

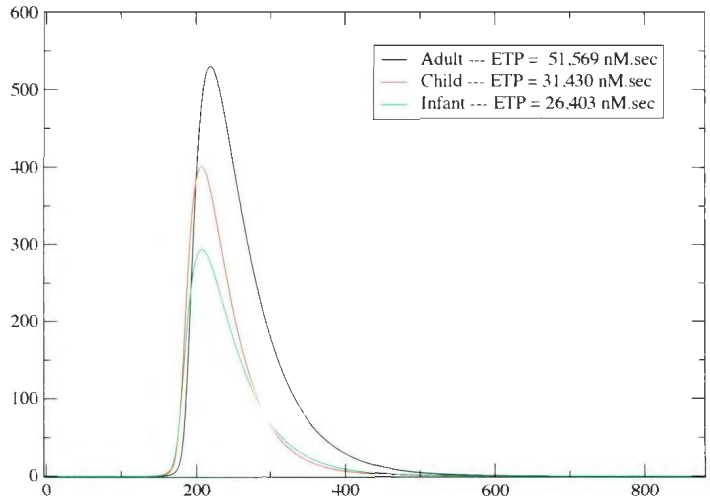

Figure 3.8: Simulated thrombin generation with initial data taken from Andrew and Paes [1987], and Andrew et al. [1988, 1992] (see Table 3.2) but with factor X concentration changed to the adult value for all age groups.

Table 3.8 shows the ETP values, peak time, peak, and initiation phase length for thrombin generation with factor X changed to the adult value for all age groups.

Table 3.8: Factor X test results for Andrew and Paes [1987], and Andrew et al. [1988, 1992] data.

	ETP (nM·sec)	Peak (nM)	Time to Peak (seconds)	Initiation phase (seconds)
Adult	51,569	530	219	175
Child	31,430	401	207	168
Infant	26,403	293	208	167

Changing the child and infant factor X levels to the adult level increases the ETP values to 31,430 nM·sec and 26,403 nM·sec respectively (see Tables 3.4 and 3.8). There is also a corresponding increase in the peak level of thrombin generated for both age

groups. The time to peak decreases for both age groups when factor X concentration is increased to the adult value, which points to a shorter initiation phase as higher factor X is likely to speed up the common pathway leading to the formation of thrombin.

As expected, the increase in factor X levels for the child and infant increases the thrombin generated. However, of note is the shortening of the initiation phase for both the child and the infant, leading to a faster initiation (168 seconds and 167 seconds respectively) than the adult. Comparing with Figure 3.5, it is clear that the infant initiation is the more affected of the two. The change in ETP is similar for both age groups.

3.6.0.3 Propagation phase

In the propagation phase prothrombin is activated and converted to thrombin by Va-Xa [van't Veer and Mann, 1997], and the majority of thrombin produced is due to the positive feedback activation of factors V and VIII by thrombin. The amount of thrombin produced is therefore dependent on the prothrombin amounts of 622 nM, 1141 nM and 1400 nM [Andrew et al., 1990], and also on Va-Xa. Since prothrombin is never completely exhausted, Va-Xa becomes the limiting factor in thrombin production. The infant has the least prothrombin concentration of all the three age groups, while the adult has the greatest. This could contribute to explaining why we have the least thrombin generated in the infant, and the greatest in the adult (based on ETP values, see Figure 3.5).

Figure 3.9 shows the simulated change in prothrombin concentration over time for the three age groups. Prothrombin conversion to thrombin begins at the end of the initiation phase, thereafter reducing prothrombin concentration. However, the prothrombin concentration is never reduced to zero which is consistent with experimental observations.

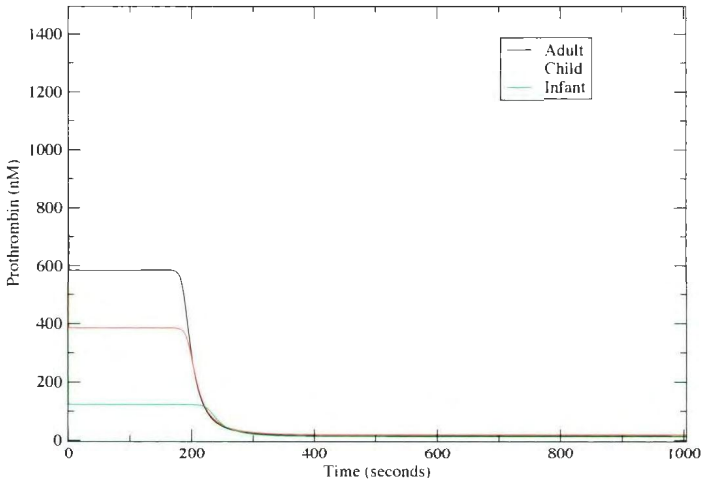

Figure 3.9: Simulated prothrombin concentration in three age groups, with initial data taken from Andrew and Paes [1987], and Andrew et al. [1988, 1992] (see Table 3.2) showing that prothrombin is not a limiting factor.

Figure 3.10 below shows the effects of changing child and infant prothrombin concentration values to adult values.

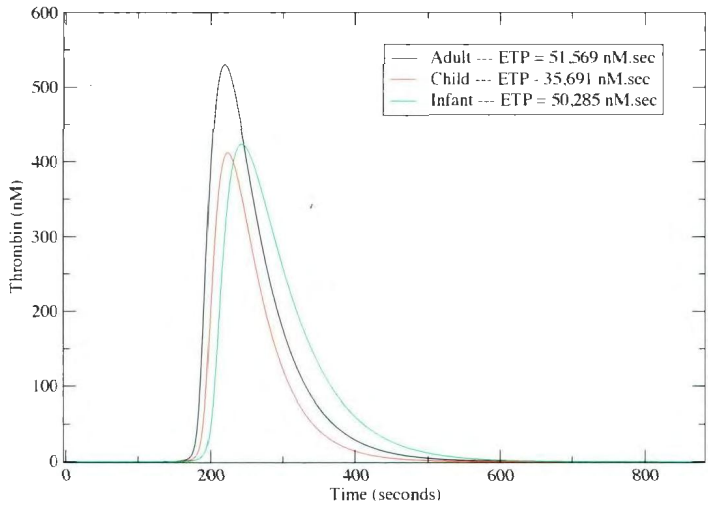

Figure 3.10: Simulated thrombin generation, with initial data taken from Andrew and Paes [1987], and Andrew et al. [1988, 1992] (see Table 3.2) but with prothrombin concentration set to the adult value for all age groups.

As expected, the increase of prothrombin levels to adult values increases the ETP values from 28,900 nM·sec to 35,691 nM·sec and 22,290 nM·sec to 50,285 nM·sec for the child and infant respectively. This increase in ETP is very large (twice the original ETP value) for the infant, reflecting the sensitivity of infants to prothrombin levels, likely due to the lower anticoagulant amounts. While there is an increase in peak thrombin of 58 nM (354 nM to 412 nM) and 203 nM (220 nM to 423 nM) for both the child and infant respectively, the infant has a notably greater increase and higher peak. There is hardly any change in the time to peak value for the child (223 seconds to 224 seconds) while for the infant the time to peak value decreases (261 seconds to 244 seconds).

3.6.0.4 Termination phase

The termination phase occurs when the thrombin generated begins to decrease after reaching the peak. This phase is controlled by the anticoagulants, and continues until

the thrombin concentration reaches zero. The termination of the adult and child thrombin generation looks similar, but is sharper than that of the infant profile (see Figure 3.5 and Table 3.5). This could be a result of the higher levels of most inhibitors in children and adults compared to infants.

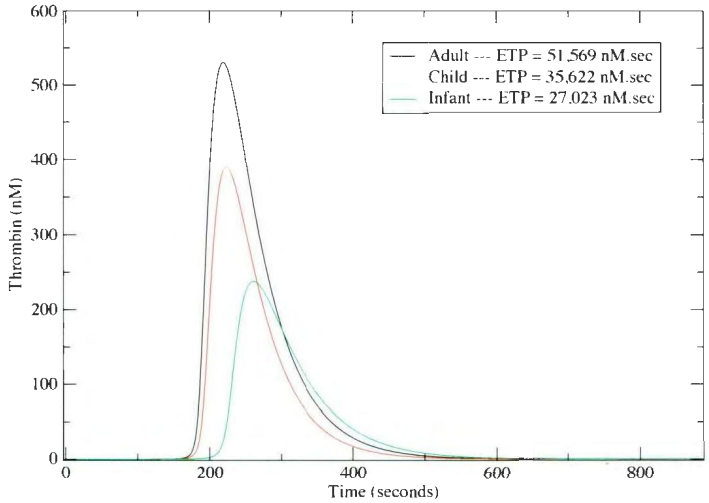

Figure 3.11: Simulated thrombin generation, with initial data taken from Andrew and Paes [1987], and Andrew et al. [1988, 1992] (see Table 3.2) but with α_2-macroglobulin concentration set to the adult value for all age groups.

α_2-macroglobulin is an important inhibitor in children, therefore we investigate its influence on thrombin generation in children and infants by setting its concentration to adult levels (see Figure 3.11). When α_2-macroglobulin is changed to adult values for the child and infant (a reduction of α_2-macroglobulin since it is higher in children and infants than adults) there is an increase in ETP values from 18,087 nM·sec to 35,622 nM·sec for the child and from 22,290 nM·sec to 27,043 nM·sec for the infant, both reflecting an increase in thrombin generated (see Table 3.9). There is hardly any change in the time to peak (224 nM and 261 nM for the child and infant respectively), suggesting that α_2-macroglobulin has no significant effect on the time to peak and

most likely the initiation phase of thrombin generation. There is a corresponding increase in peak levels of 18 nM (220 nM to 238 nM) and 36 nM (354 nM to 390 nM) for the infant and child, as seen with most ETP changes. Notably, due to the decrease in α_2-macroglobulin, the termination phase for both the child and infant becomes less sharp relative to the simulation with the original α_2-macroglobulin levels (see Figure 3.5).

Table 3.9: Model results for α_2-macroglobulin concentration set to the adult value for all age groups.

	ETP	Peak	Time to Peak	Initiation phase
	(nM·sec)	(nM)	(seconds)	(seconds)
Adult	51,569	530	219	175
Child	35,622	390	224	183
Infant	27,023	238	261	213

To measure the effect of the α_2-macroglobulin inhibition, the model was run for Table 3.2 data, with α_2-macroglobulin set to zero. Table 3.10 shows the ETP comparisons, including the change in ETP when α_2-macroglobulin is absent.

Table 3.10: Change in ETP with the removal of α_2-macroglobulin for Andrew and Paes [1987], and Andrew et al. [1988, 1992] data.

	ETP (nM·sec)	ETP without α_2-macroglobulin (nM·sec)	ETP change (%)
Adult	51,569	72,708	41
Child	31,430	47,294	64
Infant	26,403	41,818	88

The thrombin generated with the inhibition of α_2-macroglobulin is 41%, 64%, and 88% (of the thrombin without) for adults, children and infants respectively. α_2-macroglobulin has the greatest effect on the infant ETP.

3.7 Simulation using the Monagle et al. data

Figure 3.12 shows the thrombin profiles for all three age groups based on the Monagle et al. [2006] data (see Table 3.3).

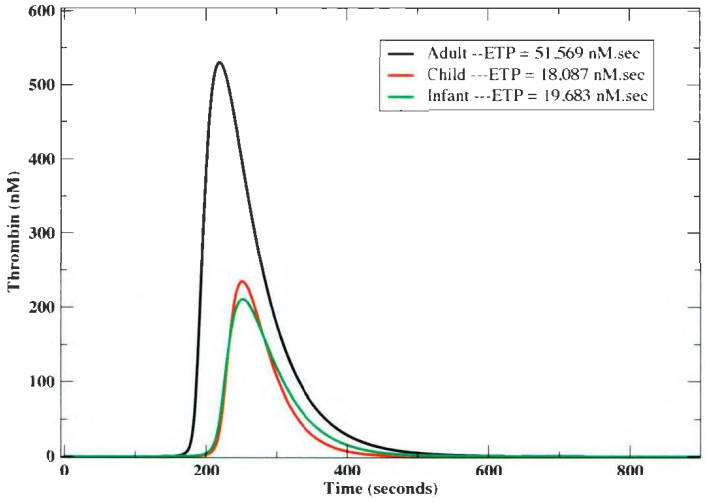

Figure 3.12: Simulation of thrombin generation in three age groups using the Monagle et al. [2006] data in Table 3.3.

Table 3.11 shows model results for ETP, time to peak, peak thrombin, and the duration of the initiation phase for three age groups, based on the results shown in Figure 3.12.

Table 3.11: Model results for Monagle et al. [2006] data.

	ETP	Peak	Time to Peak	Initiation phase
	(nM·sec)	(nM)	(seconds)	(seconds)
Adult	51,569	530	219	175
Child	18,087	235	251	211
Infant	19,863	197	256	207

Table 3.12 shows the propagation and termination phase slopes for these simulations. The magnitude of both slopes increase from infants to adults.

Table 3.12: Thrombin propagation and termination phase slopes for the Monagle et al. [2006] data.

	Propagation phase (nMs^{-1})	Termination phase (nMs^{-1})
Adult	18.26	-4.91
Child	9.09	-2.94
Infant	7.38	-2.18

3.7.0.5 Thrombin generated and peak

From Figure 3.12, we see that the infant thrombin peak is lower than both the adult and child thrombin peak levels. This peak pattern does not correspond to the amount of thrombin generated as measured by ETP values of 51,569 nM·sec, 18,087 nM·sec, and 19,683 nM·sec for adults, children, and infants respectively. The prothrombin concentration in infants and children is 49% and 81% of that of adults, while the calculated ETP values for the infant and children are 39% and 35% of adults respectively. Although the prothrombin levels reflect a trend consistent with developmental haemostasis for the Monagle et al. [2006] data, the ETP values for the child and infant do not conform to the 50% to 75% of adult thrombin range reported in the literature [Andrew et al., 1990, Vieira et al., 1991, Shah et al., 1992, Andrew et al., 1994b, Monagle, 2004]. However, thrombin generation is still delayed and reduced in children and infants compared to adults.

3.7.0.6 Initiation phase

For the Monagle et al. [2006] data the initiation phase for the child and infant profile is delayed, similar to the infant profile for the Andrew and Paes [1987], and Andrew et al. [1988, 1992] data. The initiation phase for the child and infant profile is 211 seconds,

36 seconds longer than the adult initiation phase of 175 seconds. The similar initiation phase length for the child and infant profiles is reflective of the minimal difference in the amounts of the factors most influential in the initiation phase, factors V and VII in the Monagle et al. [2006] data.

3.7.0.7 Propagation phase

Although the child profile has a higher thrombin peak of 235 nM compared to the infant peak of 197 nM, the infant ETP is greater than that of the child profile. The child ETP is 35% of the adult value of 51,569 nM.sec while the infant ETP is 38%. These observations seem to be inconsistent with developmental hemostasis. Immediately after initiation, the continuous downregulation of thrombin by AT and the Protein C system begins [Mann et al., 2009]. In the Monagle et al. [2006] data we have significantly higher amounts of both AT and Protein C, this could be the reason for the observations above. In addition, the low factor VIII concentration for the child could also be playing a role in this observation. The time to peak for the child and infant are close at 251 seconds and 256 seconds respectively, although both are longer than that of the adult at 219 seconds. The propagation phase is much steeper for the adult at 18.26 nMs^{-1}, approximately double the slope of 9.09 nMs^{-1} and 7.38 nMs^{-1} for the child and infant, respectively.

Prothrombin plays a critical role in thrombin generation where it is converted to thrombin in the common pathway. For the Monagle et al. [2006] data (see Table 3.3) the infant prothrombin concentration is less than half the adult, and the child concentration is about 81% of the adult. To investigate the sensitivity of prothrombin concentration levels to thrombin generation in infants and children, we set the prothrombin concentration to adult levels. Figure 3.13 shows the effects of increasing the

prothrombin values in children and infants to adult levels.

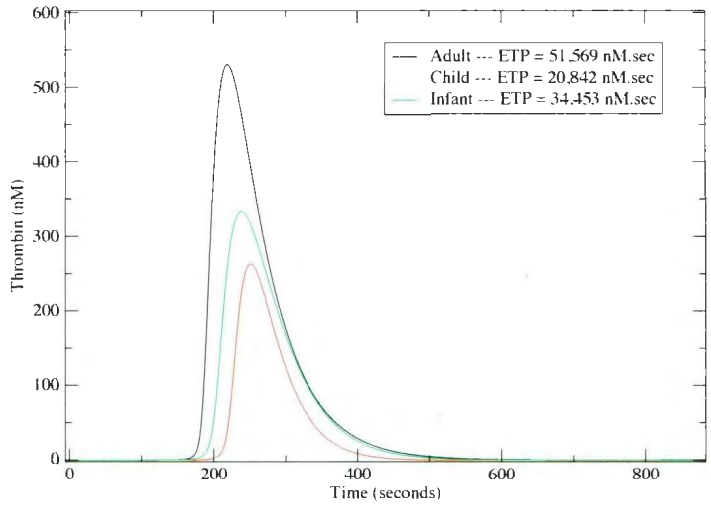

Figure 3.13: Simulated thrombin generation, with initial data taken from Monagle et al. [2006] (see Table 3.3) but with prothrombin concentration set to adult levels for all age groups.

As expected, the increase of prothrombin to adult values increases the ETP values for both the child (18,087 nM·sec to 20,842 nM·sec) and infant (19,863 nM·sec to 34,453 nM·sec) profile, indicating an increase in thrombin generated. However, this increase in ETP is very large for the infant, nearly twice the original value. The infant prothrombin increase to the adult value, which is about twice the infant value, increases the ETP to almost twice the amount. Similary, for the child, the prothrombin to ETP proportions are comparable (1:1.24 compared to 1:1.15). There is an increase in the peak values for both the child (235 nM to 264 nM) and the infant (197 nM to 333 nM), with the increase in the infant more than 1.5 in maguitude. There is hardly any change in the time to peak for the child (251 nM to 252 nM), with a slight decrease in the infant time to peak (256 nM to 252 nM). The infant seems to be particulary more sensitive to the prothrombin level than the child.

3.7.0.8 Termination phase

The termination portion of the curve for the child profile is sharper than that of the infant (Figure 3.12), reflecting the higher levels of anticoagulants in the child data (see Table 3.3). As the infant profile terminates, it approaches zero nM thrombin at a more gradual slope of 2.18 nMs^{-1}, hence contributing to the higher ETP value compared to the child. The adult termination slope of 4.91 nMs^{-1} is the steepest, reflecting the higher Protein C and Protein S levels.

Protein C is an important inhibitor of thrombin through its interaction with Protein S and Tm, forming the activated Protein C system. However, its inhibitory activity with varying age is unknown [Monagle and Massicotte, 2011]. To investigate further we set Protein C concentration to adult values for all age groups. Figure 3.14 shows the simulation of thrombin generation but with Protein C concentration set to adult values for the child and infant.

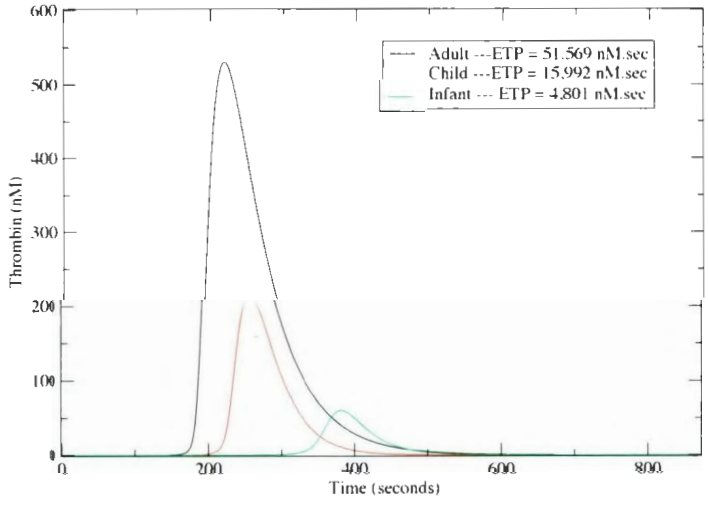

Figure 3.14: Simulated thrombin generation in three age groups, with initial data taken from Monagle et al. [2006] (see Table 3.3) but with Protein C concentration set to adult values for all age groups.

When the level of Protein C is increased to the adult level of 60 nM in both the child and infant, there is a decrease in ETP values from 18,087 nM·sec to 15,992 nM·sec for the child, and 19,863 nM·sec to 4,801 nM·sec for the infant. For the child, a 9.1% increase in Protein C leads to a 11.6% decrease in ETP while for the infant, a 185.7% increase in Protein C gives a 75.8% decrease in ETP. The change in the infant ETP is equivalent to a four-fold decrease. There is no significant change in time to peak in the child profile, while there is an increase in the time to peak for the infant from 256 seconds to 383 seconds. Similarly, the peak levels for both the child (235 nM to 215 nM) and infant decrease, with a decrease of more than 3 times for the infant profile (197 nM to 60 nM).

It is clear from Figures 3.12 and 3.14 that the increase in Protein C lowers the thrombin generated for both children and infants. However, the infants seem to be more sensitive to this increase, as evidenced by a more than four-fold decrease in thrombin generated (19,683 nM·sec to 4801 nM·sec). In addition, Protein C seems to delay thrombin generated as the initiation phase increases for both children and infants. Although the activity of Protein C with varying age is unknown, the model indicates that an increase in Protein C will result in decreased and delayed thrombin generation.

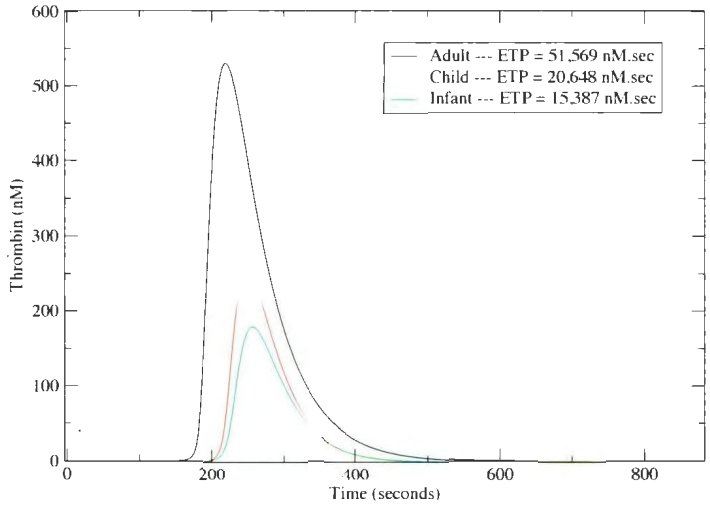

Figure 3.15: Simulated thrombin generation in three age groups, with initial data taken from Monagle et al. [2006] (see Table 3.3) but with AT concentration set to adult values for all age groups.

AT is a major inhibitor of thrombin and activated factor X in both adults and children and its effects in infants and children is thought to be compensated for by the higher levels of α_2-macroglobulin [Ignjatovic et al., 2011]. When the AT level is decreased by 15.8% to adult levels for the child, there is a slight increase (of 14.2%) in ETP values, from 18,087 nM·sec to 20.648 nM·sec, while there is a 22.5% decrease (19,863 nM·sec to 15,387 nM·sec) in the infant ETP from a 26.3% increase in AT. Changes in AT for both child (15.8%) and infant (26.3%) lead to corresponding similar changes of ETP of 14.2% and 22.5%, respectively. Peak values change similarly, with an increase in the child (235 nM to 253 nM) and decrease in the infant (197 nM to 180 nM). There is no significant change in the time to peak for either age group (251 seconds to 253 seconds for the child and 256 seconds to 257 seconds for the infant), suggesting that AT has an insignificant effect on the initiation phase.

The effect on thrombin generation of a change in AT concentration is not significant when compared to the effect of changing Protein C. This seems to agree with the

hypothesis that AT activity in children and infants is largely compensated for by α_2-macroglobulin [Monagle and Massicotte, 2011]. Overall, both AT and Protein C seem to affect the ETP and initiation phase of thrombin.

3.7.1 Discussion

In the propagation phase of the Monagle et al. [2006] simulation (see Figure 3.12), less thrombin is produced for the child and infants when compared to the Figure 3.5 simulation based on the Andrew and Paes [1987], and Andrew et al. [1988, 1992] data. The lower thrombin generated is observed regardless of the higher factor X levels (64 nM and 120 nM versus 75 nM and 133 nM) and the same factor V level (14 nM and 17 nM) in the Monagle et al. [2006] simulation (see Figure 3.12). This observation can be attributed to the higher anticoagulants AT and Protein S in the Monagle et al. [2006] data (see Table 3.3). Simulation of thrombin generation using the Monagle et al. [2006] data for the age-dependent coagulation protein levels gives results that are not totally consistent with conclusions by Andrew et al. [1992]. The ETP values do not increase with age, as shown in Table 3.11, rather, the infant ETP is greater than the child's. In Figure 3.12 the amount of thrombin (ETP) generated by the infant is greater than that generated by the child even though the thrombin peak is greater for the child than the infant. However, the initiation phase is still delayed for both the child and the infant relative to the adult profile, and their ETP values are less than 50% of the adult value of 51,569 nM.sec. One notable difference between the Figure 3.12 simulation based on the Monagle et al. [2006] data and the Andrew and Paes [1987], and Andrew et al. [1988, 1992] simulation in Figure 3.5 is the factor VIII concentration of the child. With a factor VIII amount of 0.5 nM for the child and 0.7 nM for the infant in the Monagle et al. [2006] data, greater thrombin is likely to be

generated for the infant than the child, as reflected by the model result in Figure 3.12. To investigate the effects of the factor VIII difference, we changed the level of factor VIII from 0.5 nM to 0.7 nM in the Monagle et al. [2006] child data, and the results are presented in Figure 3.16 and Table 3.13. The ETP value increases to 23,684 nM·sec for the child, which is greater than the infant. This is consistent with the developmental haemostasis pattern, although this ETP value is still lower than the ETP value for the child in the Andrew and Pacs [1987], Andrew et al. [1988, 1992] data (see Table 3.4). The child ETP is now 43% of the adult, while the ETP of the infant (18,292 nM.sec) is 35% of the adult. This percentage (43%) is an increase from the Figure 3.16 value of 39%, although it still falls short of the reported 50% by Andrew et al. [1992], it is closer to the 56% of the simulation based on Table 3.2 data (see Figure 3.5). The lower ETP percentage values can be explained by the higher levels of AT and Protein S in Table 3.3, 4038 nM and 436 nM, versus 3774 nM and 289 nM in Table 3.2, respectively. A shortening of the initiation phase is also observed in this change.

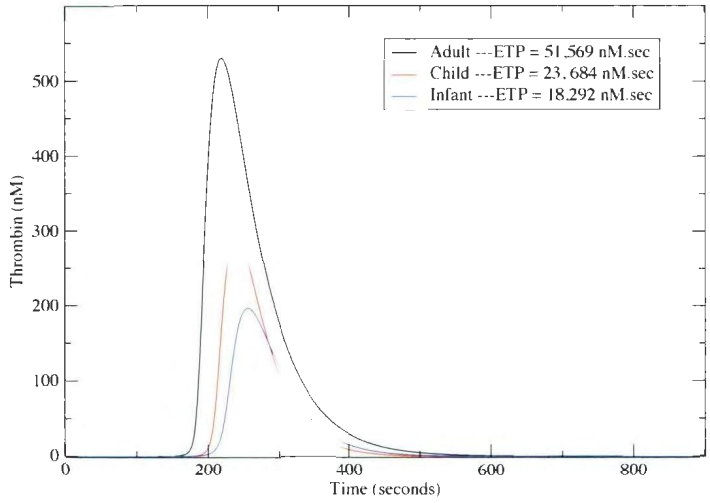

Figure 3.16: Simulation of thrombin generation in three age groups, using the Monagle et al. [2006] data in Table 3.3. with the factor VIII amount changed from 0.5 nM to 0.7 nM in the child.

Table 3.13 shows the propagation and termination phase slopes for the infant, child and adult age groups based on the Monagle et al. [2006] data, with the concentration of factor VIII changed from 0.5 nM to 0.7 nM in the child. The magnitude of the slopes increase from infants to adults.

Table 3.13: Thrombin propagation and termination phase slopes for the Monagle et al. [2006] data with the factor VIII changed from 0.5 nM to 0.7 nM in the child.

	Propagation phase (nMs^{-1})	Termination phase (nMs^{-1})
Adult	18.66	-4.87
Child	11.23	-3.42
Infant	6.67	-1.90

Here, we observe a significant increase in the magnitude of the propagation and termination slopes for the child values, from 9.09, and -2.94 to 11.23, and -3.42 respectively. This is in line with what we would expect.

3.8 Conclusion

The model simulations are consistent with the observations from empirical experiments found in the literature in as far as reduction of thrombin generation and delayed initiation phase is concerned. Child and infant thrombin generation is shown to be decreased and delayed compared to adult thrombin generation. Given the inconsistencies in the second set of data (from Monagle et al. [2006]) we choose the first set of data (from Andrew and Paes [1987], Andrew et al. [1988, 1992]) to investigate drug interactions with thrombin in the next chapter.

Chapter 4

Drug interactions with thrombin generation

4.1 Introduction

One of the main reasons for any model is to investigate how external factors can affect the modelled system. It is without doubt that developmental hemostasis affects the interaction of anticoagulant drugs with the coagulation system [Ignjatovic et al., 2011]. For this reason, we are also interested in gaining insight into how drugs used to treat blood coagulation problems affect thrombin generation. ATH, a preparation of a covalent conjugate of human AT and standard heparin [Berry et al., 1998], is an anticoagulant with a strong potential as an inhibitor of thrombin and other coagulation proteins. In this chapter, we attempt to introduce drug interactions into our model, specifically the interaction of ATH with thrombin, Xa, VIIa, IXa and XIa.

4.2 ATH in the model

ATH can inhibit several of the activated coagulation factors (enzymes) directly by forming an enzyme-ATH complex or by catalyzing the reaction between the enzyme and antithrombin

$$\text{enzyme} + \text{ATH} \xrightarrow{k_{on}} \text{enzyme-ATH} \quad \text{(direct method)} \tag{4.1}$$

$$\text{enzyme} + \text{AT} + \text{ATH} \xrightarrow{k_{on}} \text{enzyme-AT} + \text{ATH} \quad \text{(indirect/catalysis method).} \tag{4.2}$$

The major coagulation enzymes that would be inhibited directly by ATH during the coagulation cascade are IIa, Xa, IXa, VIIa, XIa, and XIIa. All except XIIa are included in our model. For this study, we focus on the direct method shown in Equation (4.1). The rates of reaction (second-order) are shown in Table 4.1:

Table 4.1: ATH rates of reaction as reported by Patel et al. [2007]

Species	k_{on} ($nM^{-1}sec^{-1}$)
Thrombin (IIa)	$k_{II} = 0.043$
Xa	$k_{Xa} = 0.00358$
VIIa	$k_{VIIa} = 0.00017$
IXa	$k_{IXa} = 0.000355$
XIa	$k_{XIa} = 0.000365$

Following the format in Equation (4.1), five ATH reactions with the respective coagulation factors and rate constants (shown in Table 4.1) are translated into ODEs and added to the model.

4.3 Simulation results

Figure 4.1 shows the adult thrombin profiles at different arbitrarily chosen levels of ATH concentration from 0 nM to 25 nM, and attempts to illustrate the effect of ATH on the adult blood coagulation system.

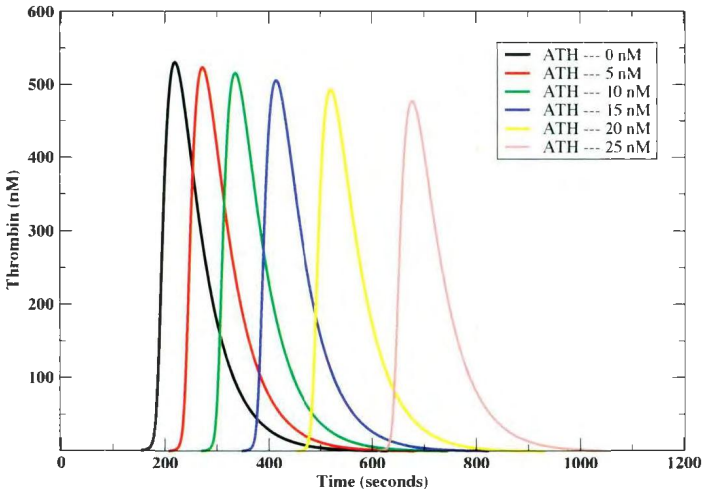

Figure 4.1: Simulated thrombin generation in adults when ATH is added.

Table 4.2 shows a summary of the effects of ATH on the simulated adult system with measurement of ETP, peak thrombin, peak time, and ETP change.

Table 4.2: Model results for adults with varying levels of ATH added.

ATH (nM)	ETP (nM·seconds)	Peak (nM)	Peak Time (seconds)	ETP change %	Initiation phase (seconds)
0	51,568.9	530	219	0.00	175
5	51,125.3	524	273	0.86	229
10	50,622.2	516	336	1.84	292
15	50,022.0	506	415	2.94	371
20	49,291.0	493	520	4.42	476
25	48,344.4	478	677	6.25	633

The initiation phase is lengthened from 175 seconds at 0 nM ATH to 633 seconds at

25 nM ATH, as shown in Table 4.2. ETP values decrease from 51,568.9 nM·sec at 0 nM ATH to 48,344.4 nM·sec at 25 nM ATH, with an ETP percentage change of 6.25% occurring from 0 nM to 25 nM ATH. Further increase of ATH in the model system leads to an increase in ETP change with each consecutive addition of ATH, eventually resulting in no thrombin produced. For example, the adult ETP change increases with each addition of 5 nM ATH as follows, 0.86%, 0.98%, 1.19%, 1.46%, and 1.92%. This observation is the same for the children and infants. The total amount of ATH added up to the point where no thrombin is produced is called the cut-off point. Thrombin peak also decreases with increasing ATH levels, while the peak time increases with increasing ATH levels from 219 seconds (0 nM ATH) to 677 seconds (25 nM ATH), mirroring the lengthening of the initiation phase. Overall the largest effect of the addition of 0 – 25 nM ATH was the delayed initiation phase of thrombin generation. This is expected from anticoagulant drugs as they delay (by inhibition) the 'short burst' of thrombin (during initiation) and thereby delay the commencement of the propagation phase where most thrombin is generated.

Figure 4.2 shows the thrombin profile in children at different levels of ATH.

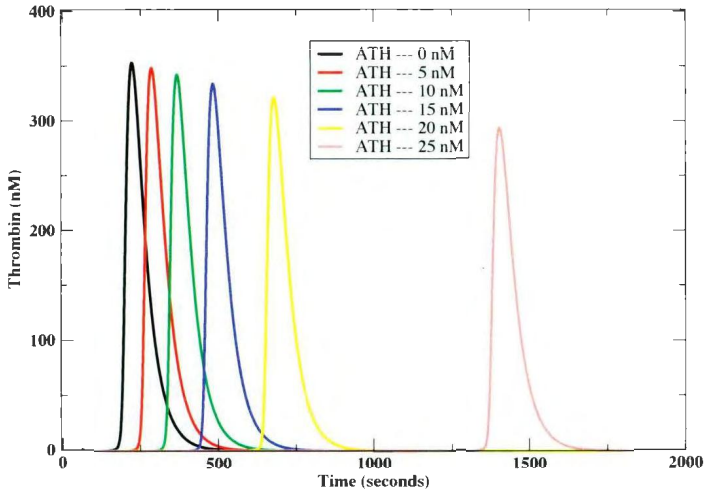

Figure 4.2: Simulated thrombin generation in children when ATH is added.

Table 4.3 summarizes the results of adding ATH at varying concentrations to the simulated child coagulation system.

Table 4.3: Model results for children with varying levels of ATH added.

ATH (nM)	ETP (nM·seconds)	Peak (nM)	Peak Time (seconds)	ETP change %	Initiation phase (seconds)
0	28,899.4	354	224	0.00	184
5	28,627.5	349	288	0.94	248
10	28,286.2	343	370	2.12	330
15	27,837.3	334	485	3.68	445
20	27,196.8	322	679	5.89	639
25	22,733.0	293	1407	21.34	1366

We observe a similar trend in the child system as in the adult, with a prolonged initiation time with the addition of ATH. However, by 25 nM ATH the lengthening of the initiation phase in children (1366 secs) is double that in adults (633 seconds), while the initiation phase with 0 nM ATH is relatively close in adults and children (175 secs vs 184 secs). There seems to be a large jump in the initiation time as ATH is

increased from 20 nM to 25 nM possibly due to the ATH levels approaching a cut-off point. The ETP values decrease from 28,899 to 22,733. with the greatest ETP change at 25 nM ATH of 21.43% compared to an ETP change of 6.25% in adults.

Figure 4.3 shows the thrombin profile in infants at different levels of ATH.

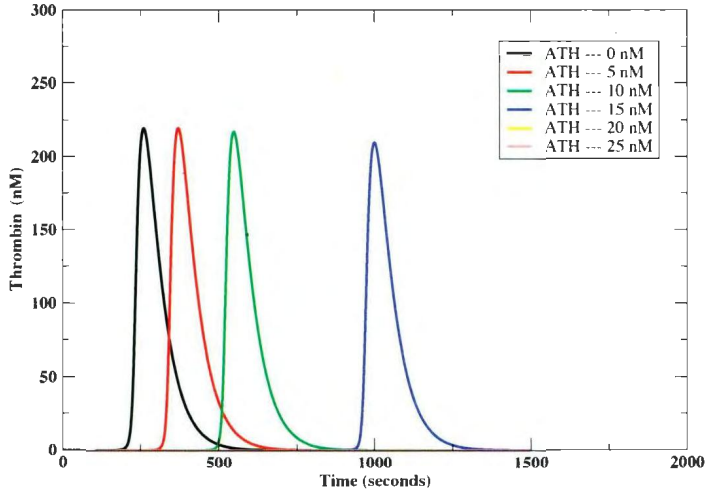

Figure 4.3: Simulated thrombin generation in infants when ATH is added.

Table 4.4 shows a summary of the effects of ATH on the simulated infant system with measurement of ETP, peak thrombin, peak time, and ETP change.

Table 4.4: Model results for infants with varying levels of ATH added.

ATH (nM)	ETP (nM·seconds)	Peak (nM)	Peak Time (seconds)	ETP change %	Initiation phase (seconds)
0	22,290.4	220	261	0.00	216
5	22,332.1	220	372	-0.19	327
10	22,199.3	217	550	0.41	505
15	21,778.9	210	1000	2.36	955
20	17,052.2	152	13,266	23.45	13,209
25	12,313.9	106	31,356	44.76	31,297

The initial addition of ATH (5 nM) seems to increase the thrombin generated in the infant by 0.19%, as measured by the ETP. This is out of line with the general observation. After the addition of 10 nM ATH, the next increment to 15 nM increases the initiation phase greatly to 955 seconds from 505 seconds, with both a lower ETP (21,778.9 nM·sec), and a thrombin peak of 210 nM. The next two graphs, with ATH concentration equal to 20 and 25 nM are not visible in the 0 – 2000 seconds time window as shown. This is due to the sudden increase in the initiation phase from 955 seconds to 13,209 seconds at 20 nM and finally, to 31,297 seconds at 25 nM ATH.

To further investigate the point (ATH concentration) where thombin generation is eventually reduced to zero, we perfomed additional simulations incrementing ATH until we arrived at zero nM thrombin or an ETP (area under graph) of below 100 nM·sec, which we consider sufficiently low. Table 4.5 below shows these points (cut-off points) and their peak thrombin observed for the adult, child, and infant model results.

Table 4.5: Thrombin generation cut-off points for the three age groups.

	ATH (nM)	Peak (nM)	ETP (nM·seconds)	Time (seconds)
Adult	150	8.38×10^{-2}	83.21	567,276
Child	100	1.15×10^{-2}	39.02	368,661
Infant	75	2.09×10^{-3}	27.05	285,996

Alternatively the cut-off points can also be observed by looking at the thrombin peaks as ATH is added to the system in Figure 4.4 below.

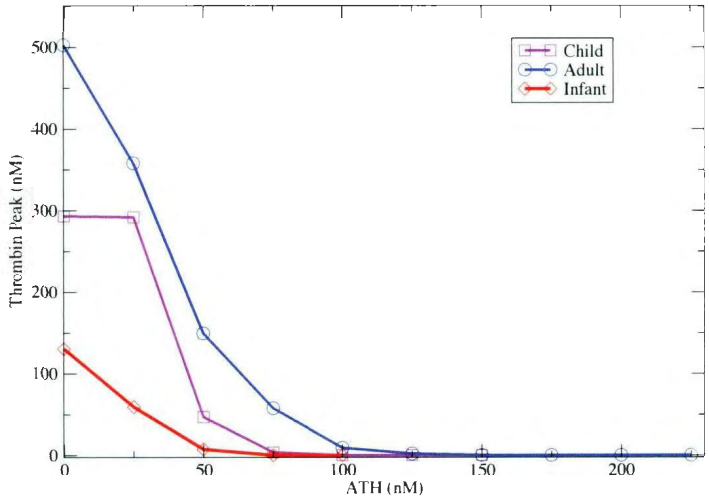

Figure 4.4: A plot of thrombin peaks versus ATH concentration for the three age groups.

The graph (Figure 4.4) shows the thrombin peaks versus ATH concentration in increments of 25 nM leading up to 225 nM. From this figure, we see that thrombin peaks decrease with increasing ATH. Different age groups reach the 'zero thrombin peak' at different times, with the infant reaching at the earliest time and the adult taking much longer. Due to the long simulation times required to obtain the cut-off points, the time increment used for data output was increased from 0.5 seconds to 10 seconds.

4.4 Discussion

Generally, for all age groups there is a significant increase in the initiation phase (delay) with increasing ATH values as seen in Figures 4.1, 4.2 and 4.3 and Tables 4.2, 4.3 and 4.4. There is also a slight decrease in the thrombin peak as the ATH concentrations increase from 0 nM to 25 nM (except for 0 nM to 5 nM ATH in the infant). The amount of thrombin generated also decreases with an increase in the ATH con-

centration, with the exception of a single case in the infant values, where the ETP experiences a slight (0.19%) increase with the addition of 5 nM ATH. In this instance, the thrombin peak remains the same at 220 nM.

4.4.1 Comparison of ATH in adults versus children.

The addition of ATH leads to a greater change in the thrombin generated in the child than for the adult in all cases of ATH concentration tested, as shown in Tables 4.2 and 4.3. The cut-off point for ATH was determined to be 150 nM for the adult versus 100 nM for the child, which is about 67% of the adult cut-off point. The cut-off point would be the ATH concentration required to eventually result in no thrombin production. Our study shows that ATH concentration cut-off points for adults, children and infants are distinctly different, as shown in Table 4.5. Given the differences observed, it is likely that the thrombin generation system's response to ATH is age-related. However, clinically relevant concentrations of ATH rather than arbitrary levels, need to be considered for a better foundation for dose-finding studies.

4.4.2 Comparison of ATH in adults versus infants.

Five nM of ATH in the infant system increases the ETP value of the child by 0.19%. This behavior is not consistent or in-line with other values and may be attributed to other unknown factors in the model. However, after 5 nM, the trend is consistent with what we observed in adults and children. The ETP values for infants decrease with the addition of ATH, with an ATH cut-off point occurring at 75 nM, after which little or no thrombin generated.

Further investigation into the value of ATH concentration at which this cut-off point

occurs revealed a value of 75 nM. This value is 50% of the adult cut-off point of 150 nM.

4.4.3 Comparison of ATH in children versus infants.

From Figures 4.2 and 4.3, we see that ATH inhibition in children and infants is clearly different from that of adults (see Figure 4.1), but similar to each other at these ATH levels. Both figures have a notable initiation delay compared to the adult results, at 15 nM for the infants and 25 nM for the children. The child cut-off value is higher than the infant's. Both have long initiation phases but the infant is longer, for example, at 15 nM ATH, the initiation phase for the child is about 445 seconds while that of the infant is almost 955 seconds. After observing the initiation phase delays of 31,297 seconds for the infant and 1366 seconds for the child, it is expected that the duration of the initiation phase will increase until a cut-off point is reached, at which point the initiation phase is non-existent. For the infant profile, the addition of 75 nM leads to little thrombin which is indicative that the cut-off point is within the proximity of that value. For the cut-off points, the x-axis requires an extension to 350,000 seconds as the initiation phase increases with decreasing thrombin curves (decreasing ETP and peak).

Table 4.6 summarizes the slopes for the propagation and termination phases for all three age groups with varying levels of ATH. The magnitude of the slopes for both the propagation and termination phases for the adult, child and infant age groups decrease with increasing levels of ATH.

Table 4.6: A summary of the propagation and termination phase slopes with varying levels of ATH in adults, children and infants.

ATH	Adult Slopes (nMs^{-1})		Child Slopes (nMs^{-1})		Infant Slopes (nMs^{-1})	
(nM)	Propagation	Termination	Propagation	Termination	Propagation	Termination
0	18.81	-5.08	12.96	-4.10	6.97	-2.00
5	18.11	-4.27	12.24	-2.90	6.68	-1.46
10	17.77	-3.94	12.18	-2.63	6.56	-1.34
15	17.35	-3.90	11.70	-2.57	6.34	-1.33
20	17.08	-3.80	11.26	-2.42	3.91	-1.22
25	16.41	-3.52	10.03	-2.08	2.46	-0.81

As ATH concentration increases, less thrombin is generated leading to lower thrombin peaks and decreased propagation slopes. This is expected as ATH is an inhibitor and its increase arrests the thrombin generation curve much quicker than lower ATH levels. The propagation slope changes from 18.81 to 16.41 (13%), 12.96 to 10.03 (23%), and 6.97 to 2.46 (65%) for the adult, child and infant, respectively, while for the termination slopes the changes are -5.08 to -3.52 (31%), -4.10 to -2.08 (49%) and -2.00 to -0.81 (60%). The changes in both the propagation and termination slopes are more profound with decreasing age, that is, the younger the individual, the more sensitive the slopes are to ATH changes.

4.4.4 Comparison of ATH reactions.

At an arbitrary ATH concentration of 10 nM, we ran the model with each one of the five ATH reactions individually, to investigate how each reaction of a coagulant protein with ATH affected the thrombin generation outcomes. Simulations for the adults, children and infants were performed, with results presented in Tables 4.7, 4.8,

and 4.9 below. The tables show the ETP, peak, and time to peak (TTP) for the different coagulation factors for the three age groups at 10 nM ATH, as well as the percentage change of each from the original (no ATH) value.

Table 4.7: Inhibition by 10 nM ATH for each of the factors in the adult.

Factor	ETP (nM·sec)	ETP change %	Peak (nM)	Peak change %	TTP (sec)	TTP change %
IIa	51,013	1.08	523	1.32	284	29.68
Xa	47,709	7.49	490	7.55	240	9.59
VIIa	51,569	0.00	530	0.00	219	0.00
IXa	51,565	7.76×10^{-3}	530	0.00	219	0.00
XIa	51,213	6.90×10^{-1}	525	0.94	220	0.46
Original	51,569	0.00	530	0.00	219	0.00
All rxns	50,622	3.70	516	2.64	336	53.42

Table 4.8: Inhibition by 10 nM ATH for each of the factors in the child.

Factor	ETP (nM·sec)	ETP change %	Peak (nM)	Peak change %	TTP (sec)	TTP change %
IIa	28,608	1.01	349	1.41	296	31.14
Xa	25,687	11.12	323	8.76	252	12.50
VIIa	28,900	0.00	354	0.00	224	0.00
IXa	28,897	0.01	354	0.00	224	0.00
XIa	28,607	1.01	350	1.13	225	0.45
Original	28,900	0.00	354	0.00	224	0.00
All rxns	28,286	2.12	343	3.11	370	65.18

Table 4.9: Inhibition by 10 nM ATH for each of the factors in the infant.

Factor	ETP (nM·sec)	ETP change %	Peak (nM)	Peak change %	TTP (sec)	TTP change %
IIa	22,356	-0.30	220	0.00	370	41.76
Xa	18,526	16.89	197	10.45	312	19.54
VIIa	22,290	0.00	220	0.00	261	0.00
IXa	22,289	4.49×10^{-3}	220	0.00	261	0.00
XIa	21,881	1.83	216	0.45	262	0.38
Original	22,290	0.00	220	0.00	261	0.00
All rxns	22,199	0.41	217	25.00	550	110.71

In all three age groups, the potential inhibitory effect of ATH seems to be the strongest

in its reaction with factor Xa. This is evidenced by the lowest ETP values of 47,709 nM·sec, 25,687 nM·sec, and 18,526 nM·sec, and the highest ETP changes of 7.49%, 11.12%, and 16.89% for each of the age groups, compared to the other four factors within the group. The thrombin peaks follow a similar trend as ETP, the higher the peak, the greater the ETP, with the greatest effect seen with factor Xa. However, the time to peak presents a different trend, it does not correspond with either the thrombin peaks or the ETP values. The longest time to peak and greatest time to peak change (29.68%, 31.14%, and 41.76%) is from the reactions of ATH with thrombin for the adult, child and infant at 284 seconds, 296 seconds, and 369.5 seconds respectively.

Based on the percentage change values in Tables 4.7, 4.8, and 4.9, the factor VIIa, IXa and XIa reactions with ATH seem to generate approximately the same amount of thrombin at similar peak and time to peak values. The inhibitory effect of ATH on thrombin generation seems to be affected predominately by the ATH reactions with factor Xa and factor IIa (thrombin). The reaction with factor IIa seems to delay thrombin generation while that with factor Xa reduces the peak of thrombin generated. The 'all reactions' simulation seems to generate the same amount of thrombin as the individual factor reactions with ATH but it is delayed in generating that same amount.

4.4.5 Comparison with original model (no ATH).

For the adult, thrombin generated for each of the added ATH reactions (see Table 4.8) is lower than for the original model (51,569 nM·sec) as measured by ETP values except for the reaction with VIIa (which gives the same amount of ETP as the original model). This is expected as each ATH inhibition reduces the concentration of each factor, leading to less thrombin being generated. For the factor Xa reaction with

ATH, the ETP level of 47,709 nM·sec is the lowest of all individual factor reactions with ATH. The observation is the same for the child where thrombin generated gives an equal ETP value (28,900 nM·sec) for the factor VIIa reaction with ATH compared to the thrombin (28,900 nM·sec) generated by the model with no ATH inhibition. The factor Xa reaction with ATH has the lowest thrombin generated in children (25,687 nM·sec) compared to the original model (28,899 nM·sec). For the infant, the thrombin generated is similar to the original model (22,290 nM·sec) for factors VIIa and IXa but lower for factors X and XIa. However, compared to the model without ATH inhibition (original model), the ATH reaction with factor IIa gives a higher ETP (22,356 nM·sec) for the infant. When we have a higher ETP for a factor's reaction with ATH than the corresponding ETP for the original model, this means that the inhibition works to increase the thrombin generation for some reason, while if we have a lower ETP the inhibition works to decrease the thrombin generated as expected.

The reason the reaction of factor Xa and ATH gives the lowest thrombin generated as measured by ETP values is mostly likely due to factor Xa's critical importance in the coagulation system. Factor Xa plays an important role in the conversion of prothrombin to thrombin after forming a complex Xa-Va with factor Va. Hence its reduction would have a significant impact on thrombin generated in the propagation phase.

4.4.6 Comparison of all ATH reactions added with each ATH-factor reaction addition

For the adult, the thrombin generated when all five factor reactions with ATH are included is lower than for each individual case, except for the ATH reaction with factor Xa. This means that the inhibition by ATH, with all factor reactions included,

is much more effective in lowering thrombin levels than each individual reaction with ATH, except for the factor Xa. We observe the same for the child profile but the infant is different. For all three age groups we observe that three factor reactions (IIa, VIIa and IXa) with ATH each have an ETP value higher than when all ATH reactions are included, meaning their individual reactions with ATH are less effective compared to the overall inhibition. The ATH reactions with XIa and Xa seem more effective at lowering thrombin generated as evidenced by the lower ETP values than all the other factor reactions with ATH.

When we have higher ETP values for each ATH-factor reaction added compared to that of the fully inhibited model, this (according to the model) means the collective inhibitions are more powerful than individual factor reactions with ATH (IIa, VIIa, IXa and XIa for the adult and child) as expected in most cases. On the other hand, if the ETP values are lower that could mean the collective inhibitions are less effective in the inhibition of thrombin generation (Xa for all age groups and IXa for only the infant). In all age groups, ATH inhibition of factor Xa alone seems to be the most effective in lowering thrombin even when compared to simulations with all five factors reacting with ATH. This could be explained by factor Xa having no competition in binding to ATH in the simulation.

4.5 Conclusion

The addition of ATH to the blood coagulation system seems to lead to a change in all the measured outcomes included in this particular study. These changes are different for each age group, suggesting an age-related difference in the initiation, propagation and termination of thrombin generation. Although in all cases the ETP

values decrease with increasing ATH concentrations, the rate of the decrease in ETP is different, as shown in Tables 4.2, 4.3 and 4.4 and reflected by the ETP percentage change. It is clear that the rate of decrease in ETP decreases with age, suggesting lower doses of ATH in children and infants for the same effect as in adults. With the frequency of neonates and children receiving anticoagulant therapy being higher than in the past [Monagle and Newall, 2012], age-related responses to anticoagulants have become more important when considering dosing strategies in children and infants.

Chapter 5

Conclusion

5.1 Summary

Quantitative differences in the coagulation system throughout childhood are well documented. However, the impact of these differences on anticoagulants drugs is not fully known. In this thesis we used a mathematical model in an attempt to answer the following questions:

- How is thrombin generation different in different age groups?
- How does the inhibitor ATH affect the thrombin generation in both children and adults?

Using the model, thrombin generation in children was investigated, including the effect of the potential anticoagulant complex, ATH. Ordinary differential equations (ODEs), based on enzyme reaction kinetics theory, were employed to describe the cascade of reactions known to lead to thrombin generation, including kinetic reactions describing the interaction with ATH.

The model results agree with the view that thrombin generation is delayed and reduced in children, which is in line with the developmental haemostasis principle. ATH has different effects on thrombin generation depending on the age group, as shown by the age-related changes in ETP values, and also the thresholds in response to ATH concentration levels. It is likely that administration of ATH would therefore require different dosage levels for different age groups.

5.2 Limitations and future work

Few experiments have been conducted on the use of new anticoagulant drugs to treat thrombotic complications in children. Data largely adopted from clinical trials performed on adult populations are usually relied upon. More well designed prospective trials are required to establish the optimal therapy for children with thrombotic problems. Direct comparison of the model results with literature is difficult as there is hardly any experiments with comparable data that describe both pathways.

Following the study described in this work, a number of research areas could be suitable for future work.

- Further validation of the model could be done.
- There are two ways in which ATH can interact with blood coagulation, direct and indirect. Further investigation into the indirect method (see Equation (4.2)) is necessary to fully ascertain the possible impact of ATH. Furthermore, the reversible reaction

$$\text{ATH} + \text{IIa} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{ATH=IIa} \xrightarrow{k_{\text{on}}} \text{ATH-IIa}, \tag{5.1}$$

could be tested in the model [Chan et al., 1997], where '=' in the complex ATH=IIa denotes a non-covalent bond.

- With over 150 reaction rate constants in the model, it would be beneficial to identify the constants to which the model's predictive capacity is most sensitive by employing sensitivity analysis [Saltelli et al., 2008, Danforth et al., 2009]. This would enable, where possible, rate constant improvements in those specific reactions that yield the greatest increase in predictive capacity.

5.3 Conclusions

To our knowledge, this is the first attempt to investigate thrombin generation in children using a mathematical model and also the first to attempt to describe the effect of the anticoagulant ATH on the adult and child coagulation system. This is a significant fundamental step in trying to understand ATH interaction with thrombin generation so as to find new anticoagulants that are effective, and also to help in determining appropriate dosage levels for clinical trials in the future. We conclude that the simulation results suggest a higher ability of ATH to suppress thrombin formation in children than in adults, thus lower amounts of ATH might be required in children undergoing antithrombotic therapy.

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